



A chromosomal inversion predicts the expression of sex steroid-related genes in a species with alternative behavioral phenotypes

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ABSTRACT

In white-throated sparrows, a chromosomal rearrangement has led to alternative phenotypes that differ in sex steroid-dependent behaviors. The rearrangement has captured the genes estrogen receptor alpha and 5-alpha reductase, making these genes strong candidates for mediating the behavioral phenotypes. We report here that of the two genes, expression of estrogen receptor alpha mRNA differs between the morphs and predicts behavior to a much greater extent than does expression of 5-alpha reductase mRNA. Differentiation of estrogen receptor alpha, therefore, is likely more important for the behavioral phenotypes. We also found that in some brain regions, the degree to which testosterone treatment affects the expression of steroid-related genes depends strongly on morph. A large morph difference in estrogen receptor alpha mRNA expression in the amygdala appears to be independent of plasma testosterone; this difference persists during the non-breeding season and is detectable in nestlings at post-hatch day seven. The latter result suggests a substrate for organizational effects of hormones during development.

1. Introduction

A critical step towards understanding the evolution of social behavior is identifying the underlying genetic and neuroendocrine mechanisms. This task has been made difficult by the complexity of social behavior and its polygenic basis. Species with alternative behavioral phenotypes, particularly those for which the phenotypes are linked to known hormonal or genetic variation, represent a powerful opportunity to understand how such mechanisms contribute to behavioral evolution.

The white-throated sparrow (*Zonotrichia albicollis*) is a common North American songbird with alternative phenotypes that differ in plumage coloration, hormone levels, and social behavior (Horton et al., 2014a, 2014b; Maney, 2008; Maney et al., 2015; Tuttle, 2003). This species occurs in two plumage morphs, white-striped (WS) or tan-striped (TS), which are equally prevalent among both sexes (Fig. 1). WS birds express higher levels of vocal and physical aggression in response to territorial intrusions and invest more effort in extra-pair mating than do TS birds (Horton et al., 2014b; Tuttle, 2003). In contrast, TS birds spend more time investing in mate guarding and provisioning of nestlings (Horton et al., 2014b; Kopachena and Falls, 1993; Tuttle, 2003). Like in other North American sparrows, these behaviors also differ by sex such that males sing more than females, and females provision

young more often than do males. This combination of both morph and sex differences means that WS males are the most territorial and least parental, WS females and TS males are similar in their aggressive and parental provisioning, and TS females are the least aggressive and most parental (Horton et al., 2014b). This interaction of sex and morph indicates that the basis of these alternative behavioral phenotypes is autosomal rather than linked to sex chromosomes.

Whether a bird is WS or TS depends on the presence or absence, respectively, of a chromosome-level rearrangement on chromosome 2, called ZAL2^m (Sun et al., 2018; Thorneycroft, 1966, 1975; Tuttle et al., 2016). TS birds are homozygous for the standard ZAL2 chromosome without the rearrangement whereas WS birds are heterozygous, with one copy of ZAL2^m and one copy of ZAL2 (Thomas et al., 2008; Thorneycroft, 1966). The equal proportion of heterozygous and homozygous individuals is maintained by almost complete disassortative mating (Falls and Kopachena, 2010); nearly all breeding pairs consist of a WS and a TS bird (Tuttle et al., 2016). Because the chromosome is in a near-constant state of heterozygosity (see Horton et al., 2013), recombination is suppressed between ZAL2 and ZAL2^m (Huynh et al., 2011; Thomas et al., 2008; Tuttle et al., 2016). Suppression of recombination has caused the sequences of ZAL2 and ZAL2^m to diverge by at least 1% (Sun et al., 2018).

The white-throated sparrow is a particularly promising species to

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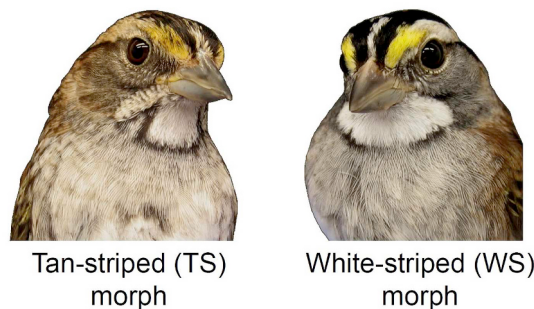


Fig. 1. White-throated sparrows occur in two plumage morphs, tan-striped (TS) and white-striped (WS). The morphs differ with respect to endocrine profiles and social behavior (Horton et al., 2014a). Photo credit B. Horton. Reprinted with permission of the Society of Integrative and Comparative Biology (see Maney et al., 2015).

model the interactions between genes, endocrine state, and behavior because the behaviors that differ between the morphs are steroid-dependent. During the breeding season, territorial aggression and parental provisioning in songbirds depend on plasma levels of testosterone (Goodson et al., 2005; Hegner and Wingfield, 1987; Schlinger and Callard, 1990; Wingfield, 1985), and the behavioral differences between white-throated sparrow morphs are most pronounced during periods of territory establishment when circulating testosterone and estradiol are highest (Spinney et al., 2006). Furthermore, during the breeding season, plasma levels of testosterone, dihydrotestosterone, and estradiol are higher in WS males than in TS males, and testosterone and estradiol are higher in WS females than in TS females (Horton et al., 2014b). These results strongly suggest that the behavioral polymorphism has an endocrine basis.

The morph differences in plasma hormone levels, however, cannot fully explain morph differences in behavior in this species. When plasma levels of sex steroids were experimentally equalized between the morphs, morph differences in aggressive behavior and singing persisted (Maney et al., 2009; Merritt et al., 2018), suggesting that the morphs differ with respect to steroid sensitivity or metabolism. In fact, several genes related to steroid sensitivity (i.e., receptors) and metabolism (i.e., enzymes) are located within the rearrangement on chromosome 2 and differ in sequence between ZAL2 and ZAL2^m. These genes include ESR1 and SRD5A2, which encode estrogen receptor alpha and 5-alpha reductase, respectively. Morph differences in expression of these genes may result in differential steroid action.

Previously, we showed morph-dependent expression of estrogen receptor alpha mRNA in several brain regions relevant to social behavior. In addition, the levels of estrogen receptor alpha mRNA in some of these regions predicted levels of territorial singing and parental provisioning better than morph itself (Horton et al., 2014a). In the present series of studies, our goal was to understand the factors that drive differential expression of estrogen receptor alpha and therefore possibly the evolution of the behavioral polymorphism in white-throated sparrows. First, we asked whether morph differences in the expression of estrogen receptor alpha are associated with morph differences in the expression of 5-alpha reductase. This enzyme irreversibly converts testosterone to the more active androgen, dihydrotestosterone, making it unavailable for conversion to estradiol. Because the gene for 5-alpha reductase has been captured inside the ZAL2^m rearrangement, we hypothesized that like estrogen receptor alpha, it would be differentially expressed and predictive of behavior. We also measured mRNA levels for aromatase, an enzyme that irreversibly converts testosterone to estradiol, and which is not inside the ZAL2^m rearrangement. Because we measured the expression of these mRNAs in sets of brain sections from the animals in our previous study on estrogen receptor alpha (Horton et al., 2014a), we could additionally test for correlations of expression among all three genes.

Because morph differences in the expression of sex steroid-related genes could be caused by morph differences in plasma estrogens or androgens, in a second study we treated non-breeding, laboratory-housed WS and TS males with testosterone and quantified the expression of two steroid-related genes inside the rearrangement, estrogen receptor alpha and 5-alpha reductase, and two genes outside it, aromatase and androgen receptor. This study allowed us to test (1) whether morph differences in the expression of steroid-related genes in free-living, breeding birds might be explained by morph differences in plasma testosterone; (2) whether morph differences in the expression of these genes persist even in the face of equal plasma testosterone in the two morphs; and (3) whether morph differences in the expression of these genes persist in the non-breeding season. Thus, we sought a much clearer understanding of the relationships among expression of these genes in the brain and seasonal changes in circulating sex steroids.

Finally, because sex steroid hormones are widely understood to have organizational effects on the brain early in life, morph differences in adult behavior in this species could be caused by morph differences in expression of steroid-related genes early during development. In a third study, we tested for morph differences in the expression of sex steroid-related mRNAs in the brains of WS and TS nestlings. Together, these three studies allowed us to test the effects of morph on the expression of behaviorally relevant genes located inside and outside the rearrangement, in sparrows at multiple life history stages.

2. Methods

2.1. Study 1: gene expression in free-living adults

2.1.1. Rationale

Previously, using *in situ* hybridization, we showed that the expression of estrogen receptor alpha in adult, breeding birds differs between the morphs in multiple brain regions related to social behavior (Horton et al., 2014a). In the present Study 1, we performed *in situ* hybridization on alternate sets of brain sections from the same birds used by Horton et al. to test whether the expression of two other steroid-related genes, 5-alpha reductase and aromatase, also differs by morph and whether it is correlated with the expression of estrogen receptor alpha or morph-dependent behaviors.

2.1.2. Behavioral observations and tissue collection

Free-living white-throated sparrows were behaviorally characterized and collected near Argyle, Maine, USA under appropriate state and federal permits. For each bird we collected data on either song rate (N = 12 TS males, 13 WS males; 7 TS females, 6 WS females) or parental provisioning (N = 11 TS males, 10 WS males; 9 TS females, 11 WS females). We quantified song rate in response to a simulated territorial intrusion (STI; Horton et al., 2014b, 2012) during the early stages of breeding at the peak of territorial behavior. During the nestling stage, at a different location on the site, we quantified the rate at which the parents provisioned the nestlings (number of trips to the nest per hour to feed nestlings) by placing cameras near nests. These behaviors (song rate in both sexes and parental provisioning in males) differ significantly between the morphs (Horton et al., 2014b) and are significantly correlated with expression of estrogen receptor alpha mRNA (Horton et al., 2014a) as measured via *in situ* hybridization. The methods for each type of behavioral test were as previously described (Horton et al., 2014b, 2012). Approximately 24 h after the last observation, birds were captured in mist nets. Birds for whom song rate was quantified were captured by using playback to lure them into the net. Average time to capture was 6.1 min $\pm$ 0.93 SE. There were no effects of sex (F = 1.629, p = 0.211) or morph (F = 0.758, p = 0.390) on time to capture, and no interaction between sex and morph (F = 0.127, p = 0.724). Parental animals were captured by placing a mist net near the nest. Brains were harvested, frozen on dry ice, and shipped to Emory University (Atlanta, GA, USA).

2.1.3. *In situ* hybridization and quantification of signal

Brains were cut coronally into seven sets of 20 μm sections. Estrogen receptor alpha mRNA was labeled in one set, and the results previously published (Horton et al., 2014a). We used an identical protocol (^{35}S -labeled riboprobes) to label 5-alpha reductase mRNA and aromatase mRNA (Supplemental Table 1; for detailed methods, see Leung et al., 2011). Briefly, brains were sectioned at 20 μm onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). After sections were defrosted, delipidated, acetylated, and dehydrated, 10E-7 mCi of riboprobe in 100 mL hybridization buffer was applied to each slide. Slides were then coverslipped and incubated overnight in a mineral oil bath (see Supplemental Table 1 for hybridization temperatures). The following day, the slides were washed, treated with RNase, washed again (see Supplemental Table 1 for wash temperatures) and dehydrated. Dry slides were placed into film cassettes against Kodak BioMax maximum-resolution film and protected from light (see Supplemental Table 1 for exposure times). The film was then developed in a Konica SRX101-A developer and scanned at 2500 dpi using a digital Epson V700 scanner. The slides were then Nissl-stained in toluidine blue and scanned at the same resolution.

The sequence of each riboprobe was confirmed via cloning and Sanger sequencing; the sequence used for 5-alpha reductase did not span known polymorphisms between ZAL2 and ZAL2^m. *In situ* hybridization with complementary sense probes produced no labeling in each case. We labeled 5-alpha reductase mRNA in five separate runs of *in situ* hybridization, four for the males (N = 21 TS, 19 WS) and one for the females (N = 7 TS, 5 WS; we did not test for sex differences in this study). We labeled aromatase mRNA in a subset of these males (males sampled at the height of territorial behavior when morph differences in singing behavior were peaking, N = 11 TS, 13 WS), in two runs of *in situ* hybridization. We did not label aromatase mRNA in females. We did not measure protein levels or enzyme activity, here or in Studies 2 or 3.

We quantified 5-alpha reductase mRNA in seven brain regions (see Supplemental Figs. 1–5 for locations of sampling), chosen on the basis of clear morph differences in the expression of estrogen receptor alpha in these animals (Horton et al., 2014a). These regions included the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH), the medial bed nucleus of the stria terminalis (BSTm), the anterior hypothalamus (AH), the medial preoptic area (POM), and the lateral septum (LS). Also included was nucleus taeniae of the amygdala (TnA), a region for which nomenclature has been in flux (see Reiner et al., 2004; Horton et al., 2014a; Mello et al., 2019). Prior to 2017, the region was thought to be homologous to the mammalian medial amygdala (see Reiner et al., 2004) and we have referred to it as such in previous work (Horton et al., 2014a; Maney et al., 2015; Zinzow-Kramer et al., 2015). This homology has now been called into question (Vicario et al., 2017; Mello et al., 2019); nonetheless, TnA shares many features with the medial amygdala of mammals. In addition to expressing sex steroid receptors and aromatase, the songbird TnA is heavily interconnected with both sensory regions and the hypothalamus (Cheng et al., 1999). In zebra finches, TnA lesions disrupt the ability to adjust singing behavior to social context (Ikebuchi et al., 2009), suggesting an important role in the perception of social cues. In the present paper we refer to it as TnA, with the caveat that new nomenclature was very recently proposed (Mello et al., 2019). We quantified aromatase in the same seven regions, with the exception of LS, in which we did not visibly detect expression of that gene.

We used ImageJ to measure the average gray value of each region of interest in the films, correcting for background by subtracting the gray value of a nearby region without discernible label (Horton et al., 2014a; Maney et al., 2003; Matragrano et al., 2011). The regions of interest were identified using major landmarks (see Supplemental Figs. 1–5) visible in the corresponding Nissl-stained sections. All signal was measured bilaterally in two consecutive sections, which were ~120 μm apart since multiple sets of tissue were cut and only one set was run for each gene. The values from those two samples were then averaged to arrive at a single number per region per gene for each individual. In

each case, we took care to quantify the signal in precisely the same locations in which estrogen receptor alpha expression had been quantified in each animal, by overlaying the images of consecutive sections in which each different mRNA was labeled.

2.1.4. Data analysis

Data from the *in situ* hybridizations (corrected gray values) were square root transformed to reduce heteroscedasticity and non-normality. Results of Shapiro-Wilks and Levene's tests before and after transformation are given in Supplemental Tables 2 and 3. We then tested for morph differences in all brain regions in SPSS (Version 25) using a separate MANOVA to test for the effects of morph for each sex and each gene and controlling for the effect of *in situ* hybridization run and year of collection when applicable. When a significant effect was detected by the MANOVA, we performed follow-up univariate F tests for each region, controlling for *in situ* hybridization run and year of collection. Within each sex, we used Pearson partial correlations to test for significant correlations between the expression of 5-alpha reductase and aromatase within each brain region, controlling for morph, *in situ* hybridization run, year of collection, and plasma values of testosterone and estradiol (Horton et al., 2014a). Using previously published data from the same animals and brain regions (Horton et al., 2014a), we also tested for correlations, controlling for the same variables, between expression of each of these mRNAs and estrogen receptor alpha mRNA. Finally, we tested for correlations between mRNA expression and behavior using Pearson partial correlations in SPSS, holding morph and year of collection constant. For both gene-by-gene correlations and gene-behavior correlations, we corrected for multiple testing using a sequential Bonferroni correction (Rice, 1989).

2.2. Study 2: effects of testosterone on gene expression in males

2.2.1. Rationale

In Study 1, we measured gene expression in unmanipulated animals. In Study 2, we tested whether the expression of mRNAs that are differentially expressed between the morphs is sensitive to manipulation of testosterone. If so, then morph differences in plasma testosterone, which we previously reported for the sample of birds in Study 1 (Horton et al., 2014a), could explain morph differences in the expression of sex steroid-related mRNAs.

2.2.2. Hormone manipulation and tissue collection

To test our hypothesis, we used alternate sets of tissue from a previous study (Grozhiik et al., 2014) in which we manipulated plasma testosterone in male, laboratory-housed white-throated sparrows. Thirty-five adult males (N = 20 TS, 15 WS) were captured via mist-netting on the campus of Emory University during fall migration. Birds were housed on short photoperiod (8.5 h of light) to ensure their gonads remained regressed and endogenous steroid hormone levels remained low (Wolfson, 1958). Half of the males (N = 10 TS, 8 WS) received subcutaneous silastic capsules (length 12 mm, I. D. 1.46 mm, O. D. 1.96 mm; Dow Corning, Midland, MI, USA) filled with testosterone (Steraloids, Newport, RI, USA) and sealed at both ends with silicone adhesive. The remaining males (N = 10 TS, 7 WS) received empty capsules. Because the birds were maintained on short days, the control males experienced naturally low levels of plasma testosterone typical of the non-breeding season. Two weeks after the capsules were administered, brains and blood were collected. We measured testosterone, dihydrotestosterone, and estradiol in each sample using radioimmunoassay. In the testosterone-treated males, plasma levels of all three hormones were significantly higher than in the control males, were typical of free-living breeding males, and did not differ between the morphs. See Grozhiik et al. (2014) for further details.

2.2.3. *In situ* hybridization

Brains were cut into seven sets of 20 μm sections as in Study 1. Because our primary goal was to explore mechanisms underlying the

previously described morph differences in estrogen receptor alpha, we first ran *in situ* hybridization for that gene (see [Supplemental Table 1](#) for riboprobe sequences). In an alternate set of sections, we labeled 5-alpha reductase mRNA as described above for Study 1. Because sets of remaining tissue from these birds were limited at this point, we labeled aromatase mRNA in a subset of the birds (testosterone group: 6 TS, 6 WS; control group: 6 TS, 5 WS) and androgen receptor mRNA in another subset of the birds (testosterone: 5 TS, 6 WS; control: 6 TS, 5 WS). Expression of androgen receptor mRNA was not quantified in Study 1, but it was added in Study 2 to gain a more complete understanding of the local context for sex steroid action. All quantification of signal was done as described above for Study 1, in the same regions of interest.

2.2.4. Data analysis

Data were square-root transformed as for Study 1. Results of Shapiro-Wilks and Levene's tests before and after transformation are given in [Supplemental Tables 4 and 5](#). All analyses were performed in SPSS. We performed an omnibus 2-way MANOVA for each gene, which included all brain regions, to test for effects of morph and treatment on gene expression. If the MANOVA revealed a significant effect of morph or treatment or an interaction between morph and treatment, post-hoc F-tests were performed for each brain region as appropriate on the basis of the MANOVA results. If we found significant effects or interactions in the F-tests, we followed up with Fisher's LSD pairwise tests to test for effects of morph within treatment and vice versa, as appropriate on the basis of the F-test results. As in Study 1, we tested for gene-by-gene correlations using partial Pearson correlations, correcting for the performance of multiple correlations per gene-gene pair using a sequential Bonferroni correction ([Rice, 1989](#)), and controlling for morph and treatment.

2.3. Study 3: gene expression in nestlings

2.3.1. Rationale

Steroid hormones are important modulators of neural development in songbirds, particularly during the first week of post-hatch life, and may contribute to behavioral differentiation ([Adkins-Regan et al., 1994](#); [Jacobs et al., 1999](#); [London et al., 2006](#); [Moore, 1991](#); [Perlman and Arnold, 2003](#); [Rhen and Crews, 2002](#); [Schlinger et al., 2001](#)). In Study 3, we tested for morph differences in the expression of sex-steroid related mRNAs early during development, at post-hatch day 7. In this study, we used quantitative real-time PCR (qPCR) to quantify the expression of mRNAs.

2.3.2. Tissue collection

The nestlings used in Study 3 were the offspring of adults used in Study 1. We collected a total of 68 nestlings from 17 different nests (N = 14 TS males, 19 WS males; 16 TS females, 19 WS females) over two consecutive field seasons. Brains were dissected from the skulls, frozen rapidly on dry ice, and shipped to Atlanta where they were stored at -80°C until sectioning. Brains were mounted inside a cryostat to be sectioned in the coronal plane. Next, 60 μm sections were removed until a region of interest was exposed. The region was then microdissected with a punch tool (0.5 mm or 1 mm depending on the region) inserted to a depth of ~ 400 – $600\ \mu\text{m}$. We punched four different regions of interest: TnA, dorsal hypothalamus (Dhyp, containing AH and PVN), ventral hypothalamus (Vhyp, containing VMH), and POM. The BSTm and LS regions were not sampled in Study 3. The 0.5 mm punches of TnA tissue from each hemisphere of the brain were combined into one sample, while the 1.0 mm punches of dorsal and ventral hypothalamus and POM were centered on the midline and thus contained tissue from both hemispheres. The punch of the dorsal hypothalamus was bounded on the dorsal side by the anterior commissure, and the ventral punch was immediately ventral to the dorsal punch.

2.3.3. RNA extraction and qPCR

We extracted RNA from each punch using either the Qiagen Allprep RNA/DNA micro kit with modifications ([Zinzow-Kramer et al., 2015](#)) or the Qiagen miRNeasy micro kit with modifications ([Zinzow-Kramer et al., 2014](#)). To produce cDNA, we performed reverse transcription using the Transcriptor First Strand cDNA synthesis kit (Roche) with random hexamer primers, then diluted the reaction to 20 ng/ μl for qPCR. We designed exon-spanning primers ([Supplemental Table 6](#)) for use with Roche Universal Probe Library (UPL) for estrogen receptor alpha, 5-alpha reductase, aromatase, and androgen receptor, then verified the amplified sequences via cloning and sequencing. qPCR was performed using a Roche LightCycler 480 Real-Time PCR System in triplicate for each sample on a 384-well plate as previously described ([Zinzow-Kramer et al., 2014](#)). Amplification efficiencies were calculated via standard curves and averaged between 90 and 110%, depending on the gene. Using the LightCycler 480 Software Version 1.5.0, we calculated crossing point (Cp) values using the Abs Quant/2nd Derivative Max method. We normalized the expression of each gene of interest to the geometric mean (formula: $(\text{Cp}_{\text{target}}/\text{Cp}_{\text{reference1}} \times \text{Cp}_{\text{target}}/\text{Cp}_{\text{reference2}})^{1/2}$) of two reference genes, peptidylprolyl isomerase A (PPIA) and transferrin receptor (TFRC; [Pfaffl, 2001](#); [Vandesompele et al., 2002](#)), which have been previously validated for use in white-throated sparrow brain tissue ([Zinzow-Kramer et al., 2014](#)). The expression of these reference genes is stable in brain tissue across sex and morph in this species ([Zinzow-Kramer et al., 2014](#)).

2.3.4. Data analysis

All data were log transformed with base 10. Results of Shapiro-Wilks and Levene's tests before and after transformation are given in [Supplemental Tables 7 and 8](#). All analyses were performed in SPSS. We performed separate omnibus MANOVAs for each gene to test for effects of morph and sex, controlling for year of collection and nest of origin. If the MANOVA revealed significant effects or interactions, we followed up with univariate F-tests for each brain region. If the effects of morph, sex, or their interaction were significant in the F-tests, we performed post-hoc pairwise comparisons between the morphs within sex and vice versa using Fisher's LSD tests.

2.3.5. Replication of morph differences in adults

Because the known morph differences in mRNA expression in adults were detected using *in situ* hybridization (e.g. [Horton et al., 2014a](#)), we verified that the method we used in the current study in nestlings, qPCR, would allow us to detect those effects in adults as well. We therefore conducted qPCR to quantify expression of the same mRNAs in the same brain regions in adults. Free-living males (N = 8 TS males, 8 WS males) and females (N = 6 TS females, 9 WS females) were collected as described above for Study 1. All of these adults were collected early in the breeding season during the peak of territorial activity and song playback was used to lure them into nets. Average time to capture was 6.1 ± 0.89 SE min. There were no effects of sex ($F = 0.167$, $p = 0.602$), morph ($F = 0.678$, $p = 0.686$) on time to capture, and no interaction between sex and morph ($F = 0.138$, $p = 0.713$). The brains were dissected from the skulls and frozen as in Study 1 ([Zinzow-Kramer et al., 2015](#)). Brains were shipped frozen to Emory University (Atlanta, GA, USA) and sectioned at 300 μm on a cryostat. Regions of interest were dissected using a 0.5 mm or 1.0 mm punch tool, and RNA was extracted from the punched samples as described above for the nestlings. For these adults, the samples of dorsal and ventral hypothalamus were pooled to increase the yield of RNA per sample. Expression of estrogen receptor alpha, 5-alpha reductase, aromatase, and androgen receptor mRNA was quantified using qPCR as described for the nestlings. Data were analyzed also as described for the nestlings, with the exception that we did not control for nest or year of collection; all adults were collected in a single field season.

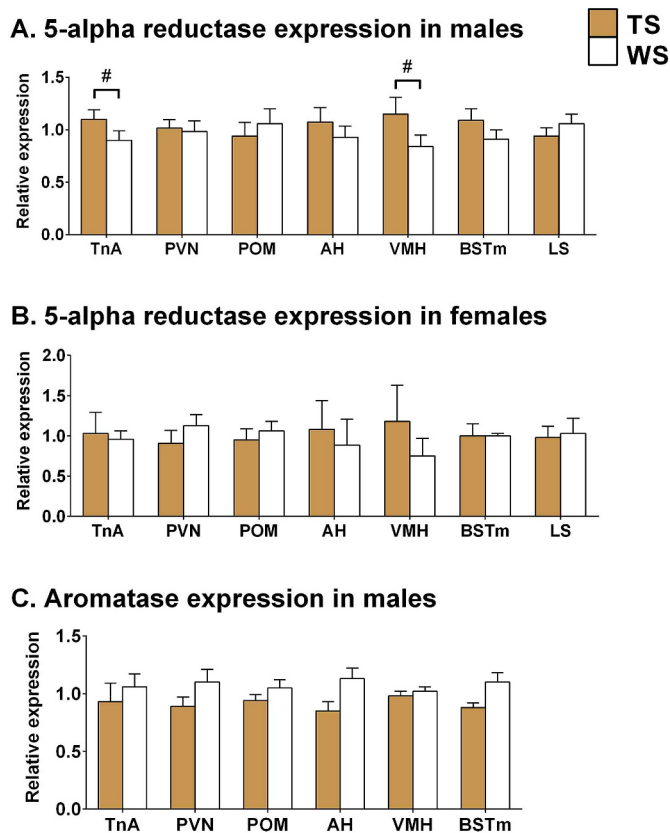


Fig. 2. Morph differences in expression of 5-alpha reductase (A, B) and aromatase (C) mRNA in the brains of in male (A, C) and female (B) white-throated sparrows. mRNA levels were quantified using *in situ* hybridization. Within region, all values were normalized to the series mean for both morphs combined, such that 1.0 on the Y-axis represents the mean corrected gray value for each region. This normalization allowed plotting the data from all of the regions on the same axis. Thus, region-to-region variation in signal is not represented here. Trending pairwise differences ($0.05 < p < 0.10$) are marked with number signs for TnA ($p = 0.056$) and VMH ($p = 0.065$). TnA, nucleus taeniae of the amygdala; PVN, paraventricular nucleus; POM, medial preoptic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; BSTm, bed nucleus of the stria terminalis, medial portion; LS, lateral septum.

3. Results

3.1. Study 1: gene expression in free-living adults

Expression of 5-alpha reductase and aromatase mRNA, as detected using *in situ* hybridization, in males is plotted in Fig. 2, as well as expression of 5-alpha reductase in females. The omnibus MANOVA for 5-alpha reductase expression in males showed a significant effect of morph ($F = 3.889$, $p = 0.005$), but post-hoc F-tests revealed no significant morph differences in any brain region. We found only trending morph differences in TnA ($F = 3.886$, $p = 0.056$) and VMH ($F = 3.601$, $p = 0.065$), in both cases expression was marginally higher in TS than WS birds. There was no main effect of morph on 5-alpha reductase expression in females ($F = 1.789$, $p = 0.341$). Post-hoc analyses were therefore not conducted for females. For aromatase mRNA expression in males, the omnibus MANOVA revealed no overall effect of morph ($F = 1.253$, $p = 0.332$). No post-hoc tests were conducted.

We used our previously published data on the expression of estrogen receptor alpha mRNA in these birds (Horton et al., 2014a) to test for correlations between expression of that gene and the two genes quantified here. We also tested for a correlation between 5-alpha reductase mRNA and aromatase mRNA. After correction for multiple testing, mRNA expression was not correlated for any gene pair within any

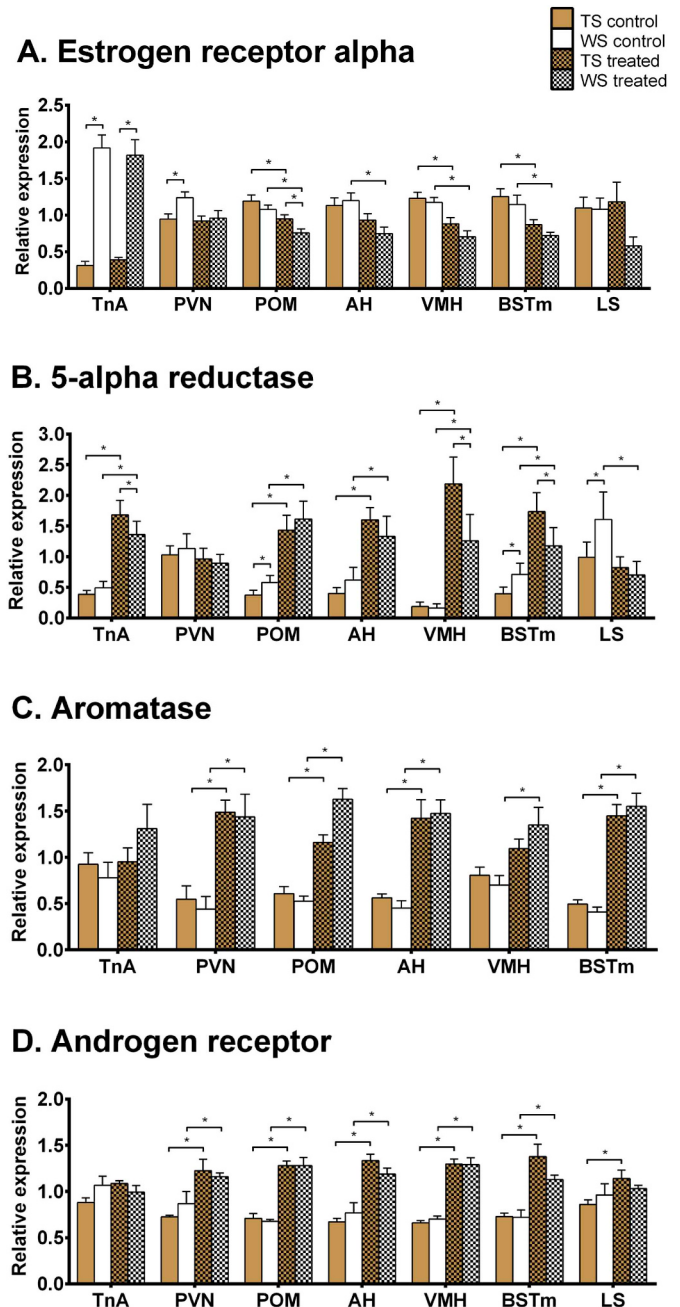


Fig. 3. Effects of testosterone treatment and morph on the expression of (A) estrogen receptor alpha mRNA, (B) 5-alpha reductase mRNA, (C) aromatase mRNA, and (D) androgen receptor mRNA in male white-throated sparrows treated with testosterone-filled or blank implants. mRNA was labeled using *in situ* hybridization. Values for each region are normalized to the mean of all males for each region, with means and SEMs for normalized values shown. For abbreviations, see Fig. 2. Significant pairwise differences ($p < 0.05$) are marked with asterisks.

region for either sex (Supplemental Table 9). Additionally, neither song rate nor parental provisioning was predicted by 5-alpha reductase or aromatase mRNA expression in any region after correcting for multiple testing (Supplemental Table 10).

3.2. Study 2: effects of testosterone on gene expression in males

The effects of testosterone treatment on the expression of estrogen receptor alpha, 5-alpha reductase, aromatase, and androgen receptor mRNAs, as detected using *in situ* hybridization, are plotted in Fig. 3 and

Table 1

F and p values for the MANOVAs (labeled below as omnibus), univariate F-tests, and post-hoc pairwise comparisons in Study 2. mRNA was labeled using *in situ* hybridization. Significant effects are shown in bold.

Gene	Univariate F-tests within gene and region						Post-hoc pairwise comparisons (p)				
	Region	Morph		Treatment		Morph x Treatment		TS vs. WS		Control vs. Treated	
		F	p	F	p	F	p	Control	Treated	TS	WS
Estrogen receptor alpha	Omnibus (all regions)	38.239	< 0.001	5.013	< 0.001	0.940	0.499				
	TnA	296.38	< 0.001	0.316	0.578	–	–	< 0.001	< 0.001	–	–
	PVN	6.198	0.019	3.961	0.056	–	–	0.013	0.742	–	–
	POM	6.412	0.017	26.923	< 0.001	–	–	0.218	0.010	0.003	< 0.001
	AH	0.251	0.620	12.067	0.002	–	–	–	–	0.128	0.003
	VMH	1.712	0.201	27.613	< 0.001	–	–	–	–	0.003	< 0.001
	BSTm	2.232	0.146	31.026	< 0.001	–	–	–	–	< 0.001	< 0.001
	LS	2.683	0.112	2.851	0.102	–	–	–	–	–	–
5-alpha reductase	Omnibus (all regions)	7.064	< 0.001	78.136	< 0.001	2.941	0.024				
	TnA	0.018	0.894	229.065	< 0.001	6.538	0.016	0.083	0.006	< 0.001	< 0.001
	PVN	0.453	0.506	1.542	0.224	0.044	0.835	–	–	–	–
	POM	11.886	0.003	157.873	< 0.001	0.288	0.595	0.012	0.176	< 0.001	< 0.001
	AH	0.022	0.884	46.121	< 0.001	2.272	0.142	–	–	< 0.001	0.003
	VMH	5.122	0.031	125.886	< 0.001	4.671	0.039	0.783	< 0.001	< 0.001	< 0.001
	BSTm	0.121	0.730	44.513	< 0.001	7.771	0.009	0.023	0.004	< 0.001	0.015
	LS	4.812	0.036	12.921	0.001	5.230	0.029	0.005	0.340	0.301	< 0.001
Aromatase	Omnibus (all regions)	2.961	0.053	28.443	< 0.001	1.608	0.235				
	TnA	–	–	1.884	0.187	–	–	–	–	–	–
	PVN	–	–	31.058	< 0.001	–	–	–	–	0.003	0.002
	POM	–	–	93.619	< 0.001	–	–	–	–	< 0.001	< 0.001
	AH	–	–	59.568	< 0.001	–	–	–	–	< 0.001	< 0.001
	VMH	–	–	14.665	0.001	–	–	–	–	0.126	0.005
	BSTm	–	–	130.543	< 0.001	–	–	–	–	< 0.001	< 0.001
	Androgen receptor	Omnibus (all regions)	1.793	0.179	40.189	< 0.001	0.812	0.594			
TnA		–	–	1.080	0.313	–	–	–	–	0.060	0.512
PVN		–	–	22.626	< 0.001	–	–	–	–	0.001	0.021
POM		–	–	129.282	< 0.001	–	–	–	–	< 0.001	< 0.001
AH		–	–	57.439	< 0.001	–	–	–	–	< 0.001	0.001
VMH		–	–	156.570	< 0.001	–	–	–	–	< 0.001	< 0.001
BSTm		–	–	47.474	< 0.001	–	–	–	–	< 0.001	0.002
LS		–	–	5.654	0.029	–	–	–	–	0.018	0.459

the F and p values for the statistical tests are shown in Table 1. Interaction plots are shown in Supplemental Fig. 6. The omnibus MANOVA for estrogen receptor alpha (Fig. 3A), which included data from all brain regions, showed significant effects of morph ($F = 38.239$, $p < 0.001$) and treatment ($F = 5.013$, $p = 0.001$), but no interaction between morph and treatment ($F = 0.940$, $p = 0.499$). Thus, expression of this gene differed according to morph and was affected by testosterone treatment, but the treatment did not affect the morphs differentially. F-tests for each region revealed that testosterone treatment decreased the expression of estrogen receptor alpha mRNA in all regions except TnA, PVN, and LS. Additionally, we found that expression was higher in WS than TS birds in TnA and PVN, but that morph difference was reversed in POM. In POM, expression was higher in TS than WS birds. These morph differences were consistent with what we previously reported in free-living birds (Horton et al., 2014a).

For 5-alpha reductase mRNA (Fig. 3B), the omnibus MANOVA showed significant effects of both morph ($F = 7.064$, $p < 0.001$) and treatment ($F = 78.136$, $p < 0.001$) as well as a significant interaction between the two ($F = 2.941$, $p = 0.024$). In other words, the expression of this gene depended on morph and was affected by testosterone treatment, but the treatment had different effects in the two morphs. The F-tests showed that expression was higher in TS than WS birds in POM and VMH, but higher in WS than TS in LS (see Table 1). The F-tests showed further that testosterone treatment increased expression in all brain regions except PVN and LS; in the latter region, testosterone decreased expression. Thus, regulation of expression seemed to differ in LS from that in the other regions. The region was also unique in that

there was a morph difference in the blank-treated birds that was not present after testosterone treatment (the same pattern was observed in POM but we could not detect an interaction between morph and treatment in that region). In TnA and VMH, there was no morph difference in the blank-treated birds but the testosterone treatment resulted in higher expression in TS than WS birds. In BSTm, a morph difference in the blank-treated birds actually changed direction with treatment, from higher in WS to higher in TS. These results paint a complex picture of regulation of 5-alpha reductase expression.

The effects of testosterone treatment on the expression of aromatase mRNA are shown in Fig. 3C. The omnibus MANOVA showed a significant effect of treatment ($F = 28.443$, $p < 0.001$), but only a marginal effect of morph (WS higher; $F = 2.961$, $p = 0.053$) and no interaction ($F = 1.608$, $p = 0.235$). F-tests showed that testosterone treatment significantly increased expression of aromatase mRNA in nearly all regions we examined except TnA (Table 1).

The effects of testosterone treatment on the expression of androgen receptor mRNA are shown in Fig. 3D. The omnibus MANOVA showed a significant main effect of treatment ($F = 40.189$, $p < 0.001$), but no main effect of morph ($F = 1.793$, $p = 0.179$) or interaction between the two factors ($F = 0.812$, $p = 0.594$). F-tests showed that testosterone treatment increased expression in all regions except TnA (Table 1).

Finally, we tested for correlations among all four of the genes in each region while controlling for morph and treatment. After Bonferroni correction, mRNA expression was not correlated for any gene pair within any region (Supplemental Table 11).

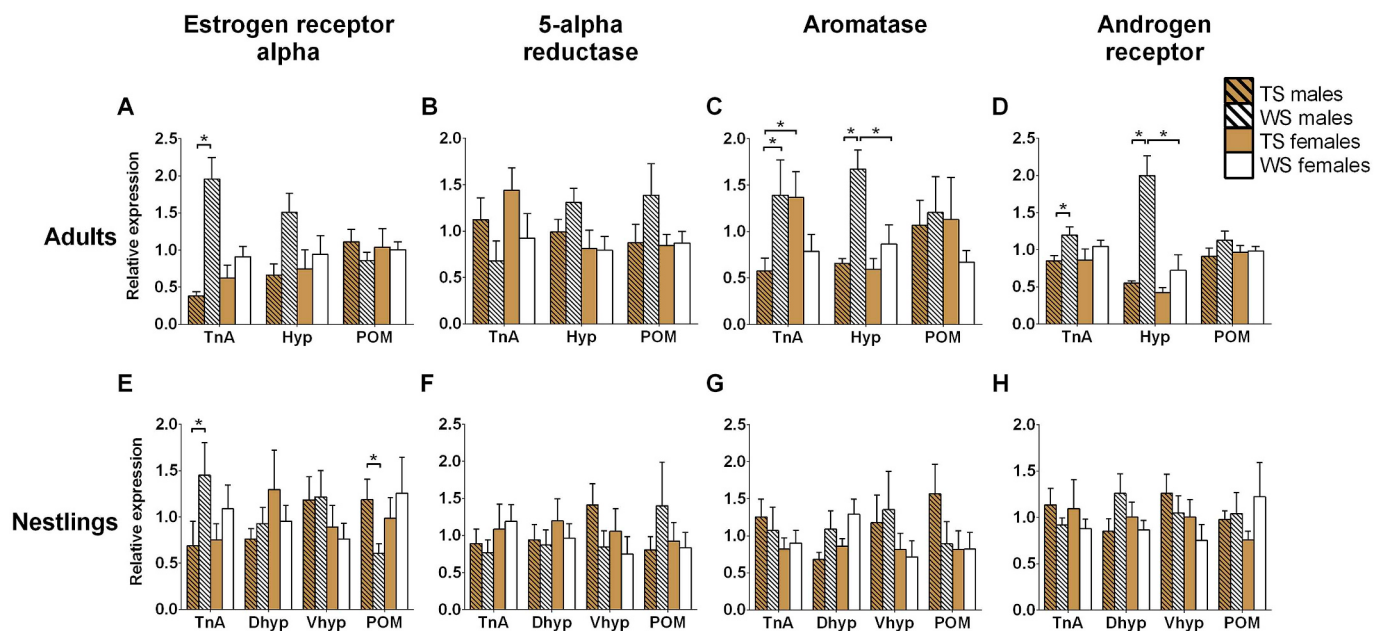


Fig. 4. Expression of estrogen receptor alpha (A, E), 5-alpha reductase (B, F), aromatase (C, G), and androgen receptor (D, H) mRNA in the brains of adult (A–D) and nestling (E–H) white-throated sparrows. mRNA levels were quantified using qPCR. Values for each region are normalized to the region-specific mean of all adults or all nestlings and means and SEMs for normalized values are shown. * $p < 0.05$ for the pairwise comparison. TnA, nucleus taeniae of the amygdala; HYP, hypothalamus (separated into dorsal hypothalamus, Dhyp, and ventral hypothalamus, or Vhyp, for nestlings); POM, medial preoptic area.

3.3. Study 3: gene expression in nestlings

In Study 3 we tested for morph differences in expression of the same four steroid-related genes in nestlings. Because we used qPCR rather than *in situ* hybridization for this study, we first performed the same quantifications in adults in order to confirm that we can detect morph differences using this method. The data for the adults are shown in the top panel of Fig. 4, and for the nestlings in the bottom panel. Interaction plots are shown in Supplemental Figs. 7 and 8. The F and p values for the statistical tests are shown in Table 2 for adults and Table 3 for nestlings.

For estrogen receptor alpha, the overall MANOVA showed a significant main effect of morph in adults ($F = 9.889$, $p < 0.001$) and nestlings ($F = 4.339$, $p = 0.007$) but no effect of sex at either age (adults: $F = 0.845$, $p = 0.483$; nestlings: $F = 0.443$, $p = 0.777$). There was no interaction between morph and sex at either age (adults: $F = 1.962$, $p = 0.147$; nestlings: $F = 0.774$, $p = 0.551$). Using post-hoc univariate F -tests, we found significant morph differences in estrogen receptor alpha expression in adult TnA and adult hypothalamus (Table 2; Fig. 4). In nestlings, we found a significant morph difference in TnA and POM (Table 3, Fig. 4).

The omnibus MANOVA for 5-alpha reductase mRNA showed a

Table 2

F and p values for MANOVAs (omnibus tests), univariate F -tests, and post-hoc pairwise comparisons for adults in Study 3. mRNA was labeled using qPCR. Significant effects are shown in bold.

Gene	Effects of morph and sex						Post-hoc pairwise comparisons (p)			
	Region	Morph		Sex		Morph x Sex	TS vs. WS		Male vs. Female	
		F	p	F	p		Male	Female	TS	WS
Estrogen receptor alpha	Omnibus (all regions)	9.889	< 0.001	0.845	0.483	1.962	0.147			
	TnA	33.939	< 0.001	–	–	–	–	< 0.001	0.075	–
	Hyp	5.435	0.027	–	–	–	–	0.067	0.163	–
	POM	0.065	0.800	–	–	–	–	–	–	–
5-alpha reductase	Omnibus (all regions)	1.222	0.323	1.480	0.245	5.046	0.007			
	TnA	–	–	–	–	4.536	0.042	0.216	0.094	0.055
	Hyp	–	–	–	–	0.834	0.368	–	–	–
	POM	–	–	–	–	2.576	0.120	–	–	–
Aromatase	Omnibus (all regions)	4.59	0.011	3.014	0.049	3.492	0.030			
	TnA	0.166	0.687	0.799	0.379	9.707	0.004	0.013	0.080	0.012
	Hyp	12.568	0.001	8.549	0.007	2.333	0.137	0.001	0.197	0.345
	POM	0.270	0.608	0.286	0.597	0.000	0.997	–	–	–
Androgen receptor	Omnibus (all regions)	10.887	< 0.001	7.091	0.001	2.391	0.093			
	TnA	7.278	0.012	0.674	0.419	–	–	0.032	0.120	–
	Hyp	18.461	< 0.001	16.892	< 0.001	–	–	0.001	0.191	0.362
	POM	1.554	0.223	0.020	0.888	–	–	–	–	–

Table 3

Results of tests for the effects of sex and morph on expression of estrogen receptor alpha in nestlings. F and p values for the MANOVA (omnibus test), univariate F-tests, and pairwise comparisons for nestlings in Study 3 are shown. mRNA was labeled using qPCR. Significant effects are shown in bold.

Effects of morph and sex						Post-hoc pairwise comparisons (p)				
Region	Morph		Sex		Morph x Sex		TS vs. WS		Male vs. Female	
	F	<i>p</i>	F	<i>p</i>	F	<i>p</i>	Male	Female	TS	WS
Omnibus (all regions)	4.339	0.007	0.443	0.777	0.774	0.551				
TnA	9.124	0.005	–	–	–	–	0.004	0.397	–	–
Dhyp	0.144	0.706	–	–	–	–	–	–	–	–
Vhyp	0.390	0.536	–	–	–	–	–	–	–	–
POM	10.551	0.002	–	–	–	–	0.041	0.328	–	–

significant morph by sex interaction in adults ($F = 5.046$, $p = 0.007$) but not in nestlings ($F = 0.161$, $p = 0.955$). Neither the nestlings nor adults exhibited significant effects of morph (adults: $F = 1.222$, $p = 0.323$; nestlings: $F = 0.444$, $p = 0.775$) or sex (adults: $F = 1.480$, $p = 0.245$; nestlings: $F = 0.086$, $p = 0.986$). Because we detected no significant effects of morph or sex in expression of 5-alpha reductase mRNA in nestlings, we did not conduct post-hoc ANOVAs (Table 3; Fig. 4). Following up using F-tests in adults, we found a significant interaction between sex and morph in TnA (Table 2; Fig. 4).

The omnibus MANOVA for aromatase mRNA in adults showed significant main effects of morph ($F = 4.59$, $p = 0.011$) and sex ($F = 3.014$, $p = 0.049$), and there was an interaction between morph and sex ($F = 3.492$, $p = 0.030$). We subsequently found significant effects of morph and sex in the hypothalamus and a significant interaction between morph and sex in TnA (Table 2; Fig. 4). In nestlings, there were no significant main effects of morph ($F = 0.308$, $p = 0.870$) or sex ($F = 1.359$, $p = 0.274$), nor a morph by sex interaction ($F = 0.591$, $p = 0.672$). No post-hoc F-tests or pairwise tests were conducted in nestlings.

Lastly, we found significant effects of morph ($F = 10.887$, $p < 0.001$) and sex ($F = 7.091$, $p = 0.001$) on the expression of androgen receptor mRNA for adults but not in nestlings (morph: $F = 0.950$, $p = 0.449$; sex: $F = 0.303$, $p = 0.874$). Additionally, we found no significant interactions between morph and sex at either age (adults: $F = 2.391$, $p = 0.093$; nestlings: $F = 0.643$, $p = 0.636$). Subsequent F-tests showed that in adults, there were significant effects of morph in the hypothalamus and TnA (Table 2; Fig. 4). We also found a significant effect of sex in the hypothalamus (Fig. 4). In nestlings, because the omnibus MANOVA showed no effects of morph, sex, or morph by sex interaction in expression of androgen receptor, no univariate F-tests were conducted.

4. Discussion

4.1. Overview

In white-throated sparrows, differentiation of genes inside a chromosomal rearrangement has led to the evolution of two plumage morphs, TS and WS, with alternative behavioral phenotypes (Falls and Kopachena, 2010; Maney, 2008; Maney et al., 2015; Tuttle et al., 2016). Because the behaviors that differ between the morphs are widely known to depend on sex steroids in related songbirds (Ketterson and Nolan, 1992), the genes inside the rearrangement that encode sex steroid-related proteins, such as receptors or enzymes that metabolize sex steroids, likely represent the evolutionary substrate of this behavioral polymorphism. In a previous study, Horton et al. (2014a) showed that the neural expression of estrogen receptor alpha, a gene inside the rearrangement, differs between the morphs and predicts territorial singing and parental provisioning. We therefore hypothesized that the well-established morph differences in these two behaviors could be explained by differential estrogen receptor alpha expression. There is,

however, another steroid-related gene inside the rearrangement: 5-alpha reductase, which metabolizes testosterone into dihydrotestosterone, a non-aromatizable androgen. In the current study, we explored the possibility that this gene is also differentially expressed and predicts behavior. In addition, we quantified the expression of two steroid-related genes outside the rearrangement: aromatase, which converts testosterone into estradiol, and the androgen receptor. This study thus provides a more complete picture of how the rearrangement affects the expression of genes in the steroid pathway and therefore the likely neuroendocrine substrate underlying the behavioral polymorphism.

4.2. Patterns of 5-alpha reductase and aromatase expression in the context of estrogen receptor alpha

In our previous study, we used *in situ* hybridization to show that the expression of estrogen receptor alpha differs between the morphs, in both sexes, in a number of brain regions important for social behavior, including TnA, PVN, POM, AH, VMH, BSTm, and LS (Horton et al., 2014a). In the current study, we used the same method in alternate brain sections from the same sample of birds to test for morph differences in the expression of 5-alpha reductase (Study 1). Morph differences in the expression of this enzyme could mean that more testosterone is metabolized to dihydrotestosterone in one morph than the other, making that hormone less available to estrogen receptors in one morph. We also tested for morph differences in the expression of aromatase, an enzyme that metabolizes testosterone to estradiol. Because this gene is not inside the rearrangement, its genetic sequence does not differ between the morphs; however, we reasoned that up- or down-regulation in the expression of estrogen receptor alpha or 5-alpha reductase in one morph could cause compensatory regulation of aromatase in the same regions (Fusani et al., 2001; Saldanha et al., 2000).

Contrary to our predictions, we found that neither 5-alpha reductase nor aromatase mirrored the differential expression of estrogen receptor alpha. For 5-alpha reductase mRNA (Fig. 2A and B), although there was a main effect of morph in the omnibus test in males, we were able to detect only a few marginal trends in individual brain regions. In contrast, all of the regions had shown robust morph differences in estrogen receptor alpha (Horton et al., 2014a). Similarly, we did not detect any effects of morph on the expression of aromatase mRNA in any region (Fig. 2C). With respect to aromatase, there are two caveats to consider. First, we included fewer birds than in the assays for estrogen receptor alpha and 5-alpha reductase, so our power to detect a significant effect of morph was not as great. Second, measuring levels of mRNA gives little information about the actual activity of these enzymes (Fusani et al., 2000) and activity can change both seasonally (Fusani et al., 2000; Soma et al., 2003; Wacker et al., 2010) and rapidly (Balthazart et al., 2006) in the avian brain.

Although we did not detect morph differences in the expression of 5-alpha reductase or aromatase in any particular brain region, it was still possible that the expression of one or both genes is directly related to

the expression of estrogen receptor alpha. We were able to test this hypothesis because we quantified the expression of 5-alpha reductase (Fig. 2A and B), aromatase (Fig. 2C), and estrogen receptor alpha (Horton et al., 2014a) in alternate sections from the same animals. After corrections for multiple tests and controlling for plasma hormone levels, we found no correlations among the three genes (Supplemental Table 3). Overall, the results of Study 1 show that the expression of estrogen receptor alpha cannot be predicted by the expression of 5-alpha reductase or aromatase. Morph differences in the expression of estrogen receptor alpha are more likely explained by cis-regulatory variation in the ESR1 gene itself (Horton et al., 2014a).

We previously showed that expression of estrogen receptor alpha in TnA and PVN was correlated with territorial singing in the males used for Study 1 (Horton et al., 2014a). Similarly, expression in POM was correlated with parental provisioning in males. In the present study, we detected no correlations between these behaviors and expression of either 5-alpha reductase or aromatase (Supplemental Table 4; note that our approach to correct for multiple comparisons was conservative). These results bolster our hypothesis that differentiation of the ESR1 gene is more important for the differentiation of behavior in this species than is differentiation of the gene encoding 5-alpha reductase, despite the fact that both genes are located inside the chromosomal rearrangement that defines the morphs.

A potential caveat of our approach is that our measurement of 5-alpha reductase and aromatase mRNA was limited to the regions in which we previously quantified estrogen receptor alpha mRNA (see Supplemental Figs. 1–5). These regions were chosen on the basis of the estrogen receptor alpha expression, not the expression of the other two genes, which had different distributions in the brain. This approach had two advantages in that it allowed us to test for compensatory changes within precisely the same regions, as well as to test for correlations in expression among the mRNAs we quantified. A disadvantage, however, is that we could have missed expression that predicts behavior.

4.3. Effects of testosterone on gene expression

In songbirds, expression of steroid-related genes can vary seasonally (Fraleay et al., 2010; Soma et al., 2003; Wacker et al., 2010). During the breeding season, plasma testosterone is higher in WS than TS birds (Horton et al., 2014b; Spinney et al., 2006). Morph differences in the expression of any one gene, therefore, could be attributable to regulation by testosterone (Foidart et al., 1999; Fusani et al., 2001; Soma et al., 1999). In our previous study of estrogen receptor alpha, we attempted to account for such regulation by controlling for plasma sex steroids in our statistical models (Horton et al., 2014a). Because we sampled testosterone at only one time point, however, we could not account for variation in plasma testosterone over time, which could affect expression. In the present study (Study 2), we directly tested whether the expression of steroid-related genes is regulated by plasma testosterone. Because this study was conducted on birds in non-breeding condition with quiescent gonads, we could also test two additional hypotheses. First, we asked whether morph differences in expression persist during the non-breeding season, when plasma levels of sex steroids are low and when morph differences in territorial aggression and nestling provisioning disappear (Archawaranon & Wiley, 1988; Maney et al., 2009; Spinney et al., 2006; Wiley et al., 1993). Morph differences in the non-breeding season would indicate that the differences in expression are not dependent on breeding-typical levels of these hormones, nor are they likely driven by differential expression of behavior. Second, we asked whether morph differences in expression persist in the face of breeding-typical but equal plasma levels of testosterone; in other words, whether testosterone up- or downregulates the expression of these genes differently in the two morphs. We found evidence supporting each of these hypotheses, as outlined below.

We previously showed morph differences in the expression of estrogen receptor alpha during the breeding season in all of the brain

regions we investigated in this study (Horton et al., 2014a). In the present study, we found evidence that some, but not all, of these differences may be caused by a morph difference in testosterone. In non-breeding, laboratory-housed males, testosterone treatment decreased expression of estrogen receptor alpha in POM, AH, VMH, and BSTm (Fig. 3A); in free-living, breeding birds, expression is higher in TS than in WS in the same regions (Horton et al., 2014a). This result is consistent with the hypothesis that for these regions, the morph differences observed in breeding, free-living birds could be explained, at least in part, by the fact that WS birds have higher levels of plasma testosterone during the breeding season. In contrast, our results did not support that hypothesis for the remaining regions, TnA and PVN. In free-living birds, the direction of the morph difference in these regions is opposite to that for the other regions; expression is higher in WS than TS birds (Horton et al., 2014a). In this study, testosterone decreased expression in PVN and had no effect in TnA (Fig. 3A). Thus, morph differences in plasma testosterone cannot easily explain morph differences in expression in these regions, particularly TnA.

Expression of 5-alpha reductase, aromatase, and androgen receptor was uniformly upregulated by testosterone in nearly all regions (Fig. 3B, C, D). This result is interesting because in the free-living birds in Study 1, WS birds had higher testosterone than TS birds (Horton et al., 2014b), yet we did not detect morph differences in the expression of these apparently testosterone-dependent genes (see Fig. 2A, B, C; androgen receptor was not measured in Study 1). Together, these findings suggest greater sensitivity to sex steroids in one morph than the other; in other words, expression of 5-alpha reductase may be more sensitive to upregulation by steroids in TS than WS birds. We see evidence of this phenomenon in the results of Study 2. In that study, all of the treated birds received identical doses of testosterone and there was no morph difference in the resulting plasma levels (see Grozhik et al., 2014). Nonetheless, testosterone altered gene expression to a greater degree in TS than WS birds, resulting in morph differences in the treated group. These results suggest that the two alleles of the gene encoding 5-alpha reductase, which resides inside the chromosomal inversion, may interact in different ways with androgen receptors. For example, androgen response elements on the promoter of this gene may contain SNPs that affect transcription efficacy (see Horton et al., 2014a). This type of mechanism may explain why we could not detect robust morph differences in the expression of 5-alpha reductase in free-living, breeding birds (Fig. 2) as well as why equalizing plasma levels of testosterone could not equalize expression of that gene (Fig. 3). Mechanisms such as this may also explain why morph differences in behavior persist, even in the face of equalized plasma sex steroids (Maney et al., 2009).

The results of Study 2 should also be considered in light of findings by Merritt et al. (2018), who studied rapid effects of estradiol on aggression in white-throated sparrows. They reported that a bolus oral dose of estradiol rapidly facilitated aggression in WS but not TS birds. Because all of the birds in that study were in non-breeding condition, this result suggested that differential sensitivity to estradiol persists into the non-breeding season. In the current Study 2, which was conducted on males in non-breeding condition, the most striking morph difference in expression was that for estrogen receptor alpha in TnA (Fig. 3A). Estrogen receptor alpha was differentially expressed in PVN as well. Both of these populations of receptors, in TnA and PVN, predict the magnitude of territorial aggression in free-living birds of this species (Horton et al., 2014a). Taken together, our data suggest that these populations of receptors are strong candidates for mediating the morph-specific effects of exogenous estradiol reported by Merritt et al. (2018) and morph differences in aggression generally in this species.

4.4. Early onset of morph differences in gene expression

Because the alternative phenotypes in this species have a genetic basis (Thornycroft, 1966, 1975; Maney, 2008; Maney et al., 2015;

Tuttle et al., 2016), it is tempting to conclude that morph differences in gene expression in the brain are causal for behavioral differences and not the other way around. It is of course possible, however, that the morph differences that we have detected in the brain are the result, not the cause, of morph differences in behavior. Engaging in social behavior such as territorial aggression strongly upregulates expression of many genes in the same brain areas we studied here (Mukai et al., 2009). In order to control for possible confounding effects of engaging in aggression, in Study 1 we collected tissue at least 24 h after the last STI (Horton et al., 2014a). We could not, however, control for any differential behavior occurring naturally just prior to tissue harvest. In Study 3, we tested for morph differences in nestlings at post-hatch day 7, two days before the natural fledge date (Falls and Kopachena, 2010). We found that the morph difference in estrogen receptor alpha in TnA was detectable even at this early age, well before the emergence of territorial aggression (Fig. 4E). Similarly, we found that the expression of estrogen receptor alpha in POM was higher in TS than WS nestlings, again mirroring the morph difference reported in adults (Horton et al., 2014a). In adults, estrogen receptor alpha expression in TnA and POM strongly predicts territorial singing and parental provisioning, respectively (Horton et al., 2014a). Because the morph differences in expression emerge before the behaviors (Fig. 4E; see also Fig. 3A for expression in birds in non-breeding condition), our findings show that these differences are not likely caused by engaging in the behaviors. Rather, estrogen receptor alpha expression in these regions is more likely to be a trait that does not depend on behavior or plasma testosterone—in other words, this trait is more likely to be controlled by cis-regulatory variation between ZAL2 and ZAL2^m.

Exposure to sex steroids during development is widely understood to interact with genotype to have organizational effects on the brain, permanently altering behavior (McCarthy and Arnold, 2011). Our data from Study 3 suggest very early differentiation of the estrogen receptor alpha population in TnA, suggesting a possible mechanism by which early action of steroids could lead to morph differences in adult behavior. The early hormonal milieu in white-throated sparrows is unknown, but there are several potential sources of sex steroids during development, including yolk (Schwabl, 1993), plasma (Hutchison et al., 1984; Wingfield et al., 1980) and brain (London et al., 2003; Tam and Schlinger, 2007). Thus, individual variation in the expression of estrogen receptors could have important, long-lasting effects on the development of the brain and behavior. We show here that in nestlings, estrogen receptor alpha expression is WS-biased in TnA, and TS-biased in POM, providing opportunity for organizational effects of estradiol in these regions.

4.5. Comparison between two methods of mRNA quantification

Our data from adults (Studies 1 and 2) and our data from nestlings (Study 3) were generated via two different methods: *in situ* hybridization and qPCR, respectively. In order to confirm that these two methods produce comparable results, we performed qPCR on a separate sample of adults in Study 3. We were able to replicate some, but not all, of the results obtained using *in situ* hybridization (Horton et al., 2014a). The morph difference in estrogen receptor alpha expression in TnA was replicated (Fig. 4A), but expression in the microdissected samples from hypothalamus did not depend on morph. These punches contained at least three distinct nuclei that were examined individually by Horton et al. (2014a) and in Study 1: PVN, AH, and VMH (see Supplemental Figs. 3 and 4). Estrogen receptor alpha is differentially expressed in all three regions, but in different directions; whereas expression is WS-biased in PVN, it is TS-biased in the other two regions (Horton et al., 2014a). Thus, it is possible that combining these regions prevents detection of a morph difference in a particular direction. We were unable to detect a morph difference in estrogen receptor alpha in adult POM, although it was detected in nestlings (Fig. 4E).

Our *in situ* results differed from our qPCR results also with respect to

aromatase. Whereas we found no morph differences in aromatase expression anywhere in the brain with *in situ* (Fig. 2C), with qPCR we found striking morph differences in TnA and hypothalamus (Fig. 4C). We believe that this discrepancy may reflect the location of the regions sampled in the respective studies. The region known as TnA is actually made up of two distinct regions (Mello et al., 2019); the ventral portion contains the population of estrogen receptor alpha. The dorsal portion, on the other hand, contains the densest expression of aromatase in the songbird brain (Soma et al., 2003). When we quantified aromatase in Study 1, we were careful to sample from the ventral region because we were interested in how aromatase expression relates to the morph difference in estrogen receptor alpha. Our microdissected punches of TnA may, however, have contained at least part of the dorsal portion. Similarly, as noted above, our microdissected samples of hypothalamus contained regions that were not sampled with *in situ* hybridization, leaving open the possibility that the morph difference in aromatase expression detected with qPCR was driven by expression in a region we did not sample with *in situ*.

5. Conclusions

In previously published work, we linked differentiation of the gene encoding estrogen receptor alpha to differences in mRNA expression, aggression, and parenting in white-throated sparrows (Horton et al., 2014a). We have hypothesized that differentiation of this gene has played a causal role in the evolution of the behavioral phenotypes in this species. In the current study, we present further evidence that the behavioral phenotypes are mediated, at least in part, by cis-regulation of estrogen receptor alpha. In Study 1, we showed that the expression of another steroid-related gene inside the inversion, 5-alpha reductase, does not differ strikingly between the morphs as does expression of estrogen receptor alpha. Further, unlike estrogen receptor alpha, 5-alpha reductase was not correlated with territorial singing or parental provisioning. In Study 2, we tested the extent to which these genes are regulated by plasma testosterone, and therefore the extent to which they may be regulated seasonally. Our results showed that in general, testosterone treatment increased the expression of 5-alpha reductase, aromatase, and androgen receptor, and decreased the expression of estrogen receptor alpha. There was, however, one clear exception: in TnA, a region in which expression of estrogen receptor alpha strongly predicts territorial singing, expression of that gene was unaffected by testosterone treatment and differed strikingly between the morphs in both testosterone-treated and control groups. This result suggests that estrogen receptor alpha expression in TnA may explain the rapid effects of estradiol on behavior in birds in non-breeding condition (Merritt et al., 2018) and may be important for the behavioral polymorphism in this species. In Study 3, we showed that this morph difference in gene expression is detectable even in nestlings, before the development of territoriality, suggesting a possible substrate for organizational effects of estradiol on behavior. Overall, our results show no evidence that differentiation between ZAL2 and ZAL2^m has had trans-regulatory effects on steroid-related genes either inside or outside the rearrangement. Instead, they support a model in which differentiation of estrogen receptor alpha may play a primary role in the evolution of alternative behavioral phenotypes in this species. Future work should interrogate the causality of the cis-acting regulatory elements, both epigenetic (Bentz et al., 2016) and genetic (Horton et al., 2014a), that might drive morph differences in estrogen receptor alpha expression during development and adulthood.

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Authors' contributions

DLM, BMH, and KEG conceived and designed the study. BMH and KEG helped to collect the samples and perform the molecular work. DMH, KEG and YH helped to collect the data. KEG and DLM drafted the manuscript. All authors approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mce.2019.110517>.

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