Integrative and Comparative Biology

Integrative and Comparative Biology, volume 55, number 2, pp. 323–331
doi:10.1093/icb/icv005

Society for Integrative and Comparative Biology

SYMPOSIUM

Estrogen Receptor Alpha as a Mediator of Life-History Trade-offs

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From the symposium "Neurohormones, Brain, and Behavior: A Comparative Approach to Understand Rapid Neuroendocrine Function" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2015 at West Palm Beach, Florida.

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Synopsis Trade-offs between competitive and parental strategies often are mediated by sex steroids. The mechanisms underlying steroid signaling and metabolism may therefore serve as targets of disruptive selection that leads to alternative behavioral phenotypes. White-throated sparrows exhibit two color morphs that differ in both competitive and parental behavior; white-striped (WS) birds engage in more territorial singing, whereas tan-striped (TS) birds provision nestlings more often. Although WS birds have higher levels of plasma testosterone (T) and estradiol than do TS birds, experimental equalization of these hormones does not abolish morph differences in singing. Neural sensitivity to sex steroids may differ between the morphs because the gene for estrogen receptor alpha (ERα) has been captured by a chromosomal rearrangement found only in the WS birds. We recently showed that expression of this gene differs between the morphs and may drive the behavioral polymorphism. First, the ERα promoter region contains fixed polymorphisms that affect transcription efficiency *in vitro*. Second, in a free-living population, local expression of ERα depends strongly on morph and predicts both territorial singing and parental provisioning. Differential ERα expression is particularly striking in the medial amygdala; WS birds have three times more ERα mRNA than do TS birds. This difference persists during the nonbreeding season and is unaffected by exogenous T treatment. Finally, preliminary data generated by RNA-seq confirm that ERα expression in MeA is both differentially expressed and correlated with territorial singing. Together, these results suggest that ERα may be a target of disruptive selection that leads to alternative behavioral strategies. Our future directions include a more detailed analysis of the ERα promoter regions to determine the molecular basis of differential expression as well as gene network analyses to identify genes connected to ERα.

The white-throated sparrow as a model of life-history trade-offs

Disruptive selection that drives incompatible traits into alternative phenotypes is most likely to act on genes with multiple functions. Such genes include those that encode the action or regulation of hormones (Ketterson and Nolan 1992; Finch and Rose 1995; Rhen and Crews 2002; Sinervo and Svensson 2002; Nijhout 2003; Hau 2007; McGlothlin and Ketterson 2007; Miles et al. 2007). For example, trade-offs between territorial aggression and parenting (Trivers 1972) are thought to be mediated by sex steroids such as testosterone (T). In many species of fish, birds, rodents, and primates, high levels of circulating androgens are associated with increased intrasexual competition manifested as aggression or mating effort, whereas low levels are associated with increased parenting effort (e.g., Ketterson and Nolan 1994; McGlothlin et al. 2007). In humans, paternal care and fatherhood often have been associated with low plasma levels of T (Storey et al. 2000; Wynne-Edwards 2001; Fleming et al. 2002; Gray et al. 2002; Gray 2003), and high T levels associated with male–male aggression and competition (Booth et al. 1989; Bernhardt et al. 1998; Book et al. 2001).

In this review, we describe our ongoing research with a model organism particularly well-suited for understanding the mechanisms underlying the evolution of life-history trade-offs: the white-throated sparrow (*Zonotrichia albicollis*). This seasonally breeding songbird is ordinary in most respects;

Advanced Access publication April 7, 2015

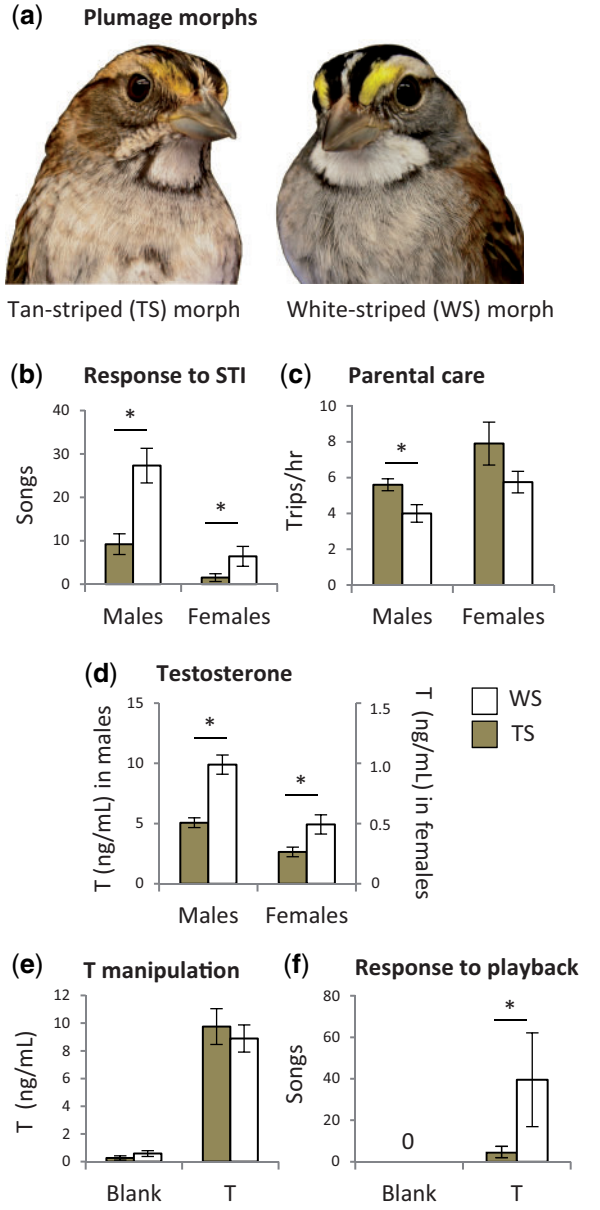

Fig. 1 Morph differences in plumage, behavior, and hormones in white-throated sparrows. (**a**) Tan-striped (TS) and white-striped (WS) birds occur at equal rates. (**b**) WS birds of both sexes respond to STI with more singing than do TS birds. (**c**) TS males provision young in the nest at greater rates than do WS males (data from first brood). (**d**) Early in the breeding season, WS birds of both sexes have higher plasma testosterone (T) than do TS birds. (**e**) When T was manipulated in captive, non-breeding males to equalize T between the morphs, WS males nonetheless sang more in response to song playback than did TS males (**f**). (b), (c), and (d) redrawn from Horton et al. (2014a) and (e) and (f) from Maney et al. (2009) with permission.

it is abundant throughout its range in eastern North America, defends territories during the breeding season, and is socially monogamous. Over the past several decades, however, research on this species has unearthed distinctive features. Within any population, about half of the birds have white and black stripes on the crown and a clear white throat. The rest have brown and tan stripes and a streaked throat. Before about 1970, field guides often labeled the two color types as male and female or adult and immature (e.g., Peterson 1961). Both of those categorizations, however, were incorrect. The variation in plumage corresponds to morphs, or alternative phenotypes. Once they molt into adult plumage (Fig. 1a), individuals are either "tan-striped" (TS) or "white-striped" (WS). Morph is fixed for life; individuals of this species do not switch between phenotypes. Breeding pairs typically consist of one TS and one WS bird (reviewed by Falls and Kopachena 2010). Together with the plumage morphs, this disassortative mating system makes this species unique among songbirds.

The plumage morphs are interesting to behavioral biologists because they correspond to behavioral phenotypes that differ with respect to song rate and parental care. In both sexes, the WS birds sing at higher rates than do the TS birds (Fig. 1b). Song is used in this species primarily to defend territory, and most findings of morph differences in singing were obtained in the context of simulated territorial intrusion (STI; Lowther 1962; Jones 1987; Kopachena and Falls 1993a; Horton et al. 2014a). WS females sing at high rates compared with females of related species, whereas TS females rarely sing. In WS male × TS female pairs, therefore, most of the singing is done by the male whereas in pairs of the other morph type, the male and female share the singing about equally.

During the parental phase of the breeding season, males and females both bring food to the nestlings. The rate at which they do so, however, depends on morph—particularly for males. TS males provision their young more often than do WS birds (Horton et al. 2014a; Fig. 1c). Other authors have reported the same effect in females as well (Knapton and Falls 1983; Kopachena and Falls 1993b; cf. Horton and Holberton 2010). Interestingly, we have observed a morph difference in parenting only during first broods of the season. TS and WS males invest equally in the provisioning of replacement broods (Horton et al. 2014a). This finding is consistent with shifts in parental investment as the season progresses in other species (Biermann and Robertson 1981; Robinson et al. 2010). We hypothesize that late in the season, because fewer females are fertile, WS males gain little by investing in extra-pair copulations over nestling provisioning.

Endocrine correlates of behavioral polymorphism

The two morphs lie at either end of a continuum, with investment in the defense of resources and in mating success at one end and investment in current offspring at the other (Trivers 1972). In other words, they exemplify the classic trade-off between investing in territoriality and mating effort versus parental care, which in many birds is likely mediated by T (Ketterson and Nolan 1992; Hau 2007). In white-throated sparrows and related species, exogenous T treatment increases territorial singing and decreases parental behavior (Silverin 1980; Wingfield 1984; Hegner and Wingfield 1987; Schoech et al. 1998). These are precisely the behaviors that differ according to morph, so T is an excellent candidate for mediating morph differences in behavior—along with other hormones and neuropeptides that are modulated by T.

Plasma levels of T do not differ between the morphs during the non-breeding season (Schlinger 1987), when behavior is not polymorphic (Harrington 1973; Watt et al. 1984; Schlinger 1987; Schwabl et al. 1988; Piper and Wiley 1989; Dearborn and Wiley 1993; Wiley et al. 1999). During the breeding season, however, when morph-dependent behaviors emerge, T is higher in WS than in TS birds of both sexes (Spinney et al. 2006; Swett and Breuner 2009; Horton et al. 2014a). This difference is most pronounced early in the breeding season, when the birds are competing for territories (Fig. 1d). Plasma levels of the hormone estradiol (E2), which is synthesized from testosterone, are also higher in WS birds than in TS birds (Horton et al. 2014a). Because both singing and parental provisioning depend on plasma T (Silverin 1980; Wingfield 1984; Hegner and Wingfield 1987; Schoech et al. 1998; Lynn et al. 2009), it is tempting to speculate that morph differences in plasma levels of these hormones completely explain morph differences in both singing and parenting. Correlation, however, does not imply causation. In males, plasma T increases in response to STI (Wingfield and Hahn 1994) or to the presence of receptive females (Moore 1982; Dufty and Wingfield 1986; Wingfield and Monk 1994). A one-way causal effect of T on social behaviors, therefore, may not completely explain either polymorphic behavior or the inverse relationship between singing and parenting effort in this species.

To test whether morph differences in plasma steroids can explain the differences in behavior, we experimentally equalized plasma T in males (Maney et al. 2009). We treated non-breeding birds, in which plasma levels of sex steroids were low and the gonads were regressed, with silastic implants that elevated plasma T to levels typical of a WS male early in the breeding season. After treatment, plasma T did not differ between the morphs (Fig. 1e). We hypothesized that if morph differences in behavior can be explained by differences in T, we should not see any morph differences in behavior in this sample. We then performed song playbacks in the laboratory and recorded the birds' vocal responses. Untreated, non-breeding birds did not sing in response to the playback (Fig. 1f). T-treated birds, however, did sing, and the WS birds sang at higher rates than did the TS birds (Fig. 1f). E2-treatment of non-breeding females produced the same result: WS females sang more than did their TS counterparts. This study showed clear evidence that morph differences in singing behavior cannot be completely explained by the differences in plasma levels of sex steroids in either sex. The results suggest an alternative hypothesis: that the brains of TS and WS birds differ in their sensitivity to sex steroids.

Estrogen receptor alpha polymorphism

In order to ask how the brains of the two morphs differ, we turn to their genetics. The plumage polymorphism has a genetic basis first described decades ago by Thorneycroft (1966, 1975), who compared the karyotypes of TS and WS birds. He noted that whereas all TS birds had two copies of a submetacentric chromosome 2, all WS birds had at least one copy of a metacentric homolog. He hypothesized that the metacentric arrangement, later denoted by Thomas et al. (2008) as $ZAL2^m$, came about via a pericentric inversion. Using mapping and population genetics techniques, Thomas et al. (2008) showed that in fact $ZAL2^m$ contains at least two pericentric inversions (Fig. 2a), and that recombination is profoundly suppressed within the rearranged region. The resulting limited gene flow has caused the $ZAL2^m$ to diverge from the ZAL2, and the two haplotypes are now 1% different from each other (Huynh et al. 2011). Thus, odds are relatively high that alleles on each haplotype are expressed at different levels or even encode different proteins.

The differentiating region of $ZAL2^m$ provides an excellent target for investigating the genetic basis of the behavioral polymorphism in this species. Which genes inside the rearrangement are already known to affect aggression and parenting in songbirds? Steroid-sensitive behaviors often are correlated with the expression of steroid receptors (Cushing et al. 2004; Trainor et al. 2006; Ball and Balthazart 2008;

Ketterson et al. 2009; Rosvall et al. 2012). In dark-eyed juncos (*Junco hyemalis*), a close relative of white-throated sparrows, aggressive responses to STI were positively correlated with the expression of estrogen receptor alpha (ERα in the ventral telencephalon [Rosvall et al. 2012]). Thus, variation in the expression of an important steroid receptor predicted variation in a steroid-dependent behavior—an intuitive result, given that the receptor confers sensitivity to E2, a major metabolite of T. In white-throated sparrows, the gene for ERα (ESR1) has been captured by the rearrangement on ZAL2^{m} (Fig. 2a; Thomas et al. 2008) making it a prime candidate for driving variation in territorial singing and parenting behavior.

To determine whether the functioning of the receptor itself may have been affected by the rearrangement, we sequenced the coding region of the ESR1 gene on both ZAL2 and ZAL2^{m}. We found two differences that affect the coding sequence of the receptor protein. The first was in the activation function (AF-1) domain, where several known coactivators bind, and the other in the ligand binding domain. These changes in the protein sequence of ERα are unlikely to significantly impact its function, however, because the substitutions found in the ZAL2^{m} isoform are the same residues found in functional estrogen receptors in other species (Horton et al. 2014b). We therefore turned our attention to the upstream regions likely to contain promoters.

In contrast to the coding sequence, the promoter sequences upstream of the start site differ substantially between the haplotypes. Several of these changes occurred at putative binding sites for transcription factors. The ZAL2^{m} allele, for example, has gained a binding site for Pbx-1, a transcription factor that affects the expression of estrogen receptors in humans (Cheung et al. 2009). This and many other changes in the *ESR1* promoter sequence may drive differences in ERα expression. We tested this hypothesis *in vitro* by cloning the putative promoter regions into constructs containing a luciferase reporter gene. In HELA cells, the level of transcription was higher from the ZAL2^{m} promoter than from the ZAL2 promoter (Horton et al. 2014b; Fig. 2b). Because mRNA transcription depends on many factors that may not have been available in our *in vitro* preparation, we could not conclude from this study that the ZAL2^{m} promoter is more effective. We could, however, conclude that the promoter sequence we analyzed contains enough variation to affect transcription, which set the stage for *in vivo* comparisons.

Expression of ERα in free-living birds

To test for differential regulation *in vivo*, we quantified ERα mRNA expression in the brains of

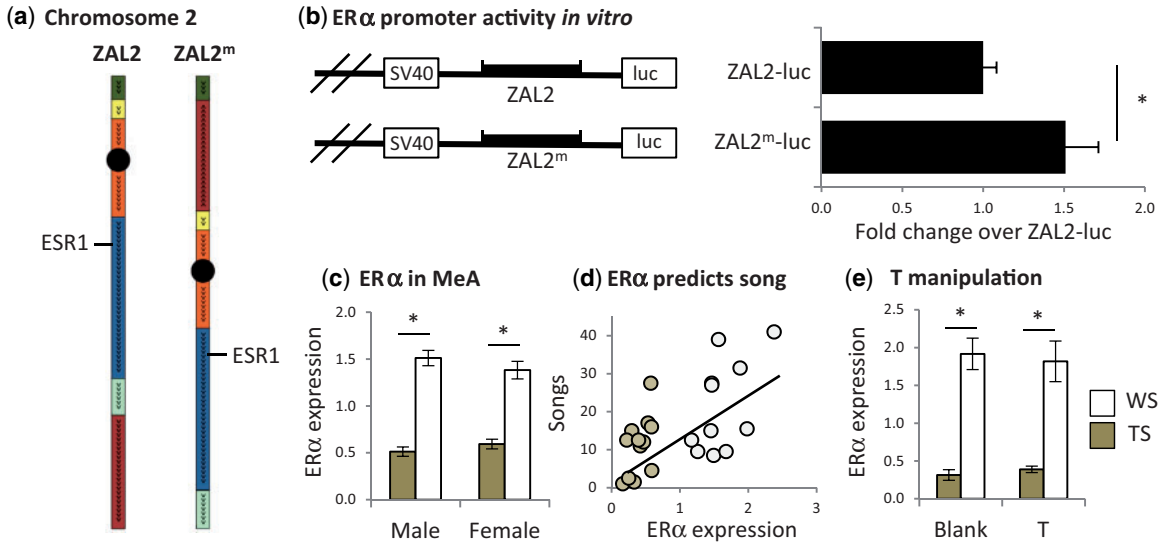

Fig. 2 The expression of estrogen receptor alpha (ERα) mRNA has been affected by a chromosomal rearrangement. (**a**) The ESR1 gene has been captured by a rearrangement that consists of at least two pericentric inversions (see online version for color; Thomas et al. 2008). (**b**) In HeLa cells, a ZAL2^{m} promoter construct inserted upstream of luciferase (luc) resulted in significantly more expression compared with the ZAL2 construct. (**c**) ERα mRNA expression in the medial amygdala (MeA) is significantly higher in WS than in TS birds of both sexes. (**d**) ERα expression in MeA significantly predicts STI-induced singing in free-living males. (**e**) When testosterone (T) was equalized between the morphs in captive males, the morph difference in MeA ERα expression persisted. (b), (c), and (d) redrawn from Horton et al. (2014b) with permission.

behaviorally characterized, breeding white-throated sparrows near Argyle, ME. We performed *in situ* hybridization using a radiolabeled riboprobe that did not span any SNPs—in other words, a riboprobe exactly complementary to both the ZAL2 and ZAL2^{m} mRNAs. Using this technique, we found significant differences in ERα mRNA expression in eight regions of the brain known to be involved in social behavior. We will focus here on the most striking difference: ERα mRNA levels were nearly three times higher in WS than in TS birds in the medial amygdala (MeA; Fig. 2c). In both sexes, levels of MeA mRNA were nonoverlapping between the morphs; in other words, males and females could be accurately assigned to a morph simply by looking at ERα expression in MeA (Horton et al. 2014b).

The MeA is part of an interconnected social behavior network (Newman 1999; Goodson 2005) consisting of steroid-sensitive regions that contribute to a variety of social behaviors, including aggression and parenting. In animals that rely heavily on pheromonal communication, it receives massive projections from the accessory olfactory system (Scalia and Winans 1975). In birds, perhaps because they rely on vocal communication, MeA receives input from the auditory thalamus. This input reaches higher auditory association areas via the primary auditory cortex (Field L) and via MeA. MeA also receives input from the song control system, and thus connects vocal areas, the auditory system, and the social behavior network (Cheng et al. 1999). Lesions of MeA disrupt behavioral responses to social signals in ring doves and Japanese quail (Cheng et al. 1999; Thompson et al. 1998), and inhibit male-directed song in zebra finches (Ikebuchi et al. 2009). We therefore asked whether the profound morph difference in ERα expression in MeA was related to singing behavior. In males, we found a strong correlation between the expression and the vocal response to STI (Fig. 2d). In females, we found a nonsignificant trend in the same direction; our inability to demonstrate this relationship was likely caused by our lower sample size for females. Follow-up analysis in males demonstrated that ERα expression in MeA predicted singing even better than did morph. In other words, when morph was controlled, the correlation between ERα expression and singing persisted; in contrast, when the variation in ERα was controlled, the effect of morph on singing disappeared. Importantly, ERα expression predicted singing even when plasma levels of T and E2 were controlled in the analysis, further supporting our hypothesis that morph differences in singing behavior in this species cannot be explained simply by plasma levels of either sex steroid.

To confirm that morph differences in ERα mRNA do not depend on plasma T, we performed a second T-manipulation study. Non-breeding males of both morphs received subcutaneous silastic capsules containing either nothing or enough T to mimic plasma levels typical of WS males early in the breeding season. We quantified ERα mRNA expression in MeA after 2 weeks, during which time T reliably stimulates singing in captive males (Maney et al. 2009; Grozhik et al. 2014). Our results showed clearly that T-treatment had no effect on ERα expression in that region in either morph (Fig. 2e). This result suggests that morph differences in ERα expression in MeA in our free-living population (Fig. 2c) cannot be attributed solely to the differences in plasma T (Fig. 1d). Rather, changes in the promoter sequence may cause differential rates of ERα transcription on the ZAL2 and ZAL2^{m} alleles (Fig. 2b). In other words, the rearrangement itself, or more precisely the resulting differentiation of ESR1, may cause differential expression of ERα mRNA by altering transcription efficiency.

Figure 2d shows a second interesting finding: a large effect of morph not only in the T-treated group but also in the control group. This result is curious because to date, no morph differences in behavior have been reported in non-breeding populations. In winter flocks and in laboratory-housed birds held on short days, morph does not predict dominance rank or aggression (Harrington 1973; Watt et al. 1984; Schlinger 1987; Schwabl et al. 1988; Piper and Wiley 1989; Dearborn and Wiley 1993; Wiley et al. 1999). In contrast, when birds are held on long days and undergo gonadal recrudescence, WS birds engage in significantly more aggression than do their TS cage-mates and outrank them on average (Watt et al. 1984; Maney and Goodson 2011). Morph differences in dominance and aggression therefore seem to depend on season (Maney and Goodson 2011). The large morph difference in ERα in MeA in non-breeding birds (Fig. 2c) suggests that although the morphs may not differ in aggressive behavior during the fall, a neurological substrate certainly exists to support such differences. Perhaps differences are not manifested in the fall because plasma T is negligible and aromatase activity is suppressed (Soma et al. 2003; Meitzen et al. 2007), meaning that local levels of E2 in MeA may be quite low. We are currently testing whether exogenous administration of E2 can rapidly affect behavior in non-breeding birds (see Heimovics et al. 2014) and whether those effects

could depend on morph, as ERα expression in MeA would suggest.

Life-history trade-offs and the medial amygdala

Could life-history trade-offs be mediated entirely in the MeA? Cushing et al. (2004, 2008) provided some evidence for this hypothesis. They worked with two populations of prairie voles (*Microtus ochrogaster*), one from a monogamous, biparental population in Illinois and other from a population in Kansas with less male parental care and more promiscuity. Compared with the Illinois males, the Kansas males had higher levels of ERα mRNA expression in MeA (Cushing et al. 2004). Importantly, experimental overexpression of ERα in MeA profoundly inhibited parental behavior and increased interest in novel females (Cushing et al. 2008). Together with these and other studies in rodents (e.g., Murakami et al. 2011), our work on white-throated sparrows suggests that ERα in MeA may underlie the evolution of a suite of complex correlated traits that constitute a ''personality'' (Wolf 2007) or ''behavioral syndrome'' (Bell 2007) that maximizes territoriality and mate-seeking while minimizing prosocial behaviors such as monogamy and parenting. In white-throated sparrows, ERα in MeA was negatively correlated with parental provisioning, but that effect disappeared when plasma T and E2 were controlled (Horton et al. 2014b). Manipulation of plasma T did not affect ERα expression in MeA (Fig. 2d), but T could regulate expression in another region to affect behavior. For example, ERα expression in the medial preoptic area predicts parental provisioning (Horton et al. 2014b). If ERα expression in this region depends on plasma T, as it does in other species (e.g., Lisciotto and Morrell 1993), morph differences in that expression may be explained by differing levels of plasma steroids. We are currently testing this hypothesis.

Disruptive selection and chromosomal structure

The sequestration of life-history strategies into alternative phenotypes in white-throated sparrows resembles the evolution of behaviors that are sexually differentiated. The behaviors associated with the ZAL2^m chromosome, namely higher territorial aggression and lower parental provisioning, are the same behaviors that differ by sex in this and many other species. It is interesting, therefore, to compare the ZAL2^m chromosome to sex chromosomes. First, because of the disassortative mating system, each breeding pair consists of one bird with and one without ZAL2^m. Second, because WS–WS pairs are rare, the ZAL2^m is in a near-constant state of heterozygosity—not unlike the mammalian Y chromosome. This situation has suppressed recombination and created one of the largest blocks of linkage disequilibrium ever described in a vertebrate (Thomas et al. 2008). A major difference between ZAL2^m and the mammalian Y, however, is that ZAL2^m seems to be a healthy chromosome—although it is differentiating from its counterpart, it is not degenerating. We have not detected disrupted genes, repetitive elements, or other signatures of chromosome degeneration (Davis et al. 2011). That the chromosome appears healthy suggests that ZAL2^m homozygotes occur in numbers sufficient to support recombination and gene flow.

We do know that the ZAL2^m/2^m genotype is not lethal because homozygotes, although rare, appear healthy. Out of approximately 700 birds genotyped in our laboratory since 2005, we have collected one ''superwhite'' bird with two copies of ZAL2^m (Horton et al. 2013). Because she was a hatch-year female, she should have had rather dull plumage for a WS bird (Piper and Wiley 1989). She was as bright as an adult male, however (she is pictured in Fig. 1a, on the right). She was extremely vocal and aggressive, dominating opponents in behavioral tests. Her phenotype was thus an exaggerated version of a typical ZAL2/2^m heterozygote, supporting the hypothesis that alleles inside the ZAL2^m rearrangement confer high aggression. Two other superwhite birds have been described in the literature (Thorneycroft 1975; Falls and Kopachena 2010), but their behavior was not characterized systematically. Considering all genotyping done in our laboratory and by others (e.g., Romanov et al. 2009), three superwhite birds have been found among 1700 birds genotyped—a frequency of less than 1/500. Homozygotes are thus indeed rare, but apparently common enough to prevent degeneration of the rearranged chromosome (Davis et al. 2011). Mechanisms that prevent greater rates of homozygosity are not well-understood, but are likely to involve strong biases in mate choice and high aggression between males and females of the WS morph.

Future directions

Given that the ZAL2^m rearrangement contains approximately 1000 genes (Thomas et al. 2008) and because a primary adaptive advantage of inversions is to bind together two or more co-adapted alleles (Dobzhansky 1970), we are under no illusion that

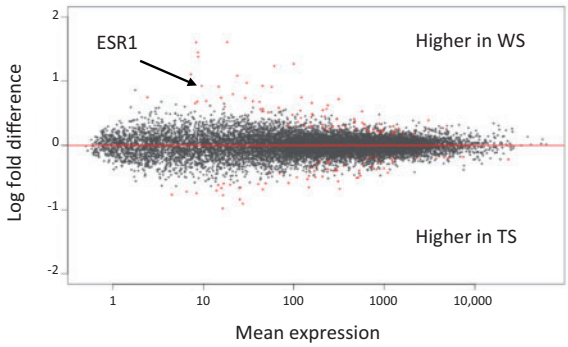

Fig. 3 Analysis of RNA-seq data for all transcripts in the medial amygdala of 10 WS and 9 TS free-living males. Genes shown in red (see online version), which include ESR1, are differentially expressed.

behavioral polymorphism in the white-throated sparrow is caused by a single gene. In addition to ESR1, genes for gonadotropin receptors and a steroidogenic enzyme have been captured by the rearrangement and we are currently evaluating those. Moreover, we are now taking a discovery-based approach to identify all of the genes that are differentially expressed in the brain and that predict behavior. We recently completed an RNA-seq study of all transcripts in MeA in free-living, behaviorally characterized males (W. M. Zinzow-Kramer, unpublished data). So far, using this independent sample we have confirmed that ERα mRNA is expressed in MeA at higher levels in WS than in TS males (Fig. 3) and that its expression is correlated with the vocal response to STI. We are working now to identify networks of genes, connected to ERα and otherwise, that may function together to affect behavior.

Differentiation of the ZAL2^m rearrangement has undoubtedly led not only to altered promoter efficiency but also to nonsynonymous changes in protein-coding regions. The complete genome sequences of a TS (ZAL2/2) bird and our superwhite (ZAL2^m/2^m) are now available, so we are working with our collaborators toward a complete SNP catalog. We will soon be able to identify every potential alteration in protein function, and will work toward identifying coadapted alleles. We believe that this species will prove to be an important model for understanding not only how chromosomal rearrangements affect the genes they capture, but also how and why they may confer selective advantages and persist in populations (Dobzhansky 1970).

Summary

Alternative life-history strategies in white-throated sparrows are determined, in part, by a rearrangement on the second chromosome. Because of the disassortative mating system in this species, the rearrangement is in a near-constant state of heterozygosity that limits gene flow. As a result, the rearranged chromosome 2 is differentiating from its counterpart, and genes inside the rearrangement are accumulating SNPs that affect gene transcription. The gene encoding ERα may contribute to the behavioral phenotype, as it is expressed differently in the two morphs and predicts both territorial and parental behaviors. We hypothesize that differentiation of this gene has played a causal role in the evolution of life-history strategies in this species.

Acknowledgments

The authors wish to thank all of the people who contributed to the work discussed here, including Jamie Davis, Will Hudson, Ignacio Moore, Cliff McKee, Justin Michaud, Eric Ortlund, Sandra Shirk, Greg Tharpe, Jim Thomas, and Elaina Tuttle.

Funding

This work was supported by the National Institutes of Health [1R01MH082833 to D.L.M.] and the National Science Foundation [SMA-1306132 to W.M.Z.-K. and IOS-0723805 to D.L.M.]. SICB provided financial assistance for attendance at the symposium.

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