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## Genes located in a chromosomal inversion are correlated with territorial song in white-throated sparrows

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### Abstract

The genome of the white-throated sparrow (*Zonotrichia albicollis*) contains an inversion polymorphism on chromosome 2 that is linked to predictable variation in a suite of phenotypic traits including plumage color, aggression, and parental behavior. Differences in gene expression between the two color morphs, which represent the two common inversion genotypes (ZAL2/ZAL2 and ZAL2/ZAL2<sup>m</sup>), are therefore of potential interest toward understanding the molecular underpinnings of these phenotypes. To identify genes that are differentially expressed between the two morphs and correlated with behavior, we quantified both behavior and brain gene expression in a population of free-living white-throated sparrows. We quantified behavioral responses to simulated territorial intrusions (STIs) early during the breeding season. In the same birds, we then performed a transcriptome-wide analysis of gene expression in two regions, the medial amygdala and hypothalamus. Both regions are part of a ‘social behavior network’, which is rich in steroid hormone receptors and previously linked with territorial behavior. Using network analyses, we identified modules of genes that were correlated with both morph and STI-induced singing behavior. The majority of these genes were located within the inversion, demonstrating the profound effect the inversion has on the expression of genes captured by the rearrangement. Gene pathway analyses revealed that in the medial amygdala, the most prominent pathways were those related to steroid hormone receptor activity. Within these pathways, the only gene encoding such a receptor was ESR1 (estrogen receptor alpha). Our results thus suggest that ESR1 and related genes are important for behavioral differences between the morphs.

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## Keywords

behavioral polymorphism; RNA-seq; social behavior; weighted gene co-expression network analysis (WGCNA); estrogen receptor alpha

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## Introduction

The white-throated sparrow (*Zonotrichia albicollis*), a common North American songbird, provides a unique model to study the relationship between genes and behavior because it exhibits a chromosomal polymorphism linked to a behavioral polymorphism. In this species, the second chromosome occurs in two arrangements, ZAL2 and ZAL2<sup>m</sup> (Thornycroft, 1975). These arrangements differ at a minimum by a pair of pericentric inversions that span ~98 Mb, representing about 10% of the genome (Thomas et al., 2008). Inversions can lead to changes in genetic sequences that can have profound effects on phenotype. Although inversion polymorphisms have been described in multiple species, from *Drosophila* to humans (Kidd et al., 2008; Ma & Amos 2012; White, 1977), the consequences of such rearrangements are not completely understood. The white-throated sparrow provides a vertebrate model of an inversion polymorphism that clearly affects the brain and behavior.

Within a population, approximately half of the individuals of both sexes are heterozygous for the chromosomal arrangement (ZAL2/ZAL2<sup>m</sup>) (Thornycroft, 1975). These individuals have contrasting white and black stripes on the crown and are members of the “white-striped” (WS) morph (Lowther, 1961). The other half of the population is homozygous for the ZAL2 arrangement (ZAL2/ZAL2), a genotype that results in a “tan-striped” (TS) phenotype characterized by tan and brown stripes on the crown (Lowther 1961). Birds homozygous for ZAL2<sup>m</sup> are extremely rare, occurring at a frequency of roughly 1 in 500 (Horton et al., 2013). Their scarcity is due at least in part to a disassortative mating system; within a population, nearly all breeding pairs consist of one TS and one WS bird (Lowther 1961). WS male-WS female pairings, which are necessary to create a ZAL2<sup>m</sup>/ZAL2<sup>m</sup> homozygote, make up less than 2.5% of breeding pairs (Falls & Kopachena, 2010). This mating system maintains the ZAL2<sup>m</sup> arrangement in the population and keeps the frequency of ZAL2<sup>m</sup>/ZAL2<sup>m</sup> homozygotes very low. Because of its near-constant state of heterozygosity, recombination within the ZAL2<sup>m</sup> inversions is profoundly suppressed (Davis et al., 2011; Huynh et al., 2011). Limited gene flow between the ZAL2 and ZAL2<sup>m</sup> haplotypes has caused the accumulation of single nucleotide and insertion/deletion polymorphisms and thus genetic differentiation of the rearranged region. Davis et al. (2011) estimated that there are on the order of 1870 nonsynonymous differences between the ZAL2 and ZAL2<sup>m</sup> alleles inside the inversions, affecting approximately 1,100 genes. This genetic differentiation between the two chromosomes must be responsible for the phenotypic differences observed between the two plumage morphs.

The chromosomal rearrangement is of particular interest because it affects social behaviors (Maney, 2008; Tuttle, 2003). Individuals with the ZAL2<sup>m</sup> chromosome engage in more aggression and less parental care than individuals without it (Falls & Kopachena, 2010). For example, WS birds of both sexes sing more and are thus more vocally aggressive than their

TS counterparts (Horton et al., 2014b; Kopachena & Falls, 1993a; Lowther, 1962). TS birds, on the other hand, provision nestlings more frequently than WS birds; this effect is especially pronounced in males (Horton & Holberton, 2010; Horton et al., 2014b; Knapton & Falls, 1983; Kopachena & Falls, 1993b;). WS males seek out more extra-pair copulations than TS males, who instead guard their mates more closely (Formica & Tuttle, 2009; Tuttle, 2003). The two phenotypes represent alternative reproductive strategies (Trivers, 1972), in which individuals maximize investment in competition and mate finding or investment in mate-guarding and parenting (Tuttle, 2003). Because the strategy depends on the presence or absence of the ZAL2<sup>m</sup> chromosomal arrangement, this polymorphism represents a powerful genetic target with which to understand the molecular mechanisms underlying the associated behaviors.

In this study, we employed a genome-wide approach to identify genes associated with behavior and the chromosomal rearrangement. First, we measured natural variation in aggressive responses to simulated territorial intrusions (STIs) in a free-living population of male white-throated sparrows early in the breeding season. We then generated and analyzed transcriptomes from two brain regions thought to be important for social behavior, namely the medial amygdala (MeA, formerly called nucleus taeniae in birds; for a review of its homology see O'Connell & Hoffman, 2011) and the hypothalamus (HYP). We chose these regions because they are part of a well-known social behavior network that responds during social encounters (Goodson et al., 2005; Maney et al., 2008). In the MeA and HYP in particular, the expression of genes that confer sensitivity to sex steroids, such as estrogen receptor alpha (ER $\alpha$ ), was already known to be correlated with territorial responses (Rosvall et al., 2012) and to differ between the morphs (Horton et al., 2014a). Our goal in the current study was to expand our understanding of the genetic basis of this behavioral polymorphism beyond a handful of genes. Because the neurochemical and neuroanatomical pathways that regulate social behaviors such as aggression rely on the expression of many genes (reviewed in Anholt & Mackay, 2012; Nelson & Chiavegatto, 2001), we employed a network-based approach to identify groups of co-regulated genes (modules) with expression that is correlated with plumage morph, song rate, or physical aggression. Toward this goal, we used weighted gene co-expression network analysis (WGCNA) (Zhang & Horvath, 2005), which has previously been used to relate gene expression to physiological and behavioral traits (Drnevich et al., 2012; Filteau et al., 2013; Fuller et al., 2007; Ghazalpour et al., 2006; Hilliard et al., 2012; Langfelder & Horvath, 2008). We then examined the gene modules to identify candidate genes and molecular pathways linked to aggressive behavior. By taking this whole transcriptome approach, we aimed to better understand the molecular mechanisms underlying territorial aggression and, more generally, the effects of inversion polymorphisms on gene expression.

## Materials and Methods

### Ethical note

All studies were done with the approval of Emory University Institutional Animal Care and Use Committee, and with appropriate state (Maine Inland Fisheries and Wildlife and federal

(U.S. Geological Survey and U.S. Fish and Wildlife Service) permits, and with permission from the Forest Society of Maine.

### Behavioral Assay and Data Collection

Our study population consisted of free-living white-throated sparrows in the Hemlock Stream Forest, Argyle, Maine. So that we could correlate behavior with gene expression, we performed behavioral tests prior to tissue collection. We quantified territorial aggression during the peak of territorial behavior (7 May–3 June), after pair formation and territory establishment but prior to or early in the stage of incubation (Falls & Kopachena, 2010). Prior to behavioral observations, birds were captured in mist nets using conspecific song playback and banded with color bands and aluminum USGS identification bands. At the time of capture, morph was determined based on plumage color (Watt, 1986), and sex was determined by the presence or absence of a cloacal protuberance or brood patch. Morph was later confirmed by PCR genotyping of the DSE locus (Thomas et al., 2008; Horton et al., 2013), and sex confirmed by gonads during tissue collection (see below).

Simulated territorial intrusions (STIs) were performed as described by Horton et al. (2014b). Briefly, at least three days after they were banded, each resident male was exposed to a caged, live male decoy placed onto his territory while conspecific song was played via an adjacent speaker. We used four different male decoys, two of each morph, and four song exemplars chosen from those used in the previous study. In this species, behavioral responses to STI can depend on the morph of the intruder (Horton et al., 2012; Kopachena & Falls, 1993). To control for this potential variation, we performed two STIs on each male, on consecutive days but at the same time of day (0630–1230 Eastern Daylight Time, EDT), presenting either a TS or WS decoy. The morph of the intruder was counterbalanced such that half the pairs were presented with a TS decoy first, and the other half were presented with a WS decoy first. For all analyses, data on behavioral responses were averaged across the two STIs for each individual. Behavioral data were collected from 9 TS males and 10 WS males.

Each STI was monitored by two observers and behaviors were recorded as previously described (Horton et al., 2014b). After the male arrived in (or at) the arena (i.e. was detected within 30 m of the decoy), playback continued for ten minutes. We recorded behaviors indicative of territorial aggression in *Zonotrichia* species. The physical behaviors we measured were latency to approach (the time from start of playback until the resident male arrived in (or at) the arena), number of directed flights over the decoy, time spent within 5 m and within 2 m of the decoy, and distance of closest approach to the decoy (Kopachena & Falls, 1993a; Wingfield & Hahn 1994). We recorded three types of vocalizations that commonly occur during agonistic encounters and that signal aggression (chip calls, trills, and songs) (Collins & Houtman, 1999; Falls & Kopachena, 2010).

### Statistical analysis

Because the behavioral data were not normally distributed (based on Shapiro-Wilk tests), we tested for morph differences in behavior using the Wilcoxon Rank Sum test. To reduce the number of comparisons, we used principal component analysis (PCA) to construct a

composite physical aggression score (PC1) from three physical behaviors (*sensu* Horton et al., 2012). The behaviors used in the PCA were time within 2 m, closest approach, and flights. We chose to exclude time in 5 m because variation in this measure within the sample population was low. Physical latency scores were not recorded for trials in which the resident male was within the area at the start of playback. Because PCA does not tolerate missing data, we did not include latency in the PCA. The data on closest approach were square root transformed to achieve normality prior to PCA calculations. Statistical tests and PCA were done using JMP Statistic software (SAS, Cary NC).

### Tissue collection and RNA extraction

Because we were interested in identifying the genes and networks related to the propensity and capacity to respond to challenge rather than those induced by the act of responding and the challenge itself, we collected birds on the day following the second STI. Nineteen males (10 WS and 9 TS) were collected the day after the second STI using mist nets and song playback. Playbacks were started between 0700–1400 EDT, which was  $5.35 \pm 2.03$  hr. after sunrise, and were balanced so that birds of both morphs were collected at earlier and later time points. Average latency to capture was  $2.79 \pm 0.14$  min after the onset of the song playback, with a maximum of 4.0 min. Immediately after capture, birds were sacrificed by isoflurane overdose and brains were rapidly dissected, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . At the end of the field portion of the study, tissues were shipped to Atlanta on dry ice and stored at  $-80^{\circ}\text{C}$  until sectioning. Brains were cryosectioned in the coronal plane at  $300\mu\text{m}$  thickness and the regions of interest were microdissected from frozen sections using a 1.0 mm diameter punch tool (Fine Science Tools, Foster City, CA). The ventromedial telencephalon, which contained the medial amygdala (MeA), was sampled in each hemisphere in two consecutive sections for a total of 4 punches, which were pooled for RNA extraction (Fig. S1a). For most of these punches, MeA constituted the majority of the sampled tissue. For the hypothalamus (HYP), all punches were centered on the midline such that they contained tissue from both hemispheres. One punch was made immediately ventral to the anterior commissure and included the caudal portion of the medial preoptic area (POM), the paraventricular nucleus (PVN), and the anterior hypothalamus (AH) (Fig. S1b). A second punch was made ventral to the first and included the ventromedial hypothalamus (VMH). Both HYP punches were made in two consecutive  $300\mu\text{m}$  sections for a total of 4 punches, which were pooled for RNA extraction. RNA was extracted using the Qiagen Allprep DNA/RNA micro kit with modification, as follows. Punches were homogenized in RLT plus +  $\beta$ -mercaptoethanol using a pellet mixer. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 7.5 was added to each sample. Samples were further homogenized by passing them through a Qiashredder column (Qiagen). Phases were separated by spinning for 15 min at  $4^{\circ}\text{C}$ , and the aqueous phase was collected and further processed according to the manufacturer's protocol.

### RNA-sequencing library prep

Library preparation and sequencing were done at the Emory Yerkes Nonhuman Primate Genomics Core. The quality of total RNA was assessed on an Agilent bioanalyzer instrument. RNAseq libraries were constructed using the Illumina TruSeq RNA Sample Preparation Kit v2 according to the manufacturer's protocol. Briefly, polyadenylated

transcripts were purified on oligo-dT magnetic beads, chemically fragmented, reverse transcribed using random hexamers and incorporated into barcoded cDNA libraries. Libraries were validated by microelectrophoresis, quantified, pooled and clustered on an Illumina TruSeq v3 flowcell. The clustered flowcell was sequenced on an Illumina HiSeq 1000 in 100-base paired end-read reactions. We obtained 11–22 million reads per sample.

### Creation of an enhanced reference genome

The reference genome, downloaded from NCBI (*Zonotrichia albicollis* 1.0.1, GCA\_000385455.1, Tuttle et al., in review), was generated from a bird homozygous for the ZAL2 haplotype. In order to eliminate potential mapping bias against reads that originated from the ZAL2<sup>m</sup> chromosome, we created an enhanced reference genome that contained sequences representing both ZAL2 and ZAL2<sup>m</sup> haplotypes within the inversion. To create this enhanced version of the white-throated sparrow genome, we first performed a ~30x whole-genome shotgun sequencing of a ‘superwhite’ bird homozygous for the ZAL2<sup>m</sup> chromosome (Horton et al., 2013, SRA accession pending). Novoalign (novocraft.com, version 3.00.05) was used to map the reads to the reference genome using ‘-r RANDOM -I PE 510,150 -0 SAM’ option. To identify single nucleotide substitutions (SNPs) between the ZAL2 and ZAL2<sup>m</sup> chromosomes, Samtools (Li et al., 2009) was used to call SNPs between the reference genome and the superwhite genome. High-quality (genotype quality score = 99) homozygous non-reference SNPs were then used to generate alternative contigs representing ZAL2<sup>m</sup> sequences within the inversion (Supplemental File 1). To create these ZAL2<sup>m</sup> contigs, we first identified NCBI genome contigs that mapped to ZAL2 via orthology with zebra finch chromosome 3 (Davis et al., 2011; Romanov *et al.*, 2011) based on sequence similarity and gene annotations. We then created ZAL2<sup>m</sup> versions of the contigs that mapped within the inversion using the GATK tool FastaAlternativeReferenceMaker (McKenna, et al., 2010). We also generated assemblies of rRNA and the mitochondrial genome derived from these whole-genome shotgun sequences (Supplemental Files 2–3). Our final enhanced reference genome was composed of the NCBI reference genome (*Zonotrichia albicollis* 1.0.1) plus ZAL2<sup>m</sup> versions of contigs located within the inversion, rRNAs, and a mitochondrial genome.

### RNA-seq mapping and gene annotation

RNA-seq reads were mapped to the enhanced version of the white-throated sparrow reference genome described above. We used the STAR aligner (Dobin et al., 2013) to map the reads to the modified reference allowing each read to map to at most 2 loci (out Filter Multimap Nmax = 2). Reads that did map within the inversion to both the reference and alternative contigs were then filtered to retain just the primary mapping location. All other dual mappings were excluded. Retained mappings to the alternate contigs were relabeled to conform to the unmodified reference contig names. Reads were assigned to genes using htseq-count (Anders et al., 2015) based on NCBI RefSeq annotations.

### Network analysis

To identify groups of co-expressed genes (modules) correlated with both song rate and morph, we performed weighted gene co-expression network analysis (WGCNA) (Zhang &

Horvath, 2005) for the MeA and HYP samples separately. Network analyses were performed using R version 3.1.1 (<http://www.r-project.org>) and functions in the WGCNA library version 1.41.1 (Langfelder & Horvath, 2008). Genes with unreliable expression (defined as 0 reads in more than half the samples) were removed from the dataset. This filtering did not selectively remove genes expressed only in one morph. Out of 3191 genes removed, 3185 of the genes had 0 reads in at least 3 TS and 3 WS samples and all of the genes removed had 0 reads in at least 1 TS and 1 WS sample. After this screen, 13,426 and 13,605 genes remained in the MeA and HYP datasets, respectively. Prior to network analysis, read counts were transformed using the ‘variance Stabilizing Transformation’ function in the DESeq2 package for R (Love et al., 2014). We performed WGCNA in R using functions in the WGCNA library (Langfelder and Horvath, 2008). To create the weighted gene co-expression network, a weighted adjacency matrix was created for all pairwise gene comparisons across samples based on the absolute value of the Pearson correlation raised to the power  $\beta$ . The power  $\beta$  was determined empirically by examining the scale-free fit across a range of powers. The adjacency matrix was transformed into a Topological Overlap (TO) Matrix. A dendrogram was constructed using average linkage hierarchical clustering based on the distance measure calculated as 1-TO. Next, modules were identified by cutting the dendrogram using the dynamic Tree Cut algorithm (Langfelder et al., 2008). Finally, modules with similar expression patterns were merged. The algorithm labels each module with an arbitrarily assigned color; the grey module is reserved for genes not assigned to a proper module. The WGCNA function blockwise Modules was used to create the network and identify modules in one step. The function offers many choices of parameters for each step in the process. Using the scale-free topology criteria as described in Zhang and Horvath (2005), the power  $\beta$  was set to 4 and 7 for the MeA and HYP networks, respectively. Additional values used to create both the MeA and HYP networks were: TOM Type = unsigned, deep Split = 2, min Module Size = 30, reassign Threshold = 0, merge Cut Height = 0.25, and pam Respects Dendro = FALSE. All other parameters were left at default values.

### Statistical analysis

The WGCNA program was used to calculate the module membership (MM) for each gene, which is the correlation between a gene’s expression profile and the module eigengene (ME, the first principal component of module gene expression). To identify modules composed of genes that were highly correlated with the trait of interest, we calculated Pearson correlation coefficients between MEs and traits of interest (morph, song rate, and PC1 for physical aggression). We chose song rate (number of songs during the 10-min STI) as a measure of vocal aggression because song is the primary vocalization used to deter territorial intruders (Falls, 1988) and the rate at which it is used in territorial defense differs dramatically between morphs in our study population (Horton et al., 2014b, and Fig. 1). We also calculated gene significance (GS) as the Pearson correlation coefficient between the expression of each gene and each trait or EGR1 expression. For correlation analyses, data on song rate were square root transformed to achieve normality. Pearson correlations were computed using the WGCNA ‘cor’ function. VisAnt (Hu et al., 2004) was used to visualize the networks represented by the top modules. Intramodular connectivity (k<sub>IN</sub>), used to identify well-connected genes, was calculated for each gene as the sum of its network

connections with other module members. To compare MeA and HYP networks, the function module Preservation was used. This function computes preservation statistics for a test network (HYP) compared to a reference network (MeA). This function also computes gene membership overlap counts and Fisher's exact test p-values between modules in each network.

### Identification of genes on chromosome 2

We chose to use the annotation of the chicken (*Gallus gallus*) genome to assign genes to *Z. albicollis* chromosome 2 (ZAL2/2<sup>m</sup>) because the chicken genome was better annotated than the zebra finch genome in the Ensembl database. Chicken gene names and chromosome annotations were downloaded from Biomart ([www.ensembl.org/biomart](http://www.ensembl.org/biomart), Ensembl Genes 75). We assigned 891 genes to ZAL2/2<sup>m</sup> based on homology to genes on chicken chr3, which is orthologous to ZAL2/2<sup>m</sup> (Thomas et al., 2008). Because this analysis is based on synteny with chicken chromosome 3, any loss of synteny between the species would result in misidentifying ZAL2/2<sup>m</sup> genes. To capture ZAL2/2<sup>m</sup> genes not represented in the chicken genome, we identified a list of 32 white-throated sparrow contigs that map to ZAL2/2<sup>m</sup> (see above). A total of 1183 genes mapped to one of these contigs. It is possible that some smaller contigs representing ZAL2/2<sup>m</sup> sequences were missing from our list, which would have resulted in missing any ZAL2/2<sup>m</sup> genes located on those contigs. There was good correspondence between these two methods; 877 genes mapped to ZAL2/2<sup>m</sup> using both methods. After summing the genes that mapped using at least one method, a total of 1197 genes were assigned to ZAL2/2<sup>m</sup>.

### Gene function and pathway analysis

Gene Ontology: Gene names were converted to the corresponding zebra finch (*Taeniopygia guttata*) Ensembl IDs for gene ontology enrichment analysis using the CORNA package in R (Wu & Watson, 2009). Gene conversions were performed using ID conversion tables downloaded from Ensembl (Flicek et al., 2014). Fisher's exact test was performed using the list of genes in the network as the background. We considered terms with  $p < 0.05$  to be significantly over-enriched in the module. To prioritize enriched terms most likely to be relevant to module function, terms annotated with fewer than three genes or represented by only one gene in the module were removed from the list. To identify genes annotated with a specific term, gene names were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7, Huang et al., 2009). We used the functional annotation table to identify genes annotated with the term (e.g. "transcription factor activity"). Because zebra finch genes were poorly annotated in the DAVID database, human gene annotations were used.

Pathway analysis: Lists of gene name annotations for each module were used for Ingenuity Pathway Analysis (IPA) using the Core Analysis procedure and the human genome as background. IPA calculates canonical pathway enrichment using a right tailed Fisher's exact test. A  $-\log(p\text{-value}) > 1.3$  was considered significant.



## Results

### Behavior

Of the behaviors recorded, only song rate differed between the morphs, which is consistent with previous results from this population (Horton et al., 2014b). In our sample population, TS males sang less than WS males in response to STI (Fig. 1, Wilcoxon  $W=6$ ,  $p<0.005$ ). There was no difference between the morphs in any of the other responses to the STIs (trills, chips, flights over decoy, time spent within 5 m, time spent within 2 m, latency to respond, and closest approach; see Fig. 1). Statistical power was 0.95 for songs, 0.06 for chips, 0.05 for latency, and 0.14–0.19 for the other behaviors. Although our power to detect morph differences in behaviors other than singing was low in this study, we replicated our results from a larger study (e.g. Horton et al., 2014b). The first principal component (PC1) for physical aggression explained 73% of the variation, and did not significantly differ between the morphs (Wilcoxon  $W=58$ ,  $p=0.29$ ).

### Gene networks in the medial amygdala

WGCNA identified 17 modules in the MeA samples. To relate module expression to trait expression, we calculated the correlation between the ME of each module and the three traits of interest (morph, song rate, and PC1 physical aggression) (Fig. 2a). Correlations for only one module, greenyellow, had  $p$ -values  $<0.01$ . After Bonferroni correction (significance threshold  $\alpha=9.8e-4$ ), the greenyellow module remained significantly correlated with both morph ( $r=0.99$ ,  $p=9e-18$ ) and song rate ( $r=0.74$ ,  $p=3e-4$ ). In contrast, no modules were correlated with the PC1 for physical aggression (Fig. 2a and Table S1), or any of the individual behaviors used in the PCA (data not shown). Next, to relate expression variability in individual genes to trait variability, we calculated gene significance measures (GS) for each gene (Langfelder & Horvath, 2008). As expected, the greenyellow module, which was correlated with both morph and song rate, contained genes that were also highly correlated with both traits (Fig. 2c and Table S2). The majority of the genes in the greenyellow module (115 of 157) were located within the inversion on *ZAL2/2<sup>m</sup>* (Fig. 2c, 3a, and Table S2).

### Gene networks in the hypothalamus

WGCNA identified 83 modules in the HYP dataset. Only one module, black, had module-trait correlations with  $p$ -values  $<0.01$ . After Bonferroni correction (significance threshold  $\alpha=2e-4$ ), the ME of the black module remained significantly correlated with both morph ( $r=-0.99$ ,  $p=6e-16$ ) and song rate ( $r=-0.75$ ,  $p=2e-4$ ). As with the MeA modules, no ME was significantly correlated with PC1 physical aggression (Fig. 2b and Table S3) or any of the individual physical behaviors used in the PCA (data not shown). The black module was composed of 511 genes that were highly correlated with both song and morph, and 226 of these genes were located within the inversion on *ZAL2/2<sup>m</sup>* (Fig. 2d, 3b, and Table S4).

### The modules correlated with song and morph share chromosome 2 genes

A large percentage of both the MeA greenyellow and the HYP black module was composed of differentially expressed genes located on chromosome 2 (Fig. 3). To assess the similarity between these two modules, we used the WGCNA function `modulePreservation` to compute

preservation statistics and a cross-tabulation table between the MeA and HYP modules. Preservation statistics were summed into one variable, the  $Z_{\text{summary}}$ ; a  $Z_{\text{summary}}$  score  $>10$  was considered strong evidence for module preservation (Langfelder et al., 2011). The  $Z_{\text{summary}}$  for the MeA greenyellow module in the HYP network was  $>40$ . There was also significant overlap in gene membership between the MeA greenyellow and HYP black modules (Fig. S2). We found 85 genes located within both modules, which was significantly more than would be expected by chance (Fisher's exact test,  $p < 5.0e-80$ ), and 70 of these shared genes were located on ZAL2/2<sup>m</sup>.

### Biological pathways in the modules correlated with morph and song rate

We utilized two strategies to identify genes that may play key roles in the behavioral polymorphism in this species and thus territorial behavior generally. First, we looked for overrepresentation of genes belonging to known biological pathways. We used both pathway (IPA tool) and GO enrichment analyses to identify pathways represented by the genes in the modules that were significantly correlated with song. There was a strong correlation between module membership and GS song rate ( $r=0.65$ ,  $p < 3e-20$  MeA greenyellow;  $r=0.65$ ,  $p < 1e-62$  HYP black), which suggests a relationship between connectivity within the module and behavioral relevance. In order to identify the most behaviorally relevant pathways, we filtered the results for pathways that contained gene(s) with high module membership (top 50, Tables S2 and S4).

In the MeA greenyellow module, IPA identified 22 pathways that were significantly overrepresented, 17 of these pathways contained highly connected genes (Table 1). Most of these pathways shared the signaling molecules MAP3K7, TGFB2, NFKBIE, or SOS1. In the HYP black module, IPA identified 42 pathways that were significantly overrepresented, 24 of these pathways contained highly connected genes (Table 2).

The GO analysis identified 18 enriched terms that were represented by two or more genes in the MeA greenyellow module. Enriched GO terms involved microtubule activity (GO:0034453, GO:0003777, GO:0007018), fatty acid metabolic process (GO:0000038, GO:0006635), mitochondrion (GO:0005739), ADP binding (GO:0043531), kinase activity (GO:0000186, GO:0006469), and locomotory behavior (GO:0007626) (Table S5). We identified 74 enriched terms that were represented by two or more genes in the HYP black module. To focus on the terms most relevant for the HYP black module, we looked for terms that contained hub genes. These terms included activation of MAPK activity (GO:0004709 and GO:0000186), prenyltransferase activity (GO:0004659), isoprenoid biosynthetic process (GO:0008299), and extracellular matrix organization (GO:0030198) (Table S5).

### Identification of biologically relevant hub genes

In the second approach to identify biologically relevant genes, we used GO annotations to identify transcriptional regulators within the module. Because well-connected transcription factors may coordinate the expression of multiple genes, we searched for well-connected (aka "hub") genes within the module (Fig. 4, and Table S2 and S4) that were annotated with the GO term "transcription factor activity". In the MeA greenyellow module, we identified three genes with this GO annotation: ESR1, TAF5L, and GTF2H5. All three of these genes

were also represented in the IPA pathways identified for that module. The most well connected gene within the module, EPM2A, while not a transcription factor, was strongly connected to both TAF5L and ESR1 (see Fig. 4a). Other well-connected genes that have a role in transcription were C1D, OLIG3, and STON1. In the HYP black module, hub genes annotated with “transcription factor activity” were TAF5L, ACSS1, AGT, and MYF5. Among these genes, only TAF5L was strongly connected to the other hub genes (Fig. 4b). Our ability to speculate about the functional roles and importance of hub genes was limited, in both modules, by the information available about each gene. Well-connected genes not discussed here, particularly ones that are not annotated (e.g. LOC designations) may also play interesting roles.

## Discussion

In this study, our aim was to identify genes that correlate with behavior, and thus are candidates for mediating the effects of a chromosomal inversion on social behavior in this species. In order to accomplish this aim, we quantified territorial behavior in free-living males and subsequently analyzed gene expression in brain regions that are part of a social behavior network (Goodson, 2005) and previously linked with territorial responses in this species (Horton et al., 2014a). Using gene co-expression network analysis, we found one module significantly correlated with both morph and song rate in each of these regions. In contrast, no modules were correlated with physical aggression, a behavior that did vary among individuals but did not differ between the morphs (see also Horton et al., 2014b).

The positive correlation between module membership and gene significance for song in both the MeA greenyellow and HYP black modules indicates that genes central to the network (i.e. hub genes) are also highly correlated with song rate. Because of their centrality within the module, hub genes have the potential to be key drivers of the gene co-expression network. Some of the most well-connected hub genes in these two modules are involved in basic cellular processes likely common to most cell types. For example the gene EMP2A, which is a hub in both the greenyellow MeA and black HYP modules (Fig. 4) encodes the protein laforin, which maintains the phosphorylation state of glycogen (see Raththagala et al., 2015) and is thus key to cellular energy supply and metabolism. Another hub gene in both modules, NTPCR, hydrolyzes nucleosides (e.g. GTP, ATP) and is thus may also be important for energy homeostasis. AARS2, which is highly connected in the greenyellow module and appears in the black module as well, catalyzes the attachment of amino acid residues to tRNA (reviewed by Gillet & Felden, 2001) and is thus critical for protein translation. Each of these genes is evolutionarily ancient, with homologs found in most taxa including plants and prokaryotes, reflecting their ubiquitous roles in basic cellular functioning. Their specific roles in behavior are unclear in the context of this study, other than that they support basic functions in brain regions that are known to be active during territorial defense and other social behaviors.

In order to better understand the relationships among the genes in the two modules correlated with behavior, we conducted IPA, which reveals enrichment of gene pathways with known functions. Each of top three pathways in the MeA greenyellow module involved nuclear receptor signaling (Table 1). The glucocorticoid receptor signaling pathway

represents a particularly interesting finding because Horton & Holberton (2010) found that during the parental provisioning stage of the breeding season, TS males had lower levels of plasma corticosterone (CORT) than WS males. To test whether this hormonal difference may mediate known morph differences in parental provisioning rates, they experimentally raised plasma CORT in TS birds and blocked CORT in the WS birds. These manipulations essentially eliminated morph differences in parental provisioning rates (Horton & Holberton, 2009). Although we did not collect tissue during the parental phase, our results confirm that genes in the CORT signaling pathway are differentially expressed in the morphs and may play a role in morph-dependent behaviors.

Perhaps our most compelling finding is that the MeA module that was correlated with both morph and singing was enriched for genes in the estrogen receptor (ER) signaling pathway (Table 1). Expression of the gene that encodes estrogen receptor alpha (ER $\alpha$ ) has been shown in previous studies to be correlated with territorial aggression and parenting behavior in this and other species. For example, in dark-eyed juncos (*Junco hyemalis*), aggressive responses to STI were positively correlated with expression of ER $\alpha$  in the ventral telencephalon (containing the MeA) and negatively correlated in HYP (which in that study included POM and VMH) (Rosvall et al., 2012). In a previous study, we reported that in white-throated sparrows, ER $\alpha$  mRNA is differentially expressed in MeA and several other brain regions (Horton et al., 2014a). In that study, expression of ER $\alpha$  in both the MeA and PVN predicted song rate in response to STI, a correlation that persisted even when morph and plasma levels of testosterone and estradiol were held constant. Mediation analysis revealed in fact that singing in response to STI was better predicted by ER $\alpha$  expression in MeA than by morph (Horton et al., 2014a). In the current study, ER $\alpha$  in the MeA module was associated with both morph and singing, which replicates our previous findings and supports our hypothesis that this gene may play a key role in the behavioral polymorphism in this species and the evolution of alternative reproductive strategies generally (Maney et al., 2015; Cushing et al., 2008).

In addition to ESR1, other genes with known relationships with estrogen signaling were located in the modules correlated with singing. For example the expression of TAF5L, a transcription factor in the ER signaling pathway, was well-connected in both the MeA greenyellow and HYP black modules (Fig. 4). Thompson et al. (2012) showed that TAF5L expression was highly correlated with the expression of immediate early genes such as Egr-1 and Fos in the song control nuclei of white-crowned sparrows (*Z. leucophrys*), a congener of the white-throated sparrow. Because Thompson et al. quantified expression in the motor nuclei that are most active during singing, their results suggest that TAF5L expression may be related to activity generally; it may also be altered by increasing local concentrations of estradiol (Fong et al., 2007), which is released in nearby regions in response to hearing conspecific song (Remage-Healey et al., 2008).

When bound with ligand, ERs act via two main mechanisms: genomic and nongenomic. In the canonical genomic pathway, the receptor binds estradiol inside the cytoplasm, translocates to the nucleus and acts as a transcription factor. If ER $\alpha$  mediates morph differences in behavior *via* genomic mechanisms, we might expect it to regulate the transcription of a large number of other genes and to be well-connected in the modules

correlated with behavior. It does not, however, seem to act as a hub in either network (Fig. 4). It is possible that ER $\alpha$  may regulate morph-dependent behaviors *via* nongenomic mechanisms such as membrane-bound receptors. For example, ER $\alpha$  can act in a nongenomic fashion by interacting with the metabotropic glutamate receptor (mGluR1; Meitzen & Mermelstein, 2011). ER $\alpha$  can also induce phosphorylation of the membrane-associated protein Shc, which then interacts with two other proteins, Grb2 and SOS, to initiate intracellular events (Song et al., 2002). Genes for proteins critical to each of these nongenomic mechanisms, mGluR1 and SOS, are both found in the MeA greenyellow module meaning that like ESR1, their expression depends on morph and is linked with behavior.

When estradiol acts via nongenomic mechanisms, resulting changes in intracellular activity, such as MAPK activation and firing, are rapid and likely short-lived. Intracellular events downstream of ER $\alpha$  activation therefore may not be detectable by measuring gene expression many hours after the last STI, nor would they necessarily be correlated with behavior. We intentionally delayed sampling until 24 hours after the last STI because we were not interested in the effects of behavioral responses on gene expression, but rather the opposite – we wanted to discover genes related to the motivation to respond to challenge rather than engaging in the response itself. Other researchers have published lists of genes that are induced by STI in wild songbirds; for example of all the genes induced by STI in the hypothalamus of song sparrows (*Melospiza melodia*; Mukai et al., 2009), only two genes, TBCE and PEX13, were in a module associated with behavior in this study (HYP black module). According to Mukai et al., many other genes are selectively expressed as a result of engaging in territorial defense; we would expect to find robust morph differences in the expression of those genes immediately following STI.

Synaptic plasticity has been associated with behavioral differences between sexes in zebrafish (Wong et al., 2014) and vocal learning in zebra finches (Hilliard et al. 2012). Enriched pathways and GO terms in both the MeA greenyellow and HYP black modules can be broadly associated with synapse function or activity. In the MeA, enriched GO terms were related to energy production and vesicle transport (e.g. mitochondrion and microtubules). In the HYP black module, three of the six genes annotated with the GO term “extracellular matrix organization” are hub genes (Table S5). Both modules were enriched for genes related to retinoic acid signaling. In The HYP black module, these terms were “isoprenoid biosynthetic process”, “RAR activation”, and “VDR/RXR activation”. The MeA greenyellow module was enriched for genes belonging to the PPAR $\alpha$ /RXR $\alpha$  activation pathway. Both the extracellular matrix and retinoid signaling play important roles in synaptic function and plasticity (Frischknecht & Gundelfinger, 2012, Shearer et al., 2012). These results suggest that morph differences in vocal aggression may relate to processes involved in synaptic plasticity.

Because we did not continuously monitor the birds' behavior during the hours immediately preceding tissue collection, we must consider the possibility that the morph differences in gene expression we found were related to territorial behavior that we did not observe. Previous studies in songbirds have demonstrated that large numbers of genes are regulated by either experience (e.g., listening to song) or behavior (e.g., singing) (Drnevich et al.,

2012; Mukai et al., 2009; Whitney et al., 2014). Formica et al. (2004) have shown that WS and TS birds prefer different types of habitat; WS males choose territories with more neighbors and therefore higher frequencies of agonistic encounters than TS males (see also Formica & Tuttle, 2009). We believe that morph differences in habitat selection were unlikely to have played a key role in our findings here. First, although we cannot rule out effects of social niche partitioning, in this population the morphs are fairly evenly distributed and tend to occupy the same types of habitat (Horton et al., 2014b). Second, to determine whether any of the modules were correlated with potential neuronal responses to stimuli, we examined the expression of an immediate early gene, *EGR1*. *EGR1* expression, however, was not correlated with either of the modules associated with morph and song (Fig. 2). Note, however, that singing is not known to be correlated with *EGR1* induction in MeA (Earp & Maney, 2012), so this measure may not indicate whether birds were engaging in territorial defense. Finally, we and others have demonstrated significant morph differences in behavior in controlled conditions in the laboratory; WS males sang at higher rates and were more aggressive than TS birds (Maney et al., 2009; Maney & Goodson, 2011; Swett, 2007) even when social conditions were identical. These differences persisted despite similar levels of plasma sex steroids in the two morphs, supporting the hypothesis that morph differences in neural sensitivity to sex steroids, which could take the form of morph differences in receptor numbers or distribution, may underlie morph differences in territorial behavior.

WGCNA identified many more modules in the HYP than the MeA network, even though both networks were constructed using the same parameters. One possible explanation for the larger number of modules in the HYP network is that it reflects the more heterogeneous nature of the HYP samples. Gene expression patterns are known to depend on brain region (Balakrishnan et al., 2014; Drnevich et al., 2012; Whitney et al., 2014). Our HYP punches contained several hypothalamic nuclei (see Materials and Methods and Fig. S1) in which genes may be differentially regulated. In networks that are built using samples collected from multiple, distinct brain regions or nuclei, modules that distinguish these individual nuclei or specific brain regions can be identified (Drnevich et al., 2012; Konopka et al., 2012; Oldham et al., 2006). We were unable to perform this type of analysis with our dataset because our network was built using one sample type (HYP). Although we are unaware of genes that define only one subregion of the hypothalamus, we have previously shown that morph differences in *ESR1* expression are dependent on brain region. We previously showed, for example, that *ESR1* expression is higher in TS than WS birds in VMH and caudal POM, but higher in WS than TS birds in PVN (Horton et al., 2014a); both VMH and PVN were contained in our HYP sample. Heterogeneity may thus explain why, although we replicated our previous finding of differential expression of *ESR1* in MeA (Horton et al., 2014a), we could not detect differential expression of *ESR1* in our HYP sample. In dark-eyed juncos, expression of *ESR1* in HYP was negatively correlated with territorial aggression (Rosvall et al., 2012). Here, we were unable to detect a correlation between song rate and *ESR1* in HYP and *ESR1* was not located within the HYP black module. In future studies, it may be important to conduct RNA-seq on smaller samples limited to a single region in order to detect morph differences in gene expression within regions with distinct functions and cell types.

Our results demonstrate the profound effects of the *ZAL2/2<sup>m</sup>* rearrangement on gene expression. Our two modules of interest, MeA greenyellow and HYP black, were composed primarily of genes captured by the inversion (see Figs. 2 and 3). Standard network analysis groups genes into modules based on co-expression. The usual interpretation of these modules, which we have presented above, is that co-expressed genes are functionally related. As a result, a significant fraction of genes within a module often represent similar gene ontology categories or canonical pathways (Langfelder et al., 2011; Oldham et al., 2006). However, multiple mechanisms can influence gene-gene correlations, including higher order chromosome configuration and epigenetic regulation (Gaiteri et al., 2014). We must also consider a second interpretation; in our analysis, genes may be grouped into co-expression modules due to co-localization within the differentiating region. We did not observe enrichment of *ZAL2/2<sup>m</sup>* genes in any other modules, suggesting that the inversion was a facilitating factor in the emergence of the MeA greenyellow and HYP black modules. In support of this alternative explanation, we found that all of the chromosome 2 genes within the MeA greenyellow and HYP black modules were located within the inversion; in other words, none of the chromosome 2 genes within these modules were located in the recombining regions of chromosome 2, outside of the inversion. We also found significant overlap in gene membership between the modules, and most (70/84) of these shared genes were located within the inversion. Therefore, the traditional interpretation that genes within a co-expression network are connected because they act in the same pathway or biological processes represents only one of several possible mechanisms driving gene-gene correlations. It is also important to note that because of the large morph difference in singing in this study (Fig. 1a), it is difficult to dissociate that trait from plumage morph; the only modules correlated with singing were also correlated with morph. Placement of *ESR1* within the MeA greenyellow module confirmed that our network analysis correctly identified a gene known to predict song rate independently of morph (Horton et al., 2014a). Whether song rate is reliably predicted by other genes in the module, however, is difficult to test in this dataset because of our relatively small sample size. Follow-up studies will be necessary to evaluate the potential roles of other promising genes in territorial aggression.

The white-throated sparrow is a useful model system in which to study the consequences of an inversion on gene expression. By suppressing recombination between alleles, chromosomal rearrangements can foster the evolution of co-adapted alleles, or ‘supergenes’ (Dobzhansky, 1970). Inversions that encompass multiple genes involving a suite of phenotypic traits might therefore maintain both behavioral and color polymorphism within a species (reviewed in McKinnon & Pierotti, 2010). Alternatively, a single gene with pleiotropic effects might be responsible for both behavioral and color polymorphism. Likely candidates are transcription factors and hormones. Transcription factors have the capacity to coordinate the expression of multiple genes that share a cis-regulatory region. Hormones can activate transcription factors and act through nongenomic signaling pathways. We cannot determine from our current study whether co-adapted alleles or a small number of pleiotropic genes can explain the behavioral polymorphism. It will be interesting to determine in future studies the extent to which manipulating expression of a single gene can influence behavior in this species.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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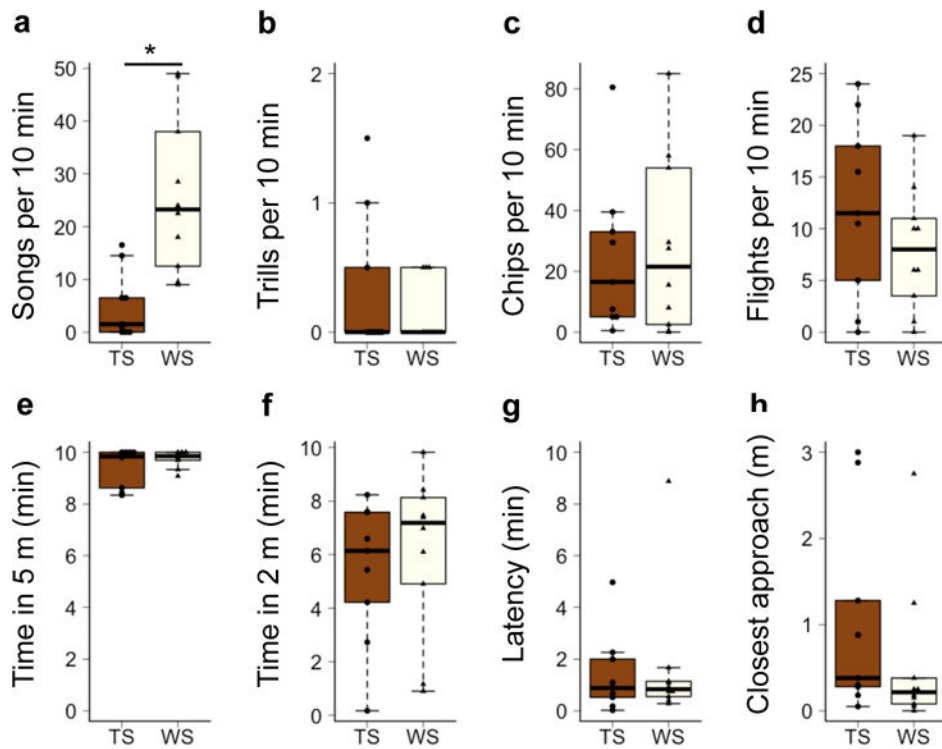


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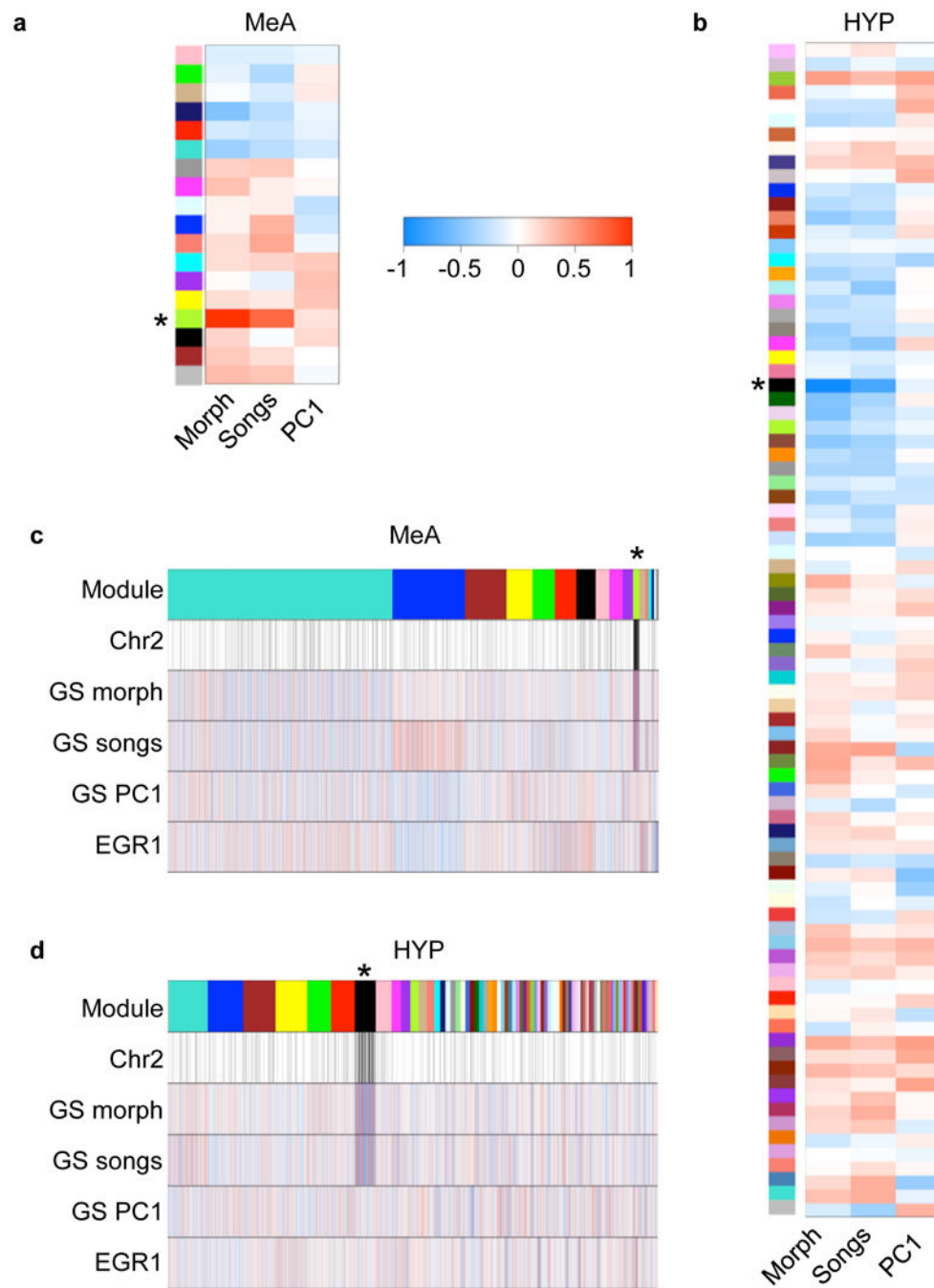
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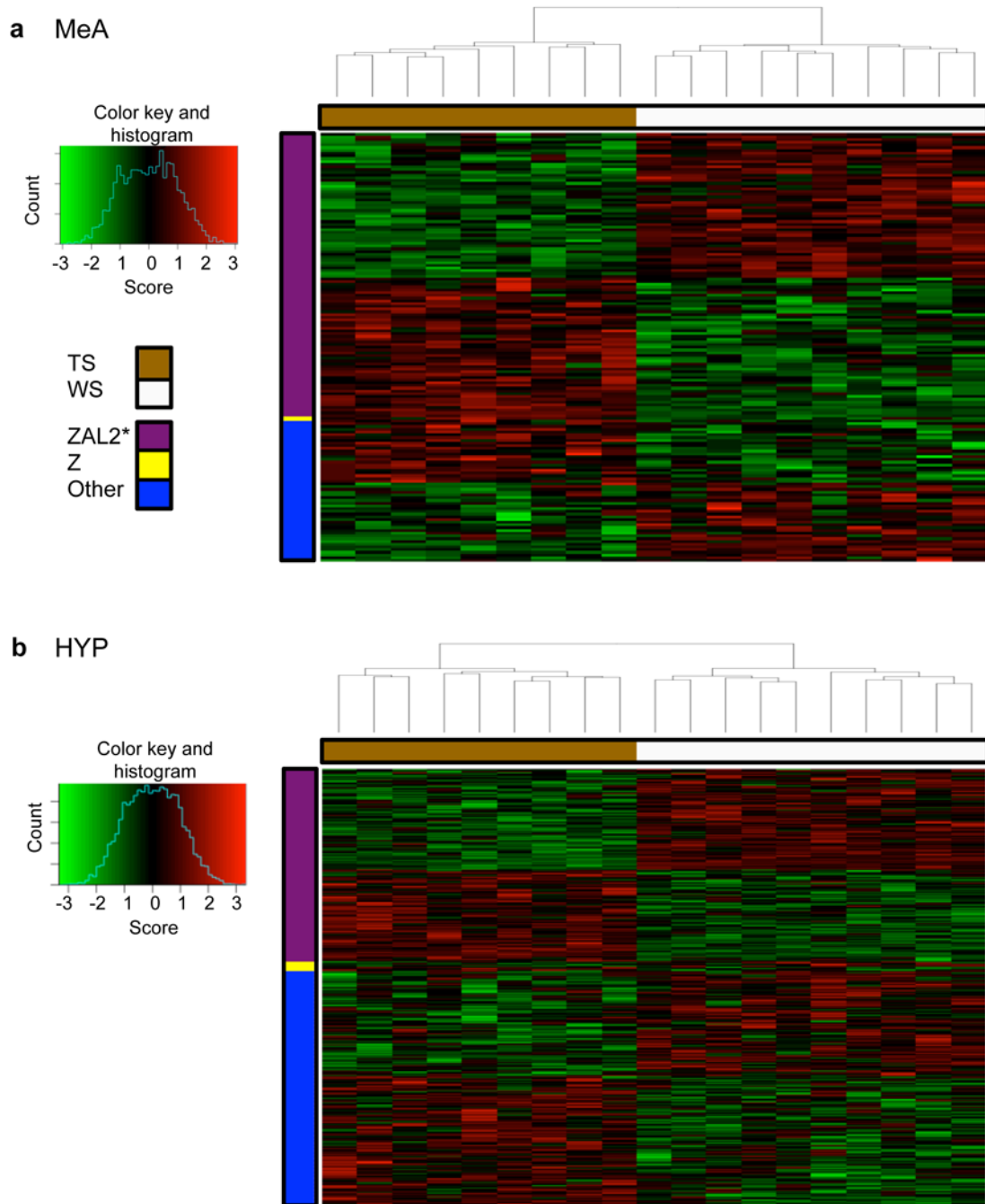
**Figure 1.**

Behavioral responses to simulated territorial intrusions. Vocal measures of aggression were average (a) number of songs, (b) number of trills, and (c) number of chips recorded per 10-min STI. Measures of physical aggression were average (d) number of flights per 10-min STI, (e) time spent within 5 m, (f) time spent within 2 m, (g) latency, and (h) distance of closest approach. Scores for individual birds are represented by circles (TS) or triangles (WS). Whiskers represent 1.5 times the inner quartile range. \* $p < 0.005$



**Figure 2.** Correlations between gene expression and traits. Correlations were calculated between module eigengenes (MEs) and morph, song rate, or PC1 for physical aggression. In the medial amygdala (a) and the hypothalamus (b), each row corresponds to a module and columns correspond to each trait. The color of each cell indicates the value of the correlation coefficient. Positive correlations are red, and negative correlations are blue, as indicated by the color scale bar. In the medial amygdala (c) and the hypothalamus (d), genes are ordered according to membership within the modules. The color bar in the top row indicates the

module to which each gene is assigned; genes assigned to the grey module are not shown. In the second row, genes located on *ZAL2/2<sup>m</sup>* are indicated by black bars. Rows 3–5 represent the correlation between the expression of each individual gene and the trait indicated (gene significance, or GS). Row 6 represents the correlation between the expression of each gene and *EGR1* expression. Each gene is represented by a line, and the color of the line indicates the correlation coefficient, as indicated by the color scale bar. Correlation and p-values are provided in Tables S1 and S3. \*Modules correlated with both morph and song.



**Figure 3.**

Heat map of expression for individual genes in the MeA greenyellow (a) and HYP black (b) modules. Columns represent samples, sorted by hierarchical clustering. Morph is indicated by the color bar at the top (tan = TS and white = WS birds). Rows represent genes, sorted by chromosome location, then by hierarchical clustering. The color bar on the left indicates the chromosome location of the genes. Genes were assigned to chromosome 2 (ZAL2, purple), chromosome Z (Z, yellow), or other (all genes not assigned to ZAL2 or Z, blue). Expression (score) is represented by the standardized residual (value-mean/std. deviation). Red,



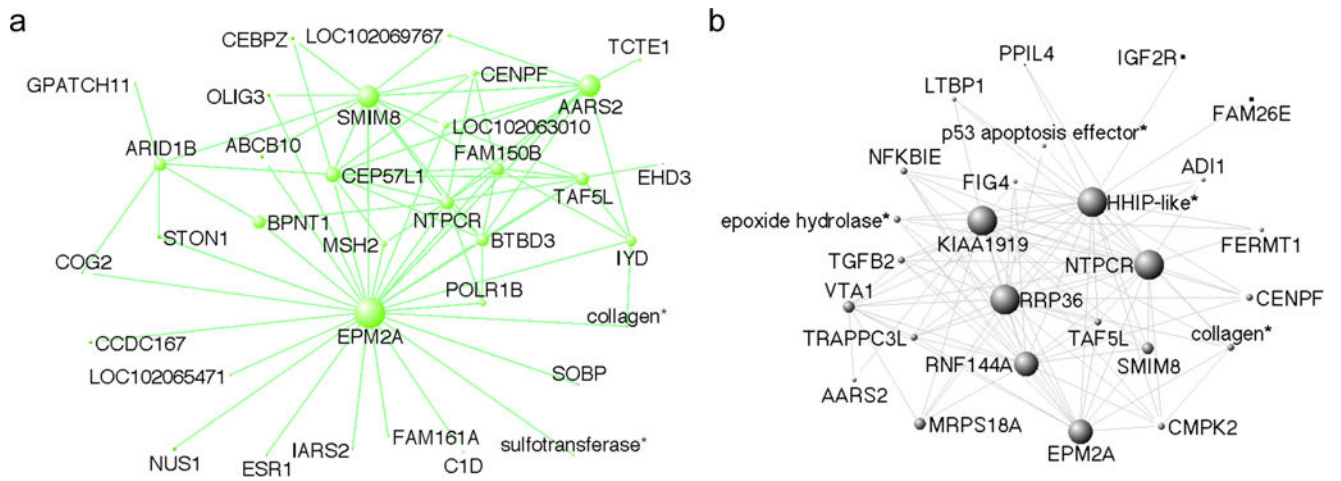
increases; green, decreases; black, no change relative to the mean. \*All ZAL2 genes located within these two modules were also located within the inversion.

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**Figure 4.** Connectivity in the (a) MeA greenyellow and (b) HYP black networks. Only the 75 (MeA) or 100 (HYP) strongest connections among the top 50 most well-connected genes (determined using kIN) are shown. Node size represents the degree of connectivity. \*Where available, gene description for LOC genes are used. See supplemental tables S2 and S4 for LOC gene designations.

**Table 1**

Pathways significantly overrepresented in the MeA greenyellow module \*

Ingenuity Canonical Pathways	$-\log(\text{p-value})$	Ratio	Genes
Glucocorticoid Receptor Signaling	3.07	0.027	MAP3K7, TGFB2, NFKBIE, GTF2H5, SOS1, ESR1, TAF5L
PPAR $\alpha$ /RXR $\alpha$ Activation	2.40	0.028	MAP3K7, ACOX1, TGFB2, NFKBIE, SOS1
Estrogen Receptor Signaling	2.19	0.031	GTF2H5, SOS1, ESR1, TAF5L
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.96	0.038	TGFB2, NFKBIE, SOS1
Hepatic Cholestasis	1.83	0.025	MAP3K7, TGFB2, NFKBIE, ESR1
Thyroid Hormone Biosynthesis	1.76	0.333	IYD
PPAR Signaling	1.76	0.032	MAP3K7, NFKBIE, SOS1
IL-17A Signaling in Fibroblasts	1.75	0.057	MAP3K7, NFKBIE
tRNA Charging	1.66	0.051	AARS2, IARS2
Role of PKR in Interferon Induction and Antiviral Response	1.64	0.050	MAP3K7, NFKBIE
Geranylgeranyldiphosphate Biosynthesis	1.64	0.250	GGPS1
Trans, trans-farnesyl Diphosphate Biosynthesis	1.54	0.200	GGPS1
IL-6 Signaling	1.52	0.026	MAP3K7, NFKBIE, SOS1
PKC $\theta$ Signaling in T Lymphocytes	1.50	0.025	MAP3K7, NFKBIE, SOS1
Assembly of RNA Polymerase II Complex	1.46	0.040	GTF2H5, TAF5L
PI3K/AKT Signaling	1.45	0.024	ITGA2, NFKBIE, SOS1
CD27 Signaling in Lymphocytes	1.43	0.039	MAP3K7, NFKBIE

\* IPA identified 24 pathways significantly enriched in the MeA greenyellow module. Only those pathways that also contain well-connected genes are shown.

Ratio: Number of pathway genes in the greenyellow module divided by the total number of genes in the pathway.

**Table 2**

Pathways significantly overrepresented in the HYP black module\*

Ingenuity Canonical Pathways	$-\log(p\text{-value})$	Ratio	Genes
RAR Activation	2.77	0.057	PRKCD, PNRC1, AKT3, MAP3K5, NT5C1B, SMAD6, TGFB2, PRKCI, GTF2H5, ERCC3
RANK Signaling in Osteoclasts	2.23	0.068	MAP3K7, AKT3, MAP3K5, NFKBIE, MAP3K4, NFATC1
Gαq Signaling	2.21	0.054	PRKCD, AKT3, PLCB4, HTR2C, PRKCI, NFKBIE, NFATC1, FNBP1
Type II Diabetes Mellitus Signaling	2.21	0.060	MAP3K7, PRKCD, AKT3, MAP3K5, SOCS5, PRKCI, NFKBIE
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	2.02	0.125	IMPA2, ITPKA, BPNT1
LPS-stimulated MAPK Signaling	1.94	0.069	MAP3K7, PRKCD, MAP3K5, PRKCI, NFKBIE
HGF Signaling	1.87	0.057	MAP3K7, PRKCD, AKT3, MAP3K5, PRKCI, MAP3K4
VDR/RXR Activation	1.83	0.064	SERPINB1, PRKCD, TGFB2, PRKCI, HES1
CD27 Signaling in Lymphocytes	1.81	0.077	MAP3K7, MAP3K5, NFKBIE, MAP3K4
Methylthiopropionate Biosynthesis	1.73	1	ADI1
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.72	0.044	MAP3K7, PRKCD, AKT3, MAP3K5, PRKCI, NFKBIE, MAP3K4, FNBP1
G-Protein Coupled Receptor Signaling	1.67	0.039	AKT3, MC3R, PLCB4, CNR1, HTR1E, HTR2C, FSHR, ADRA2C, MC5R, NFKBIE
PKCθ Signaling in T Lymphocytes	1.65	0.051	GRAP2, MAP3K7, MAP3K5, NFKBIE, MAP3K4, NFATC1
Hepatic Cholestasis	1.50	0.043	MAP3K7, PRKCD, TGFB2, PRKCI, HNF4A, NFKBIE, MAP3K4
PI3K Signaling in B Lymphocytes	1.50	0.047	AKT3, PLCB4, PRKCI, NFKBIE, NFATC1, DAPP1
GNRH Signaling	1.48	0.047	MAP3K7, PRKCD, MAP3K5, PLCB4, PRKCI, MAP3K4
Mismatch Repair in Eukaryotes	1.46	0.125	RPA1, MSH2
Erythropoietin Signaling	1.45	0.060	PRKCD, AKT3, PRKCI, NFKBIE
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	1.41	0.118	ACAT2, GGPS1
NF-κB Signaling	1.37	0.041	MAP3K7, AKT3, NTRK2, NFKBIE, TBK1, IGF2R, FGFRL1
D-myo-inositol (1,4,5)-trisphosphate Degradation	1.37	0.111	IMPA2, BPNT1
B Cell Receptor Signaling	1.34	0.040	MAP3K7, AKT3, MAP3K5, NFKBIE, MAP3K4, NFATC1, DAPP1
NF-κB Activation by Viruses	1.33	0.055	PRKCD, AKT3, PRKCI, NFKBIE
STAT3 Pathway	1.33	0.055	SOCS5, NTRK2, IGF2R, FGFRL1
Role of NFAT in Cardiac Hypertrophy	1.31	0.039	MAP3K7, PRKCD, AKT3, PLCB4, SLC8A1, TGFB2, PRKCI
NGF Signaling	1.31	0.047	MAP3K7, PRKCD, AKT3, MAP3K5, MAP3K4

\* IPA identified 42 pathways significantly enriched in the HYP black module. Only those pathways that also contain well-connected genes are shown. Ratio: Number of pathway genes in the black module divided by the total number of genes in the pathway.