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Sex chromosome gene expression associated with vocal learning following hormonal manipulation in female zebra finches

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eLife Assessment

This study is **valuable** as it provides information about the genes regulated by sex hormone treatment in song nuclei and other brain regions and suggests candidate genes that might induce sexual dimorphism in the zebra finch brain. The analysis presented is thorough and detailed. Whereas the evidence for gene regulation by hormone treatment is well supported, the evidence for an association of those genes with song learning (as written in the title) is **incompletely** supported as no manipulation of song learning or song analysis was conducted.

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Abstract

Zebra finches are sexually dimorphic vocal learners. Males learn to sing by imitating mature conspecifics, but females do not. Absence of song in females is associated with partial atrophy and apparent repression of several vocal learning brain regions during development. However, atrophy can be prevented and vocal learning retained in females when given early pharmacological estrogen treatment. To screen for candidate drivers of this sexual dimorphism, we performed an unbiased transcriptomic analysis of song learning nuclei specializations relative to the surrounding regions from either sex, treated with vehicle or estrogen until 30 days old when divergence between the sexes becomes anatomically apparent. Analyses of transcriptomes by RNA sequencing identified song nuclei-specialized gene expressed modules associated with sex and estrogen manipulation. Female HVC and Area X gene modules were specialized by estrogen supplementation, exhibiting a subset of the transcriptomic specializations observed in males. Female RA and LMAN specialized modules were less dependent on estrogen. The estrogen-induced gene modules in females were enriched for anatomical development functions and strongly correlated to the expression of several Z sex chromosome genes. We present a hypothesis where reduced dosage and expression of these Z chromosome genes suppresses the full development of the

song system and thus song learning behavior, which is partially rescued by estrogen treatment.

Introduction

Vocal learning is the ability to imitate heard sounds using a vocal organ, and is a necessary and specialized component for spoken language and song. Vocal learning is found in seven non-human clades, four mammalian and three avian, each having independently evolved the trait[1,2]. Oscine songbirds have proved to be the most tractable for studying vocal learning in the lab, with much of the field focusing on the Australian zebra finch (*Taeniopygia castanotis*). Despite ~300 million years of separation from their common ancestor[3,4], there is remarkable evolutionary convergence between songbird and human vocal learning in terms of behavioral progression, developmental effects of deafening, anatomical connectivity of vocal-motor learning circuits, sites of accelerated evolution within the genome, and genes with specialized up- or down-regulated expression in song and speech circuits relative to the surrounding motor control circuits[1,5–13]. Unlike in humans, however, vocal learning is strongly sexually dimorphic in zebra finches and many other vocal learning species[14,15]. Male zebra finches learn to produce a species appropriate song by imitating mature male conspecifics during juvenile development, while females are limited to producing innate calls[16].

The songbird vocal motor learning circuit contains four major interconnected telencephalic song control nuclei; HVC (proper name) in the dorsal nidopallium (DN); the lateral magnocellular nucleus of the anterior nidopallium (LMAN) in the anterior nidopallium (AN); the robust nucleus of the arcopallium (RA) in the lateral intermediate arcopallium (LAI; also called AId); and Area X in the striatum (Str; **Fig. 1a**)[7]. During juvenile development in zebra finch females, HVC and RA atrophy, HVC fails to form synapses in RA, and Area X never appears[14,17–24]. Amazingly, female zebra finches treated with estrogen or a synthetic analog at an early age do not exhibit song system atrophy and instead form a functional neural circuit with all the anatomical components and connections seen in males[25–28]. This “masculinized” song system allows estrogen supplemented females to imitate vocalizations, though not with the same accuracy as males[25,27,29–31]. Interestingly, lesioning female HVC prevents estrogen dependent anatomical “masculinization” of its postsynaptic targets to RA and Area X[32].

The genetic basis of this estrogen-sensitive and sexually dimorphic vocal learning in zebra finch remains largely unknown beyond the downstream recruitment of the androgen receptor (AR)[33]. However, the examination of a rare gynandromorphic zebra finch with lateralized sex chromosome composition indicates that genetically male HVC and Area X are larger than their female analogs independent of gonadal hormone production, implicating sex chromosome gene expression within the song system[34]. Unlike the mammalian X and Y, birds have a Z and W sex determination system where females are hemizygous (ZW) and males homozygous (ZZ)[35]. The relevant transcriptional machinery appears to be set up by post-hatch day 30 (PHD30), after which estrogen fails to masculinize female song nuclei or behavior and the male song system enlarges while the female song system atrophies[17,19,25,36]. Taken together with the hypothesis that vocal learning in females was lost multiple independent times amongst songbirds[15], the extant findings suggest genetic drivers of vocal learning loss associated with estrogen, sex chromosomes, and song nuclei gene expression specializations. To screen for potential genetic drivers (loci whose expression/inheritance regulate the trait) and locate their action within the song system, we performed an unbiased analysis of transcriptomes from song nuclei and surrounding motor control regions in zebra finches of either sex chronically treated with 17-β-estradiol (E2) or vehicle from hatch until sacrifice at PHD30 (**Fig. 1b**). While the birds for this study were sacrificed prior to the developmental presentation of song behavior, we have previously shown that female finches treated with E2 in the same exact way go on to produce rudimentary imitative songs as

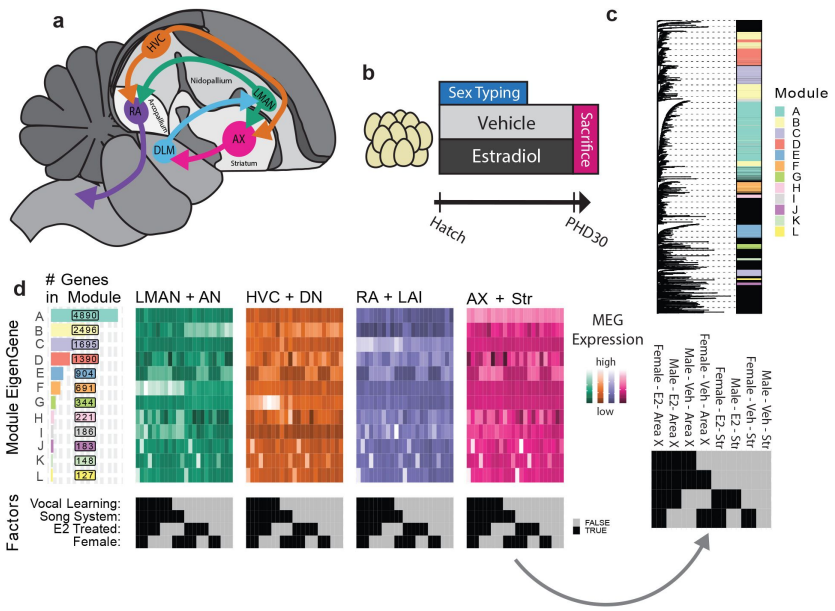


Figure 1

Song system anatomy and experimental design.

a, Diagram of song system connectivity within the adult male zebra finch brain with major telencephalic domains indicat- ed. Area X connects back to LMAN through the non-vocal specific thalamic nucleus DLM. **b,** Experimental design. Animals were treated with E2 or a vehicle from hatch until sacrifice on PHD30. **c,** WGCNA assignment of genes to modules. Left: Hierarchy computed over the transcriptome wide topological overlap matrix of gene to gene correlations in transcript abundance across samples. Right: Module assignment raster, rows are genes colored according to the assigned module, unassigned genes in black. **d,** MEG expression heat- maps arranged by module size (left) aligned to traits of interest (bottom). Each row is an MEG and each sample is a column. Samples are grouped according to neural circuit node in different colored subpanels. Color intensity encodes MEG expression as calculated by WGCNA. An example raster with sample category labels is provided at right.

adults, consistent with the known induction of vocal learning in females by E2[25,29]. We used a new zebra finch genome assembly and annotation produced by the Vertebrate Genomes Project containing both the Z and W chromosomes[37].

Results

Identification of gene expression modules

We first sought to characterize differences in song nuclei gene expression specializations relative to their immediate surrounding motor brain regions in juvenile males and females with and without E2 treatment. We used RNA-Seq data from a previous study by our lab, on the effects of E2 manipulation on the song system in both males and females[29]. This E2-treatment program masculinized females sufficiently for them to produce song as adults in a parallel cohort of birds[29]. Birds were sacrificed as juveniles at post-hatch day 30 (PHD30), after a one hour period of silence to limit activity-dependent gene expression in song nuclei and surrounding motor regions; this developmental age was chosen because it is around the time when both males and E2-treated females start to sing (e.g. subsong) and all four song nuclei are sufficiently developed to be visually apparent in histological sections[29]. The four major song nuclei (HVC, LMAN, RA, and Area X) and their adjacent surrounding motor regions (DN, AN, LAI, and Str respectively; **Fig. 1a**) were dissected using laser capture. In the case of vehicle-treated females, which lack Area X, a piece of striatum was taken from where Area X would be in males to serve as the Area X sample. In our previous study, we found that these PHD30 vehicle-treated males had larger RAs and HVCs than their female counterparts, Area X was absent in vehicle-treated females, and LMAN was similarly sized in both sexes. Following E2 treatment, E2-treated male RA was significantly smaller than vehicle-treated males; while Area X appeared in E2-treated females and HVC was significantly larger than in vehicle-treated females[29]. This past study mapped the RNA-Seq reads to an older genome assembly lacking the W sex chromosome[29].

With this data, we first remapped RNA-Seq reads (from n = 3 birds per group) to a new zebra finch assembly (GCF_008822105.2) with both the Z and W chromosomes. As many W chromosome genes are duplicated from the Z chromosome and thus highly similar in sequence, only single-mapped reads were considered to minimize misattributed Z chromosome reads. In our quality control analyses, hierarchical clustering of sample expression vectors revealed two technical outliers, one HVC from a vehicle-treated male and one RA from a E2-treated female, which we excluded from further analysis (**Fig. S1**). Similar to previous PCA and hierarchical clustering[29], the remapped RNA-Seq data expression levels with outliers removed still resulted in separation of song nucleus and surround and some E2-treated female samples, without sufficient resolution at finer group levels (**Fig. S1**). To address our questions at finer resolution and in an unbiased way, we performed weighted gene correlation network analysis (WGCNA), where we decomposed the transcriptome into actively expressed gene modules using hierarchical clustering of the gene adjacency matrix. This matrix describes the inferred structure of gene networks within our data and was calculated with a soft thresholding of the matrix of gene-to-gene expression correlations across samples (**Fig. S2**)[38]. After the genes were given initial hierarchical cluster-based module assignment, they were then iteratively reassigned to the module whose aggregate expression they best correlated with until no additional genes met the WGCNA reassignment threshold (**Fig. S3** and Methods for parameterization). This reassignment was performed to ensure that genes are matched to the aggregate measure that best represents their expression.

Of the ~21,000 annotated zebra finch genes, 13,220 were well expressed in the finch telencephalic brain regions sampled, a comparable number of genes to what we have seen expressed in adult zebra finch telencephalon[39,40]. These 13,220 were assigned to 14 co-expression modules (**Fig. S4a**). The median observation of unassigned transcripts was 22-fold lower than the median observation of module assigned transcripts (4.86 vs. 0.22 FPKM). Two modules clearly marked

single samples from two different birds (**Fig. S4a,b - arrows**), indicative of technical overfitting; these two modules (not birds) were excluded from further analysis, resulting in a more visibly diverse pattern within and across modules (**Fig. S4c,d**). The remaining 12 modules contained 12,444 genes in total; these 12 moduleswere lettered in descending order of size A through L, containing from 4,890 to 127 constituent genes each, which were dynamically expressed across brain regions and treatment groups (**Fig. 1c**). The results of module assignment can be found in supplemental_gene_module_assignment.csv.

Song nuclei specialization modules and sex differences

To understand song system transcriptional specialization in the context of the gene modules, we calculated the module eigengene (MEG) expression values for each of the 12 modules (**Fig. 1d**). MEGs are the first principle component of variance of all genes in a module and are the aggregate measure for each module's expression across samples. We then tested for statistically significant correlations between MEG expression for each module and the song nuclei specializations relative to their respective surrounds. Each song nucleus in vehicle-treated control males had unique and overlapping specialization of genes in 2 to 4 modules of the 12 (**Fig. 2a**). The male LMAN specialization relative to the surrounding AN were correlated with modules B, F and I; male HVC specialization relative to DN consisted of genes in modules B, F, and G; male RA specialization relative to LAI consisted of genes in modules C and L; and male Area X specialization relative to the surrounding Str consisted of genes in modules C, F, G, and I. Area X was the only song nucleus that did not have a specialized module unique to it relative to other song nuclei (**Fig. 2a**). In non-vocal learning juvenile females, interestingly LMAN was specialized relative to AN by the same gene modules as in males (B, F, and I) as well as an additional module G (**Fig. 2b**); RA was specialized by module A as in males, but not module L and by additional modules A and G. In contrast, neither juvenile female HVC nor Area X exhibited significant gene module expression specializations relative to their surrounds.

E2-responsive gene modules in song nuclei

We next assessed the effects of chronic exogenous estrogen on the developing song system. In the E2-treated juvenile males, the song nuclei specialized modules overlapped to those seen in vehicle-treated males (**Fig. 2a vs 2c**). Differences were that: in LMAN and Area X module I was no longer present; in RA module L was no longer present, module A appeared as seen in females;, and module E also appeared. These differences in gene module specializations in the male song system are consistent with the E2-treatment regimen used causing a slight decrease in vocal learning accuracy in males[29].

In contrast, E2-treated females more closely mirrored the gene module specializations seen in the vehicle treated male song system (**Fig. 2a vs 2d**). Specifically, in LMAN, modules B, F, and I were retained similar as in vehicle-treated males, and module D uniquely appeared; in HVC, module G expression appeared and was strongly specialized as in vehicle-treated males, but modules C and I appeared relative to vehicle-treated females (**Fig. 2b** vs 2d); in RA, module G disappeared and module C was retained as in vehicle-treated males, and module A was retained and module K appeared relative to vehicle-treated females.; and in Area X, only module C appeared specialized in the E2-treated females. That is, the transcriptional response of female song nuclei to E2-treatment appeared to be far more dramatic than in males.

We performed an additional test for E2-induced changes to gene module expression in females by comparing E2-treated song nuclei in females to the combination of E2-treated surrounds from the same animals, vehicle-treated female surrounds, and vehicle-treated female song nuclei (**Fig 2e**). This analysis represents a comparison between samples from vocal learning capable female samples and non-vocal learning female samples at each node. It revealed 1 to 3 modules in each song nucleus of E2-treated females that were a subset of some of the strongest correlated

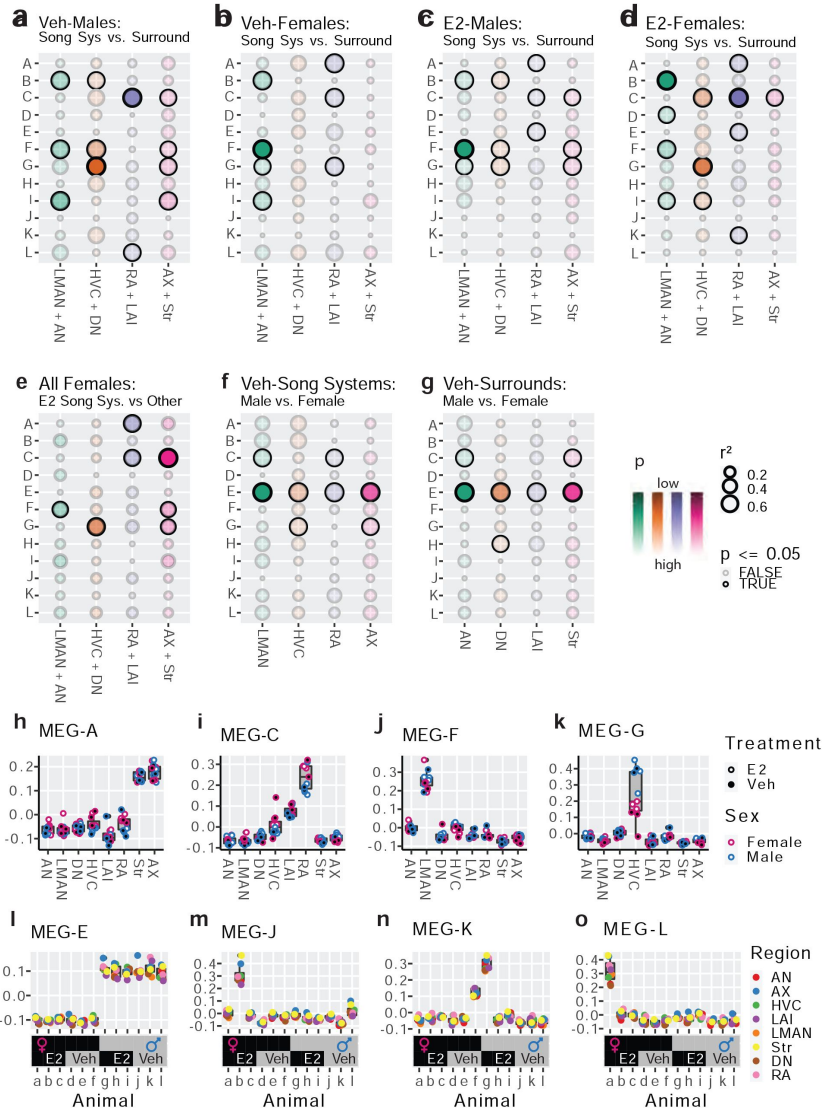


Figure 2

Association of modules to experimental variables.

a-g, Bubble plots show- ing statistical association between MEG expression and variables of interest in various sample subsets. Strength of association (r^2) is encoded by bubble size, significance (p) is encoded in the color scale with significant associations darkly bordered. Pearson correlation and Students t test, alpha=0.05. Plots show the associations between gene modules (rows) to; **a-b,** vehical treated song system specializations, comparing MEG expression in song system samples from either sex to their appropriate surrounding controls; **c-d,** E2-treated song system specialization, same comparison as **a-b** but within E2-treated samples; **e,** female vocal learning capacity after E2, comparing E2 treated female song system compo- nents to all other female samples from that circuit node; **f,** sexual dimorphism within the song system, comparing vehicle treated male and female song system components; **g,** sexual dimorphism within the surrounding control regions, comparing the vehicle treated male and female surrounding control samples. Each neural circuit node is considered separately (columns). **h-k,** Expression of modules with strong region specific expression. Module A is highly expressed in Str and Area X samples, with addtional differences between RA and LAI (**h**); Module C is highly expressed in the arcopallium, especially RA, with some increaase in HVC (**i**); Module F is expressed highly only in LMAN regardless of sex or treatement (**j**); Module G is only highly expressed in HVC, where it differs both by sex and treatment (**k**). **l-o,** Expression of selected module eigengenes by animal (top) aligned to their respective experi- mental variables (bottom), color indicates region. The sex chromosome enriched module E was highly expressed in all male samples and depleted in all female samples regardless of brain region or pharmacological treatment. Module J, K, and L eigengenes were each highly expressed in samples from one (J and L) or two (K) animals across all brain regions sam- pled.

modules found in control males (**Fig. 2a vs 2e**). These were: module F in LMAN; module G in HVC; module C in RA; and modules C, F and G in Area X. That is, these are four core modules for song nuclei that are the most sensitive to E2-induction or enhancement in females and most associated with the presence of vocal learning in females.

Gene modules for telencephalic sex differences

We next tested whether some modules could be explained by sex differences in the brain, regardless of song nuclei presence or vocal learning status. We compared vehicle-treated male and female song nuclei and surrounds separately, and found that module E strongly correlated with higher expression in all male brain regions relative to females (**Fig. 2f,g**). Among the song nuclei, module G eigengene expression was significantly higher in male HVC and Area X; conversely, module C expression was higher in female LMAN and RA (**Fig. 2f**). Among the surrounds, module H eigengene expression (not significant in any other comparison) was significantly higher in female DN and module C was significantly higher in female AN and Str (**Fig. 2g**). These findings indicate that module E contains genes that could be explained by a broad transcriptome sex difference in the brain, whereas the expression of other modules are more specific to brain region and/or treatment.

Gene modules with region and bird specific expression

We next quantified the magnitude of module expression differences and observed several gene modules with clear region-specific expression patterns (**Fig. 2h-k**). Module A, which appeared specialized in RA relative to LAI in several comparisons, was highly expressed in all Area X and Str samples, regardless of treatment or sex (**Fig. 2h**). Interestingly, *FOXP2*, a gene critical for spoken-language and vocal learning in humans[41] and songbirds[42], was a potent member of module A, correlating with the eigengene at $r^2 = 0.92$ across all samples. Module C, which was specialized to RA, HVC, and Area X relative to their surrounds and sexually dimorphic in LMAN and Str, was most highly expressed in arcopallial samples, especially RA, with some increase in HVC (**Fig. 2i**). Module F, which was specialized to LMAN relative to its surround in all such comparisons, was highly expressed exclusively in LMAN regardless of sex and E2-treatment (**Fig. 2j**). Module G, which was specialized to HVC in a sex- and E2-dependent manner, was only highly expressed in HVC samples, with males having higher expression than females, and females further split by treatment (**Fig. 2k**).

We also checked if genes in specific modules were enriched in their expression in specific animals or divisions of animals regardless of region. The module E eigengene was highly expressed in all male samples and lowly expressed in all female samples regardless of brain region or treatment (**Fig. 2l**), consistent with brain sex differences (**Fig. 2f,g**). At the other extreme, three small modules showed higher expression specific to individual birds: module J was highly expressed in all samples of animal ‘b’, an E2-treated female (**Fig. 2m**); module K was highly expressed across all samples of animal ‘f’ vehicle-treated female and at roughly half-dose in animal ‘g’, an E2-treated male (**Fig. 2n**); module L was highly expressed in animal ‘a’, another E2 treated female (**Fig. 2o**). These findings indicate that the three smallest modules, J, K and L, although with some patterning to RA for the later two (**Fig. 2a,d**), are strongly animal specific in their expression. As we can think of no source of technical variation that would produce a broadly distributed shift in the neural expression of a single gene module, this variation is likely attributable to biological interindividual variation, whether this be genetic or from life history we cannot say from the present data.

Functional enrichment of specialized modules

We sought to understand the cumulative biological function of genes among the modules. To do this, we mapped the zebra finch genes to their 1:1 human orthologs where possible and then used human gene annotation to examine the Gene Ontology (GO) functions enriched within each

module's constituent genes. Of the 12,444 module assigned genes, 7,909 (63%) had 1:1 human orthologs annotated in Ensembl. We found GO terms significantly enriched in module G (**Table 1**), which included "DNA binding transcription factor activity", "cell differentiation", "anatomical morphogenesis", "cell-to-cell signaling", and "positive regulation of multicellular organism growth", indicating that module G genes specialized in male and E2-treated female HVC and Area X potentially act to integrate and differentiate late born neurons. Other significantly enriched terms were "extracellular matrix structural component", "external side of the plasma membrane", and "extracellular space", indicating that this module may also act to restructure the extracellular matrix, perhaps to accommodate new cells. Module E, which was differential between the sexes for both song nuclei and surrounds, had 6 significantly enriched terms, of which 3 pertained to DNA damage repair: "nucleolus", "transcription, DNA templated", and "U2-type precatalytic spliceosome" (**Table 2**). These results in module E indicate that there is a sexually dimorphic gene expression program broadly distributed across the finch telencephalon that likely acts within the nuclear environment. A full table of GO enrichments by module can be found in supplemental data (supplemental_sig_go_enrich.csv).

Gene modules enriched for human speech associated genes

We next asked if any of the modules were enriched for genes previously determined to be convergently specialized in songbird song nuclei and human speech brain regions[8,40]. These gene lists included the transcriptional convergence between RA and human dorsal laryngeal motor cortex (dLMC), HVC and dLMC, LMAN and dLMC, Area X and the anterior caudate, and Area X and the anterior putamen (Gedman et al. Fig3B,D[9]). We found that module B, specialized to both LMAN and HVC (**Fig. 2a**), was enriched for the convergently specialized genes in human dLMC (**Fig. 3a**) known to match the uppers layers of the cortex[9]. Module C, specialized to RA (**Fig. 2a,i**), was enriched for the genes convergently specialized in dLMC and RA (**Fig 3a**) known to match the lower layers of the cortex[9]. Module A, highly expressed in Area X and Str (**Fig. 2h**), was enriched for genes convergently specialized in Area X and human anterior striatum (both caudate and putamen; **Fig. 3a**). Module I, specialized to Area X (**Fig. 2a**), was also even more strongly enriched for the same convergences as module A (**Fig. 3a**). These findings indicate that the genes previously identified as convergently regulated between songbird song brain regions and human speech brain regions are components in the larger specialized gene networks identified here using WGCNA.

Gene modules enriched for specific chromosomes

We next determined whether any modules were enriched in genes from specific chromosomes. We performed a bootstrapped enrichment analysis, randomizing the mapping between genes and modules 50,000 times to approximate null distributions. P values for each chromosome-module pairing were then FDR corrected. To do this as accurately as possible, we performed this analysis using revised chromosomal assignments and structure from the most recent zebra finch genome assembly, bTaeGut_1.4.pri, which better resolves the microchromosomes[43]. Surprisingly, we found that each of the 12 modules were enriched for genes from at least one chromosome (**Fig. 3b**). The most striking was module E genes, which were enrinched on the Z and W sex chromosomes, with nearly all W expressed genes and ~⅔ of Z expressed genes being members of module E. This is consistent with module E exhibiting higher expression in male samples regardless of treatment or region (**Fig. 2d-e**). For the autosomes, we observed significant enrichments of two HVC specialized gene modules on chromosome 1A: module B (specialized in male HVC and human dLMC); and module G (specialized in male HVC and E2-treated female HVC and sexually dimorphic in vehicle-treated HVC). These results are particularly interesting given that zebra finch chromosomes 1 and 1A are believed to be the result of a songbird-specific fragmentation of the ancestral chromosome 1 found in chickens[44,45].

Table 1

Module G functional enrichment analysis.

Significantly enriched GO terms within the 1:1 human orthologs of module G. Lists full GO term, GOid, and uncorrected p value calculated by GAGE.

Module G: Significantly Enriched GO

GOterm	GOid	p
DNA-binding transcription factor activity, RNA polymerase II-specific	GO:0000981	0.012
cell differentiation	GO:0030154	0.013
DNA-binding transcription factor activity	GO:0003700	0.015
extracellular matrix structural constituent	GO:0005201	0.024
inflammatory response	GO:0006954	0.026
anatomical structure morphogenesis	GO:0009653	0.03
external side of plasma membrane	GO:0009897	0.036
extracellular space	GO:0005615	0.037
DNA-binding transcription activator activity, RNA polymerase II-specific	GO:0001228	0.04
cell-cell signaling	GO:0007267	0.041
positive regulation of multicellular organism growth	GO:0040018	0.042
transmembrane transporter activity	GO:0022857	0.043

Table 2

Module E functional enrichment analysis.

Significantly enriched GO terms within the 1:1 human orthologs of module G. Lists full GO term, GOid, and uncorrected p value calcu-lated by GAGE.

Module E: Significantly Enriched GO

GOterm	GOid	p
nucleolus	GO:0005730	0.024
transcription, DNA-templated	GO:0006351	0.031
DNA repair	GO:0006281	0.037
damaged DNA binding	GO:0003684	0.044
U2-type precatalytic spliceosome	GO:0071005	0.047
cellular response to DNA damage stimulus	GO:0006974	0.048

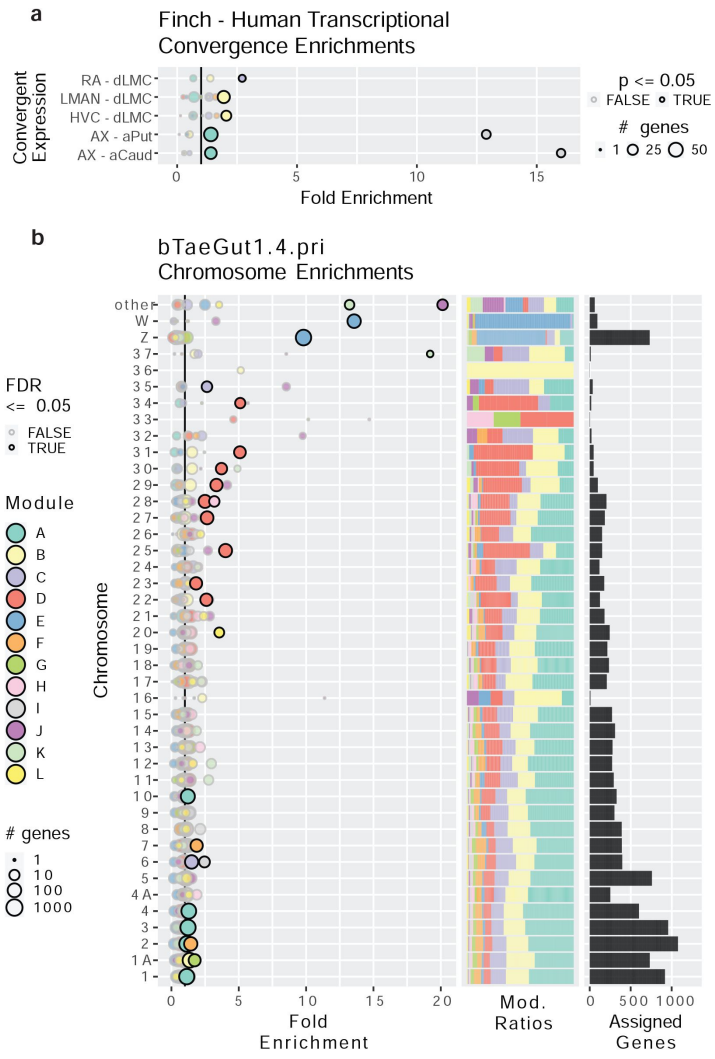


Figure 3

Gene module enrichments for human convergent signature and for chromosomes.

a, Enrichment of genes previously found to be convergently differentially expressed in the human laryngeal motor cortex and the pallial song nuclei or convergently expressed between the human vocal striatum and Area X. Bubble size linearly encodes the number of genes in each convergence module pairing. Significance was assed using a one-tailed GAGE test, similar to GO ontologies, alpha=0.05. Significant enrichments are darkly bordered and opaque. Values to the right of the vertical black line indicate above random chance. **b,** Enrichment of genes from specific chromosomes. Left, fold enrichment of modules onto zebra finch chromosomes in the newest genome assembly available; center, the portion of module assigned trascripts from each chromosome per module; right, the number of module assinged genes per chromosome. Each row is a chromosome with each bubble representing the enrichment of transcripts from that chromosome in one of the gene module defined by WGNCA. Values to the right of the vertical black line indicate above random chance. The size of the bubbles indicates the log10 transformed number of genes in each chromosome module pairing. Significance was assessed using an FDR corrected boot- strapped test of observed enrichment for each module chromosome pairing based on 50,000 randomizations of genes into modules. Significant enrichments are darkly bordered and opaque. **a-b** use the same color scale for modules.

Module A, the largest module, which was highly expressed in Area X and adjacent striatum, was enriched across 5 macro-chromosomes (chr1, 2, 3, 4, and 10). Module F, which was strongly expressed in LMAN, was enriched on chromosomes 2 and 7. Modules C (most strongly expressed in the arcopallium and part of RA and Area X specializations) and I (a component of the LMAN specialization) were enriched on chromosome 6 and microchromosome 35. Module D, a component of the LMAN specialization in E2-treated females, was enriched across 8 small microchromosomes (chr22, 23, 25, 27, 28, 29, 30, 31). Most (4 of the 5) of the smallest modules in gene counts were enriched in the microchromosomes: Module H, which was sexually dimorphic only in DN, was enriched on microchromosome 28; module K was significantly enriched on microchromosome 37 and in the "other" category, which includes all remaining unnamed DNA scaffolds in the assembly such as further microchromosomes; module J was also enriched in "other".

Sex chromosome gene expression across regions

To better understand the relationship between the sex chromosomes and module E, we examined the distribution of membership in module E with the sex chromosomes separated. WGCNA allows us to consider gene membership in a module as a continuous variable, rather than a binary variable, by correlating each gene's expression profile to the module eigengene. Doing this for module E, we found that Z transcripts were positively correlated to the module eigengene while W transcripts were anticorrelated (**Fig. 4e**). This is consistent with Z chromosome transcripts were generally lower expressed in females relative to males, while W chromosome transcripts were only expressed in female brains. This general reduction in female Z chromosome transcript abundance within module E is consistent with the finding that diploid Z chromosomes in male birds do not have one copy inactivated to compensate for gene dosage, unlike the X inactivation in female mammals[34,46].

Given the sexually dimorphic expression of module E across brain regions and the enrichment of the sex chromosomes within that module, we separately compared the expression of sex chromosome genes in the non-vocal motor surrounds of vehicle-treated control males and females regardless of WGCNA assignment to better understand sex chromosome expression without the influence of vocal learning specialization or E2 response. To do this, we first tested for correlated expression between each sex chromosome transcript to the animals' sex within each region such that female-enriched transcripts were positively correlated and male-enriched transcripts were anticorrelated with sex (**Fig. 4f,g**). In these gene sets, we identified between 73 and 82 significantly expressed W chromosome genes and between 433 and 527 significantly depleted Z chromosome genes for each of the surround regions in males relative females. Examining the union of these gene sets revealed that a total of 95 W and 694 Z chromosome genes were differentially expressed between sexes in at least one brain region, 62% and 65% of annotated sex chromosome genes respectively. Conversely, the intersection of these regional gene sets contained 58 significantly expressed W chromosome genes and 306 significantly depleted Z chromosome genes in all non-vocal regions in females, representing 38% and 29% of annotated sex chromosome genes, respectively (**Fig. 4h-i**, **Table S2**). This indicates that there is both a large broadly distributed set of sex enriched/depleted sex chromosome genes and regional patterned sex chromosome gene expression.

Modeling vocal learning and sex chromosome module interactions

As it is unlikely that the gene modules act independently of each other, we sought putative interacting genes between two modules of interest, module G specialized in HVC and Area X and module E dominated by sex chromosome genes. To do this, we again replaced binary in-or-out module membership with continuous module membership by correlating each genes' expression to the relevant module eigengene[38]. This allowed us to quantify the extent to which any gene was associated with any module, regardless of initial assignment[33]. Looking across all assigned genes for our modules of interest, we identified two outlier genes, *PDE8B* and *HABP4*, which were

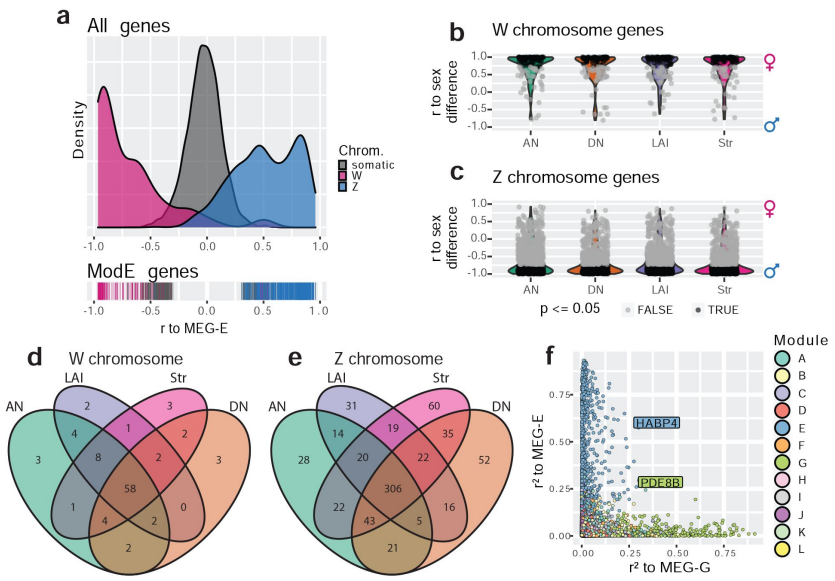


Figure 4

Brainwide signatures of sex chromosome expression.

a, Distribution of continuous membership in module E across all module assigned genes (top) and module E assigned genes (bottom) based on corelation of expression to the module eigengene (Pear- son r to MEG-E) with sex chromosomes separated. **b-c**, Distribution of sex chromosome gene expression correlations to the sex difference in vehicle treated finches. Postive correla- tions indicate female biased expression while anticorrelations indicate male biased expres- sion. Significance was assessed in each region using an upper-tailed student's correlation test for W chromosome transcripts (**b**) and lower-tailed for Z chromosome transcripts (**c**), with significant correlations in black, alpha=0.05. **d-e,** Venn diagrams intersecting the significantly sex difference correlated genes across non-vocal surround regions for the W and Z chromo- somes respectively. **f,** Comparison of continuous membership in module E (r2 to MEG-E, y-axis) and module G (r2 to MEG-G, x-axis) across all 12,444 module assigned genes.

the most E associated gene assigned to module G and the most G-associated gene assigned to module E, respectively (**Fig. 4j**). Both genes were significantly upregulated in male HVC relative to its surround, but not in female HVC of either treatment. *PDE8B* catalyzes the hydrolysis of the second messenger cAMP and mutations to the gene cause an autosomal dominant form of striatal degeneration in humans[47]. *HABP4* is an RNA binding protein, known to repress the expression and subsequent DNA binding of *MEF2C*[48], a Z chromosome transcription factor which has undergone accelerated evolution in songbirds[49] and whose repression by *FOXP2* is critical for cortico-striatal circuit formation in mice related to vocal behaviors[50]. Both *PDE8B* and *HABP4* are found on the Z chromosome and *HABP4* was one of the 694 Z transcripts significantly reduced across all brain regions in vehicle-treated females relative to males (**Table S2**). These findings again indicate that Z chromosome genes may be subject to multiple gene regulatory programs: the broadly distributed brain transcript reduction driven by reduced sex chromosome copy number; and the specialized upregulation in HVC for vocal learning behavior.

Sex chromosome dosage effects by module

To better understand the relationship between gene modules, sex chromosome gene expression levels, sex chromosome dosage, and song nuclei specializations, we directly compared the abundance of Z and W transcripts between vehicle-treated male and female samples in surrounds and song nuclei. After averaging across samples for each brain region, and combining them for all brain regions, Z chromosome transcripts were present in both females (pink) and males (blue) as expected (**Fig. 5a**). Although more Z chromosome genes were assigned to module E, many were assigned to the other modules (**Fig. 5a**), consistent with our chromosome mapping without enrichment analysis (**Fig. 3b**). However, there were clear male vs female expression differences apparent for Z chromosome genes in module E, but no obvious sex differences for Z chromosome gene expression in other modules. In contrast, W chromosome transcripts were mainly expressed in females (pink) and not males (blue), and were generally restricted to module E (**Fig. 5b**), also consistent with our chromosome enrichment analysis (**Fig. 3b**). To further quantify these effects outside and inside of the song system, we computed the percent of total expression for each sex chromosome gene which came from male samples (male_avg/(male_avg+female_avg)) and compared these distributions to those predicted by chromosome dosage. The expected average percentages based upon dosage are 66.6% male expression for Z chromosome genes (2 Zs in males vs. 1 Z in females; **Fig. 5c-f**, red lines) and 0% male expression for W chromosome genes (0 W in males vs 1 W in females; **Fig. 5g-j**, red lines).

Module E assigned Z chromosome genes were expressed at almost exactly the ratio predicted by Z chromosome dosage on average; 65.1% measured versus 66.6% predicted, and the most abundantly expressed transcripts (largest circles) were expressed closest to the dose prediction in both surrounds (**Fig. 5c,d**) and song nuclei (**Fig. 5e,f**). For non-E modules, their Z chromosome transcripts were intermediate between equal expression and the prediction from chromosomal dosage in surrounds (**Fig. 5c,d**), with Z chromosome genes in module D and H were the least male biased across regions (**Fig. 5e,f**). One major difference between surrounds and song nuclei was the Z chromosome gene expression levels again in module G. The 25 Z chromosome genes in module G were more male biased in song nuclei compared to surrounds (**Fig. 5c,d vs e,f**) and were the only set of Z chromosome genes that were male biased above the predictions from dose on average (**Fig. 5e**). These findings indicate that the expression levels of Z chromosome genes in module E were predominantly dose regulated, with the abundance of the RNA dictated by the presence versus absence of those specific chromosomes regardless of brain region sampled. However, Z chromosome genes in the other modules whose expression is enriched for one or several brain regions, animals, or treatments exhibited varying degrees of dosage compensation to overcome the Z chromosome difference.

In contrast, W chromosome genes assigned to module E had on average 6.8% male read mapping counts (median 0.5%; **Fig. 5g,h**). Non-module E assigned W genes had 33.0% male reads mapped (median 32.7%; **Fig. 5g,h**). As the values should be 0% reads from males mapped to the W

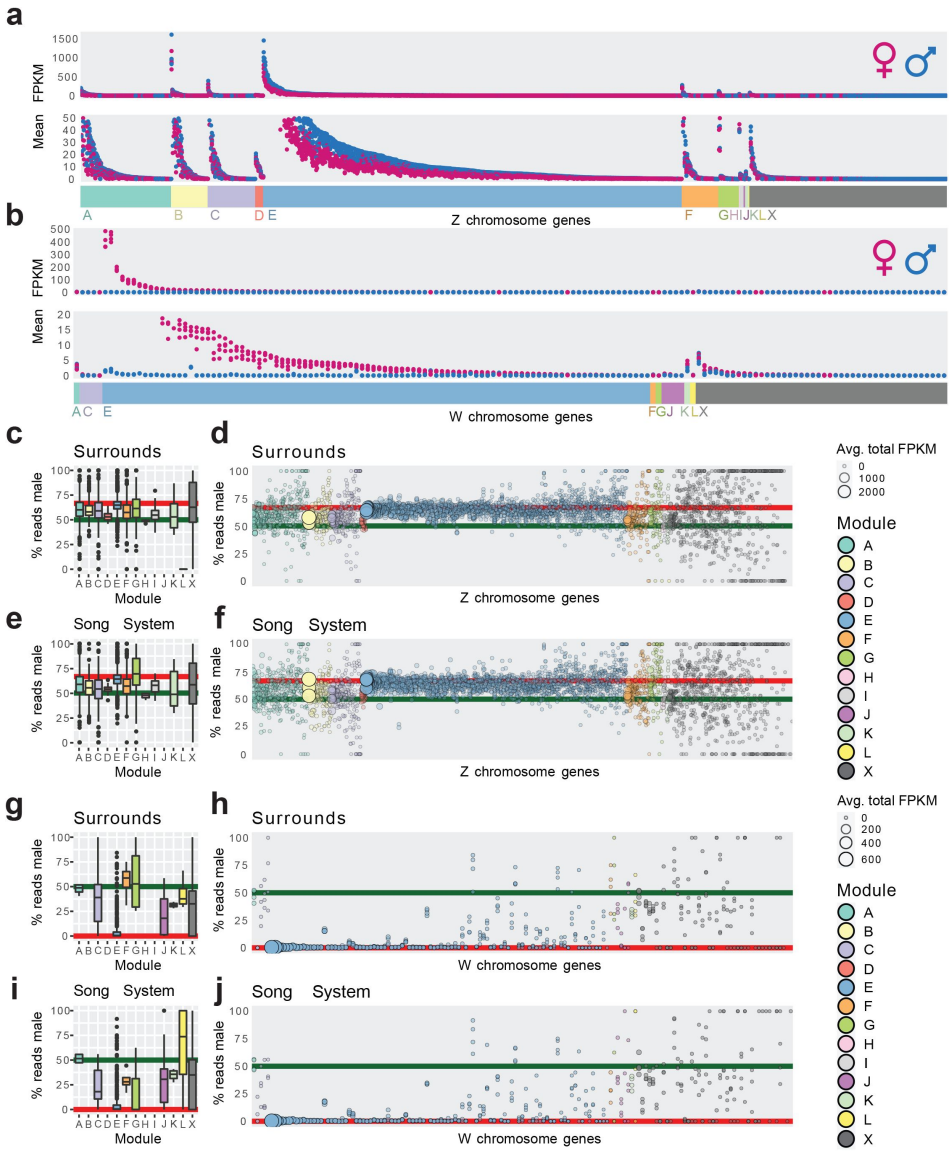


Figure 5

Sex chromosome dosage effects by module.

a, Scatter plot of transcript expression abundance for all Z chromosome genes (y-axis) ordered by module assignment and then expression (x-axis). Each dot is one gene with expression level measured as the average of one brain region in vehicle-treated male birds (blue, n= 3)) or female birds (pink, n = 3)). The upper graph shows all genes while the lower graph has a cut-off y axis to better show lowly expressed genes. **b,** similar as **a**, except for all W chromosome genes. **c,** Boxplots showing the distribution of the percent reads from male surrounds per Z chromosome gene from **a** (y-axis) and their module assignment (x-axis). Red line indicates the male read percentage expected for Z chromosome genes, 66.6%; green line indicates equal expression between sexes. **d,** Bubble plot of the underlying Z chromosome single gene data from **c** X-axis is ordered according by module assignment, encoded by bubble color, and expression. Bubble size and opacity indicates cumulative average expression (male avg. FPKM + female avg. FPKM), with higher expressed genes being larger and more opaque. **e-f,** same as **c-d** but for song nuclei. Note that the Z chromosome genes of module E are expressed on average at almost exactly the sex ratio predicted by dosage while Z chromosome genes in other modules show some degree of compensation. **g-j,** same as **c-f**, except for W chromosome genes. Red line indicates the male read percentage expected for W chromosome genes, 0%; green line indicates equal expression between sexes.

chromosome, we believe this male read mapping is from less divergent transcript paralogs from the Z chromosome that map to the W chromosome. Examining the data at the level of single genes, we observed that module E assigned W genes were far more likely than non-module E genes to show no- or little- putative Z paralog mapping (more genes on or near the 0% line; **Fig. 5g,h**). Within module E, the genes with the lowest expression (smaller circle size) were the most likely to have putative paralogous expression (the lowest ~⅓ of these genes contributed to the majority of expression above 0%, **Fig. 5h**). We observed similar results in module E versus other modules for W expression in song nuclei (**Fig. 5i,j**). While the male read mapping of the W genes in modules F, G, and L do appear to shift in the song system relative to the surrounds, each of these modules contains a single W gene and all three of these genes are lowly expressed (**Fig. 5b**).

These results demonstrate that rather than being confounded by chromosome dose, WGCNA allowed us to resolve the effects of dose in an unbiased way. Module E grouped together the un-dosage-compensated Z chromosome and W chromosome genes across brain regions. In contrast, the Z chromosome genes placed in modules specialized for one or more song nuclei had some level of dosage compensation in males. This compensation appeared regionally patterned for Z chromosome genes in module G (enriched in 2 or more song nuclei depending on treatment), which were specialized beyond the normal chromosome dosage only within the song system. Average expression values of all sex chromosome genes for each brain region from vehicle-treated animals can be found in supplemental_z_chromosome_express.csv and supplemental_w_chromosome_express.csv.

Candidate gene drivers of HVC specialization in E2-treated females

To reduce the 344 genes in module G to the putative drivers of HVC development, we next examined the relationship of membership in module G (correlation to MEG-G) to gene expression specialization in HVC at the level of single genes. We did this by testing for correlations between individual gene expression and the specialization of HVC in males (vehicle- and E2-treated) or E2-treated females in each of the following four comparisons: 1) male HVC specialization relative to the surround; 2) E2-treated female HVC specialization relative to the surround; 3) male HVC relative to female HVC in vehicle-treated controls; and 4) E2-treated female HVC specialization relative to vehicle-treated female HVC. For all four comparisons, we found the higher the correlation of module G genes to the MEG-G, the higher the correlation with the vocal learning specialization (**Fig. 6a-d**). This means that their expression was higher in male or E2-treated female HVC relative to the appropriate non-vocal learning controls across all comparisons. We identified genes of interest as being strongly correlated ($r^2 \geq 0.5$) to both the module G eigengene and vocal learning specialization (**Fig. 6e-h**, higher magnification view of colored boxes in **Fig. 6a-d**). All genes of interest exhibited a positive correlation with song nuclei gene expression specializations across all four comparisons (**Fig. 6a-d**). We next generated a core gene list from module G that correlates with both in E2- and sex-dependent HVC expression by intersecting these four vocal learning specialized gene sets (**Fig. 6i**). We found a core set of 14 genes that strongly marked vocal learning capable HVC in all comparisons and strongly correlated with the aggregate of module G expression (**Table 3**, Fig. S6). The results of each individual comparison can be found in supplemental_hvc_specializations.xslx.

We examined the chromosomes of origin for these 14 core genes and found that three (*GHR*, *RGS7BP*, and *THBS4*) were on the Z sex chromosome (**Fig. 6j**). This was a >3-fold significant enrichment over chance of Z chromosome transcripts ($p = 0.009$, upper-tailed hypergeometric test) against a background of module assigned genes. This result was statistically significant regardless of the background gene set, when using all genes (~21,000) or only module G members (344). These three Z chromosome module G genes in HVC not only exhibited >50% reduced expression in control female HVC relative to males, but also exhibited upregulated expression in male HVC relative to the surrounding DN regardless of treatment and upregulation in E2-treated female HVC relative to either the surround or vehicle-treated female HVC (Fig. S6). In the context of our cross-region sex chromosome analysis (**Fig. 3c-f**), only *RGS7BP* was sexually dimorphic outside of the

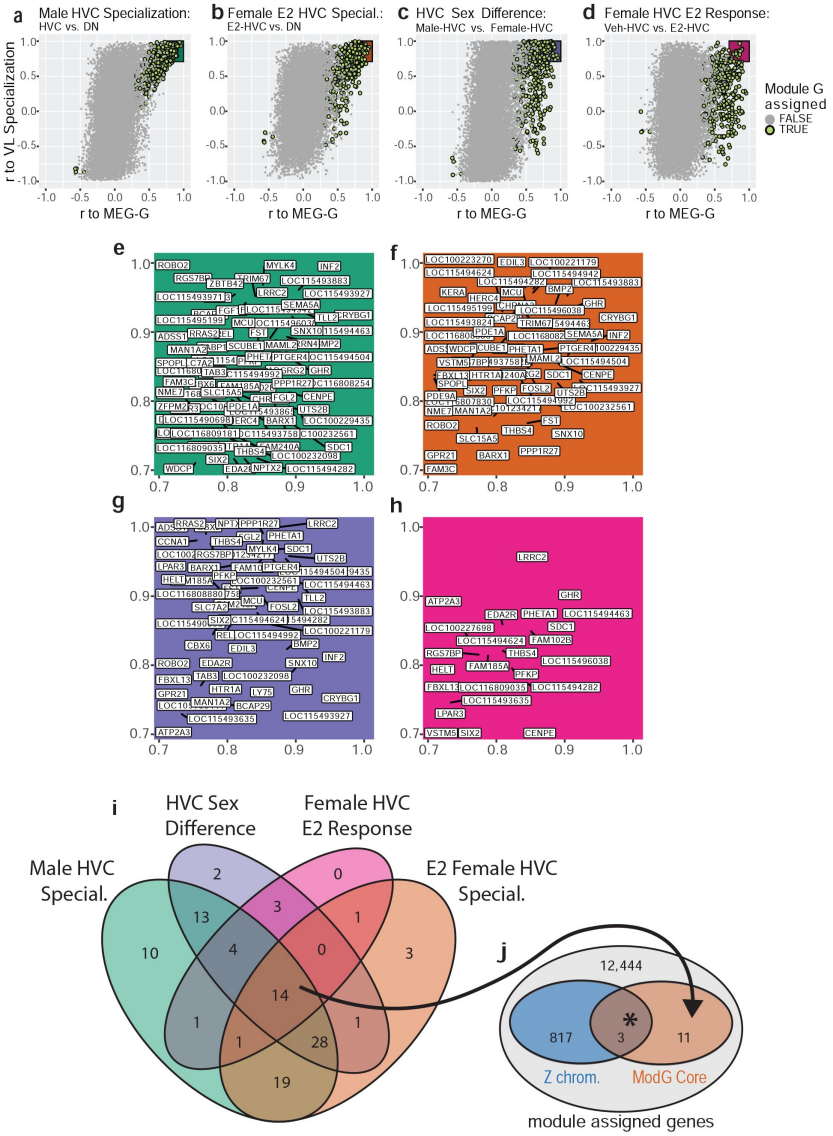


Figure 6

Identification of core genes in module G and their association to the Z chromosome.

a-d, Single gene continuous membership in module G (x-axis; Pearson r to MEG from module G) for all assigned genes vs correlation to vocal learning in masculine or mas-culinized HVC relative to samples from non-vocal learning females in each of the four com-parisons; **a,** male song system membership, comparing individual gene expression in male HVC samples of either treatment to expression in the surrounding DN; **b,** female vocal learn-ing capacity after E2, comparing E2 treated HVC to all other female DN or HVC samples; **c,** sexual dimorphic gene expression within the song system, comparing vehicle treated male and female song system components; **d,** estradiol responsive gene expression in female HVC, comparing E2 treat and vehicle treat female HVC samples. Each point is a gene colored by module assignment, the shaded area indicates gene of interest criteria for each comparison. **e-h,** Blowup of shaded regions in **a-d** respectively showing genes of interest from each comparison. **i,** Identification of core genes by intersecting the four gene sets of interest. **j,** Enrichment of Z chromosome transcripts within the core genes. * indicates p = 0.0087 by an upper- tailed hypergeometric test.

Module G: Core VL Genes

Z Chromosome	Other	cont.
GHR	CENPE	LOC115494624
LRRC2	EDA2R	PFKP
RGS7BP	FAM102B	PHETA1
THBS4	FBXL13	SDC1
	LOC115494282	SIX2
	LOC115494463	

Table 3

Core genes of module G specialization to vocal learning HVC.

Putative drivers of module G specialization to vocal learning capale HVC. Intersection of the four gene of interest sets (**Fig. 4b**), separating the significant enrichment of genes from the Z sex chromosome.

song system, being absent in all female surround regions. Neither *GHR* (growth hormone receptor) nor *THBS4* were significantly depleted in any surround region comparison. Taken together, these results indicate that the 3 core genes in vocal learning capable HVC on the Z chromosome are subject to additional E2 sensitive transcriptional regulation in HVC, separate from the Z chromosome transcript reduction seen throughout the female brain.

Of the 14 core genes total, several have been previously studied in the brains of other species which may inform their role in vocal learning systems. *THBS4* encodes a secreted extracellular-matrix glycoprotein necessary for appropriate neuronal migration in the mouse[51] and is elevated 6-fold in the human cortex compared with non-vocal learning primates[52]. Human *EDA2R* was recently identified as a top correlate of cognitive performance and brain size[53]; it was also found in a human GWAS study that correlated it with circulating estrogen and testosterone levels[54]. Rare mutations in human *PHETA1* lead to Lowe oculocerebrorenal syndrome, that includes pathophysiology in seizures, mental retardation, and structural brain abnormalities[55,56]. *SIX2* is a homeobox domain containing transcription factor that governs early brain and craniofacial development and provides neuroprotection from dopamine injury[57,58]. *GHR* encodes a transmembrane receptor whose activation controls cell division[59]. The gene which encodes GHR's ligand, growth hormone (GH), is interestingly duplicated and undergoing accelerated evolution in the genomes of songbirds, is upregulated in the zebra finch auditory forebrain following the presentation of familiar song, and exerts anti-atrophic influence during chicken development[60–62]. Based on these findings, we consider *GHR* as the most likely candidate gene related to the E2 sensitive atrophy of HVC in females.

Discussion

The present study seeks to further our understanding of sexually dimorphic vocal learning in zebra finches by comparing the gene expression specializations in the song system between male and female birds at PHD30 and in response to E2-treatment, at the onset of atrophy of the female vocal circuit. The birds were given either a vehicle or E2 from hatching, which induces rudimentary vocal learning behavior in females where it would otherwise be absent. In control females, HVC appeared unspecialized at the level of gene module expression, with no significantly differentially expressed MEGs compared to the surrounding nidopallium. However, in E2-treated females, HVC exhibited a subset of the observed male HVC gene expression specializations. Similarly, in the vehicle-treated females, the striatum located where Area X would be also lacked any specialized gene module expression, but the E2-treated female Area X had a subset of specialized gene expression as in males. This contrasts with RA and LMAN which were similarly specialized in males and females in the absence of E2-treatment. Given that lesions of HVC prevent the emergence of Area X in E2-treated females[32], these results support a model of zebra finch development where transcriptomic masculinization of female HVC by E2 is a critical event which facilitates the emergence of female Area X and ultimately endows these females to produce some rudimentary learned song.

How did E2 treatment produce the transcriptomic effects we observed with module G in female HVC and what are the implications regarding sexually dimorphic vocal learning in the zebra finch? One possibility is that this process begins with increased estrogen receptor (ER) activity within developing HVC cells after being provided surplus activating ligand[63], followed by altered transcription of ER targets in the genome. Module G contained the androgen receptor (**Table S1**), which is believed to be a major downstream effector of the E2 response in female zebra finches[33]. This initial transcriptional loading of the system would then have been processed by gene regulatory networks within each cell, spreading in effect through the transcriptome. It is possible that differential module G expression arose purely from traditional gene regulatory networks, where transcription factors form complex, elaborate feedback networks with themselves and the genes they regulate. However, this framework fails to explain

why the core 14 vocal learning correlated genes from module G in HVC were enriched for transcripts from a single chromosome: the Z sex chromosome with halved copy number in females.

We hypothesize that the Z chromosome genes identified here are co-regulated, necessary components of a growth-enabling transcriptional program downstream of ER, represented by module G. This module G transcriptional program could be specialized to developing male HVC by the expression of patterning genes, such as *SIX2*, early in development and maintained through persistent *GHR* signaling. We propose that these Z chromosome transcripts in module G are reduced in females by lower haplotype dosage during development and thus fail to specialize female HVC. Due to lower abundance of gene products from module G, female HVC may be unable to accommodate new neurons during juvenile development and fail to facilitate emergence of its downstream target Area X. Similarly, without a sufficiently developed HVC, RA lacks one of its major inputs and may subsequently atrophy. We propose that E2 masculinizes female song behavior by increasing the abundance of these module G transcripts in HVC, increasing specialized HVC growth, and facilitating emergence of HVC's other major target, Area X (**Fig 7**). This model of sex chromosome influenced song system development is consistent with recent work comparing male and female zebra finch transcriptomes from RA at young juvenile (PHD20) and young adult (PHD50) ages in un-manipulated birds (Friedrich et al. 2022)[64]. While that study proposed that the role of the sex chromosome in maintaining transcriptomic sex differences diminishes across development as the proportion of specialized genes that originate on the sex chromosomes diminishes, this effect was driven by large increases in differentially expressed autosomal genes rather than by any reduction in sex chromosome dimorphism; the percentage of differentially expressed Z chromosome genes increased from 28% at PHD20 to 39% at PHD50[64]. This leads us to conclude that sexually dimorphic Z chromosome expression in juveniles precedes the sexually dimorphic expression of the autosomes seen in adults. This is consistent with our hypothesis that sufficient expression of select Z chromosome gene products (GHR, etc..) is necessary for subsequent autosomal song system specializations (module G). Further, our results are consistent with GH's known role in avian neuroprotection, with elevated signaling associated with the survival of chicken neurons during rounds of pruning in the developing retina[65].

Our results help refine the traditional notion that hormonal signaling organizes the brain to produce sexually dimorphic behaviors independent of neuronal sex chromosome content[66,67]. Instead, our data indicate that sexual dimorphism of the zebra finch song learning ability was likely established by the interaction of sex hormone signaling and sex chromosome gene expression within HVC during development. These findings are thematically similar to work in the Four Core Genotypes mouse model[68], where chromosomal and gonadal sex are separable by translocating the sex determining *Sry* gene. In these mice, sex chromosome composition regulates sexually dimorphic brain gene expression, circuit anatomy, and behaviors[68–71], though the sex chromosome genes responsible remain unknown. Additional experiments manipulating the candidate genes implicated here in developing HVC to both mimic and prevent the action of E2 in female zebra finches are needed to test these hypotheses.

In addition to these vocal learning focused results, performing unbiased gene network analysis with samples taken throughout the zebra finch telencephalon in both sexes revealed a surprisingly strong relationship between the modular organization of telencephalic gene expression and the chromosomal structure of the genome. Each of the 12 modules identified by WGCNA were enriched for genes from at least one chromosome. Of these significant enrichments, those on the micro- and sex- chromosomes stood out as particularly strong. Module E encompassed the majority of W and Z chromosome genes and was sexually dimorphic in its expression in all sampled regions. Indeed, we found that roughly ⅓ of sex chromosome transcripts are significantly sexually dimorphic in all non-vocal brain regions, and roughly ⅔ are sexually dimorphic in at least one region. This half-brain-wide and half-regionally-patterned sexual dimorphism observed from the sex chromosomes may act as a substrate for the evolution of

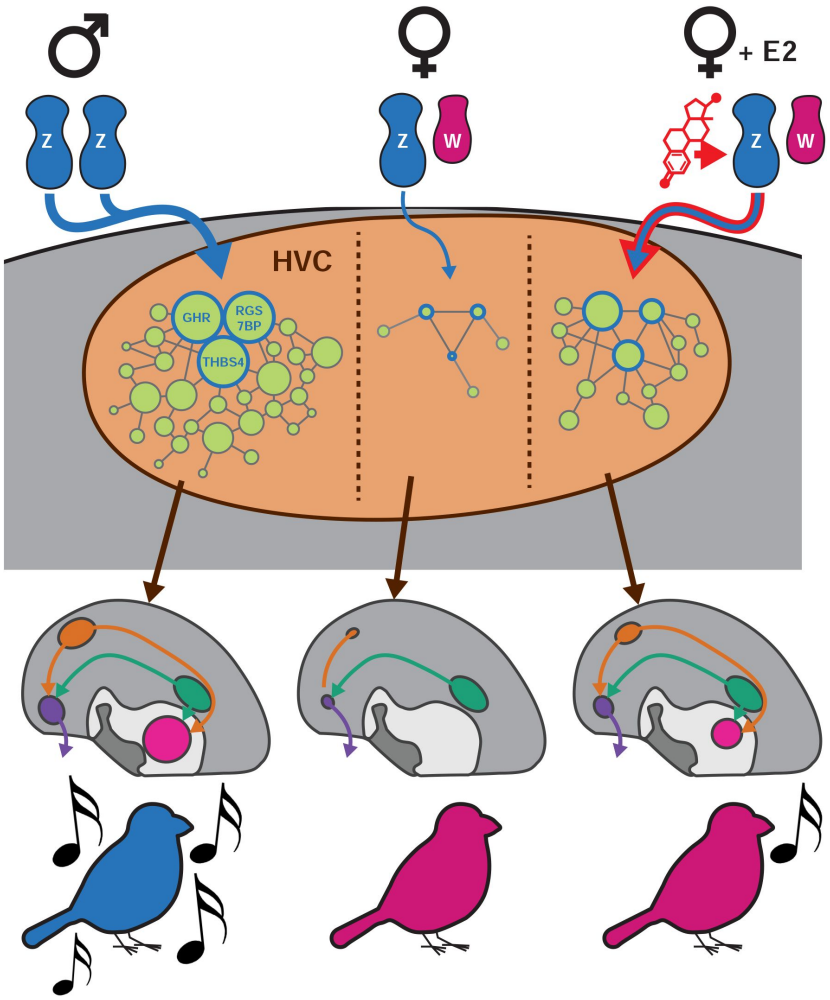


Figure 7

Proposed model of sexually dimorphic zebra finch vocal learning.

We propose that estradiol treatment in female zebra finches masculinizes song behavior by over-coming insufficient Z sex chromosome dosage in HVC to increase the expressed of tran- scripts normally depleted in females. The Z chromosome genes upregulated by E2 are com- ponents in a larger proliferative genetic program which prevents HVC atrophy in males and allows for its expansion late in development. The upregulation of these genes allows for the increased specilization of the gene networks they participate in, promoting HVC development sufficiently to enable rudimentary vocal learning in females.

sexually dimorphic behaviors generally, with brain-wide shifts in expression providing a transcriptional background for evolution of sex-specific patterning in additional sex chromosome genes. These regionally patterned sex chromosome genes could then recruit gene expression networks from somatic chromosomes, resulting in the sex specific anatomical patterning of gene expression from throughout the genome. This general model is consistent with local reduction of GHR and other Z chromosome transcripts in the developing HVC lineage, leading to a loss of specialized module G expression in female HVC from somatic chromosomes. This hypothesis of an interaction between sex chromosomes and autosomes linked to the gain and loss of vocal learning in one sex can be tested in future studies.

Materials and Methods

Animal Handling and Sample Preparation

The 96 samples used in the present analysis are the E2 or vehicle treated subset of a previously published RNAseq dataset[29]. We briefly re-describe our methodology here. All animal procedures were approved by the IACUC of Duke University.

E2 (Sigma E1024-1G) was dissolved in DMSO (100mg/mL) and then diluted in olive oil (1mg/mL). 30-50uL of E2 sample or DMSO only vehicle was applied to the flank of male and female zebra finches daily from PHD0-PHD14 and on alternating days from PHD15-30 (n=3 per sex-treatment combination). We have previously shown that this treatment program is sufficient to induce song system masculinization in E2 treated female zebra finches[29].

On PHD30, animals were sacrificed following one hour of dark isolation. Animals were anesthetized by isoflurane inhalation and rapidly decapitated. Brain hemispheres were dissected, embedded in OCT, and flash frozen in an ethanol and dry ice slurry. Sections were taken from the right hemisphere coronally at 14um onto polyethylene naphthalate (PEN) membrane slides for RNA isolation and adjacent sections taken on charged glass slides for histology or in-situ hybridizations. From the PEN membrane slides, song nuclei and surrounding control regions were laser capture microdissected (LCM) using an ArcturusXT LCM system (Nikon) guided by a Nissl stained tissue series for each animal. No sample pooling was performed, each sample originated from a single bird. This is 8 samples worth of RNAseq data per bird for 12 birds, providing 3 samples per sex-treatment-region combination, roughly a terabyte of read data.

RNA was extracted from the LCM isolated tissue samples using the Arcturus Picopure kit (Applied Biosystems KIT0204) following manufacturer's instructions. RNA quality was assessed using an Agilent 2100 bio-analyzer and the RNA 6000 pico kit (Agilent 5067-1513). Next, cDNA was synthesized using the SMART-Seq v4 Ultra Low input RNA Kit (Takara 634892). Sequencing libraries were made with the NEBNext Ultra II DNA Library Prep kit (New England Biolabs E7645L) and cleaned-up using SPRIselect beads (Beck-man Coulter B23317). Libraries were sequenced by Novogene Co., Ltd. on the Novaseq 6000 platform (Illumina) and S4 flow cells resulting in 150bp paired-end reads.

RNAseq read mapping and quality control

RNAseq reads were first trimmed to remove adapters and low quality base calls using Trimmomatic[72] and then mapped to a high-quality Vertebrate Genomes Project (VGP) female zebra finch nuclear genome (bTaeGut2.pat.W.v2, GCF_008822105.2)[37] using STAR (v2.7.1)[73]. Uniquely mapped reads were then tallied at the level of genes using Rsubread::featureCounts (R-3.6.3)and then counts normalized to fragments per kilobase of transcript per million mapped reads[74]. Multi-mapped reads were rejected as they have a higher probability of being associated with technical artifacts of sequencing or genome assembly. Read based quality control was performed with FastQC (Babraham Bioinformatics) with reports prepared by MultiQC (Python-

3.5.5)[75]. This workflow was automated by the CountMatrix pipeline (*https://github.com/mattisabrat/CountMatrix/*). We next removed two outlier samples (one male vehicle HVC and one female vehicle RA) based upon hierarchical clustering of the sample space before computing gene-to-gene correlations (**Fig. S1**).

Gene Module Identification

All remaining analyses were completed in R-4.2 unless otherwise specified. Data was wrangled in the tidyverse, and custom visualizations produced with ggplot, ggdendro, VennDiagram, RColorBrewer, and ggpubr[76,77]. Unsigned topological overlaps between genes (gene-to-gene correlations) were calculated in a single block with WGNCA::blockwiseModules with a soft thresholding power (β) of 6 based on scale free topology fit and model connectedness as described in the WGNCA vignette (**Fig. S2**)[38]. Next we determined an appropriate WGCNA parameterization quantitatively and qualitatively by sweeping the module size and tree cut height parameters of WGNCA::recutBlockwiseTrees. We selected our model for analysis by examining the resulting gene assignments and sample-sample distance matrices. We were able to increase or decrease the proportion of genes assigned to WGCNA modules by lowering or raising the minimum module size parameter, respectively. We found that setting the minimum size parameter below 100 genes included more genes, but with models that increasingly over-fit single samples, producing obvious outliers in the distance matrix (**Fig. S3**). Correspondingly, raising the minimum size parameter beyond 100 included fewer genes, but did not greatly reduce the number of outlier samples. Based on this we selected 100 as the minimum module size, parameterizing to explain as much transcriptomic variance as possible while minimizing the number of technically overfit samples (**Fig. S2-4**).

Module association to vocal learning

Module eigengenes (MEGs) from each module were correlated against binarized song system membership, vocal learning capability, or sex and the statistical significance of each correlation assessed using WGCNA::corPvalueStudent. This was done in the following sample subsets by node: male samples broken out by treatment; female samples broken out by treatment; all female samples; song system components from either sex treated with vehicle; and surrounding control regions from either sex treated with vehicle. Within each of the 4 sex-treatment combinations we compared the song system components to surrounds at each node. Within all female samples we compared the vocal learning capable E2-treated song system elements to all other female samples from each node. Within the vehicle song systems and vehicle surrounds we compared between male and female finches for each region.

Module gene ontology and convergent vocal learning gene expression signature enrichment

Module assigned zebra finch genes were mapped to their 1:1 human orthologs where possible, dropping unmapped or multi-mapped genes, using orthofindR::getOrthos (*https://github.com/ggedman/orthofindR*) which wraps Ensembl's biomaRt. Uncorrected p values for the enrichment of human gene ontology terms within the human orthologs of module G were calculated using generally applicable gene set enrichment (GAGE) implemented in gage::gage[78]. To determine if the genes previously shown as convergently differentially expressed in the zebra finch song system and human speech brain regions mapped to specific modules, we treated these five gene lists identically to GO terms and tested for their significant enrichment across the human orthologs of each module also using GAGE.

Analysis of sex chromosome gene expression independent of vocal learning or E2 treatment

Sex chromosome transcripts, regardless of WGCNA module assignment, were examined in the vehicle-treated non-vocal learning producing surround samples for each node. To find the most consistently expressed and depleted W and Z chromosome genes respectively, we correlated expression of each sex chromosome transcript with sexual dimorphism within each region, such that expressed W genes would be positively correlated and depleted Z chromosome genes would be anticorrelated. We computed correlations and p values using the WGCNA corAndP function; upper-tailed for the W chromosome and lower-tailed for the Z chromosome. Genes significantly expressed or depleted across regions were then intersected to identify consistently regulated transcripts across the telencephalon.

Module enrichment on chromosomes

To associate modules to chromosomes, we bootstrapped FDR corrected p values for the enrichment of each chromosome-module pairing by randomizing the mapping of genes to modules 50k times and calculating the fold-enrichments observed on each chromosome from each module in each randomization to empirically determine the null distributions. The calculation of bootstraps and p values was performed in Python-3.5.5 and parallelized using joblib's Parallel.

Identification of core module G genes in HVC and Z chromosome enrichment

We defined genes of interest as the subset of significantly (T test for correlation: $p \leq 0.05$) vocal learning capability correlated genes in HVC whose expression correlated to module eigengene G (MEG-G) across the dataset at $r^2 \geq 0.5$ and to vocal learning at $r^2 \geq 0.5$ in at least one of the four vocal learning comparisons in HVC, calculated using WGCNA::corAndP. These comparisons were: all male HVC samples against all male DN samples; female E2 treated HVC against all other female samples at the node, including vehicle treated HVC; Vehicle treated male HVC against vehicle treated female HVC; and E2 treated female HVC against vehicle treated female HVC. We defined core genes as those meeting this criteria for all four HVC vocal learning comparisons. We tested the statistical significance of Z chromosome enrichment in this core gene list with an upper-tailed hypergeometric test, implemented in phyper, where each core gene is a sampling event without replacement from module assigned genes.

Data and Code Availability

All raw data for this experiment is available on the NCBI Sequence Read Archive (accession: PRJNA698257). The count matrix, quality control results, and analysis code is available online (*https://github.com/mattisabrat/sex-and-song*).

Supplementary figures and tables

Figure S1

Outlier sample detection by hierarchical clustering.

Two samples (a vehicle-treated male HVC sample and an E2-treated female RA sample, in red) form single sample branches in the hierarchical clustering tree, indicative of technical outliers unlikely to fit the correlational structure of the larger dataset. Samples were removed prior to gene network construction and module detection.

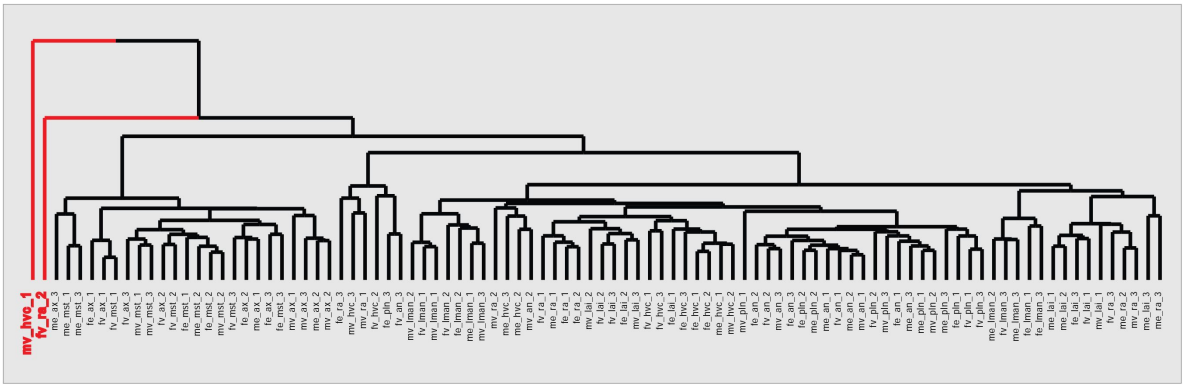
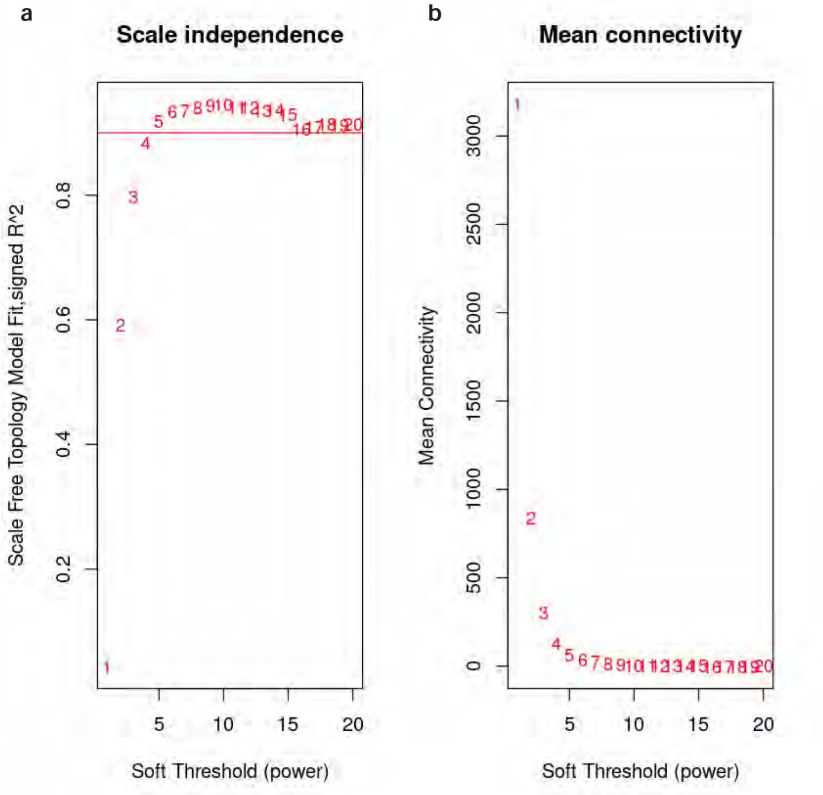


Figure S2

Selection of soft thresholding power for WGCNA model.

Soft-thresholding power (beta, x-axis) is the exponent to which each element in the gene-to-gene correlation matrix is raised during adjacency matrix calculation. **a,** Scale-free fit index (y-axis) as a function of the soft-thresholding power (x-ax- is). Horizontal line indicates a fit of 90%. **b,** Mean connectivity (degree) in the network model (y-axis) as a function of the soft-thresholding power. We selected a power of 6 as it is on the knee of both plots and above the 90% scale free fit criteria.



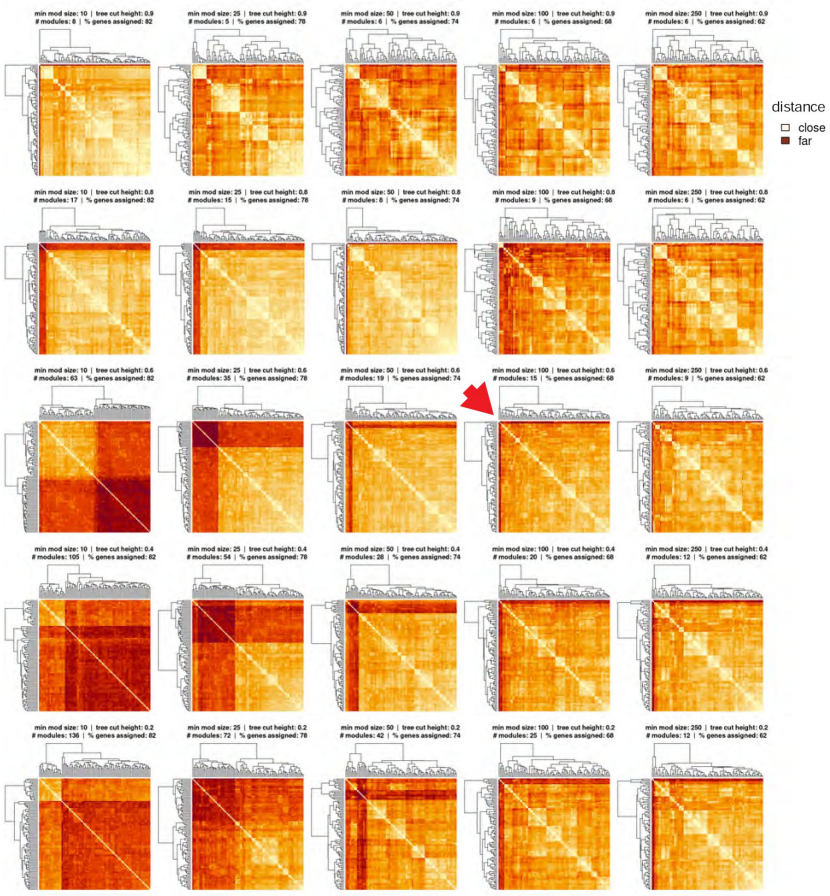


Figure S3

Selection of minimum module size and tree cut height parameter values for WGCNA model.

Each plot shows the sample distance matrix that results from the parameters in the plot title. Titles also show the number of modules found in each resulting model and the percentage of genes in the finch genome assigned. Minimum module size increases across columns (left to right: 10, 25, 50, 100, 250) and tree cut height decreases down rows (top to bottom: 0.9, 0.8, 0.6, 0.4, 0.2). Red arrow indicates selected model. Model was selected to explain as much transcriptomic variance as possible while minimizing the number of technical- ly overfit samples.

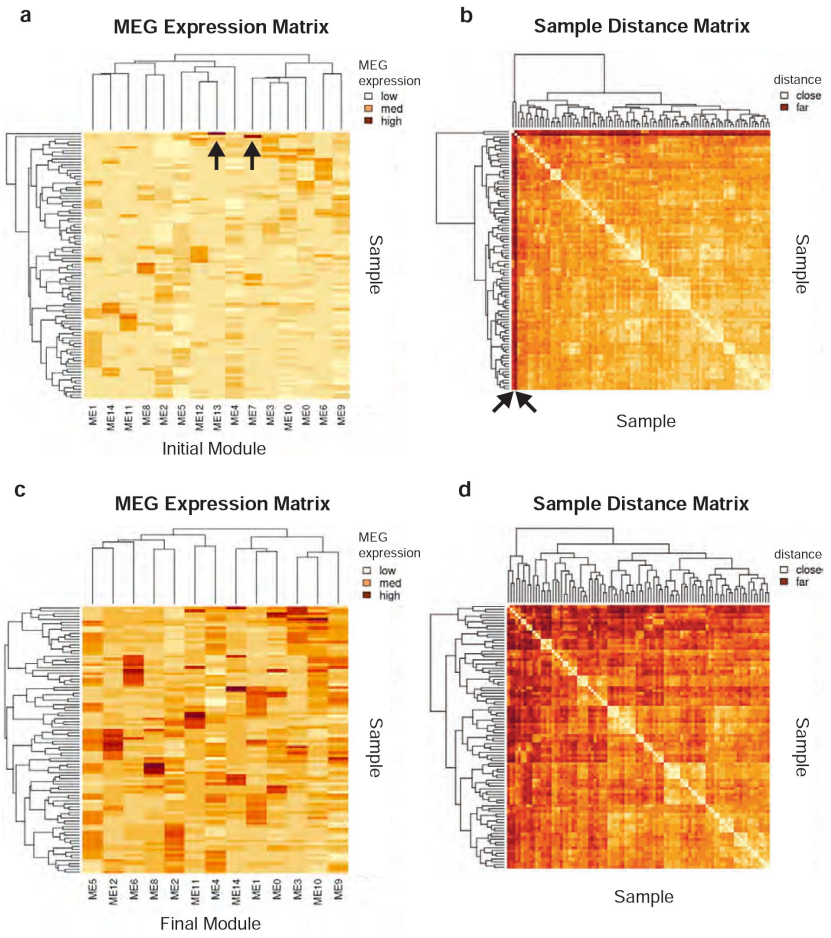


Figure S4

Initial module overfitting to single samples.

a, Module eigengene (MEG) 7 and 13 are both highly expressed only in sinlge samples, indicated by black arrows. **b,** This overfitting causes these samples to be deep outliers in the sample-sample distance matrix, distant from all samples but tthemselves, indicated by black arrows. **c,** Removing these module eigengenes from the set prevents these samples from behaving as outliers in the distance matrix (**d**). These overfit modules were removed prior to module lettering and statis- tical analysis.

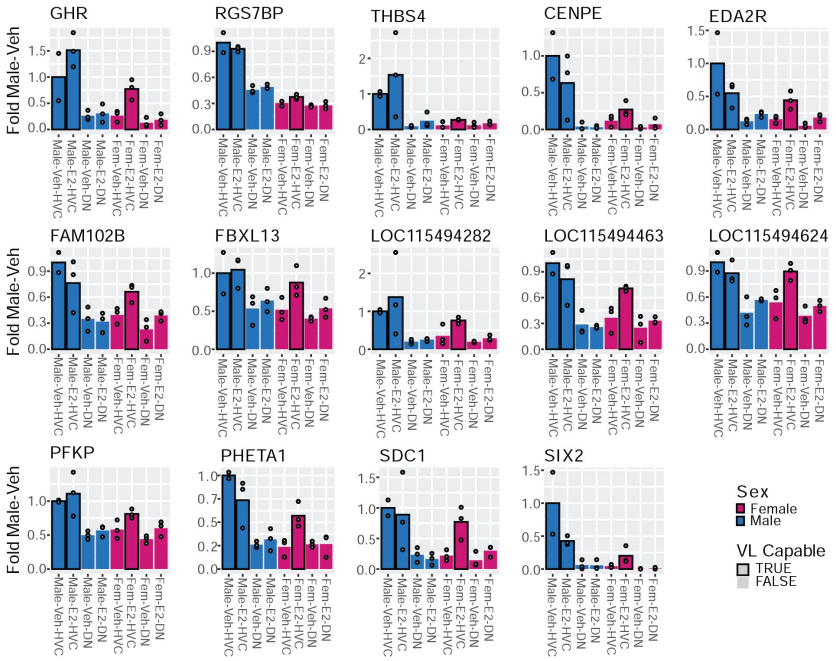


Figure S5

Expression of module G core genes in HVC and surrounding dorsal nidopallium.

Each of the 14 core genes show reduced expression in female HVC relative to the male with an increase in expression in response to E2 treatment. Bar represents mean with individual data points shown. This transcriptional response to E2 is not seen in the surrounding DN.

Z chromosome								W chromosome	
ABHD17B	CPLANE1	FOCAD	LOC100224449	MED18	PLIN2	SERF1A	TMEM171	CHD1W	LOC116806914
ACAA2	CREB3	FSD1L	LOC100224709	MIER3	PLPP1	SETBP1	TMEM267	LOC100189940	LOC116806917
ACO1	CSNK1G3	FUT10	LOC100225594	MIR2954	POLR1E	SETD9	TMEM38B	LOC100190109	LOC116806918
AGGF1	CWC27	FXN	LOC100226145	MOCS2	PPIC	SGTB	TNPO1	LOC100190380	LOC116806920
AGTPBP1	CZH18orf25	GALT	LOC100226207	MPDZ	PPIP5K2	SHC3	TOPORS	LOC115492298	LOC116806923
AK3	CZH18orf32	GIN1	LOC100229438	MRPL17	PPWD1	SHLD3	TPGS2	LOC116806641	LOC116806940
AMACR	CZH5orf51	GNE	LOC100230245	MRPS27	PSIP1	SLC30A5	TRAPPC13	LOC116806651	LOC116806947
ANKRA2	CZH5orf63	GNG10	LOC100230481	MRPS30	PTAR1	SLC38A9	TRIM23	LOC116806853	LOC116806950
ANKRD55	CZH9orf40	GOLM1	LOC105760834	MRPS36	PTBP3	SLC44A1	TSTD2	LOC116806854	LOC116806951
AOPEP	CZH9orf64	GOLPH3	LOC105760853	MTAP	PTCD2	SLF1	TTC33	LOC116806857	LOC116806966
AP3B1	CZH9orf85	GRIN3A	LOC115490934	MTREX	PTGR1	SMAD2	TTC37	LOC116806862	LOC116806973
APBA1	DCP2	HABP4	LOC115490942	MTX3	PUM3	SMAD7	TUSC1	LOC116806863	LOC116806978
APTX	DCTN3	HACD4	LOC115490943	MYORG	RAB3C	SMARCA2	TUT7	LOC116806864	LOC116806979
ARHGEF39	DDX58	HAUS1	LOC115490975	NAA35	RAD1	SMC5	TXN	LOC116806866	LOC116806985
ARRDC3	DHX29	HAUS6	LOC115490991	NDUFAF2	RAD17	SMIM15	UBAP1	LOC116806867	LOC116806986
ATG12	DNAJA1	HEXB	LOC115491002	NDUFS4	RASA1	SNAPC3	UBAP2	LOC116806869	LOC116806988
ATP5F1A	DNAJC21	HINT1	LOC115491073	NFIB	RCL1	SNCAIP	UBE2R2	LOC116806870	LOC116806990
ATP5ME	DTWD2	HINT2	LOC115491151	NIPBL	REEP5	SNX18	UBQLN1	LOC116806871	LOC116806993
BDP1	ECPAS	HMGCR	LOC115491190	NLN	RFK	SNX2	UGCG	LOC116806872	SMAD4
BRIX1	ELAC1	HMGCS1	LOC115491229	NMRK1	RFX3	SNX24	UHRF2	LOC116806874	
BTF3	ELAVL2	HNRNPK	LOC115491243	NOL6	RGMB	SNX30	UTP15	LOC116806876	
C9orf72	ELP1	HSD17B4	LOC115491251	NSA2	RGP1	SPIN1	VCP	LOC116806879	
CAAP1	EMC4	HSDL2	LOC115491282	NTRK2	RGS7BP	SPTLC1	WDR36	LOC116806881	
CARNMT1	ERBIN	IDUA	LOC115491283	NUDT12	RHOBTB3	SREK1	WDR41	LOC116806882	
CBWD1	ERCC6L2	IL6ST	LOC115491288	NUP155	RIC1	SREK1IP1	WDR70	LOC116806883	
CCDC112	ERCC8	INIP	LOC116806725	PAIP1	RICTOR	SRFBP1	XPA	LOC116806886	
CCDC171	ERMP1	IPO11	LOC116806757	PAM	RIOK2	SRP19	ZCCHC9	LOC116806887	
CCNH	EXOSC5	ITGA2	LOC116806794	PARP8	RIT2	ST8SIA5	ZDHHC21	LOC116806888	
CDC37L1	FAM172A	KATNAL2	LOC116806830	PCGF3	RNF170	STARD4	ZFAND5	LOC116806889	
CDK7	FAM174A	KDM4C	LOC116806836	PELO	RNF180	STOML2	ZFR	LOC116806892	
CEMIP2	FAM219A	KIAA0825	LSM5	PGM5	RNF20	SUB1	ZFYVE16	LOC116806894	
CENPH	FANCG	KIAA1328	LYRM7	PHAX	RNF38	SYT4	ZNF131	LOC116806898	
CENPK	FBXL17	KIF2A	LYSMD3	PIAS2	RPL17	TARS1	ZSWIM6	LOC116806902	
CEP78	FBXO4	LMBRD2	MACIR	PIGG	RPL37	TBCA		LOC116806904	
CERT1	FCHO2	LNPEP	MAP3K1	PIGO	RPP25L	TENT2		LOC116806905	
CETN3	FEM1C	LOC100190031	MARVELD2	PIK3C3	RPS23	THAP1		LOC116806906	
CHD1	FER	LOC100217858	MBLAC2	PIP5K1B	RPS6	TMED7		LOC116806909	
CLTA	FKTN	LOC100219485	MCCC2	PJA2	SCAMP1	TMEM161B		LOC116806910	
COMMD10	FNTA	LOC100221697	MCTP1	PLAA	SECISBP2	TMEM167A		LOC116806911	

Table S1

Sex chromosomes consistently repressed or expressed across regions.

Lists Z and W chromosome genes from **Fig. 3e-f** which exhibited sexually dimorphic expression in vehicle-treated, non-vocal learn- ing related samples across brain regions.

Gene	G	Gene	G	Gene	G	Gene	G	Gene	G	Gene	G	Gene	G	Gene	G
CRYBG1	0.96	EDA2R	0.81	LOC105759411	0.74	LOC115491275	0.68	TWIST1	0.64	LOC115490816	0.58	ATG3	0.51	LOC116808453	0.43
INF2	0.94	LOC115494624	0.81	LOC116807830	0.74	CCBE1	0.68	OPN4	0.63	SLC25A4	0.57	COG5	0.51	CDT1	0.43
LOC115493927	0.92	THBS4	0.81	LOC115490698	0.74	MPP5	0.68	LOC115491180	0.63	IGF2	0.57	GRB2	0.51	LOC115496033	0.43
TLL2	0.91	REL	0.81	LOC115493635	0.73	SLC16A3	0.68	TMEM201	0.63	SLIT3	-0.57	LOC115493910	0.50	INSYN2B	0.43
LOC100229435	0.90	LOC115493865	0.81	ADSS1	0.73	LOC116807734	0.68	TTC28	0.63	LOC100220690	0.57	LOC100223718	0.50	LOC100222957	0.43
LOC115493883	0.90	ZBTB42	0.81	LOC100219000	0.73	SLC13A1	0.68	LOC116808070	0.63	LOC115497877	0.57	SLC25A48	0.50	LOC116809294	0.42
GHR	0.90	BARX1	0.81	ROBO2	0.73	VAV2	0.68	CDCP2	0.63	GFRA1	0.57	ACSF2	0.50	LOC116806919	0.41
SNX10	0.89	LOC100232098	0.80	VSTM5	0.73	SNTG1	0.68	ONECUT1	0.63	TNKS	-0.56	VIT2	0.50	LOC116807261	0.41
LOC115494463	0.89	FAM240A	0.80	LOC115493971	0.72	LOC115491133	0.68	LOC100220758	0.63	CH25H	0.56	WT1	0.49	LOC100220760	0.41
SEMA5A	0.89	LOC115491108	0.80	SPOPL	0.72	TMSB15B	0.67	IL1R1	0.63	WWC2	0.56	LOC115495752	0.49	LOC116807756	0.40
CENPE	0.89	CABP1	0.80	LOC116807829	0.72	AR	0.67	LOC116808082	0.63	LOC116809281	0.56	GABRB1	0.49	TRNAC-GCA_8	0.40
UTS2B	0.89	BCAP29	0.80	DNAH10	0.72	LOC116808110	0.67	YEATS4	0.62	BBS5	0.56	SEPTIN3	0.49	TGIF1	0.40
BMP2	0.89	LOC101234217	0.80	HELT	0.71	POU1F1	0.66	HPRT1	0.62	MCM10	0.56	FLNC	0.49	SHMT1	0.40
LOC115494504	0.88	HERC4	0.79	NME7	0.71	LGMN	0.66	LOC101233072	0.62	TSC22D1	0.56	LOC115494122	0.48	MYPN	0.40
SDC1	0.87	LOC115493758	0.79	PDE9A	0.71	RSPO4	0.66	DIO3	0.62	LOC116808493	0.56	SCGN	0.48	SFRP4	0.40
LRRN4	0.87	FAM185A	0.79	FBXL13	0.71	LOC100221042	0.66	LOC116807890	0.62	AZIN1	0.55	LOC115495359	0.48	LOC116808124	0.39
LOC100232561	0.87	HTR1A	0.78	LOC116808880	0.71	LOC100222749	0.66	LOC115491135	0.62	MARK1	-0.55	LOC115495608	0.48	MIR2984	0.39
LRRC2	0.86	GBX2	0.78	ACSL1	0.71	TACC2	0.66	LOC115496139	0.62	TOM1	0.55	CDYL	0.47	LOC115497917	0.39
LOC115496038	0.86	SIX2	0.78	PTX3	0.71	LOC115495472	0.66	TNFRSF13C	0.62	GMDS	0.55	LOC100222730	0.47	HOXA11	0.38
PTGER4	0.86	PDE1A	0.78	LOC100228395	0.71	MUC2	0.65	ATP11B	0.62	ACKR3	0.55	LOC100220430	0.47	LOC115493492	0.38
MAML2	0.85	LOC116809181	0.78	LOC100222099	0.70	LOC115496281	0.65	LOC116806804	0.61	FAM110A	0.55	TPPP3	0.47	LOC116808892	0.38
LOC100221179	0.85	RGS7BP	0.77	LOC115495208	0.70	LOC105758841	0.65	LOC115494393	0.61	LOC116807701	0.54	LOC100218771	0.47	MMRN2	0.37
LOC115494942	0.85	SLC15A5	0.77	TMC5	0.70	CHRNA5	0.65	ASB15	0.61	LOC116808425	0.54	LOC115496073	0.46	LOC100223936	0.37
LOC116808254	0.85	RRAS2	0.77	LOC116808614	0.70	DSG2	0.65	FOXO3	0.61	NIF3L1	0.54	LOC100218321	0.46	ZNF367	0.37
PPP1R27	0.85	CBX6	0.77	LOC115493739	0.70	NEK10	0.65	LOC100189947	0.61	CHRNB2	0.54	LOC100227844	0.46	LOC115491274	0.37
PHETA1	0.85	LOC100227698	0.77	LOC100232469	0.70	ST6GAL2	0.65	LOC116808684	0.61	PITX3	0.54	NME2	0.46	ACOT11	0.36
MYLK4	0.85	MAN1A2	0.76	GLI2	0.70	DAPL1	0.65	EIF4E3	0.61	MFAP4	0.54	LOC115491148	0.46	BHLHA15	0.36
FGL2	0.85	TAB3	0.76	SLCSA8	0.70	GPHN	0.65	SHISA8	0.61	LOC116808247	0.54	LOC115497656	0.46	LOC115493789	0.35
FOSL2	0.85	SLC7A2	0.76	LOC115490696	0.70	COL19A1	0.64	LOC101233680	0.60	CD83	0.53	PKHD1	0.46	LOC115498168	0.35
FAM102B	0.85	KERA	0.76	LOC100221052	0.70	DMXL2	0.64	TRAPPC9	0.60	CDADC1	0.53	MUC6	0.46	LOC116808559	0.35
ADGRG2	0.85	CCNA1	0.76	LOC116806729	0.70	LOC115494903	0.64	LOC115495769	0.60	PROB1	0.53	LOC115494032	0.46	LOC116808141	0.35
FST	0.84	LMTK2	0.76	UTP25	0.70	CTCFL	0.64	LOC116806809	0.59	LOC100221733	0.53	LOC116808435	0.46	LOC115494754	0.34
LOC115494282	0.84	WDCP	0.75	LOC100219379	0.70	LOC115496575	0.64	LOC115497201	0.59	LOC115492991	0.53	HAO2	0.45	LOC116809267	0.34
MCU	0.84	LOC115493751	0.75	SLC35C2	0.70	LOC116808083	0.64	EYA4	0.59	PKP4	0.53	LOC100223745	0.45	VWA3B	0.34
TRIM67	0.84	LOC100223270	0.75	FFAR4	0.69	ARHGAP24	0.64	LOC100225408	0.59	FOXL3	0.52	LOC115492560	0.45	MIR204-2	0.33
LOC115494992	0.84	SPTLC3	0.75	LOC100223369	0.69	DIPK1A	0.64	LOC100222387	0.59	ASB10	0.52	BRCA1	0.45	GLDN	0.33
LY75	0.83	LOC115495199	0.75	CPA6	0.69	HMMR	0.64	LOC115495471	0.59	LOC115490948	0.52	OPN1SW	0.44	LOC115496310	0.33
FGF18	0.83	LOC116809035	0.75	LOC116809117	0.69	LOC100218870	0.64	LOC116807851	0.58	LOC115496648	0.52	MAP2	-0.44	HPD	0.32
NPTX2	0.82	DMRT1	0.75	CCDC82	0.69	LOC116808795	0.64	LOC100230156	0.58	PGM3	0.52	FOSB	0.44	LOC115491248	0.32
PFKP	0.82	FAM3C	0.74	CCDC146	0.69	TRNAU1AP	0.64	GRIP1	0.58	LOC116807831	0.52	DAPP1	0.44	LOC101233536	0.32
CHRNA3	0.82	LOC115493824	0.74	GPR155	0.69	PDE8B	0.64	LOC115493350	0.58	ITGA9	0.52	TTK	0.44	PLA2G4E	0.31
SCUBE1	0.82	LPAR3	0.74	LOC100227652	0.68	LOC115493570	0.64	HTR1F	0.58	LOC115494131	0.52	LOC105758677	0.44	LOC115492139	0.31
EDIL3	0.81	GPR21	0.74	NFKBIZ	0.68	HEY1	0.64	MUSTN1	0.58	POT1	0.51	TK1	0.43	PRDM13	0.31

Table S2

ModuleG constituent genes.

Lists all genes assigned to module G by and their continuous membership in module G (Pearson r to MEG from moduleG)

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Additional information

Author contributions

MHD performed all analyses, generated all plots, and wrote the paper; HNC gathered all data and edited the paper; HM and EDJ edited the paper.

Additional files

supplemental_sig_go_enrich.csv

supplemetal_w_chrom_express.csv

supplemetal_z_chrom_express.csv

supplemental_gene_module_assignment.csv

supplemental_hvc_specializations

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Reviewer #3 (Public review):

Summary:

Davenport et al have investigated how a masculinizing dose of estrogen changes the transcriptomes of several key song nuclei song and adjacent brain areas in juvenile zebra finches of both sexes. Only male zebra finches sing, learn song, and normally have a fully developed song control circuitry, so the study was aimed at further understanding how genetic and hormonal factors contribute to the dimorphism in song behavior and related brain circuitry in this species. Using WGCNA and follow-up correlations to re-analyze published transcriptome datasets, the authors provide evidence that the main variance of several identified gene co-expression modules significantly correlates with one or some of the factors examined, including sex, estrogen treatment, regional neuroanatomy, chromosomal placement, or vocal learning, noting that the latter is largely based on inference due to expression in song control nuclei.

Strengths:

Among the main strengths are the thorough gene co-expression module and correlation analyses, and the inclusion of both song nuclei and adjacent areas, the latter serving as sort of controls for areas that are not dimorphic and likely broadly present in birds in general. In situ hybridization data discussed in a previous publication (Choe et al., Hormones and Behavior, 2021) provides some support for the neuroanatomical specializations of gene expression. It is also significant that the transcriptome re-analysis was performed with an improved genome assembly that also includes the sex chromosomes, thus expanding the Z/W chromosome gene analyses in Friedrich et al, Cell Reports, 2022. The most relevant finding is arguably the identification of some modules where gene expression variation within song nuclei correlates with hormonal effects and/or gene location on sex chromosomes, which are present at different dosages between sexes. Sex differences in gene expression in areas that are not song nuclei may also bring insights into functions other than song behavior or vocal learning. The study also shows how a published RNA-seq dataset can be reanalyzed in novel and informative ways.

Weaknesses:

The validation of the inferred direction of regulation in the identified co-expression modules is limited to the in situ data mentioned above. Further evidence that representative genes in the main modules differ in expression when comparing sexes or E2- vs VEH-treated tissues using independent samples and/or methods would provide further validation and enhance rigor. Most importantly, E2 is known to exert various actions on brain physiology and neuronal function. Because there was no manipulation of candidate genes, nor assessment/manipulation of vocal behavior or vocal learning, an involvement of the identified candidate genes in setting up the sexual dimorphism of the song system or song behavior was not directly tested in this study. For the latter reason, the implication of the Title (..."gene expression associated with vocal learning...") is not well supported. While novel insights were gained into brain expression of Z chromosome genes, it cannot be excluded that the higher male expression of some Z genes may not affect brain cell function and thus may not require active compensation (as discussed for nucleus RA in Friedrich et al, Cell Reports, 2022).

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Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This study is useful as it provides further analysis of previously published data to address which specific genes are part of the masculinizing actions of E2 on female zebra finches, and where these key genes are expressed in the brain. However the data supporting the conclusion of masculinizing the song system are incomplete as the current manuscript is a re-analysis of differential gene expression modulated by E2 treatment between male/female zebra finches without manipulation of gene expression. The conclusions (and title) regarding song learning are also incompletely supported with no gene manipulation or song analysis. Importantly, the use of WGCNA for a question of sex-chromosome expression in species without dosage compensation is considered inadequate. As the experimental design did not include groups to directly test for song learning, and there was also no analysis of song performance, these data were also considered inadequate in that regard.*

We are sorry the editor felt the manuscript so incomplete and inadequate. Though the tone of this assessment seems more severe than the below reviewer comments, we are also happy to see that the editor has considered our paper further for a revised publication, based on the reviewer's comments. We address the editor's comments as follows:

While we agree that manipulation of some of the genes we discovered, whose expression levels are E2-sensitive in the song system, would take the study further in validating some proposed hypothesis in the discussion of the paper, we don't think the outcome of gene manipulations would change the major conclusions from the results of the paper. In this study we performed estrogen hormone manipulations, with causal consequences on gene expression in song nuclei and associated song behavior. In a way this is analogous to gene manipulations, but manipulating directly the action of estrogen. The categories of genes impacted, and the differences among the sex chromosomes wouldn't change.

For the comment on WGCNA being inadequate for addressing questions on sex chromosome expression in species without dosage compensation, we think the evidence in our data does not bear that out. One main result of this paper is the separation of Z chromosome transcripts whose expression is most strongly regulated by chromosomal dosage (WGCNA module E) across regions from those subject to additional sources of regulation in song nuclei (other modules). It seems to us that rather than being confounded by the lack of dosage compensation, WGCNA allowed us to better resolve the effects of dosage on different genes within the sex chromosomes. We have added a new figure more directly examining sex chromosome transcript abundance within different modules. Briefly, we found that module E assigned Z chromosome genes exhibited almost exactly the male-biased expression ratio expected from no dosage compensation while the Z chromosome genes in song nuclei assigned to other modules were expressed below the dosage predicted value, consistent with module E containing those genes whose expression are most strongly regulated by dose across all brain regions sampled.

At its core, WGCNA finds sets of correlated genes. The biological reality of the zebra finch transcriptome is that Z chromosome expression is largely anti-correlated with W chromosome due to dosage. However, this dosage effect is not felt equally by all genes and WGCNA provides an unbiased computational framework which can be used to separate dose from other potential sources of gene regulation. This is why roughly ⅓ of Z chromosome genes are not assigned to module E; for example the growth hormone receptor is assigned to module G based on its correlation with genes upregulated within HVC.

> *"As the experimental design did not include groups to directly test for song learning, and there was also no analysis of song performance, these data were also considered inadequate in that regard."*

Concerning the comment on no analysis on song performance in the paper, all such analyses were conducted on our previous study on the same animals (Choe et al. 2021, Hormones & Behavior). The birds considered here were sacrificed at PHD30, prior to the onset of learned song behavior. However, females treated with E2 the same at the same time and allowed to mature into adulthood, went onto to develop rudimentary song. Further, induction of rudimentary song learning in females following E2 treatment has been well established since the early '80s. We have added the following text toward the end of the intro to make this more clear:

"While the birds for this study were sacrificed prior to the developmental presentation of song behavior, we have previously shown that female finches treated in exactly the say way with E2 go on to produce rudimentary imitative songs as adults (Choe et al 2021), consistent with the known induction of vocal learning in females by E2 (REF)."

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Overall, this is a wonderfully designed and executed study that takes full advantage of new resources, such as the most complete zebra finch genome assembly yet, as well as the latest methods. I have very few suggestions as to the improvement of the manuscript. They are as follows:*
>
> *Results Section:*
>
> *In the paragraph "Identification of gene expression modules in song nuclei":*
>
> *"The E2-treated females in this study had similarly sized song system nuclei as males, indicating that E2 treatment prevented atrophy."*
>
> *Clarify if this comparison is to treated and/or untreated males.*

We thank the reviewer for their comment. The relative differences in the song nuclei sizes between the E2-treated females and the other groups is more complex that our original sentence implied. We have revised the main the text as follows

“In our previous study, we found that estradiol treatment in PHD30 females caused HVC to enlarge and Area X to appear when it normally does not develop in females, but both at sizes less than in untreated or treated males.The sizes of PHD30 female LMAN RA were already the sizes as seen in males, as the later has not atrophied yet at this age(25).”

> *In the paragraph "Sex- and micro-chromosome gene expression across the telencephalon": "These animal and chromosome specific shifts in the transcriptomes could represent the systemic effects of allelic chromosomal structural variation..."*
>
> *The authors should clarify the meaning of a"llelic chromosomal structural variation" in this context, as it is an unusual phrase. Major chromosomal structural variation seems unlikely to produce these effects. Is it also possible that animal-specific modules with brain-wide higher could also result from laboratory contamination between all samples from one animal? This is not too likely but perhaps should be acknowledged or ruled out.*

We have removed the word allelic, which was unnecessary. We can’t envision how laboratory contamination could occur such that all of one animal’s samples would be affected to produce the observed result which is module and chromosome specific. An animal wide effect could emerge during sacrifice, but we can think of no reason that would affect these modules and not others. Rather, the most likely explanation is biological natural difference between animals. We have added this consideration of alternative explanations.

> *In the section "Candidate gene drivers of HVC specialization in E2-treated females":*
>
> *When discussing GHR's role in cell growth and proliferation, the authors' argument could be expanded by including the documented role of GH signaling in anti-apoptotic protection of neurons from rounds of neural pruning during development as documented in the chicken, e.g. • Harvey S, Baudet M-L, Sanders EJ. 2009. Growth Hormone-induced Neuroprotection in the Neural Retina during Chick Embryogenesis. Annals of the New York Academy of Sciences, 1163: 414-416. https://doi.org/10.1111/j.1749-6632.2008.03641.x*

We thank the reviewer for sharing this publication with us.. We have added the following sentence to our discussion with the above citation. “Further, our results are consistent with growth hormone’s known role in avian anti-apoptotic protection, with elevated signaling associated with the survival of chicken neurons during rounds of pruning in the developing

retina."

> *The authors' argument of the relevance of the passerine GH duplication would be strengthened by citing:*
>
> *• Rasband SA, Bolton PE, Fang Q, Johnson PLF, Braun MJ. 2023. Evolution of the Growth Hormone Gene Duplication in Passerine Birds, Genome Biol Evol, 15(3) https://doi.org/10.1093/gbe/evad033. Greatly expands on the Yuri et al. paper cited by characterizing of the molecular evolution of these genes across hundreds of avian species, supporting positive selection on multiple amino acid sites identified in both ancestral and duplicate (passerine) growth hormone.*
>
> *• Xie F, London SE, Southey BR et al. 2010. The zebra finch neuropeptidome: prediction, detection and expression. BMC Biol 8, 28. https://doi.org/10.1186/1741-7007-8-28 The authors report significantly different expression of the ancestral GH gene in the adult male zebra finch auditory forebrain after different song exposure experiences.*

We have amended the results section sentence and added all suggested citations. The sentence now reads: "The gene which encodes growth hormone receptor's ligand, growth hormone, is interestingly duplicated and undergoing accelerated evolution in the genomes of songbirds (Rasband et al 2023); the GH ligand has been found to be upregulated in the zebra finch auditory forebrain following the presentation of familiar song (Xie et al 2010)."

> *Figures:*
>
> *- Figure 1B. "Duration of sex typing" being a shorter bar compared to the others is not fully explained in the experimental design. Presumably at the end of this time period, the sex is non-invasively, phenotypically evident. I suggest an arrow pointing to the PHD/PHD range when sex is apparent in plumage/anatomy.*
>
> *- Figure 4. Caption appears to be truncated; "across all... genes"?*

Fixed

> *- Figure 5. For 5E, 5F, 5G, 5H, consider enlarging the plots so overlapping gene symbols are readable. Alternately, smaller numbers or symbols could be used with a key in areas where overlapping symbols are hard to prevent.*

We agree that these are not the easiest to read; we originally offset the symbols in R to minimize overlaps, but it can only do so much for the more crammed panels. We have now added a supplemental .xlsx file with the underlying data from each of the 4 tests for readers that want to examine the data in more detail.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Since WGCNA methods will inherently draw together sex-chromosome genes into the same module in systems without dosage compensation, I suggest the authors rerun the WGCNA using only female samples and only male samples. Then identify the composition of modules that differ between E2 and vehicle-treated females and compare these genes to males. Then from male WGCNA identify the composition of modules that differ between E2 and vehicle-treated males and compare to female modules.*

We thank the reviewer for their suggestions. However, we believe it is not as strong as the approach we used, which is grouping data from both sexes in the WGCNA analyses in a study that is looking for sex differences. The reviewer's proposed approach amounts to computing

modules twice (once per sex), determining song system specialized modules and E2 responsive modules in both settings, then intersecting the two sets to find corresponding modules, all done to prevent the non-dose compensated sex chromosome genes from being drawn into the same module.

While WGCNA does group the majority of sex chromosome genes into module E, it does not categorize them all this way (Fig 3). The module classification instead differentiates those sex chromosome genes whose expression are most explained by chromosome dosage / sex across regions (modE) from those whose expression is controlled by other sources of regulation; for an example of the latter, the growth hormone receptor (GHR) is one of several Z chromosome genes classified into modG as its expression better correlates with the genes specialized to HVC than it does with the majority of dosage-dependent Z chromosome genes found in modE. Further, to remove biological sex as a variable in a WGCNA analysis that is focused on sex differences seems counterintuitive.

Instead, to quantitatively address the reviewer's concern, we conducted additional analyses, that led to an added new figure, associated text, and tables, that better describes sex/chromosome dosage effects on the abundance (FPKM) and expression ratios of sex chromosome transcripts by module irrespective of brain region (Fig. 5). We find that the Z chromosome genes in modE were expressed at the expected chromosome dosage in the non-vocal surrounding regions (65.06% observed vs 66.6% expected) while in other modules, other Z chromosome genes were expressed at intermediate levels between equal expression and the expected chromosomal dosage. For example, the Z chromosome content of modules D and H exhibited near equal expression between sexes. Within the song system, Z chromosome gene content of modG was highly expressed in males beyond what is expected from chromosome dosage, consistent with modG's male-specific upregulation in song nuclei relative to surrounds in the absence of E2. These results better demonstrate that in our WGCNA on the combined dataset we are able to separate those Z chromosome genes whose expression is predominantly dosage controlled from those subject to additional regulation such as song system specialization.

> *Fig. S3 Legend: 'Black arrow' -> 'Red arrow'*

Change made.

> *Fig. S5 - What part of the figure shows the 'human convergent signature'? Also, simply listing the number of genes mapped to a chromosome is misleading to readers unfamiliar with the zebra finch genome, you should either provide the number of genes on each chromosome or present as corrected by that number.*

Fig. S5 was the same type of analyses in Fig. 3 but with an older zebra finch genome assembly, where we had not included the panel a for enrichments with genes convergent in expression between songbird song regions and humans speech brain regions. However, we see that Fig. S5 was not adding any new important information to the paper, so we removed it.

For the chromosome analyses in Fig. 3b, we provide both the total raw number of module assigned genes broken down by chromosome (The black bar plots on the right) as well as a statistical fold-enrichment value of modules per chromosome. Given the number of genes per chromosome and genes per module in our data, we computed the fold-enrichment for each intersection (observed intersection size / expected intersection size). To test for the significance of these enrichments, we bootstrapped FDR corrected p values for the enrichment of each chromosome-module pairing by randomizing the mapping of genes to modules to construct a null distribution of fold enrichments for each intersection. Our intent was not to describe the size of the chromosomes themselves, information readily available

elsewhere, but to show the disproportionate chromosomal origins of the gene sets considered by this study. Performing this enrichment test using all annotated genes per chromosome would artificially increase enrichment values and make the analysis less conservative by confounding the results with the inherent enrichment for “brain function” in the assigned genes relative to all genes.

> *At several places you say "we correlated expression of each sex chromosome transcript with sexual dimorphism within each region, such that expressed W genes would be positively correlated and depleted Z chromosome genes would be anticorrelated." What was the sexual dimorphism that was being correlated with? Is this the eigengene?*

We thank you for this comment. Our language was less clear than it could be. We tested for correlations of both the eigengene and the individual gene expression profiles with the biological sex of the animals. We have changed the text to:

“To do this, we tested for a correlation between the expression of each sex chromosome transcript to the animals’ sex within each brain region. We found that female-enriched transcripts were positively correlated with sex and male-enriched transcripts were anticorrelated (Fig. 4f,g).”

> *Fig. 4A: The 'true/false' boxes and animal A-L is confusing and unnecessary. I'd suggest just using M and F (or sex symbols) with a horizontal line below each set of 3 for respective E2 and Veh.*

Change made.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *General comments:*
>
> *After the initial characterization of the datasets and module identification, it is quite hard to follow the logic of the data presentation in the various other Results sections or to clearly understand how they relate to the main stated goal to identify factors related to sex differences in vocal learning. The most relevant findings relate to the presumed actions of hormone treatment and sex chromosome gene dosage in song nuclei, whereas analyses of other brain areas, other chromosomes, or speech-related genes serve more as controls and/or appear as distractions from the main theme. A suggestion to increase the clarity of the presentation and potential impact of the study is to change the order of the presentation, focusing first on the specific analyses and comparisons that most directly speak to the main goals of the study, and then secondarily and more briefly presenting the controls or less related comparisons.*

The reviewer’s suggestion for the results section organization is exactly what we had tried to do. We opened the first paragraph on identification of modules, then presented the song nuclei specific modules, followed by E2-changes to those modules; and the followed by other specific results for the remainder of the paper, including module enrichments to specific chromosomes. The reviewer mentioned our analyses of “other brain areas” (which we assume to mean the non-vocal surround regions), other chromosomes (which we assume means autosomes) and speech-related genes as controls were a distraction in the paper; but within our analysis, these other brain regions are essential controls needed to assess the song-system specificity of any observed sex differences observed from the very first paragraphs of the results; the autosomes were not controls for sex chromosome results, but primary results in of themselves; the overlap with speech-related genes was also not a control, but a novel discovery. We have revised these points in the paper to make them

clearer, and revised some of the section titles and transitions between sections to help increase clarity of the main storyline of the paper.

> *A related comment is that many of the inferences drawn from the WGCNA analysis were quite complex, thus independent verification of some predictions would be quite valuable. For example, consider the passage: "In non-vocal learning juvenile females, interestingly LMAN was specialized relative to the AN by the same gene modules as in males (B, F, and I) as well as an additional module G (Fig. 2b); RA was specialized by module A as in males, but not module L and by additional modules A and G. In contrast, neither juvenile female HVC nor Area X exhibited significant gene module expression specializations relative to their surrounds." Providing in situ hybridization verification of these regional gene expression predictions with a few representative genes seems quite feasible given the group's expertise and would considerably strengthen confidence in the module-based inferences.*

We performed in-situ independent validation of 36 candidate genes in our first study with this dataset (Choe et al 2021). We now mention this validation in the revised paper. The reviewer's selection of one of our sentences though made us realize that our grammar used to explain the results was not as clear as it needs to be. We thus cleaned up the grammar of our module descriptions so that it should be communicated with less complexity, the main issue noted by the reviewer.

> *Because this is a re-analysis of a previously published dataset, the authors should more explicitly describe somewhere in the Discussion how the present analysis advances the understanding of sex differences in songbird neuroanatomy and behavior beyond the previous analysis.*

We have added an additional sentence into the discussion more clearly separating the results of the current study from our previous work.

> *Specific comments:*
>
> *Abstract:*
>
> *There is evidence (from Frank Johnson's lab) that RA does not completely atrophy in female zebra finches, but is still present with more preserved connectivity than previously thought, possibly related to non-singing function(s). A term like 'marked reduction' of female RA may more accurately reflect the current state of knowledge.*

We have changed the text to “partial atrophy”.

> *The term "driver" is undefined and unclear at this point of the paper; a clear definition for "driver" is also lacking in the Intro.*

We now define “driver” or “genetic driver” as understood to mean “a genetic locus whose expression and/or inheritance strongly regulates the trait of interest”.

> *When citing the literature on studies that identified "specific genes with specialized up- or down-regulated expression in song and speech circuits relative to the surrounding motor control circuits", the authors should also cite studies from other labs (e.g. Li et al., PNAS, 2007; Lovell et al, Plos One 2008; Lovell et al, BMC Genomics 2018; Nevue et al, Sci Rep. 2020), to be accurate and fair.*

Citations added

> *For clarity, the authors should explicitly formulate the hypothesis they are proposing at the end of the Summary.*

We thank the reviewer for this comment. We have replaced the final sentence of the summary with: “We present a hypothesis where reduced dosage and expression of these Z chromosome genes changes the developmental trajectory of female HVC, partially preventable by estrogen treatment, contributing to the loss of song learning behavior.”

> *Introduction:*
>
> *Vocal learning is arguably the ability to imitate 'vocal' sounds, this could be clarified here.*

We have amended the sentence to “Vocal learning is the ability to imitate heard sounds using a vocal organ…”

> *Given they are currently considered sister taxa, can the author briefly explain what is the basis for assuming that songbirds and parrots independently evolved vocal learning?*

Although songbirds and parrots belong to a monophyletic clade, they are not sister taxa. There are two clades separating them that are vocal non-learners. We have cited the reference that demonstrated this (e.g. Jarvis et al 2014 Science).

> *Why use Taeniopygia castanotis rather than the more broadly used Taeniopygia guttata?*

Zebra finches were recently reclassified and T.castanotis is now more accurate. The Indonesian Timor zebra finch retained T.guttata while the Australian finch, used here, was classified as T.castanotis.

> *The authors state: "...vocal learning is strongly sexually dimorphic in zebra finches and many other vocal learning species" and cite Nottebohm and Arnold, Science, 1978. That landmark paper only shows dimorphism in song nuclei (not learning) in two songbird species. The authors should provide citations for other species and behavior, or modify the statement.*

We have added an additional citation (Odom et al.) to this sentence which covers the phylogeny more broadly.

> *The authors refer to the nucleus RA as being located in the lateral intermediate arcopallium (LAI). Other labs have described this domain as the dorsal part of the intermediate arcopallium, thus AId or AID (Mello et al., JCN, 2019; Yuan and Bottjer, J Neurophys 2019; Yuan and Bottjer, eNeuro, 2020; Nevue et al., BCM Genomics, 2020). The authors should acknowledge this discrepancy in nomenclature so that data and conclusions can be more readily compared across studies.*

We thank the reviewer and agree that this is helpful. We have added a note at the first mention of LAI.

> *The authors state that data from the gynandromorph bird described by Agate et al implicates "sex chromosome gene expression within the song system" as involved in the song system sexual dimorphism. That study, however, only rules out circulating gonadal steroids, and while suggesting a cell-autonomous mechanism like sex chromosome*

> *genes, it does not necessarily exclude other brain-autonomous factors like sex differences in local production of sex steroids.*

We say that this study "implicated" sex chromosome gene expression, which is accurate per the results and discussion of that study. We are unsure what "brain autonomous factors like sex differences in local production of sex steroids" means?. "Brain autonomous" and "local production" in the brain seem contradictory in this context?

> *Results:*
>
> *The authors state that "the E2-treated females in this study had similarly sized song system nuclei as males, indicating that E2 treatment prevented atrophy". Can they clarify whether the VEH-treated females actually had smaller RAs than E2-treated females or VEH-treated males at this age? This is still quite early in development and it is unclear to what extent RA's marked sexual dimorphism in adults or later developmental ages has already taken place in untreated (or VEH-treated) birds. A related comment is that the authors state later on: "We interpret these findings to indicate that: LMAN and RA atrophy later in juvenile female development..." Does this mean these nuclei actually did not show the marked decreases predicted earlier in the text? Clarifying this point would be helpful.*

We thank the reviewer for pointing out this discrepancy, which reviewer #1 asked for clarification as well. RA size at this age is similar in males and females. However, HVC and Area X is smaller and absent respectively in females and E2 treatment partially prevents this atrophy. The text now reads:

"In our previous study, we found that estradiol treatment in PHD30 females caused HVC to enlarge and Area X to appear when it normally does not develop in females, but both at sizes less than in untreated or treated males.The sizes of PHD30 female LMAN RA were already the sizes as seen in males, as the later has not atrophied yet at this age(25)."

> *The authors acknowledge that area X is absent in untreated and VEH-treated females. Could they please clarify how area X and the surrounding stratal tissue that excludes area X were identified for laser capture dissections in juvenile females?*

We have added the following statement to the main text portion discussing the dissections.

"In the case of vehicle-treated females which lack Area X, a piece of striatum from the same location of where Area X is found in males was taken. "

> *Some passages in Results discussing the authors' interpretation of the modules seem quite speculative and possibly belong instead in the Discussion. For example: "... that module A and G genes could be associated with the start of this atrophy; HVC and Area X are likely the first to atrophy or not develop; and lack of any gene module specialization in them at this age could mean that they would be more sensitive to estrogen prevention of vocal learning loss."*

As suggested, we have removed this text from the results; these ideas were already presented in the Discussion. We have merged the resulting small paragraph with the preceding paragraph.

> *The authors state: "To assess the effects of chronic exogenous estrogen on the developing song system, we first performed a control analysis of modules in the E2-treated juvenile males." How can an assessment of estrogen effects be a "control" analysis? Does this refer to a contrast with females? Please clarify the language here.*

The reviewer is correct, that E2 treatment in males should not be considered a control experiment. We removed the word “control”.

> *When discussing the GO-enriched terms for module G, it is unclear how the authors reached the conclusion about "proliferative", as the enriched terms do not refer to processes more directly indicative of proliferation like "cell division" or "cell cycle regulation". Rather, these terms seem more related to differentiation and growth, which do not necessarily imply proliferation. The authors also refer to "HVC proliferation" later on in the Discussion. However, there is conclusive evidence from several labs that proliferative events associated with postnatal neuronal addition and/or replacement in song nuclei occur in the subventricular zone, not in song nuclei like HVC itself, and that the growth of song nuclei largely reflects cell survival, as well as growth in size and complexity under the regulation of sex steroids.*

We agree that “proliferative” may have been a poor word choice here. We did not mean to indicate that cell division was occuring in HVC itself. Instead we meant to indicate that HVC is able to accommodate the new born neurons from the SVZ. We have replaced the word “proliferative” throughout. In the instance the reviewer mentions specifically we replaced it with,“...potentially act to integrate and differentiate late born neurons.”

> *With regard to module E, referring to a telencephalon-wide sexually dimorphic gene expression program seems quite a stretch, given that only a few regions were sampled and compared between sexes. These related statements should be toned down.*

We have replaced “telencephalon-wide” with “more distributed across the finch telencephalon” and other similar language in each instance.

> *The following passage is very speculative and should shortened and/or moved to the Discussion: "Based on the findings in these gene sets, we hypothesize that without excess estrogen in females, HVC expansion is prevented by not specializing the growth and neuronal migration promoting genes in module G to the HVC lineage by late development. This is potentially enacted by depleting necessary gene products from the Z sex chromosome, such as GHR, which are already present in only one copy."*

We have deleted this portion of the text, as the idea is already present in the discussion.

> *Figure 5: To this reviewer, the comparisons of sex differences and of female response to E2 are the most relevant and informative ones, whereas the regional differences between song nuclei and surrounds refer to different cell populations and cell types where other processes may be occurring, independently of what occurs in song nuclei. It thus seems like the intersection analysis in panel 5i may be subtracting out important "core genes" in terms of E2 effects and/or sex differences in the most relevant cell populations, i.e. in this case within song nucleus HVC.*

Song learning and the vocal learning brain regions are specialized behaviors and associated nuclei which have a set of hundreds of specialized genes compared to the surrounds. Our previous findings shows that E2 drives the appearance of these specializations in female zebra finches. Thus, we considered this the most interesting question to focus on, which we have further highlighted. Nevertheless, in response to the reviewers suggestion, we have added a .xlsx supplemental file containing the results from each of the individual tests so readers may examine any single comparison, or set of comparisons, in more detail.

> *Discussion:*

> *It is unclear what the term "critical period" refers to in: "during the critical period of atrophy for the female vocal circuit"; please clarify.*

We agree that our language was nebulous. We have replaced it with “as several male song control nuclei begin to expand and female nuclei partially atrophy”

> *In: "HVC appeared unspecialized at the level of gene module expression in control females", does "unspecialized" refer to a lack of difference in gene expression when compared to surroundings? Please clarify. The same comment applies to other uses of "unspecialized" in this paragraph.*

Yes, unspecialized means lack of difference in gene expression in the song nucleus. To clarify this point, we have reworked that and the following sentence as follows:

“HVC appeared unspecialized compared to the surrounding nidopallium at the level of gene module expression in control females, with no significantly differentially expressed MEGs . However, in E2-treated females, HVC exhibited a subset of the observed male HVC gene expression specializations. Similarly, the vehicle-treated female striatum located where Area X would be also lacked any specialized gene module expression, but the E2-treated female Area X exhibited a subset of the male Area X specializations, consistent with the known absence of Area X in vehicle-treated females and presence in E2-treated females.”

> *The authors state: "...we surprisingly found that the most specialized genes were disproportionately from the Z chromosome", when discussing module G in HVC. Why is this so surprising? In a sense, this could be taken as consistent with the findings of Friedrich et al, 2022, where sex differences in the RA transcriptome were predominantly Z related on 20 dph. Arguably 20 dph is still quite close to 30 dph in the present study, when compared to 50 dph in Friedrich et al, when autosomes predominate.*

Our bioRxiv was originally posted in July 2021, prior to the publication of Friedrich et al, 2022; however we had previously added to our discussion that several of our results are consistent with the observations of Friedrich et al..

We have a different interpretation of Z chromosome gene results in Friedrich et al.. While the percentage of specialized genes from the Z chromosome decreased, the absolute number of specialized Z chromosome genes actually increased over this interval. In Fig. 3a from Friedrich et al. it appears that ~28% of Z chromosome genes were sexually dimorphic in their expression in RA at PHD20 but that ~39% of Z chromosome genes were similarly dimorphic at PHD50. We interpret this result as the Z chromosome genes being among the earliest genes differentially expressed between the sexes, not that their differential expression or role ever subsequently decreased. We have reworked this portion of the discussion to make our point more clear:

“This model of sex chromosome influenced song system development is consistent with recent observations comparing male and female zebra finch transcriptomes from RA at young juvenile (PHD20) and young adult (PHD50) ages in un-manipulated birds (Friedrich et al. 2022)57. While that study proposes that the role of the sex chromosome in maintaining transcriptomic sex differences diminishes across development, as the proportion of specialized genes that originate on the sex chromosomes diminishes, this effect was driven by large increases in differentially expressed autosomal genes rather than by any reduction in sex chromosome dimorphism; the percentage of differentially expressed Z chromosome genes increased from PHD20 (28%) to PHD50 (39%) (Friedrich et al). This leads us to conclude that sexually dimorphic Z chromosome expression at juvenile ages precedes the sexually dimorphic expression of the autosomes seen in adults. This is consistent with our hypothesis

that sufficient expression of select Z chromosome gene products (GHR, etc..) is necessary for subsequent autosomal song system specializations (modG)."

Further, when we write "When examining the module G HVC specialization induced by E2-treatment in female HVC, we surprisingly found that the most specialized genes were disproportionately from the Z chromosome" we are referring to the upregulation of module G by E2 in female HVC, not the sex difference described in RA by Friedrich et al. which only utilized un-treated RA samples and thus is more likely related to our observations of module E.

> *The term "sexual dimorphism" has been more traditionally used for sex differences that are very marked, like features that are highly regressed or absent in one sex, most often in females. Quantitative differences in gene expression, including dosage differences like those related to module E, are more appropriately described as sex differences rather than dimorphisms. That usage would be more consistent with most of the literature, and thus preferable.*

We did a google search for common definitions, and found more the opposite. Sexual dimorphism being used more often as differences of degree (with the zebra finch example as one of the top hits), and sex differences being used often as more absolute differences (like presence vs absence of the Y chromosome). Further, as in the reviewer's first sentence, the definition of sexual dimorphism is a sex difference. That is, the two phrases can be interchangeable. Thus, we prefer to keep sexual dimorphism.

> *Several references are incomplete or seem truncated, like 9 and 10.*

Fixed

> *Table S2: Please examine and take into account the W gene curation presented in Table S3 of Friedrich et al., 2022.*

We have added additional supplementals (supplemetal_w_chrom_express.csv and supplemetal_z_chrom_express.csv) of the data provided in new Fig 5 incorporating the curation information from Table S3 from Friedrich et al.

> *Data availability:*
>
> *Genes for all the main modules identified should be presented in a Supplemental Table, or through a link to a stable data repository.*

We have added an additional Supplemental Table supplemental_gene_module_assignment.csv with this information.

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