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To Tame a Songbird

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Madza Yasodara Farias Virgens

2023

ABSTRACT OF THE DISSERTATION

To Tame a Songbird

by

Madza Yasodara Farias Virgens

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 20126

Professor Stephanie A. White, Chair

Darwin was amongst the first to argue for the existence of parallels between birdsong and human language. In his book, The Descent of Man, he expresses that "the sounds uttered by birds offer in several respects the nearest analog to language." Was he right? A robust body of evidence accrued over 100 years of prolific research in birdsong endorses the claim. This dissertation is a collection of my modest efforts towards furthering this research program.

In Chapter 1, I explore the evolutionary genomics of the Bengalese finch, a tame songbird frequently found in pet shops and equally popular among scientists studying learned vocal behaviors. Using whole-genome sequencing and population genomic approaches, I identify selection signals that differ between Bengalese finches and their wild munia ancestors. Findings from this study suggest that, as with mammalian domesticates, the domestication of the Bengalese finch involved a shift in selective regime, capable of altering brain circuits favoring the dynamic modulation of motivation and reward sensitivity over overall augmented aggression and stress responses.

Several lines of evidence demonstrate Bengalese finches have evolved more complex vocal abilities than their wild munia ancestors: adult Bengalese finches' song retains a greater degree of variability in the ordering of vocal elements; Bengalese finches exposed to multiple tutors

compose their songs from a combination of excerpts from the different tutor's songs, in contrast to their wild ancestors, who latch onto and copy the song of only one tutor; moreover, in cross-fostering experiments, Bengalese finches can learn a munia foster parent's song more efficiently than munias learn their Bengalese finches foster parent's song.

In Chapter 2, I track the Bengalese finches clues into their complex abilities in vocal learning, practice, and performance. To do so, I assess gene expression patterns in song-dedicated versus more generalist brain regions of juvenile Bengalese finches actively engaged in song learning via imitation and adult individuals past this sensorimotor learning phase, and primarily engaged in song maintenance and performance to females. Results from this study uncover co-regulatory patterns involving genes essential to motivation and reward. These patterns are developmentally and behaviorally regulated in the songbird brain, and likely contribute to motor learning, practice, and performance.

In Chapter 3, I take an evo-devo stance to the study of vocal learning, and examine evidence that developmental timing, though not sufficient, may have been key to the evolution of complex vocal production learning. I point out how a sort of change in evolutionary regime common to domestication practices may bring about delayed developmental trajectories; and discuss its effects on brain circuits for motivation and reward sensitivity, as they relate to vocal learning.

The dissertation of Madza Yasodara Farias Virgens is approved.

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University of California, Los Angeles

2023

Dedication

This dissertation is dedicated to the memory of Gene, who like me, loved birds, but for all the cat's

reasons.

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Acknowledgments

Chapter 3 is an excerpt I primarily wrote from Aamodt, CM, Farias-Virgens, M, and White SA. (2019). Birdsong as a window into language origins and evolutionary neuroscience. Phil. Trans. R. Soc. B 375: 20190060. This work was supported by grants NIH F31-MH110209, NIH T32-MH073256, the Coordination for Improvement of Higher Education Personnel (CAPES), Brazil, NSF (grant no. Bio Anthro DDRIG 1613709), the UCLA Eureka and Will Rogers Scholarships, and the Philanthropic Educational Organization's International Peace Scholarship, and NIH RO1MH070712. I acknowledge my co-authors Caitlin Aamodt and Stephanie White (contributed writing the manuscript).

Appendix I is a version of Farias-Virgens, Madza, and Stephanie A. White. "A sing-song way of vocalizing: Generalization and specificity in language and birdsong." Neuron 96.5 (2017): 958-960. I acknowledge my co-author Stephanie White (contributed to writing the manuscript).

Appendix II is the abstract for a paper I am coauthoring. Cooke EK, Fernandez A, DeFlorencio S, Farias-Virgens M, Walters KN, Van Veen JE, and White SA* I acknowledge my co-authors Elizabeth Cooke (conceived scope of study, conducted research and is the primary author of the manuscript), Ariel Fernandez, Stacy DeFlorencio, Kelli Walters, and Ed Van Veen (contributed to conducting research) and Stephanie White (conceived scope of study, conducted research and contributed to writing the manuscript).

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2019 Aamodt, C*, **Farias-Virgens, M*** and White, S. A. *co-first authors. Birdsong as a window into language origins and evolutionary neuroscience. In Philosophical Transactions of the Royal Society B. Special issue Contributions of animal communication to understanding human language. 375 (1789), 20190060

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Chapter 1 . To Tame a Songbird: The Genomics of the Domestication Syndrome in a Songbird Model Species

To Tame a Songbird: The Genomics of the Domestication Syndrome in a Songbird Model Species

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Abstract

Many domesticated animals share a syndromic phenotype marked by a suite of traits that include more variable patterns of coloration, reduced stress and aggression, and altered risk-taking and exploratory behaviors relative to their wild counterparts. Roughly 150 years after Darwin's pioneering insight into this phenomenon, reasonable progress has been made in understanding the evolutionary and biological basis of the so-called domesticated phenotype in mammals. However, the extent to which these processes are paralleled in non-mammalian domesticates is scant. Here, we address this knowledge gap by investigating the genetic basis of the domesticated phenotype in the Bengalese finch, a songbird frequently found in pet shops and a popular animal model in learning vocal behaviors. Using whole-genome sequencing and population genomic approaches, we identify strain-specific selection signals in the BF and its wild munia ancestor. Our findings suggest that similar to other animals, the evolution of the domestication syndrome in the BF involved a shift in selective regime, capable of altering brain

circuits favoring the dynamic modulation of motivation and reward sensitivity over overall augmented aggression and stress responses.

Introduction

Heightened stress and aggressive responses are significant traits of adaptive value for wild animals, manifested as impulsive reactions to perceived threats, often associated with high arousal states [1]. Domesticated animals show lower levels of reactive aggression than their wild counterparts and more consistently exhibit risk-taking behaviors in contexts where they are highly motivated, following spontaneous learned associations and/or as reinforced by domestication practices [2]. These changes are possible due to the attenuation of environmental sources of selection commonly found in the wild and often accompanied by enhanced selective pressures imposed by close socialization with individuals of the same or distinct species in the domestic setting [3].

Here, we study one such domestication case in an under-examined order, the passerine lineage. Beginning ~250 years ago, white-backed munias (WBM; *Lonchura striata*), an extant wild songbird readily found throughout East Asia, were brought to Japan by aviculturists [4]. Shortly after the first century of domestication, these artificially bred WBMs started to exhibit piebald (i.e., patchy) patterns of plumage coloration, marking the origins of today's Bengalese finches (BF; *Lonchura striata domestica*) [5] (Fig.1). Since then, BFs have become popular cage birds, known for their easy socialization and willingness to foster offspring, including those of other songbird species [6]. In addition to confirming BFs' attenuated stress response, evidenced by decreased reactive aggression and neophobia relative to their munia ancestors [7-9], studies show that adult BFs' song retains a greater degree of variability in the ordering of vocal elements than exhibited by WBM [10, 11]. These traits evolved in concert in the BF, characterizing a prototypical case of the domestication syndrome in the avian clade.

While we have amassed considerable knowledge about the behavioral, physiological, and morphological differences between domesticated and wild mammals [12] and posed specific

evolutionary hypotheses for how those changes came about [13, 14], the extent to which this is paralleled in the non-mammalian realm is under-studied. In this work, we address this gap in knowledge by investigating the genetic basis of the domesticated phenotype in the BF. Using whole-genome sequencing and population genomic approaches, we identified selection signals for which the magnitude differs noticeably between the domesticate BF and its wild munia ancestor and interrogated annotated genes within those regions of interest (ROIs). Selection signals in the WBM population locate to regions of importance for divergence and local adaptation within the songbird lineage. Variation in genes within WBM ROIs is associated with morphological and behavioral traits relating to stress and aggression. These selection signals are absent in the domesticated BF, which instead shows signals that are shared with other avian and mammalian domesticates related to reward aspects of social interactions and feeding. In line with comparisons between other domesticates and their wild counterparts, our findings suggest that the evolution of the domestication syndrome in the BF likely involved the attenuation of selective forces for augmented reactive aggression and the intensification of ones leading to dynamic modulation of motivation and reward sensitivity.

Methods

Sample collection

Blood samples were collected from 15 BFs and 15 WBMs housed in the Okanoya Lab at RIKEN-Brain Science Institute (RIKEN-BSI, Japan). BF source colonies in the Okanoya Lab originated from and are continuously supplied by various breeders in Japan and diligently maintained per standard bird breeder practices to avoid undesired effects of inbreeding. WBMs were imported from multiple wild colonies from different locations in Taiwan and bred in Okanoya lab's aviaries, following similar practices as for BFs (~5 generations at the time of sample collection for this study). To avoid immediate relatedness biases, we used individuals who were no less than two degrees related to one another within each BF and WBM populations. Blood samples were extracted by puncturing the brachial vein and collecting the resulting droplet in a capillary tube, a

well-suited method for sampling small birds with minimal injury and distress. Samples were stored at -80°C at RIKEN-BSI until transported to UC Berkeley under material transfer agreements and USDA-APHIS approval (#128913). Housing conditions and procedures conformed to RIKEN's Animal Care and Use Committee.

Whole genome library preparation and sequencing

Total DNA was extracted from individual blood samples using a DNAeasy kit (Qiagen, Germantown, MD) following protocols at the Evolutionary Genetics Laboratory at UC Berkeley. DNA quality was visually inspected by gel electrophoresis, and DNA concentrations were measured using NanoDrop spectrophotometry and a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA). Samples showing signs of degradation or yielding DNA concentrations insufficient for library preparation (< 50ng/microL) were discarded. Whole genomic double-stranded DNA from the remaining 13 BF and 12 WBM DNA samples were fragmented to a 350bp average size using a Bioruptor sonicator (Diagenode-Hologic, Denville, NJ). Sheared samples were visualized by gel electrophoresis to verify size distribution, and DNA concentrations were re-measured using a Qubit dsDNA BR Assay Kit. All 25 samples passed this quality control step and were used to prepare whole-genome libraries using a KAPA Hyper Prep PCR-free Kit (Hoffmann-La Roche, Basel, Switzerland) and TruSeq adapters (Illumina, San Diego, CA). Library sizing information was obtained by electrophoresis using an Agilent 2100 Bioanalyzer system. The 11 BF and 11 WBM libraries passing this quality control step were sequenced on seven lanes in a 4000 HiSeq system (Illumina, San Diego, CA) at UC Berkeley's QB3 sequencing facility.

Sequencing data preprocessing and quality control

The resulting paired-end sequencing reads (150bp) underwent quality-control procedures using the software package *readcleaner* (github.com/tplinderoth/ngsQC), which includes trimming adapters using *cutadapt* v1.10; pair-end read merging using pear v0.9.10; filtering contaminants identified through alignment to the human (GRCh38) and *E. coli* (ASM584v2) genomes using

bowtie2 v2.2.9 [15], and generates final read quality reports using *fastqc* v0.11.4 [16]. Clean reads were aligned to the three available chromosome-level assemblies using the Burrows-Wheeler Alignment tool: the recently published, high coverage (a) BF genome produced by the Brainard lab at the University of California San Francisco (LonStrDom1- Illumina HiSeq 2500) and (b) the zebra finch (ZF; *Taeniopygia guttata*) genome sequenced as part of the Vertebrate Genomes Project (bTaeGut2.pat.W.v2- Pac-Bio); (c) the canonical ZF shotgun genome published in 2007 (Taeniopygia_guttata-3.2.4) and continuously updated by researchers in the field. Genome indexes for each reference genome were generated using *BWA* [17] and *SAMtools* [18], along with sequence dictionaries from *Picard tools* (broadinstitute.github.io/picard/) and aligned using appropriate read group information. After alignment, duplicate reads were marked, and an additional step of local indel realignment was performed using Genome Analysis Toolkit (GATK) [19]. Indels were called from all sampled individuals (GATK-HaplotypeCaller) and used to create lists of indel sites for each sampled individual separately (GATK-RealignerTargetCreator). These were then used as targets in the realignment (GATK-IndelRealigner). Only reads with a minimum mapping quality score of 20, which were also uniquely mapped and properly paired, were used for subsequent analyses.

Another filtering step was performed to remove paralogous or repetitive sequence regions that confound short read mapping using *ngsParalog,* (github.com/tplinderoth/ngsParalog). This step involved calling single nucleotide polymorphisms (SNPs) in each BF and WBM populations separately using Analysis of Next Generation Sequencing Data (ANGSD) [20], a software package that calculates various population genetic summary statistics from genotype likelihoods. The union of the identified variant positions in each population served in estimating per-site paralog-log likelihood ratios. Likelihood ratios for each site were then compared between BF and WBM, and the highest values were retained. The -log likelihood ratio is asymptotically distributed as a 50:50 mixture of a chi-square with one degree of freedom and a chi-square distribution with zero degrees of freedom. Accordingly, the cutoff above which a given site would be considered

paralogous and excluded from future analyses was calculated as *0.5 + 0.5 * qchisq(p, df=1) = 1 – W*, where *W* = 0.05 significance level and corrected for multiple hypothesis testing using the total number of sites.

After excluding potential paralogous or repetitive regions, the sequencing data rendered an average per-site coverage depth per individual of 8x. Sites with per individual max depth of 20x (~2SD+) were further filtered, as they could be sequencing artifacts or represent paralogous and repetitive regions bypassed by the prior filtering. To avoid spurious results from missing data, sites covered in less than 9 out of 11 individuals in each population were further filtered from the data. Finally, among the remaining sites, only diallelic sites were inferred from downstream analyses.

Population genomic analyses

Inferring genetic differentiation and selection from allelic frequencies

Using the *GATK* model within *ANGSD,* we calculated genotype likelihoods from sequencing reads as the probability of the observed sequencing data *X* given the three different possible genotypes *g=0,1,2*, representing the number of copies of the minor allele in a site for an individual, *P(X|G=g)*. The allele frequency at a given site in the population is used in calculating the prior genotype probability of the same site for an individual, $P(G_{is}|p_s)$, which can be calculated under the assumption of Hardy-Weinberg equilibrium (HWE) for the three different possible genotypes $g=0,1,2$ as $P(G_{is}=0|p_s)=(1-p_s)^2$, $P(G_{is}=1|p_s)=2p_s(1-p_s)$ and $P(G_{is}=2|p_s)=p^2_s$), and used to define posterior genotype probabilities using Bayes' theorem.

To perform principal component analysis (PCA) of the genetic relationship among individuals in our populations, we used a heuristic approach implemented in *PCAngsd* [21] that does not rely on the assumption of conditional independence between sampled individuals and, in this way, can model missing data with the inferred population structure. *PCAngsd* replaces population allele frequencies with individual allele frequencies calculated from posterior expectations of genotypes

to update prior information in calculating posterior genotype probabilities. The individual allele frequencies are computed through a singular value decomposition (SVD) method, where genotypes are reconstructed using the top principal components and serve to inform prior genotype probabilities, assuming HWE in the calculation of posterior genotype probabilities and in updating genotype expectations. This procedure is iterated until individual allele frequencies converge. Finally, the covariance matrix is calculated within a probabilistic framework by summing over each individual's genotype weighted by the joint posterior probability of individual allele frequencies. Eigendecomposition of the updated estimated covariance matrix is then performed to obtain the principal components.

A series of statistics summarizing the variation or distribution of alleles within the dataset was calculated using *ANGSD realSFS*. Per site allelic frequencies were estimated for each population separately and used to estimate the proportion of alleles happening at specific frequencies in each population – 1-dimensional Site Frequency Spectrum (1D-SFS), as well as at a particular frequency in one population and at a different frequency in the other population – 2-dimensional SFS (2D-SFS). Accordingly, considering a set of *n* aligned DNA sequences, the SFS is defined as the vector that describes the frequency of *k = 1 to n* derived alleles; $\eta = (\eta_k)_{k = 0,...,n}$, where η_k is the number of sites with *k* derived alleles. Genome-wide SFSs estimated using the self-referencing BF genome were calculated from minor allelic frequencies (MAF) (i.e., folded). The 2D-SFS served as the prior in subsequent per site Fixation index (*Fst*) calculations, a commonly used population genetics statistic that measures genetic variance *between* relative to *within* populations. Per-site *Fst* estimates were calculated using Bathia *et al.* [22] derivation of Hudson's *Fst* estimator, as implemented in *ANGSD realSFS fst*.

$$F_{ST}^{Hudson} = 1 - \frac{H_w}{H_b}$$

Where considering p_1 and p_2 allelic frequencies at a given site in each population,

$H_w = p_1(1 - p_1) + p_2(1 - p_2)$ is the heterozygosity *within* populations and

$H_b = p_1(1 - p_2) + p_2(1 - p_1)$ is the heterozygosity *between* populations.

Recast by Bathia *et al.* as:

$$F_{ST} = \frac{N}{D}$$
$$N = (p_1 - p_2)^2$$
$$D = p_1(1 - p_2) + p_1(1 - p_2)$$

This estimator is preferable for smaller sample sizes as it does not depend on their ratio.

For analyses of multiple SNPs comprising genomic regions of interest, *Fst* indexes were calculated as the ratio of the sum of per site Bathia *et al.* estimations of the variance *within* (*N*) and *between* (*D*) populations.

$$\hat{F} = \frac{\sum_{k=1}^{M} \hat{N}^{[k]}}{\sum_{k=1}^{M} \hat{D}^{[k]}}$$

Using this approach, we calculated weighted *Fst* between our BF and WBM populations in non-overlapping windows spanning 100kb across the genome, as well as across all positions within a given gene's full length, and each exonic and intronic annotated boundaries (Supp. Data 1).

Folded genome-wide 1D-SFSs were used as priors in calculating various estimators describing the genetic diversity in each BF and WBM populations separately, using *ANGSD realSFS saf2theta*. These estimators measure the ratio between the nucleotide diversity observed in a population and the expected under a neutral evolution model. We calculated Waterson's (θ_W) [23] and Pairwise (θ_π) estimators of genetic diversity for each BF and WBM populations in non-overlapping windows spanning 100kb across the genome, as well as across all positions within a given gene's full-length, and within exonic and intronic annotated boundaries (Supp. Data 2).

Considering a set of n aligned DNA sequences and η the number of sites with derived alleles in each one of the sequences, the sum of the number of observed segregating (i.e., polymorphic) sites in a population can be calculated as $S = \sum_{i=1}^{n-1} \eta_i$. Waterson's estimator infers diversity in a population from the ratio between the total observed segregating sites S over the expected number of segregating sites. This estimator is more sensitive to detecting deviations in intermediary to higher frequency variants segregating in a population [23].

$$\hat{\theta}_W = \frac{S_n}{1 + \frac{1}{2} + \frac{1}{3} + ... + \frac{1}{n-1}}$$

The Pairwise estimator calculates the number of segregating sites between two sequences at a time and then averages it over all pairs in n total sequences. This estimator is more sensitive to deviations in low-frequency variants segregating in a population.

$$\hat{\theta}_\pi = \binom{n}{2}^{-1} \sum_{i=1}^{n-1} i(n-i)\eta_i$$

We also calculated the standardized difference between these two θ estimators, *Tajima's D*, which is often used for detecting selection effects in a population [24].

$$D = \frac{\theta_\pi - \theta_W}{\sqrt{\text{var}(\theta_\pi - \theta_W)}}$$

Selection of a sequence variant leads to an individual's increased chances of survival and reproduction, which frequently leads to surrounding variants also increasing in frequency within the population (i.e., selective sweep) [25]. An event like this will cause *Tajima's D* to become negative, as there will be relatively more high-frequency variants than expected under a neutral model, thus rendering the expectation of θ_π smaller than the expectation of θ_W. Therefore, one of the general signatures of selection is a loss of nucleotide diversity around a genetic variant related to the advantageous trait. This effect might result from positive selection during the sweep-up of

the newly advantageous variant or purifying selection, resulting in the purging of deleterious variants surrounding the neutral one (i.e., background selection). Positive values of *Tajima's D* arise from an excess of intermediate frequency alleles and can result from population bottlenecks, structure, and/or balancing selection.

We also performed selection scans using *SweepFinder2* [26], an implementation of a method that performs a composite likelihood ratio test for positive selection. In this method, the likelihood of the null hypothesis is calculated for each population from their respective genome-wide 1D-SFS; and the likelihood of the alternative hypothesis is calculated from a model in which a recent selective sweep alters the neutral spectrum. *SweepFinder2* selection scans were run in non-overlapping windows spanning 100kb each (Supp. Data 3).

Direct Measures of Selection

With the *dn/ds* estimates, we aimed to test if our sets of candidate genes have a differential selection signal compared to the overall genomic background. Using Fisher's exact test, we first tested if the sets of candidate genes had a greater proportion of dn/ds values greater than or equal to one than in the overall genomic background. An odds-ratio greater than one and statistically significant would be consistent with elevated signatures of positive selection in our candidate gene sets. Next, we assessed whether our sets of candidate genes have a significantly different mean *dn/ds* than the overall genomic background using a t-test, which if the mean *dn/ds* is significantly larger than found in the genomic background, would be consistent with signatures of positive selection. However, because *dn/ds* values are heavily biased by the extent of selection on synonymous substitutions, we also partitioned two classes of substitutions into fixed and polymorphic substitutions. We used these portioned counts to first calculate neutrality indices (Nis) for every gene with at least a count value of five for each cell of the 2x2 contingency table to avoid spurious results. Next, we reasoned that NIs that were significantly less than one exhibited an excess of nonsynonymous fixed difference, and NIs that were significantly greater than one exhibited an excess of nonsynonymous polymorphisms. Significance was assessed

using a Fisher's exact test and corrected for multiple comparisons using a Bonferroni correction where each gene counts as one comparison [27]. With this partitioned count data, we also calculated the proportion of nonsynonymous substitutions fixed by positive selection for each set of candidate genes and the genomic background and assessed significance using Fisher's exact test [28]. Lastly, using the partitioned count data, we measured the direction of selection (DoS) per gene and compared the mean DoS between the sets of candidate genes and the genomic background. We assessed significance using a t-test where a significant positive DoS value is evidence of adaptive evolution, and a significant negative DoS value is evidence of slightly deleterious mutations still segregating [28].

Results

The PCA of the total genetic distance among all BF and WBM individuals shows a clear separation between the two populations along PC1 (Fig.1A). This distinction is marked by a greater proportion of low-frequency genetic variants in the wild WBM population. In contrast, variants at higher-to-fixed frequencies prevail in the domesticated BF, indicating an overall loss of genetic variability within their population. (Fig.1B). We also report genomic windows and sets of enclosed genes for which observed variability deviates from expected under neutrality in each population, as evidenced by Tajima's D summary statistics (*TajD*) (Fig 2; Supp. Data 1). Genetic diversity in WBMs deviates less than expected across their genome, whereas, in BF, long stretches of the genome show either considerable loss or gain of variability. As with many domesticates, these genome-wide observations are likely due to the heightened effects of genetic drift, set off by the primary population bottleneck that marks the major domestication event [29] and furthered by domestication practices of breeding small populations in captivity.

Differences in allelic frequencies between BF and WBM are unevenly distributed across contiguous windows along individual chromosomes, allowing for the identification of local signals of differentiation between the two songbirds, as measured by the canonical fixation index (*Fst*) (Fig 3; Supp. Data 2). While the average genetic differentiation of regions within individual

autosomes is close to the genome-wide average (avg. *Fst* ~0.2), the sex chromosome Z (ChrZ) harbors a wide region that exhibits the greatest differentiation between wild and domesticated populations (> 4sd), spanning ~7% of total chromosome size (Fig 3; Supp. Data 2). The higher differentiation of ChrZ may be attributed to this chromosome's smaller effective population size due to its heterogametic mode of transmission (males = ZZ, females = ZW) [30].

Our selection scans allowed the detection of strain-specific signals in the domesticate BF and its wild munia ancestor (Fig 2; Supp. Data 3). Here, we interrogate annotated genes within those regions of interest (ROIs) and identify overlapping signals found in other wild and domesticated populations (Supp. Text 1).

Selection signals in wild munias locate to regions of importance for divergence and local adaptation within the songbird lineage

A set of genes on Chr1 exhibit a greater selection signal in WBMs relative to BFs, hereafter designated **WBMChr1-ROI**. These genes, **GPR161, TIPRL, SFT2D2**, and **TBX19**, lie within a previously reported large chromosomal inversion that underpins phenotypic variation among hoary redpolls (*Acanthis hornemanni*), common redpolls (*A. flammea*), and lesser redpolls (*A. cabaret*) [31]. Differences in the extent of brown and red pigments in plumage coloration, bill size and shape, and body size mark redpoll ecotypes. These traits vary on a latitudinal gradient from typically lighter-colored hoary redpolls, with shorter and narrower bills, more common at higher latitudes relative to lesser redpolls, which are restricted to western Europe and southern Scandinavia. Genes overlapping this sweep in our WBM population contribute to the most highly differentiated peaks within the redpolls' chromosomal inversion and contain SNPs significantly associated with ecotype identity, suggesting their role in redpoll phenotypic variation under local adaptation.

We identified a second selection signal specific to the WBM on a set of contiguous genes spanning a region of Chr2 **(WBMChr2-ROI)**. These genes show high divergence in 11 *Lonchura* munia species radiated throughout Australia and Papua New Guinea. Genes within this region,

MYO10, ZNF622, MARCHF11, and **FBXL7,** show high differentiation in four pairwise comparisons between sympatric *Lonchura* species with minimal genome-wide divergence, therefore possibly involved in generating adaptive diversity in munias in the wild [32].

We detected a third major sweep on Chr3 only in the WBM **(WBMChr3-ROI)** comprising a large set of contiguous genes and peaking at the region encompassing **THBS2, SMOC2, DACT2**, and **FRMD1**. Closer inspection shows that the wider WBMChr3-ROI1 is marked by an abrupt decay in heterozygosity and an excess of fixed sites in the WBM population. The genes with the highest sweep signals in this region, **THBS2** and **SMOC2**, code for proteins related to matrix assembly and cell adhesiveness, expressed in cartilage and bone during development and in the adult skeleton. In mammals, these genes' products are implicated in craniofacial dysmorphism, specifically brachycephaly (i.e., shortening of the anteroposterior skull length and widening along the cranial mediolateral axis) and brachygnathism (i.e., underbite) [33-35]. The roles of **THBS2** and **SMOC2** in craniofacial morphology appear to be preserved in birds, as they feature within highly differentiated regions between Darwin's finches with different beak shapes [36].

We detected a sweep signal present only in WBMs in a region encompassing the genes **TRIM24, AKR1D1,** and **PDE3A (WBMChr1A-ROI)**. *AKR1D1* encodes a steroid A-ring reductase, 5β-reductase, which performs a fundamental step in bile acid synthesis and catalyzes the inactivation of steroid hormones in the liver, where it is predominantly expressed [37]. *AKR1D1* regulates hormonal action in the liver, including glucocorticoid-mediated effects on carbohydrate and lipid metabolism associated with stress responses [38].

Signals of selection in the BF are shared with other avian and mammalian domesticates

In the domesticated BF, a broader area inclusive of **WBMChr3-ROI1** is marked by an increase in the frequency of alternative alleles, including at sites otherwise fixed in the WBM, leading to an increased number of segregating sites (*S*) (Fig 2; Supp. Data 4). The increase in *S* is maximal at the segment encompassing the gene **PRKN**. *TajD* values in the BF population abruptly decay upstream of **PRKN** and gradually reverse toward less negative values along BFChr3-ROI1

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downstream of it. In mammals and birds, this region is associated with a common fragile site within which breakpoints locate to **PRKN**. Fragile sites are more prone to mutate and frequently undergo large structural rearrangements. Accordingly, our findings of greater variability in the BF population on a region otherwise showing an increased proportion of high-frequency variants in the WBM may relate to increased genomic instability at **PRKN**. This scenario is compatible with the relaxation of selection in the domesticated versus wild strain at BFChr3-ROI1.

PRKN provides instructions for producing the parkin protein, an E3 ubiquitin ligase fundamental to mitochondrial dynamics in response to changes in cellular and systemic energy status, including those resulting from hypothalamic signals about internal states, such as stress level, reproductive status, and energy homeostasis [39, 40]. Variation in **PRKN** is frequently associated with adiposity in mammalian and poultry livestock [41-46]. In songbirds, **PRKN** is among a group of ~60 genes identified within genomic regions putatively under selection in urban relative to rural great tit populations (*Parus major*) broadly distributed across Europe [47].

We detected a major selection signal in both BF and WBM songbird strains, though noticeably greater in the domesticate than its wild ancestor, in a Chr4 region (**BFChr4-ROI1**) comprising the genes **SNCA**, **PYURF, PIGY, IGFBP7**, and **TECRL**. This region shows peaks in selection scans in several mammalian domesticates [48-55]. The gene with the greatest sweep signal difference between BF and WBM within BFChr4-ROI1, **SNCA**, encodes alpha-synuclein, a small neuronal protein predominantly localized in presynaptic terminals where it selectively binds to synaptic vesicles and regulates neurotransmitter release [56]. Changes in **SNCA** mRNA and protein levels are associated with reward aspects of food intake and social interactions [57, 58]. In birds, **SNCA** shows signals of selection in game fowls selected specifically for cockfighting compared to wild Red Jungle fowl [60]; and Kuroiler chickens, a dual-purpose Indian breed selected for the ability to scavenge food and tolerate heat [59].

Within the sex chromosome ChrZ, we found a major signal of selection present only in the BF **(BFChrZ-ROI1)** comprising the genes **SNX24, SNX2, SNCAIP, LOX**, and **SRFBP1**. Within

BFChrZ-ROI2, the gene with the greatest sweep signal, **SNCAIP**, encodes alpha-synuclein interacting protein-1, or synphylin-1, a presynaptic protein that colocalizes with alpha-synuclein in Lewy bodies seen in Parkinson's disease (PD) [60]. Though little is known about its function in the normal brain, synphylin-1 is regarded as a partner in alpha-synuclein's synaptic functions [61] and is implicated in food intake and fat deposition in mammals and birds [62-64].

Discussion

Selection signals in the WBM population locate to regions of importance for divergence and local adaptation within the songbird lineage. These selection signals are otherwise absent in the domesticated BF, which instead shows shared signals with other avian and mammalian domesticates. In what follows, we interpret the significance of our findings, considering known morphological and behavioral differences between BFs and WBMs and similar comparisons between other domesticates and their wild counterparts.

Decreased Stress and Reactive Aggression

Like other domesticates, BFs exhibit less stress than their wild counterparts, as shown by lower levels of cortisol measured in fecal samples collected throughout the day, and lower reactive aggression, as demonstrated by less frequent biting responses when provoked with a stick attached to a piezo-electric sensor [9, 65]. In mammals and birds, stress and aggression are coordinated by the hypothalamus-pituitary-adrenal axis (HPA). Therefore, genes whose expression is enriched in components of the HPA are prime candidates to underlie differences in stress and aggression between domesticated and wild animals. Based on this criterion and the existence of prior genetic associations, we identified genes of importance for reactive aggression and stress where selection signals were present in wild munias but absent in their domesticated descendants, some of which are discussed below:

In the embryonic mouse brain, the gene **TBX19 (WBMChr1-ROI)** encodes a transcription factor that marks pituitary cell lines that will later express pro-opiomelanocortin (POMC), the peptide

that elicits adrenocorticotropic hormone release [66]. Changes in **TBX19** are suggested to underlie tameness and timidity in Chinese indigenous pigs [67].

The gene **MARCHF11 (WBMChr2-ROI)** encodes an E3 ubiquitin ligase that exhibits enriched expression in the testis, consistent with its role in protein sorting in developing spermatids [68]. In mammals, this region shows selection signals, more distinctly in Bovids, where it has been associated with fertility [69]. **MARCHF11** expression is also enriched in the pituitary gland relative to other tissues. Though its function in the pituitary remains unexplored, emerging findings suggest that **MARCHF11** brain expression is regulated by social and stress-related cues tied to reproduction [70]. Key to this process is vasopressin, a hormone synthesized within the hypothalamus and stored in the posterior pituitary. Vasopressin is released to target brain regions, where it has an indirect inhibitory effect on gonadotropin-releasing hormone through the potentiation of ACTH and stimulation of cortisol secretion [71]. **MARCHF11** expression is sexually dimorphic in midbrain vasopressin-responsive neurons, which are activated by prosocial stimuli (e.g., the presence of a female mouse, leading to mating or huddling) but not by antagonistic social stimuli (e.g., the sight of a male mouse, leading to fighting or drawing back) [70]. In songbirds, neurons expressing the avian homolog of the mammalian vasopressin, vasotocin, exert effects on sex-specific behaviors, pair bonding, gregariousness, and aggression [72, 73]. These observations implicate **MARCHF11** in sex-specific reproductive physiology and behavior that discriminates between stress conditions, as regulated by vasopressin/vasotocin.

The absence of selective pressures on costly traits otherwise valuable in the wild can also elicit morphological changes in domesticated or urbanized animals [74]. Though direct morphological comparisons between BF and WBM are yet to be explored, the absence in the BF of selective signals found in their munia ancestor at genes of importance to beak shape, **THBS2** and **SMOC2 (WBMChr3-ROI)** indicate craniofacial differences between the two. This finding is consistent with controlled observations that WBMs show stronger biting to provocation than BFs, as differences in beak dimensions and/or head width are the major predictors of bite force in finches [75].

Differences between BF and WBM, and possibly associated craniofacial allometric scaling, could derive from the lack of selective pressures such as physical endurance against aggression and foraging harder and more varied materials and food in the wild.

Increased flexibility in birdsong learning and practice

Several lines of evidence demonstrate BFs have evolved more complex vocal abilities than WBMs: adult BF song retains a greater degree of variability in the ordering of vocal elements than exhibited by WBM; BFs exposed to multiple tutors compose their songs from a combination of excerpts from the different tutor's songs, in contrast to their wild ancestors, who latch onto and copy the song of only one tutor; moreover, in cross-fostering experiments, BFs learn their WBM foster parent's song more efficiently than WBMs learn their BF foster parent's song [10, 11, 76]. Song learning and production are modulated by dopaminergic innervation originating in the midbrain and projecting to specialized telencephalic nuclei in the songbird brain, including the striatal region known as Area X and cortical vocal control nuclei, such as HVC (used as a proper name) and the robust nucleus of the arcopallium (RA) [77]. Vocal learning correlates with dopamine (DA) regulation in these regions, with overall DA levels higher during juvenile development and strongly declining in adults [78]. As songbirds progress in their learning trajectory, they explore vocal gestures and adjust their song in adaptive ways (undirected singing; UDS) to obtain positive social feedback during performance directed to conspecifics (directed singing; DS). DA release is lower in contexts associated with increased song stereotypy, as in DS, relative to contexts related to increased song variability, as in UDS [79]. Therefore, evolutionary changes in song complexity likely elicit modifications in genes related to DA functions, a major neuromodulator of neuronal excitability in the song circuit. Our analysis of genetic variation in BF and WBM populations uncovered clear signals of selection in the BF on genes regulating DA synthesis, availability, and response in the songbird brain (see also **DDC** and **DRD5**; Supp. Text 1), as well as on genes implicated in axon guidance and connectivity

(**SLIT2**, **COL4A1,** and **COL4A2**; Supp. Text 1) that contribute to the differentiation and specialization of the song control circuit.

The gene **SNCA (BFChr4-ROI1)** negatively regulates DA availability within song control nuclei, where it is constitutively downregulated at both mRNA and protein levels [80, 81]. Overall low alpha-synuclein levels in these regions may serve as a protective mechanism against protein aggregation and consequent motor deficits during aging [82]. Furthermore, **SNCA** expression is developmentally downregulated during the critical period for song learning within RA and Area X's adjacent efferent nucleus, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) [81, 83]. In the adult songbird brain, alpha-synuclein is differentially regulated within Area X between vocal practice and performance, with increased levels scaling to the number of motifs sung in UDS but not during DS [84]. Though less is known about synphilin-1's function within the song circuit, **SNCAIP (BFChrZ-ROI1)** mRNA expression is significantly downregulated within Area X during UDS [85]. This result is consistent with synphilin-1's role in regulating the ubiquitin-mediated degradation of alpha-synuclein [85, 86] and suggests this interaction contributes to the modulation of song variability during practice.

Vocal learning is sexually dimorphic in the BF, with young males learning their courtship songs through social interactions with an adult male tutor. In contrast, females judge and influence the quality of male songs. When auditioning multiple males, female Bengalese finches show elevated heart rate, increased nest-building behavior, and increased serum estradiol levels in response to the most complex song [87]. This poses female selection as a driver in the BF's evolution of more complex vocal behavior. Concurrent analyses postulate possible effects of the relaxation of sensory ecology constraints on song complexity in the domestic setting, which are otherwise present in the wild [88]. Song stereotypy (i.e., linearity or simplicity) in WBM wild populations increases the higher the degree of colony mixing with another cohabitating wild finch, the spotted munia (*Lonchura punctulata*) [87]. This source of constraint is commonly lifted during domestication, thus contributing to the evolution of more complex vocal behavior in the BF, as

biased by female preference. Our study found intensified selection in the BF relative to WBM on regions comprising genes tightly linked to dopaminergic transmission, thus capable of affecting brain circuits highly relevant for song learning and production. Though these genes locate to selection signals within both the autosomes and the ChrZ, we have found that overall selection signals in the BF disproportionately affect genes in the sex chromosome. This pattern is consistent with intensified female choice or artificial selection of Z-linked male-specific traits [31]. According to extensive historical reports, BFs were never bred for their singing ability [87], leaving females' choice of more complex songs and/or of traits coupled with it as a probable and parsimonious explanation for the evolution of BF's complex vocal learning abilities. Furthermore, genes within selection signals present in the wild WBM but absent in the BF are relevant in the specialization and function of nuclei dedicated to song, either directly or by virtue of their participation in networks engaging stress and reward/motivation in the songbird brain (Supp. Text 1).

Reduced neophobia in feeding contexts

Controlled observations reveal that BF exhibit decreased levels of neophobia in feeding contexts relative to their wild counterparts, as demonstrated by their lower latency to approach the food cup in the presence of a foreign object [8]. This result can be explained by differences in food motivation between BF and WBM, to which our discovery of evolutionary changes in regions encompassing genes implicated in reward aspects of food intake may relate. *In vivo* studies suggest that the **SNCA (BFChr4-ROI1)** gene product, alpha-synuclein, regulates DA availability in response to the direct action of metabolic hormones in DA centers or downstream from homeostatic signaling in the hypothalamus [58]. Similarly, synphilin-1 is implicated in hyperphagia, first characterized in transgenic mice overexpressing human **SNCAIP (BFChrZ-ROI1)** predominantly in neurons, which unexpectedly manifested obesity resulting from increased food intake, and in the absence of PD-like symptoms [89]. Signatures of selection at **SNCAIP** have been detected in broiler chicken lines divergently bred for abdominal fat content, which

supports synphilin-1's relation to food intake and adiposity as being conserved in avian species [90].

Increased color variation

Eye and coat color tend to be fixed traits within wild species, with some variation related to age and sexual dichromatism. In contrast, individual color variation in the adult stage is characteristic of many domestic species [91, 92]. An apparent difference between BFs and their munia ancestors is plumage pigmentation. While WBMs stereotypically have dark-brown heads, tails, and wing plumage, which morphs into more visibly barred feathers on a darker to lighter gradient towards their flank and rump, BFs' plumage assumes the most varied combination of tones, with variable body distribution, including dark-brown (chocolate), lighter brown (chestnut), yellowish-brown tan (fawn), pale-yellow (crèmino) and white (albino) morphs. Historical accounts indicate that piebald plumage coloration spontaneously emerged ~140YBP and was picked up by Japanese breeders, who further developed many BF color morphs [4]. Among the genes within regions where our genome-wide scans reveal signals of selection in the BF, many show variation linked to pigmentation differences in other animals and may as well be involved in the evolution of piebald plumage coloration in BF (Supp. Text 1). Outside of the major ROIs, we found selection signals specific to the BF at the **OCA2, HERC2**, and **GABRB3** gene cluster in Chr1 **(BFChr1-LOC2)**. **OCA2** encodes the melanosomal transmembrane protein P, involved in the trafficking and processing of tyrosinase, a catalyzer of crucial steps in the melanogenesis pathway, whose absence halts melanin production [93]. OCA2 expression is regulated by an element located within an intronic region of its contiguous upstream neighbor **HERC2**, where variants are major determinants of blue/brown iris coloration and are associated with differences in skin pigmentation in humans [94]. Mutations in OCA2's mouse orthologue (i.e., the pink-eyed dilution locus), causing the lack of functional P protein, lead to a pink-eyed and light fur phenotype [93]. The BF domestication was marked by significant breeding efforts for full white morphs, leading to the development of the black-eyed and pink-eyed albino BF strains [4]. Our findings raise the

possibility of the involvement of OCA2 in BF plumage coloration, especially relevant in the development of the pink-eyed albino and other light-colored BF strains.

BF's note on developmental aspects of the domesticated syndrome

Recent years have seen the advance of an overarching hypothesis that postulates that the suite of traits shared by many domesticates derives from selective pressures affecting the initial stages of embryonic development [14]. Within this framework, the domestication syndrome predominantly results from mild changes in the differentiation and migration of neural crest (NC) cells. This transient population delaminates from the neural tube and migrates extensively throughout the embryo to form bone, cartilage, adipose tissue, endocrine cells, several types of neurons and glia, and pigment cells. Our selection scans reveal strain-specific traces of selection in regions encompassing loci implicated in NC differentiation and migration: a signal present in the BF includes **WNT7B (BFCh1A-*LOC2*)**, a member of the Wnt signaling pathway known to regulate NC specification [95]; and a selection signal present in the WBM encompassing **BAZI1B (WBMChr19-*LOC1*)**, a transcription factor involved in NC migration and maintenance [96]. These sweeps are relatively weaker, and both *loci* locate rather away from their respective signal's peak. The uncertainty of these findings prevents us from drawing conclusions about the relevance of NC changes in the evolution of the domesticated phenotype in the BF other than referring to these genes for further investigation.

Conclusion

Results from various avenues of inquiry indicate that a typical recipe for domestication includes the attenuation of selective forces for augmented reactive aggression and the intensification of ones leading to dynamic modulation of motivation and reward sensitivity. Our findings suggest that the same principles apply to tame a songbird. Major selective sweeps in the BF comprise genes essential for DA synthesis, availability, and response in the songbird brain, thus highly relevant in the specialization and function of brain nuclei dedicated to song. Our scans also reveal

selection signals specific to the BF in genes causally linked to pigmentation differences in other animals, which could similarly explain BF color morphs.

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Author Contributions

Designed research: MFV, KO, TD; performed research: MFV; analyzed data: MFV, DP, EHS; contributed to writing: MFV, SAW, DP, EHS, KO, TD.

Competing Interests statement

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

Figures, tables, and legends

Figure 1-1. **A songbird model system for understanding the domestication syndrome.** (top) The WBM (wild; blue) and the BF (domesticated; green). BF's domestication history and environmental variables in wild and domestic settings. (A) The PCA of the total genetic distance among all BF and WBM individuals shows a clear separation between the two populations along PC1; (B) BF and WBM unfolded SFSs (i.e., the frequency distribution of variants in each population). A greater proportion of low-frequency genetic variants marks the WBM population. In contrast, variants at higher-to-fixed frequencies prevail in the domesticated BF, indicating an overall loss of genetic variability within their population.
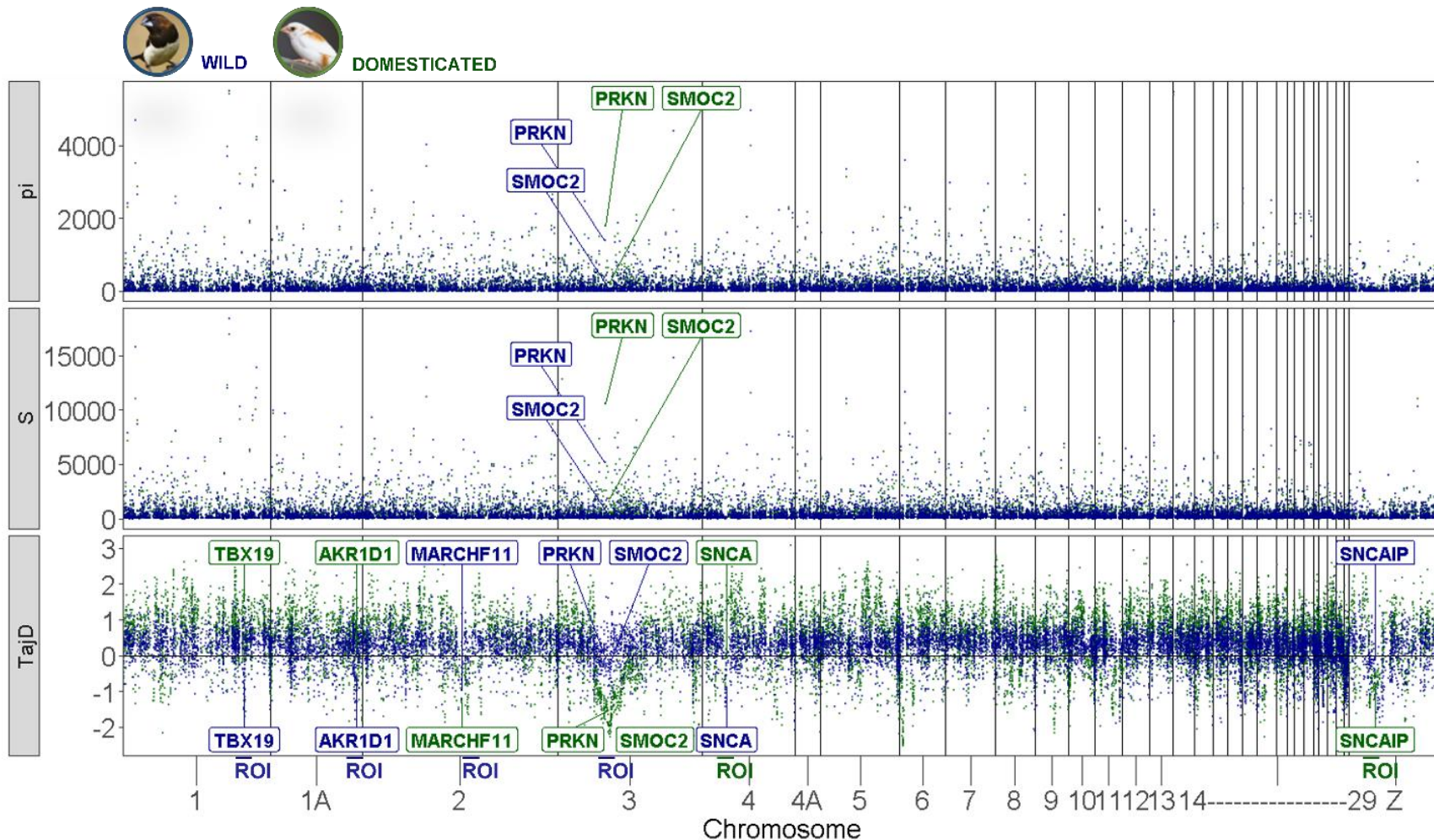
Figure 1-2. **Genetic variability in WBM and BF populations**. Each data point represents (top) the total number of Pairwise differences (*pi*) across all positions within a given gene; (middle) total number of segregating sites (*S*) across all positions within a given gene; and (bottom) observed variability relative to expected in each population, calculated as the *TajD* across all positions within a given gene. The dashed horizontal lines indicate *TajD* = 0 (variability does not deviate from expected). Labels highlight the genes within defined regions of interest (ROIs) referred to in the main text.
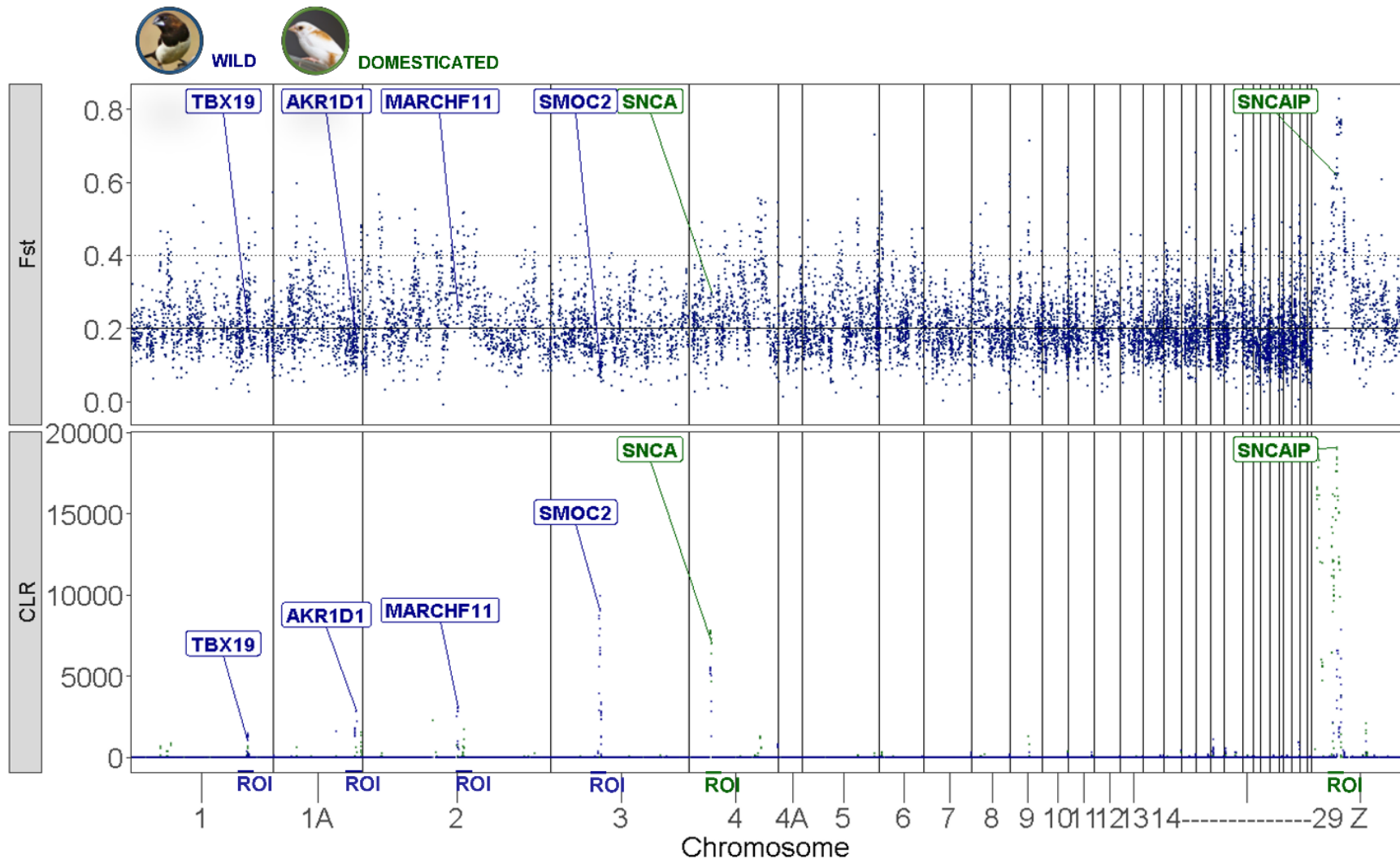
Figure 1-3. **Genetic differentiation and selection signals in WBM and BF populations**. Each data point represents a gene's (top) differentiation between the two populations, calculated as the averaged *Fst* across all positions within a given gene. The horizontal lines indicate the genome-wide averaged *Fst* ~ 0.2 (solid) and 2sd *Fst* ~ 0.4 (dotted); (bottom) composite likelihood ratio (CLR) of a selective sweep in WBM (wild; blue) and BF (domesticated; green). Labels highlight the genes within defined regions of interest (ROIs) referred to in the main text.

Supp. text 1. Other signals of selection in the BF

Sweep signals within BF's ChrZ cover a broad genomic area, including another major peak present only in the BF **(BFChrZ-ROI2)** in a region comprising a set of genes under selection in several domestic sheep populations across the globe in comparison with wild mouflon: **SLC25A46, WDR36, CAMK4, and STARD4** [97]. This sweep signal peaks at **CAMK4** which also shows signatures of introgression from wild argali to its sympatric domestic Tibetan sheep, as well as from the Asiatic mouflon to Chinese goats [98]. The **CAMK4** gene encodes two products, calspermin and Ca2+/calmodulin kinase 4. The activity of CAMK4's products triggers a wide range of biological processes, including ones relating to the development and function of mammalian reproductive organs and gametes [99]. Attesting to its central role also in avian reproduction, variation in **CAMK4** carries a strong association with the number of laid eggs in chickens [100]. However, its specific contribution to changes in reproductive traits brought by domestication remains undetermined. In addition to its central role in animal reproduction, **CAMK4** supports the differentiation and function of melanocytes and retinal pigment epithelial cells. Upregulation of **CAMK4** in melanocytes in response to intracellular increases in Ca2+ triggers downstream events that culminate in the activation of the melanocyte-inducing transcription factor, leading to the transcription of melanogenic genes [101].

Our scans detected a signal of selection present only in the BF within a region on Chr2 comprising the genes: **ABCA13, VWC2, SPATA48, IKZF1, FIGNL1,** and **DDC (BFChr2-ROI)**. This region also exhibits peaks in selection scans in several mammalian domesticates, including dogs, pigs, sheep/goats, horses, and yaks. The gene with the highest signal in this region, **SPATA48,** encodes for the spermatogenesis-associated protein 48, with testis-specific expression. In domesticated equines, variation in **SPATA48** is associated with sperm quality [102].

The gene **DDC (BFChr2-ROI)** codes for dopa decarboxylase, a key enzyme in DA biosynthesis. The regulation of DA levels within song nuclei that receive denser dopaminergic innervation from midbrain centers relative to their surroundings, such as Area X, HVC, and RA, relates to

corresponding changes in the expression of enzymes involved in DA biosynthesis, as well as DA receptors [77]. In male songbirds, **DDC** mRNA levels in the ventral tegmental area (VTA), the origin of the dopaminergic pathways, are negatively correlated with the amount of contact received from a female partner [103]. Additionally, we detected high genetic differentiation between BF and WBM for the gene encoding dopamine receptor D5, **DRD5 (BFChr4-*LOC1*)**, located immediately adjacent to a narrow sweep signal within Chr4 present only in the BF.

Outside the major ROIs, we found signals of selection present only in the BF population in genes regulating axon guidance and connectivity that contribute to the differentiation and specialization of the song control circuit. **SLIT2 (BFChr4-*LOC2*)** codes for a secreted protein-ligand that binds axonally-expressed roundabout homolog receptors (ROBOs) and guides axons away from the site of the interaction (i.e., repulsive axon guidance). Although less is known about the specific role of **SLIT2** within the song circuitry, the downregulation of its paralog SLIT1 in song nuclei RA and HVC and in the brainstem motor nucleus nXIIts is suggested to facilitate the targeting of ROBO-expressing terminals and guide the development of neuronal projections from HVC to RA, as well as direct projections from RA to nXIIts, a connection critical for learned vocalizations [104]. Lastly, we found signals of selection in the BF at collagen-encoding loci **COL4A1** and **COL4A2 (BFChr1-*LOC1*)**. These collagens are regulated by testosterone and show differential expression between male songbird brains and corresponding vestigial reminiscences in female brains in species where song is sexually dimorphic [105]. **COL4A1** expression is particularly enriched in HVC, where it peaks during sensorimotor learning (i.e., 45 days post-hatch in zebra finches) [106]. Contiguously downstream of **OCA2** is the gene **GABRB3 (BFChr1-*LOC2*)**, which encodes the β3 subunit of GABAA receptors. Consistent with its major role in neural function and development, mutations in this gene have been frequently associated with the neurodevelopmental aspects in Angelman and Prader-Willi syndromes (AS and PWS, respectively), as well as epilepsy and autism [107]. However, a potential contribution of **GABRB3** to ocular function and development is suspected, as the deletion of **GABRB3** alone causes a nearly complete loss of retinal

pigmentation due to atrophied melanosomes [108]. It has been therefore proposed that GABRB3 or elements within the gene might regulate **OCA2** expression, and impairment of this function may indirectly cause ocular hypopigmentation and visual defects, as seen in PWS in AS.

Genes in flanking regions adjacent to **BFChrZ-ROI1** are reported as highly differentiated (*Fst*) between red-shafted and yellow-shafted flickers, where SNPs are specifically associated with differences in the red/black coloration of male malar feather stripes: **FAM174A, ST8SIA4, SLCO4C1, PAM, GIN1, PPIP5K2 (BFChrZ-ROI1)** and **DCP2, REEP5, SRP19, APC, MCC, YTHDC2 (BFChrZ-ROI1)** [109]. In mammals, genes adjacent to BFChrZ-ROI1 sweep peak, **EFNA5** and **FBXL17 (BFChrZ-ROI1)**, associated with piebald coloration [110]. Of known importance for coloration, **PAM** α-amidates over 50% of all neuropeptides, including the alpha-melanocyte-stimulating hormone (α-MSH) [111]. Its activity is regulated by ATP7A, for which deletions are present in the mottled-brindled male mouse, a model for X-linked recessive disorders of copper metabolism [112]. Regulatory changes of **PAM** likely is a key molecular component of coat phenotypes in those mice mutants, and it may also contribute to the evolution of piebald plumage coloration in BF.

Variation in **SNCAIP** and adjacent genes within BFChrZ-ROI2 peak signal have been associated with differences in gray/white feather coloration between feral and domesticated geese, and in skin coloration in chickens (e.g., red/white ear lobe or shank melanin): **ZNF608, CSNK1G3, CEP120, SNCAIP, DMXL1** and **DTWD2 (BFchrZ-ROI2)** [113-115]. Interestingly, **DMXL1** SNPs are associated with vitiligo in Smith chickens, a condition that causes patchy depigmentation due to the loss of melanocytes [116]. In mammals, variation in genes adjacent to BFChrZ-ROI2 sweep peak specific to **PRDM6 (BFChrZ-ROI2)** is associated with cashmere wool type in sheep [117], and variation in **COMMD10, ARL14EPL,** and **LVRN (BFChrZ-ROI2)** associated with tabby coat coloration in cats [118].

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Chapter 2 . Neurogenetic Mechanisms for Vocal Learning, Practice, and Performance

Neurogenetic Mechanisms for Vocal Learning, Practice, and Performance

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Abstract

Learning a skilled motor behavior relies on a certain degree of iterative experimentation and performance evaluation. In this process, individuals adapt their behavior to maximize future rewards, such as in successful prosocial interactions. Both the generation of variability in motor gestures and the reinforcement learning of motor programs leading to improved performance are supported by cortico-basal ganglia circuits. These principles form the basis for vocal production learning, a trait humans and songbirds share. Here, we assess gene expression patterns of song-dedicated versus more generalist basal ganglia regions (i.e., Area X and the ventral striato-pallidum, respectively; VSP) of juvenile Bengalese finches (*Lonchura striata domestica*) actively engaged in song learning via imitation and adult individuals past this sensorimotor learning phase and primarily engaged in song maintenance and performance. Our findings reveal patterns of co-expression involving key speech- and language-related genes (e.g., FOXP1 and FOXP2), marked by high preservation across the striatum of juvenile BFs but greatly restricted to Area X in adults, thus reflecting this song nucleus' distinct functional specialization within the songbird basal ganglia.

Introduction

As for humans, a songbird's life greatly revolves around vocal production, learning, practice, and performance. Thus, both humans and songbirds evolved similar cortico-basal ganglia loops specialized to accomplish these tasks. Striatal function within the vocal neurocircuitry enables

juvenile finches to produce highly variable vocal output and evaluate the match to their tutor's song template (i.e., sensory-motor learning) [1]. The juvenile finch then gradually converges onto a stable, or crystallized, song that can be a remarkably good copy of the tutor song. Though to a limited degree, adults continue to explore vocal gestures and adjust their songs in adaptive ways to obtain positive social feedback during performance directed to conspecifics [2, 3]. Here, we identify transcriptomic patterns that distinguish a striatal region dedicated to song learning known as Area X from the more generalist striato-pallidum region ventral to it (VSP) in juvenile Bengalese finches (BF; *Lonchura striata domestica*); and interrogate their preservation in adult BFs past the critical period of vocal plasticity. We assess the generality of these spatiotemporal patterns in another songbird model species, the zebra finch (ZF; *Taeniopygia guttata*), by interrogating the extent to which they converge onto the found through our reanalysis of previously generated Area X and VSP transcriptomic datasets from juvenile and adult ZFs [4]. Finally, we extend the analysis from within our adult BF cohort and compare Area X transcriptomes collected in two natural social contexts known to elicit differences in song stereotypy, undirected singing (UD) and singing directed to females (FD) [5].

Methods

Subjects & Brain RNA-Seq data generation

All animal use was in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California, Los Angeles Chancellor's Institutional Animal Care and Use Committee.

BF pairs from our colonies were bred in individual cages to produce offspring composing the cohorts in this study. These breeding cages were placed in sound attenuation chambers when an identified candidate male offspring reached ~20d. Juvenile songbirds, still actively engaged in song learning via imitation (~65d; n = 13), and adult songbirds past this sensorimotor learning phase and primarily engaged in song maintenance (~100d; n = 13), were sacrificed by decapitation following two hours of UD singing in isolation (hereafter termed juvenile and adult

undirected singers; JUD and AUD, respectively) [4, 6]. Adult songbirds in a second group (n = 13) were sacrificed by decapitation following two hours of FD singing (AFD), elicited by placing a cage with a female adjacent to the experimental male's cage inside the same sound attenuation chamber. A different female was presented every 15 min, thus ensuring that the male was more constantly triggered to direct their songs to females [7].

Songbird brains were rapidly extracted, flash-frozen on liquid nitrogen, and stored at -80⁰C. To avoid potential artifacts relating to batch effects, once all study samples were collected, their labels were randomized across cohorts, establishing the order in which they were processed, and the same experimenter processed all samples. Individual brains were coronally sectioned on a cryostat at 30 μM until Area X became visible. Area X and outlying VSP were punched from both hemispheres using a 20-gauge Luer adapter and stored in RNAlater (Qiagen, Germantown, MD) [7]. To enable validation of punch accuracy, 30 μM sections were collected from the remaining brain, thaw-mounted, and thionin-stained for posthoc verification. Sampled tissues were lysed using QIAzol, incubated with chloroform, and centrifuged at 4⁰C. The resulting aqueous supernatant was retrieved for RNA purification using Direct-zol RNA MicroPrep Kits (Zymo Research Corp, Irvine, CA) following the manufacturer's protocol. Total RNA extracted was stored at -80⁰C until all extractions were completed and then provided to Novogene Corporation Inc (Sacramento, CA) for RNA quality assessment on an Agilent TapeStation (Agilent Technologies, Santa Clara, CA). cDNA libraries were generated from samples yielding sufficient total RNA amount and required integrity parameters (i.e., amount ≥ 0.5μg, volume ≥ 20μL, concentration ≥ 20ng/μL, RIN ≥ 8; 2 VSP-JUD and 1 VSP-AFD were discarded) using Illumina TruSeq Stranded Poly-A Prep Kits (Illumina, San Diego, CA). Libraries for each sample were sequenced in three lanes in a NovaSeq 6000 with an S4 chip (Illumina, San Diego, CA) in high output mode, generating between 15 and 35 million 150bp paired-end reads per library.

RNA-Seq data preprocessing & Gene expression analyses

We quantified gene transcription levels in the newly generated Area X and VSP RNA-Seq datasets from the individuals within BF cohorts in this study (JUD, AUD, and AFD), as well as in previously generated Area X and VSP RNA-Seq datasets from ZF cohorts (JUD; n = 19 and AUD; n= 19) [4]. Raw FASTQ files furnished by Novogene Inc. were quality-controlled using FASTQC [8], which reported high-quality reads for all samples and no adapter contamination. Using Salmon [9], we built a transcriptomic index (*salmon index*) from each of the ZF and BF reference genome assemblies and their corresponding annotations [10], to which we quasi-mapped our respective sequencing data, and quantified transcript abundance (*salmon quant –validateMappings*; TPM). Samples metadata and their transcript-level abundances were summarized to the gene level (Tximeta: *tximeta()*, *summarizeToGene()*) [11]. Genes for which expression showed no variance across all samples within a given species were removed from subsequent analysis. Estimated gene-level counts served in the differential gene expression analysis of contrasts within each BF and ZF samples: all X vs. all VSP, as well as cohorts age and singing conditions (i.e., JUD vs. AUD and AUD vs. AFD, respectively) (DESeq2; *DESeqDataSetFromMatrix()*; DESeq() *fitType = "mean"*; *results() alpha = 0.05*) [12] (*Figure 2-1*,). We then employed an iterative process for removing a given gene's expression data from individual samples that was >2.5SD from the measured across all samples, calculated the intrasample correlation (ISC) and used a hard cutoff of 2SD away from the group ISC for removal of samples from the study [4]. Quantile normalized data served in constructing weighted gene co-expression networks using the WGCNA's available R implementation [13]. We calculated soft thresholding powers (*p*) for the construction of WGCNA adjacency matrices from samples within each BF (WGCNA: *pickSoftThreshold()*; X-JUD *p*=10, -AUD *p*=12, -AFD *p*=12, and VSP-JUD *p*=12, -AUD *p*=12, -AFD *p*=14) and ZF cohorts (X-JUD *p*=11, -AUD *p*=8, and VSP-JUD *p*=8) Supp. Fig. *2-3*, Supp. Fig. *2-4*. Nine signed co-expression networks were built (WGCNA: *blockwiseModules(); minModuleSize = 100, deepSplit = 4, mergeCutHeight = 0.2, minCoreKME = 0.5, minCoreKMESize = minModuleSize/3,*

minKMEtoStay = 0.3, with other parameters set to default). Networks were iteratively constructed with genes in the grey module removed from the expression data after each round of network building and module definition [4, 6]. Intramodular connectivity (WGCNA: *intramodularConnectivity()*; kIn) and module membership, defined as the correlation to the assigned module's eigengene (WGCNA: *geneModuleMembership();* MM) were calculated for each gene for all networks. To compare patterns of gene co-expression between Area X and VSP samples within each BF and ZF species, we interrogated the preservation of gene co-expression modules defined in Area X (WGCNA: *modulePreservation()*) in the corresponding VSP expression data (X-JUD vs. VSP-JUD, X-AUD vs. VSP-AUD, and X-AFD vs. VSP-AFD), as well as across age and singing conditions (X-JUD vs. X-AUD and X-AUD vs. X-AFD, respectively). We defined sets of hub genes (kIn > 0.8) in each module within individual BF and ZF Area X and VSP networks (Supp. Data 1), which we combined with results from our module preservation analyses across comparisons to gain insight into key processes underlying vocal learning, practice, and performance.

Reproducibility of ZF gene co-expression networks

We calculated preservation scores for modules defined in ZF X-JUD networks published by Burkett et al. (2015) [4] in the corresponding expression data as re-processed in this study (WGCNA: *modulePreservation()*). This analysis revealed overall strong evidence of preservation for 19 out of the 21 X-JUD modules and 13 out of the 18 VSP-JUD modules (Z-Summary > 10) [14]. Quantifications of song variables associated with the published ZF RNA-Seq datasets were retrieved, including the number of motifs and time sung during the 2hr window preceding sacrifice (i.e., amount of singing), the percent similarity and accuracy in motif-to-motif comparisons, as indicative of overall song variability (i.e., motif identity), the bird's ability to modulate song variability in an NS-UD paradigm (i.e., variability induction) [4], and the percent similarity to the tutor's song (i.e., vocal learning). We then recalculated each module's eigengenes, defined as the first principal component of the module's expression data (WGCNA: *moduleEigengenes()*),

and assessed their Pearson correlations to each continuous song variable recorded. Significance was determined by calculating the Fisher transformation of each correlation (WGCNA: *corPvalueFisher()*). We verified the maintenance of correlations between ZF X-JUD and VSP-JUD modules in the rebuilt networks and song variables, as described in Burkett et al ($pval < 0.05$; Supp. Fig. *2-5*): X-JUD modules strongly correlated with the number of motifs sung were also present in our rebuilt networks (magenta 0.78:pval=3e-05, brown 0.64:pval=2e-03, yellow -0.71:pval=4e-04, salmon -0.68:pval=1e-03, darkgrey -0.65:pval=2e-03, and cyan -0.52:pval=2e-02), while no significant correlations were found between VSP-JUD modules and the amount of singing. Lastly, we interrogated the preservation of gene co-expression modules defined in Area X in our rebuilt networks in the corresponding VSP expression data (X-JUD vs. VSP-JUD) and confirmed Burkett and colleagues' finding that none of the ZF X-JUD modules significantly correlated with the amount of singing are strongly preserved in VSP-JUD (Supp. Fig. *2-6*) (Z-Summary > 10) [14] The direction of modules' eigengenes correlations to recorded song variables are maintained for all ZF X-JUD modules significantly overlapped between the network defined in Burkett et al. (2015) [4] and the one built through our reprocessing of the same data ($pval < 0.05$; WGCNA: *overlapTable()*). However, specific modules rendered significance to song variables other than previously reported (e.g., turquoise: song learning; 0.3: pval=0.2 motif similarity; 0.5: pval=3e-02). Among other factors, this stems from differences in composition between those networks, as genes previously found to hold the greatest significance to a particular song variable are either absent from our network (e.g., e.g., turquoise: song learning; CSGALNACT1, CCNC, EEF1E) or scattered in different X-JUD modules (e.g., STXBP6; yellow or SLC25A3; purple).

Results

Overall patterns of gene expression in the songbird basal ganglia

The PCA of the total variance in gene expression among all BF and ZF samples analyzed separately shows a clear separation between Area X and VSP along PC1 (*Figure 2-1*), highlighting the differential transcriptomic states of song-dedicated versus more generalist striatal

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regions, as accurately dissected by tissue punches. The PCA of BF Area X samples show a more clustered distribution distinguishing transcription profiles from juvenile versus adult undirected singers (X-JUD vs. X-AUD), as well as between adult males practicing versus performing a song (X-AUD vs. X-AFD) than found for VSP samples in any BF cohort (Supp. Fig. *2-1*, Supp. Fig. *2-2*). In contrast, in the reanalyzed ZF samples, Area X and VSP exhibit clustered distributions distinguishing JUD from AUD individuals. These results from both species are consistent with altered Area X transcriptional profiles before and after the critical period for sensorimotor learning and socially-induced profiles in BF; as mentioned, social modulation of gene expression was not performed in the prior ZF studies. Overall differential analysis of gene expression revealed more prominent down- than up-regulation in Area X relative to VSP, found in both BF and ZF (pCutoff = 0.05, FCcutoff = 1) (*Figure 2-1*). Genes that are downregulated in Area X relative to VSP in both species are **AKR1D1**, **PDYN**, and **CHRM5**. The differential analysis of gene expression in Area X and VSP samples between cohorts in either the BF or ZF (JUD relative to AUD, and AFD relative to AUD) did not retrieve significant results (pCutoff = 0.05, FCcutoff = 1) (Supp. Fig. *2-1*, Supp. Fig. *2-2*).

Concerning gene co-expression patterns in BF, we found increased preservation of Area X modules in VSP expression data in adults relative to juvenile birds: 14 out of the 25 X-JUD modules show evidence of preservation in VSP-JUD, whereas 25 out of the 27 BF X-AUD modules and 20 out of the 21 BF X-AFD modules show evidence of preservation in VSP-AUD and VSP-AFD, respectively (Z-Summary > 2) [14] (*Figure 2-2*). Many X-JUD modules, including ones for which there is no evidence of preservation in VSP-JUD (Z-Summary < 2) [14], show an increase in preservation in X-AUD expression data (20 out of the 25 X-JUD show evidence for preservation in X-AUD). An even greater preservation is observed between X-AUD and X-AFD expression data. Many X-AUD modules are not shared in VSP expression data, reaching strong evidence of preservation in X-AFD (25 out of the 27 BF X-AUD modules are preserved in X-AFD). Similar to the findings from BF samples, many ZF X-JUD modules deemed poorly maintained in

VSP-JUD expression data show an increase in preservation when analyzed against X-AUD expression data, including two of the modules holding solid correlations to the amount of singing in juvenile ZFs (i.e., brown 0.64:pval=2e-03 and yellow -0.71:pval=4e-04) (*Figure 2-2*).

A cross-species evaluation reveals evidence for the preservation of 11 out of the 21 ZF X-JUD modules in BF X-JUD expression data, of which two show the strongest correlations to the amount of time spent singing in juvenile ZFs (i.e., magenta 0.78: pval=3e-05 and yellow -0.71: pval=4e-04) (Supp. Fig. *2-7*). In the ZF X-AUD network, 14 out of the 16 modules show evidence of preservation in BF X-AUD expression data, of which two are significantly correlated with time spent singing in adult ZFs (i.e., green 0.48: pval=4e-02 and salmon 0.47: pval=5e-02; of note BF song data have yet to be analyzed). Differences in gene co-expression patterns within specific modules between the ZFs and BFs could result from species-specific differences. Still, they may also be attributed to biases from Burkett and colleague's experimental paradigm [4], which involved the overexpression of FOXP2 in ZF Area X, to gain insight into this gene's function during the sensorimotor phase of vocal learning, as opposed to this study's focus on investigating patterns of expression in the songbird basal ganglia in the absence of artificial manipulations.

Patterns of co-expression involving FOXP1, FOXP2, and FMR1 are developmentally and behaviorally regulated in the songbird basal ganglia

As with mammals, the songbird striatum is mainly composed of neurons signaling through the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), termed medium spiny neurons (MSNs) [15]. In our updated ZF networks, we found an essential GABAergic neuronal marker, GAD1, the gene coding for a glutamic acid decarboxylase that catalyzes GABA synthesis, as a hub member within an X-JUD module highly preserved in VSP-JUD (turquoise; kIn = 0.86) (*Figure 2-2*). Notably, FMR1 is the second most connected node within that same ZF X-JUD module (turquoise; kIn = 0.96), reproducing results from Burkett and colleagues' analyses showing FMR1 enriched co-expression across the juvenile striatum [3].

In the BF, FMR1, and GAD1 are members of distinct X-JUD modules (i.e., red and black, respectively) (*Figure 2-2*). FMR1 expression levels in Area X of juvenile BFs correlate with those of two key genes involved in vocal learning, **FOXP1** and **FOXP2** [16, 17], with all three genes assigned membership to the same X-JUD module (i.e., red). Patterns of co-expression involving FOXP1, FOXP2, and FMR1 found in BF X-JUD are preserved in VSP-JUD to a similar degree as in Area X of adult BFs. As for Area X-JUD, the robust co-expression of FOXP1 and FOXP2 in the juvenile ventral striatum is also reflected in our VSP-JUD network, where both genes are assigned to the same module (i.e., blue). FOXP1 and FOXP2 co-expression is preserved in our X-AUD network, where they figure within the same module (i.e., brown) but not in the VSP-AUD network, where they participate in different modules (FOXP1 in blue and FOXP2 in darkred). The gene GAD1 is a hub member (kIn = 0.84) within its assigned module in the BF X-JUD network, which shows weak evidence of preservation in VSP-JUD. In adult BFs, patterns of co-expression involving GAD1 are better preserved in the surrounding striatum and remain well maintained in the Area X of adult individuals across singing conditions. Conversely, co-expression patterns involving FOXP1, FOXP2, and FMR1 found in X-JUD and preserved in both VSP-JUD and X-AUD are disassociated in VSP-AUD and X-AFD.

Co-expression patterns in the songbird basal ganglia of genes directly associated with dopamine neuromodulation and Parkinsons' disease

In juvenile BFs, we found patterns of co-expression involving genes coding for protein products highly enriched in striatal MSNs, namely: **PPP1R1B**, which encodes the phosphoprotein DARPP-32 [18], a critical mediator of dopamine (DA) function; and **SNCA** encoding alpha-synuclein, a small neuronal protein predominantly localized in presynaptic terminals, where it regulates key stages of DA homeostasis, including synthesis, synaptic release, and uptake [19]. SNCA and PPP1R1B are hub members of the same X-JUD module (blue; kIn = 0.91 and 0.96, respectively), characterized by high preservation across the striatum of juvenile BFs. As for Area X-JUD, the robust co-expression of SNCA and PPP1R1B in the juvenile ventral striatum is also reflected in

our VSP-JUD network, where both genes are assigned to the same module (i.e., magenta). Patterns of co-expression involving PPP1R1B and SNCA found in X-AUD show no evidence of preservation in X-AFD, which is better shared with VSP-FD. As with FOXP1, FOXP2, and FMR1, this co-expression pattern indicates both developmental and behavioral regulation of PPP1R1B and SNCA within the songbird basal ganglia.

SNCA's product, alpha-synuclein, is also known for its role in the molecular etiology of Parkinson's disease (PD), as it colocalizes with parkin and synphilin, encoded by the genes PRKN and SNCAIP, respectively, within Lewy body brain inclusions that mark the disease [19]. The developmental and behaviorally regulated expression of SNCA in the songbird basal ganglia sparked our interest regarding the expression of these and other PD-associated genes within the songbird striatum. To this effect, we found that PRKN's co-expression pattern grants it membership in the large X-JUD module (i.e., turquoise), which is well preserved in VSP-JUD and X-AUD expression data. Patterns of co-expression involving PRKN in X-AUD show no evidence of preservation in the surrounding VSP; or in X-AFD, which shows high preservation in VSP-AFD. Curiously, SNCAIP co-expression patterns only emerge in adult BFs, where they are weakly preserved across the striatum in both singing conditions, though only reaching hub-level intramodular connectivity in X-AFD (kIn = 0.83). In the ZFs in our cohorts, patterns of co-expression involving SNCA, SNCAIP, and PRKN found in X-JUD are weakly preserved in VSP-JUD and virtually non-existent in the adult striatum.

Area X is marked by dynamic patterns of co-expression of dopamine receptors

In the BF, VSP networks are marked by the co-expression of multiple DA receptors, which often appear as hub genes within the same module (*Table 2-1*). This striking enrichment differs from Area X networks, where fewer DA receptor genes share the same co-expression module or figure among hub genes within their assigned module, as defined by the same criteria in all networks (kIn > 0.8). Noticeably, the gene coding for the dopamine receptor DRD1 is a hub member within its assigned module in VSP networks from our three BF cohorts (i.e., JUD: blue; AUD: turquoise;

and AFD: turquoise). In both VSP-JUD and -AUD, DRD1 is co-expressed with DRD2 and DRD5 within the same module. Co-expression involving DRD2 and DRD5 is shared across the striatum in juvenile BFs, with both genes assigned to the same module within each X-JUD and VSP-JUD networks (i.e., yellow and blue, respectively). The same pattern is not shared across the striatum in adult BFs, as DRD2 and DRD5 are assigned to different modules within the X-AUD network (i.e., blue and gray60, respectively) and to the same module within VSP-AUD (i.e., yellow). Patterns of co-expression of DA receptors also differ between X and VSP across singing conditions, with DRD1, DRD2, and DRD5 assigned to the same X-AFD module (i.e., yellow), while in VSP-AFD, DRD1 is assigned to a different module (i.e., blue) than the shared by DRD2 and DRD5 (i.e., red).

The mRNA expression of DRD1, DRD2, and DRD5 is knowingly highly enriched at both mRNA and protein levels in the avian and mammalian striatum [19]. In songbirds, the mRNA expression of these DA receptors is significantly upregulated in Area X relative to the surrounding striatum, while DRD3 and DRD4 either are similarly low expressed or show no differential regulation between the two striatal regions [20]. Accordingly, our data shows a more robust expression of DRD1, DRD2, and DRD5 than for DRD3 and DRD4 across the striatum.

Discussion

Our results corroborate and extend previous findings on the developmental and behavioral regulation of the expression of key genes involved in vocal learning, practice, and performance within the songbird basal ganglia. To this effect, we found evidence of robust co-expression for the transcription factors FOXP1 and FOXP2 in juvenile songbirds across the striatum [20]. Prior studies indicate that FOXP2 expression is lower in Area X relative to VSP in juvenile ZFs within a similar age range as the BFs in our juvenile cohort. In contrast, in adult birds, the relative expression between those two regions is more variable [21]. This finding is in line with ours showing that patterns of co-expression involving FOXP2 (and FOXP1) found in Area X of juvenile BFs are less preserved in adult VSP than those found in juvenile VSP, thus generating more

variability in measures of relative expression of these genes between the two brain regions in adult versus juvenile songbirds.

Our study also brings evidence of co-regulation of FOXP1 and FOXP2 with FMR1, with all three genes assigned to the same co-expression module in our BF juvenile Area X network. Patterns of co-expression involving FOXP1, FOXP2, and FMR1 identified in Area X of juvenile BFs are to some extent preserved in juvenile VSP and to a similar degree in adult Area, but not adult VSP. This indicates the differential developmental co-regulation of these genes between Area X and VSP.

In mammals, MSNs lacking FMR1's protein product show decreased dendritic spine density and lower GABAergic neurotransmission [22]. Similarly, FOXP2 loss-of-function in mice leads to increased inhibitory presynaptic strength of the striatal direct pathway due to increased GABA release, accompanied by increased expression of GAD1 [23]. Inhibitory GABAergic neurotransmission within Area X is essential in song learning and adult vocal plasticity, as it critically provides local inhibition of DA neurons, thus enabling reward-related learning [24]. We, therefore, suggest co-regulatory effects involving FOXP1, FOXP2, and FMR1 in Area X, likely associated with their involvement in GABAergic neurotransmission in this brain region.

FOXP2 mRNA levels in Area X differ between singing conditions, showing downregulation in contexts related to increased song variability, as in UD, but not in contexts associated with increased song stereotypy, as in FD [25]. The behavioral regulation of FOXP2 parallels DA release in Area X, which is increased in FD relative to UD singing [79]. Curiously, we found a pattern, unique among BF cohorts, of FMR1 and GAD1 co-expression preserved across Area X and VSP in the brains of adult BFs, collected after continuous vocal performance directed to females. This pattern is similar to that found in the Area X of individuals within the juvenile ZF cohort analyzed in this study, in which FMR1 and GAD1are also assigned to the same co-expression module. We reason that this result may derive from the artificial overexpression of

FOXP2 within Area X in most individuals within the ZF cohort, which may mimic the more homogenous FOXP2 expression across Area X and VSP seen in FD [25].

Our analyses also revealed the co-expression of genes directly involved in DA neuromodulation in both juvenile Area X and VSP, including PPP1R1B, encoding the DARPP-32, a phosphoprotein selectively enriched in medium-sized spiny neurons, where it plays a role in mediating the effects of DA [18]; SNCA, encoding for alpha-synuclein, which regulates DA availability in both mammal and avian brains [19]; and dopamine receptors DRD1, DRD2, and DRD5.

The genes PPP1R1B and SNCA show co-expression in the Area X of juvenile, not preserved in adult area X, but present in surrounding VSP in both juvenile and adult BFs. This indicates the dissociation of co-regulation between PPP1R1B and SNCA specific to Area X in adults. Accordingly, SNCA expression is developmentally downregulated during the critical period for song learning within Area X's adjacent efferent nucleus, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) [26].

The product of the gene PRKN, the U3 ubiquitin ligase parkin, regulates alpha-synuclein striatal levels. Curiously, co-expression patterns involving PRKN are noted in BF juvenile Area X, which shows strong evidence of preservation in juvenile VSP. However, PRKN does not show robust patterns of co-expression defined in our adult Area X network, as it is entirely absent from it. Instead, patterns of co-expression involving another gene associated with alpha-synuclein function, SNCAIP, emerge in the adult Area X network, as shared across the striatum and singing conditions. In the adult songbird brain, alpha-synuclein is differentially regulated within Area X between vocal practice and performance, with increased levels scaling to the number of motifs sung during UD but not FD [27]. Similarly, SNCAIP mRNA expression is significantly downregulated within Area X during UD, which suggests this interaction contributes to the modulation of song variability during practice [6]. Accordingly, we suggest that co-expression patterns involving SNCA, PRKN, and SNCAIP are differentially regulated within the songbird striatum, with co-regulatory effects between SNCA and PRKN possibly more relevant in song

learning, and ones involving SNCA and SNCAIP in modulating adult song plasticity, as associated with dopamine neuromodulation.

Lastly, our findings of shared co-expression patterns involving DRD1, DRD2, and DRD5 in the ventral striatum of both juvenile and adult BFs, not found in Area X at either age, corroborates previous evidence of DRD1 and DRD2 differential regulation in Area X already present at the sensory-motor phase of song learning [20]. Similarly, the different patterns of co-expression involving DRD1 and DRD2 within Area X between singing conditions corroborate reports of differential regulation of these genes as associated with changes in DA levels, which are lower in undirected relative to female-directed singers [21].

Conclusion

This study uncovers context-dependent and spatiotemporal transcriptional patterns in the songbird basal ganglia that likely contribute to motor learning, practice, and performance. Both birdsong and human speech share neuroanatomical analogs, which are also reflected at the transcriptional level, with certain birdsong-control brain regions sharing convergent transcriptional networks with speech-related brain regions in humans [28]. Our results, therefore, have the potential to highlight specializations related to complex communication both in birds as well as in our species.

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Author Contributions

Designed research: MFV, SAW, GX; performed research: MFV; analyzed data: MFV; contributed to writing: MFV, SAW, GX, TD.

Competing Interests statement

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.
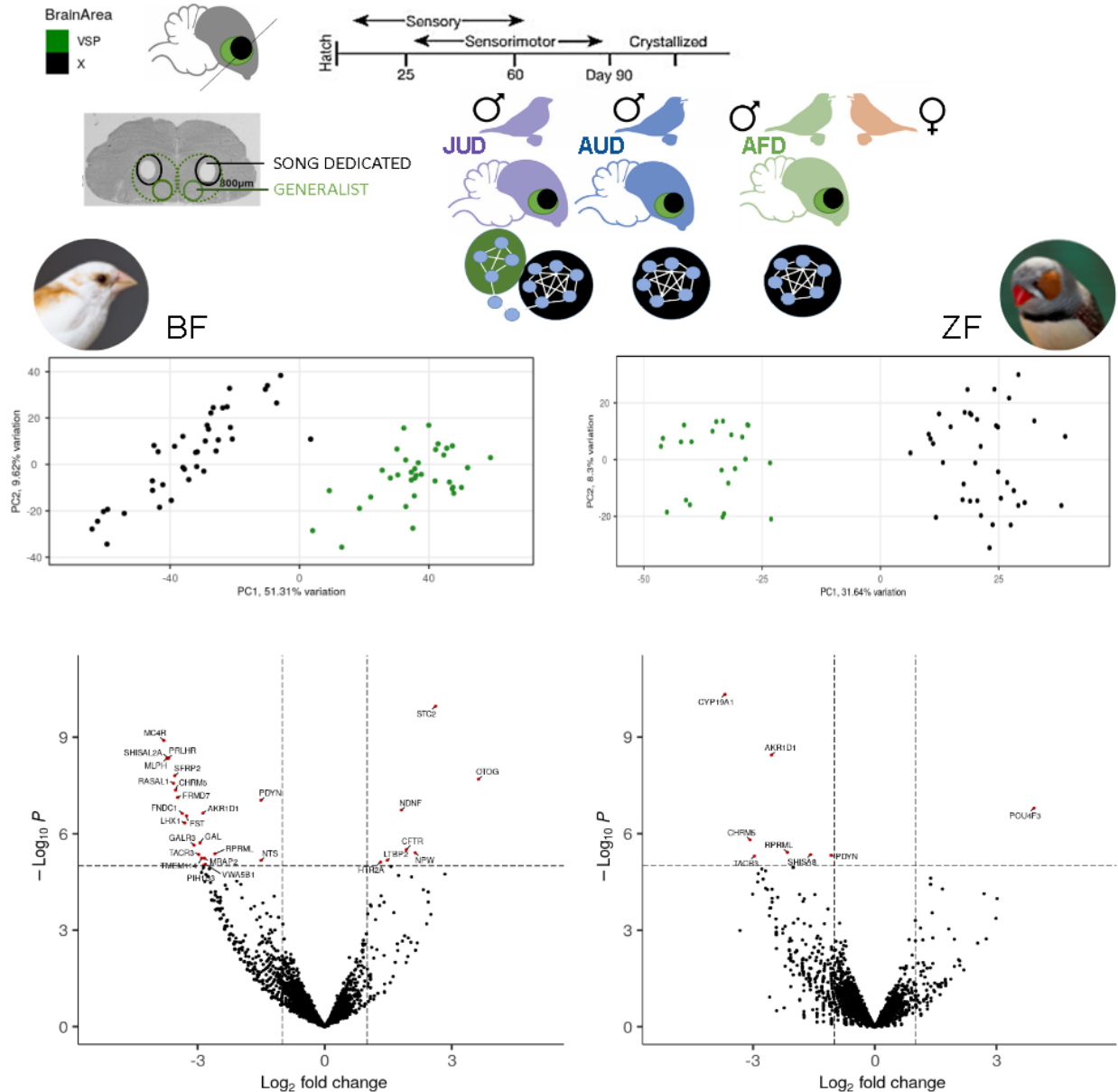
Figure 2-1. **Overall patterns of gene expression in the songbird basal ganglia.** (top) Diagrams depict a lateral view of the songbird brain indicating the song dedicated Area X (black) and the more generalist striatal area ventral to it (VSP; green); the diagonally crossing black line represents the approximated depth where Area X becomes visible to the naked human eye, and both area X and VSP can be accurately punched from the mounted brain, as illustrated in the thionin-stained coronal section. The diagrammed timeline depicts key phases and ages in birdsong ontogeny. The different colored bird icons below are aligned to the time points when Area X and VSP samples were collected from individual birds composing our cohorts (purple: JUD - ~60d juvenile male songbirds after 2hrs of singing in isolation; blue: AUD - ~100d adult male songbirds after 2hrs of singing in isolation; and green: AFD - ~100d adult songbirds after 2hrs of singing in the company of females). The analyzed Zebra finch (ZF; JUD, and AUD) datasets were previously generated, while the Bengalese finch (BF; JUD, AUD, and AFD)

datasets were newly generated for this study. (middle) The PCA of the total gene expression variance for all BF and ZF samples analyzed separately shows a clear separation between Area X and VSP along PC1. (bottom) The volcano plots show results from the differential expression analysis of all Area X samples relative to all VSP for the BF and ZF. Labels highlight significantly up- or down-regulated genes in Area X relative to VSP (pCutoff = 0.05, FCcutoff = 1).
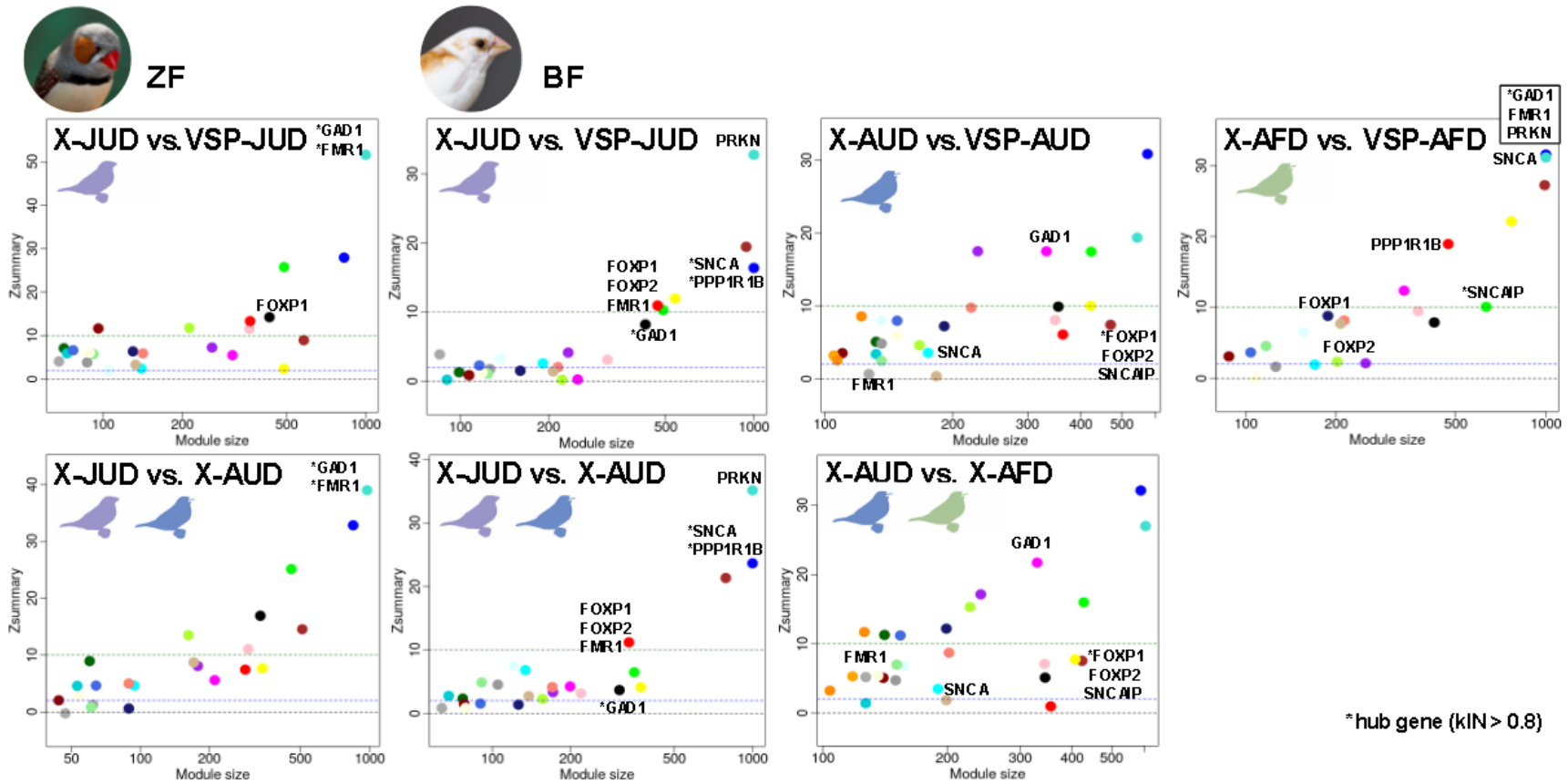
Figure 2-2. **Gene co-expression patterns in vocal learning, practice, and performance.** Each scatterplot shows the preservation scores (y-axis; Z-summary) along module size (x-axis; number of genes) for co-expression modules defined within ZF and BF Area X networks in the corresponding VSP expression data (X-JUD vs. VSP-JUD, X-AUD vs. VSP-AUD, and X-AFD vs. VSP-AFD), as well as across age and singing conditions (X-JUD vs. X-AUD and X-AUD vs. X-AFD). Dashed horizontal lines mark Z- summary = 0, Z-summary = 2, and Z- summary = 10 in each plot. A Z- summary < 2 indicates no evidence, 2 > Z- summary > 10 weak evidence, and Z- summary < 10 strong evidence of preservation. Labels highlight genes within their assigned module for which co-expression patterns are discussed in the main text.

Table 2-1. **Co-expression patterns of DA receptors in the songbird striatum†**

DA receptors	Area X			VSP		
	JUD	**AUD**	**AFD**	**JUD**	**AUD**	**AFD**
DRD1	0.55	0.40	0.28	0.89*	0.94*	0.90*
DRD2	0.73	0.88*	0.59	0.81*	0.81*	0.77
DRD3	0.74	-	-	0.46	0.40	0.60
DRD4	0.33	-	0.25	-	0.20	0.14
DRD5	0.79	0.47	0.29	0.70	0.91*	0.99*

*"-" not in the network; *hub member (kIn > 0.8)*; †Cell colors indicate the gene's assigned module within their corresponding networks whenever at least two genes populate the same module.

Supp. figures, tables, and legends

Supp. Fig. 2-1. **Overall patterns of gene expression in the BF basal ganglia.** (top) PCAs of the total gene expression variance for Area X of juvenile (JUD) and adult (AUD) BFs; and for the adult BFs in the two singing conditions (AUD and AFD). The volcano plots on the side show results from the differential expression analysis of X-JUD samples relative to X-AUD and X-AFD relative to X-AUD. No genes are significantly up- or down-regulated in either contrast (pCutoff = 0.05, FCcutoff = 1). Labels highlight genes up- or down-regulated in Area X relative to VSP (pCutoff = 0.1, FCcutoff = 1). (bottom) The same analyses were run on BF VSP samples.

Supp. Fig. 2-2. **Overall patterns of gene expression in the ZF basal ganglia**. Same analyses as described in Supp. Fig. 2 were run on X and VSP samples from the ZF juvenile (JUD) and adult (AUD) cohorts.

Supp. Fig. 2-3. **BF gene co-expression networks built in this study.** (left) Hierarchical clustering of inter samples correlations (*WGCNA: hclust() method="average"*); soft-threshold powers on the scale-independence fit index; soft-threshold power on the mean network connectivity. The same analyses were run for X and VSP samples from individuals within our BF cohorts (JUD, AUD, and AFD). (right) Area X and VSP signed weighted co-expression networks (i.e., only genes with positive expression correlation are considered connected). The clustering dendrogram of network interconnectedness is measured as the topological overlap distance between genes. Modules are defined as branches of a cluster tree resulting from the correlation strength the genes share.

Supp. Table 2-1. **Summary of network concepts for the BF gene co-expression networks built in this study.**

X-YUD — Size 9990 — Number of modules 25

	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.03445695	0.003783233	0.01804358	0.0180488
Centralization	0.05814272	0.051027585	0.09374249	0.09380664
Heterogeneity	0.47962984	2.144583235	1.37513522	1.37531599
ClusterCoef	0.13663742	0.118597986	0.15084679	0.15088621
Connectivity	344.1904559	37.79071083	180.2373078	180.2894906

X-AUD — Size 8308 — Number of modules 27

|	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.01087382	0.000132407	0.006041796	0.006043579
Centralization	0.0197551	0.006722779	0.039575152	0.039614881
Heterogeneity	0.4141977	3.674091374	1.203993055	1.20426613
ClusterCoef	0.05420837	0.027831203	0.036263022	0.036279823
Connectivity	90.3287941	1.099905846	50.18920331	50.20400985

X-AFD — Size 10055 — Number of modules 21

	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.01666014	0.000888792	0.008363213	0.008365917
Centralization	0.03682322	0.025768545	0.059390644	0.059442591
Heterogeneity	0.55749893	2.872329422	1.500054564	1.500329478
ClusterCoef	0.08510924	0.076044272	0.088374267	0.088409972
Connectivity	167.5010327	8.935917964	84.0837385	84.11093329

VSP-YUD — Size 11141 — Number of modules 22

	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.02356548	0.002012112	0.01275872	0.01276261
Centralization	0.05336054	0.035699768	0.07910906	0.0791646
Heterogeneity	0.62476466	2.422195273	1.54671363	1.54690742
ClusterCoef	0.11983503	0.094874851	0.14686996	0.14690895
Connectivity	262.5194601	22.41493064	142.1321271	142.1754257

VSP-AUD — Size 9904 — Number of modules 26

	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.01294463	0.000306268	0.006267631	0.006269857
Centralization	0.03118872	0.012717566	0.051984494	0.052037007
Heterogeneity	0.5270819	3.493224678	1.585874976	1.586217972
ClusterCoef	0.06742265	0.053379879	0.077465984	0.077505609
Connectivity	128.1906297	3.032969632	62.06835289	62.09039603

VSP-AFD — Size 10469 — Number of modules 22

|	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-Based
Density	0.01242219	0.000445961	0.006603787	0.006605906
Centralization	0.03273993	0.015288874	0.049621989	0.04966565
Heterogeneity	0.63416801	2.963535164	1.535480017	1.535716146
ClusterCoef	0.0785197	0.042673558	0.074475391	0.074500983
Connectivity	130.0354989	4.668321161	69.12844519	69.15062851
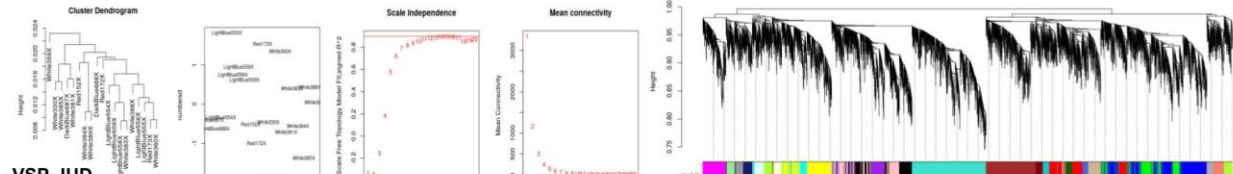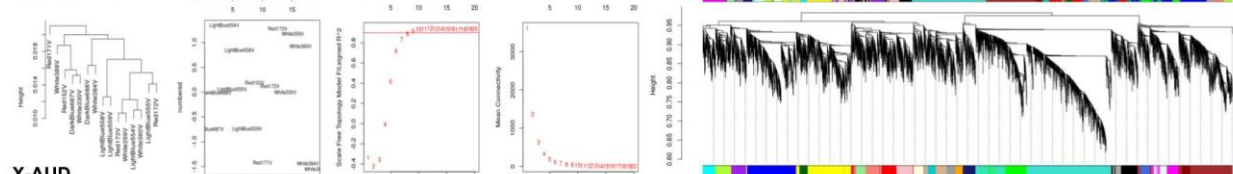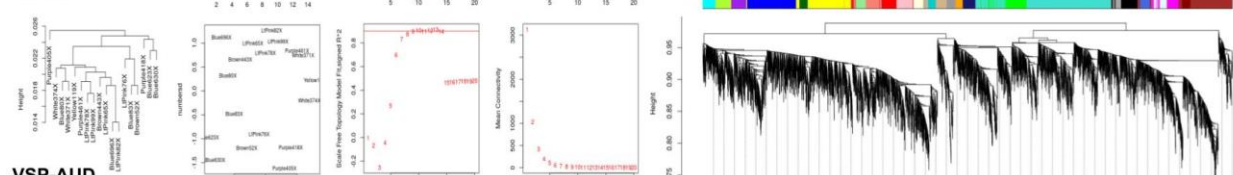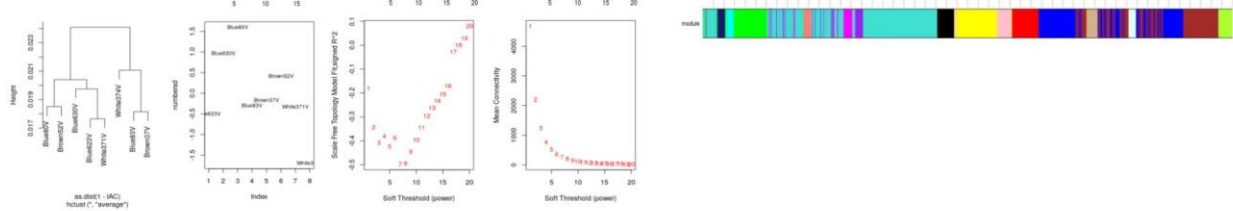
Supp. Fig. 2-4. **ZF gene co-expression networks built in this study.** Same analyses as described in Supp. Fig. 3. A ZF VSP-AUD network was not built, as our power threshold study showed aberrant behavior unable to reach free-scale topology.

Supp. Table 2-2. **Summary of network concepts for the ZF gene co-expression networks built in this study.**

X-YUD	Size	9528	Number of modules	24
	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-based
Density	0.01046215	0.000121102	0.005549932	0.00555152
Centralization	0.02358705	0.005533962	0.040116609	0.04015443
Heterogeneity	0.4793883	3.198774455	1.315621741	1.31587365
ClusterCoef	0.04574767	0.015276795	0.041400131	0.04141678
Connectivity	99.88212762	1.156160863	52.98520002	53.00036253
VSP-YUD	Size	9566	Number of modules	25
	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-based
Density	0.02842792	0.001111651	0.01794034	0.01794393
Centralization	0.03710593	0.028659433	0.08437855	0.08443602
Heterogeneity	0.31955122	2.6626072	0.95449793	0.9547237
ClusterCoef	0.08514763	0.072738459	0.06553055	0.06555707
Connectivity	271.9130521	10.63294509	171.5993755	171.6336717
X-AUD	Size	7485	Number of modules	16
	Fundamental	Eigengene-based	Conformity-Based Approximate	Conformity-based
Density	0.02463574	0.000496576	0.0185662	0.01857023
Centralization	0.04037044	0.016287391	0.06671264	0.06676098
Heterogeneity	0.35225631	2.62815937	0.79128983	0.79143093
ClusterCoef	0.06409253	0.031043841	0.0491016	0.04911275
Connectivity	184.3738478	3.716374129	138.9494208	138.9796187
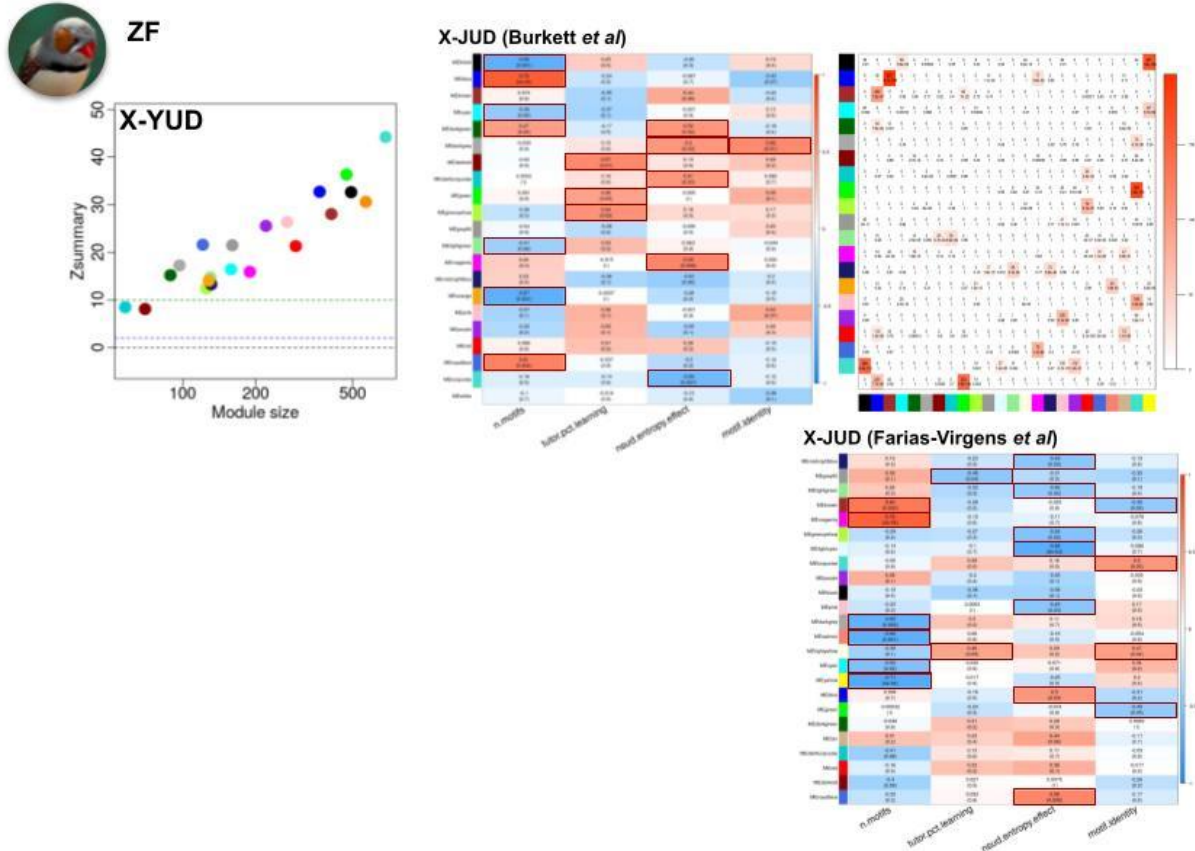
Supp. Fig. 2-5. **Reproducibility of ZF gene co-expression networks.** The scatterplot shows the preservation scores (y-axis; Z-summary) along module size (x-axis; number of genes) for ZF X-JUD modules as defined in the network published in Burkett et al. (2015) in corresponding expression data, as re-processed in this study. Dashed horizontal lines mark Z- summary = 0, Z-summary = 2, and Z- summary = 10 in each plot. A Z- summary < 2 indicates no evidence, 2 > Z-summary > 10 weak evidence, and Z- summary < 10 strong evidence of preservation. Labels highlight genes within their assigned module for which co-expression patterns are discussed in the main text. Heatmaps show correlations between co-expression modules and four song variables associated with the published ZF RNA-Seq datasets retrieved from Burkett et al. (2015) (n.motifs = number of motifs, tutor.pct.learning = percent similarity to the tutor's song, nsud.entropy.effect = induced variability in an NS-UD paradigm, and motif.identity = percent similarity and accuracy in motif-to-motif comparisons in the birds' own song). Module correlations to song variables were calculated for the ZF X-JUD published by Burkett et al. (2015) and the one built in this study. Red boxes highlight significant positive (red) and negative (blue) correlations (p < 0.05). Overlaps between modules defined in each network are also shown, with significance levels proportional to red shading.
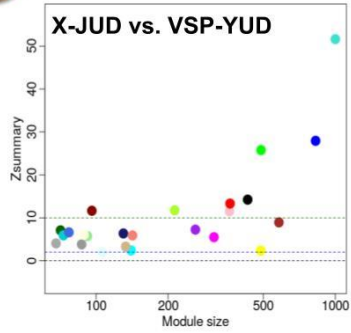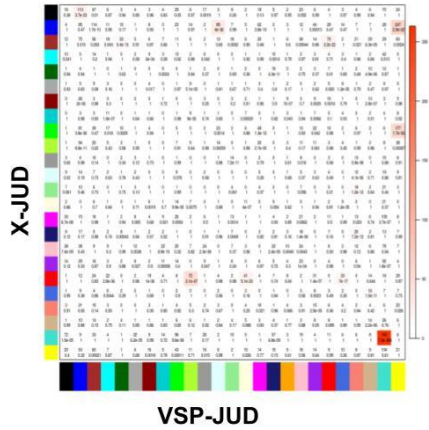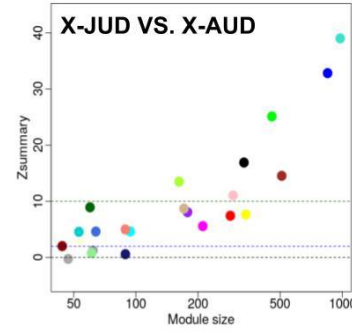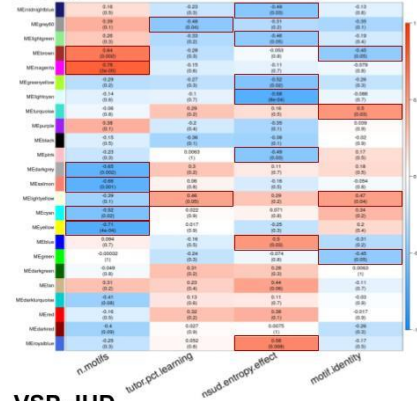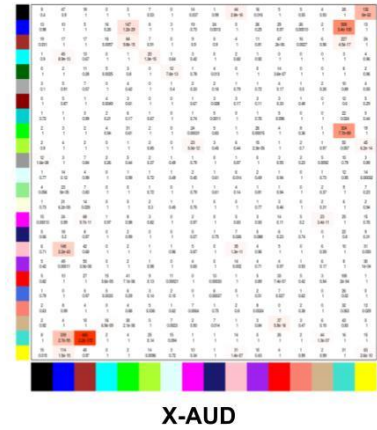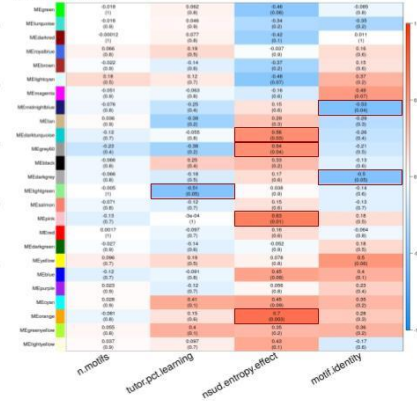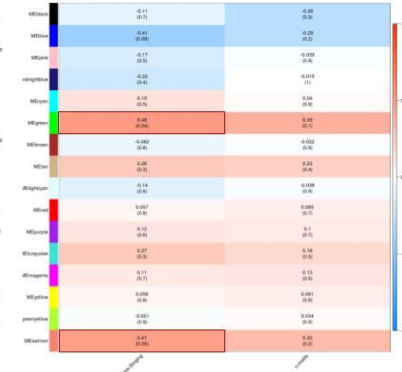
Supp. Fig. 2-6. **Analysis of preservation of gene co-expression patterns in the updated ZF networks.** Same analyses as described in Supp. Fig. 4. were run for modules defined in our rebuilt Area X network in the corresponding VSP expression data (X-JUD vs. VSP-JUD) and adult Area X expression data from (X-JUD vs. X-AUD).
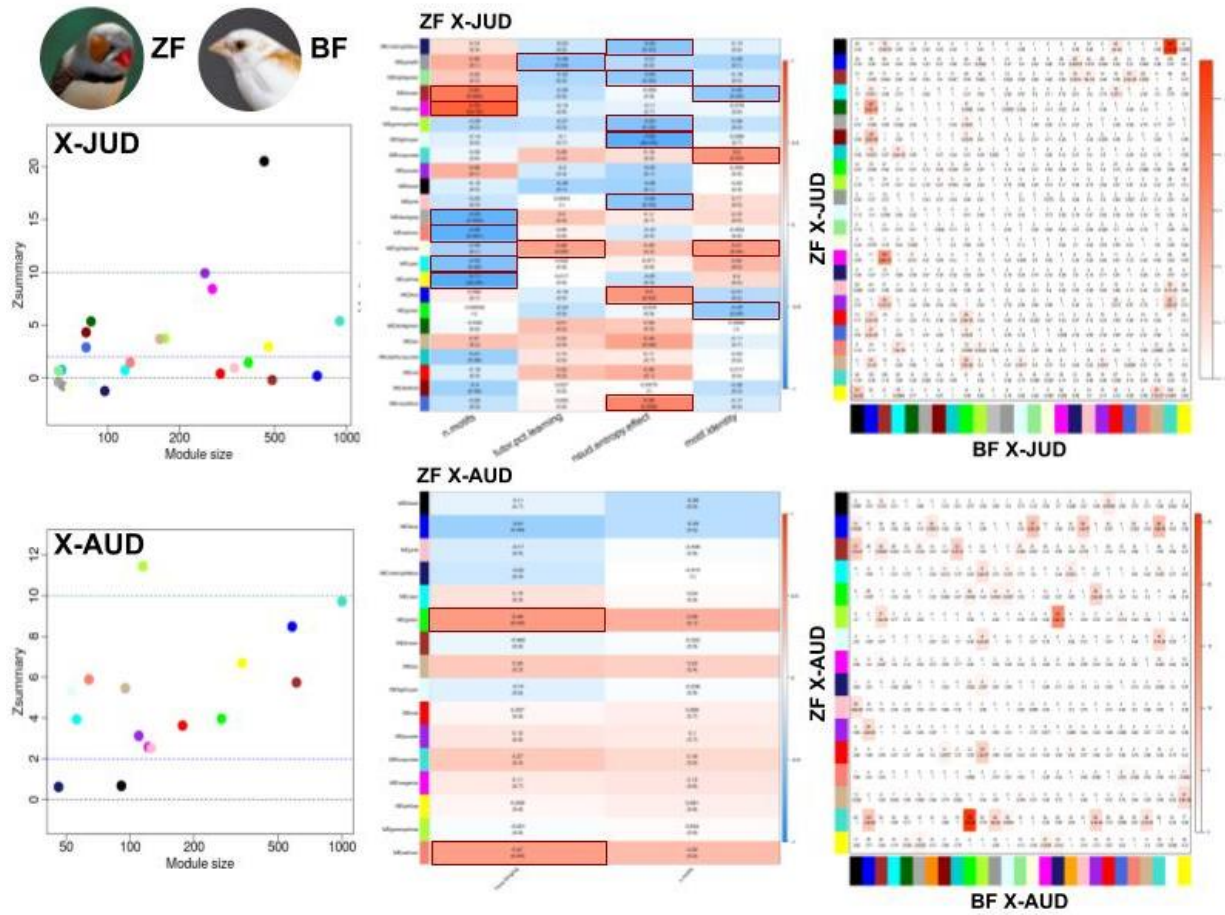
Supp. Fig. 2-7. **Cross-species evaluation of module preservation.** Same analyses as described in Supp. Fig. 4. Preservation analysis of modules defined in the updated ZF Area X network in BF expression data.

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Chapter 3 . The Slow but Rewarding Journey of Becoming a Vocal Learner

The slow but rewarding journey of becoming a vocal learner: intrinsic reward systems are central to the two-way evolutionary process between vocal learning and timing of sexual maturation

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Abstract

Humans and songbirds share the key trait of vocal learning, manifested in speech and song, respectively. Striking analogies between these behaviors include that both are acquired during critical developmental periods when the brain's ability for vocal learning peaks. Both behaviors show similarities in the overall architecture of their underlying brain areas, characterized by cortico-striato-thalamic loops and direct projections from cortical neurons onto brainstem motor neurons that control the vocal organs. These neural analogies extend to the molecular level, with certain song control regions sharing convergent transcriptional profiles with speech-related regions in the human brain. This evolutionary convergence offers an unprecedented opportunity to decipher the shared neurogenetic underpinnings of vocal learning. A key strength of the songbird model is that it allows for the delineation of activity-dependent transcriptional changes in the brain that are driven by learned vocal behavior. To capitalize on this advantage, we used previously published data sets from our lab and that correlate gene co-expression networks to features of learned vocalization within and after critical period closure to probe the functional relevance of genes implicated in language. We interrogate specific genes and cellular processes

through converging lines of evidence: human-specific evolutionary changes, intelligence-related phenotypes, and relevance to vocal learning gene co-expression in songbirds.

Introduction

In the words of the Argentinean writer Jorge Luis Borges, "'All language is a set of symbols whose use among its speakers assumes a shared past" [1]. Spoken language primarily uses sounds as symbolic vehicles, a repertoire of learned and voluntarily controlled vocal elements which can be connected in a rule-based way to form more complex sequences. Despite its human uniqueness, spoken language shares some of its necessary components with vocal behavior practiced by at least one evolutionarily distant animal group. A robust body of evidence accrued over ~100 years demonstrates striking analogies between birdsong and speech, both learned forms of vocalization.

Birdsong and speech are acquired during developmental critical periods when the brain's ability for vocal learning peaks (see Tyack, this issue). Both behaviors show similarities in the overall architecture of related brain areas, characterized by cortico-striato-thalamic loops and direct projections from cortical neurons onto brainstem motor neurons that control the vocal organs. These neural parallels extend to the molecular level, with certain song control regions sharing transcriptional profiles with speech-related regions in the human brain [2]. This offers an opportunity to decipher the shared neurogenetic underpinnings of vocal learning. This line of inquiry will additionally yield insight into human disorders of communication. Indeed, a major barrier in autism and schizophrenia research has been a lack of animal models with measurable behaviors relevant to core symptoms such as deficits in learned vocal communication [3-5]. With the advent of a well-annotated genome [6, 7], the songbird model is poised to close the gap in the available approaches for addressing language-associated neurodevelopmental disorders.

A key strength of the songbird model is that it allows for the delineation of activity-dependent transcriptional regulation driven by learned vocal behavior. To capitalize on this advantage, we used published datasets that correlate gene expression networks to features of learned

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vocalization within and after critical period closure to probe the functional relevance of genes implicated in language [8, 9]. Evolutionary changes relevant to human brain development include alterations in the timing of neuronal gene expression (e.g. heterochrony) as well as human-specific changes to the genetic sequence itself. We interrogate specific genes and cellular processes through converging lines of evidence: human-specific evolutionary changes, communication and intelligence-related phenotypes, and relevance to coordinated gene expression patterns related to learning in songbirds.

Dopamine and song variability

Within a given songbird species, there is individual variability in both the acoustic structure of the vocal units themselves, and in the order which they are sequenced. In zebra finches, a species in which song is sexually dimorphic and only males learn to sing, social context modulates song variability: it is typically reduced when a male sings to females (i.e., female-directed song - FD) relative to when he sings alone (i.e., undirected song - UD) [10]. While females prefer FD to UD song [11, 12], the more variable UD song is thought to reflect vocal-motor exploration important for motor learning and reinforcement [13]. The same benefit of variability during training leads to more efficient motor learning and performance in humans [14-16].

Along these lines, a certain degree of variation in juvenile song is thought to enable vocal learning, as young birds explore a range of vocal gestures in search for motor patterns capable of producing vocal output that matches the template built from the tutor's song [17, 18]. Similarly, in adult songbirds, variability appears fundamental for song maintenance, because it allows for the reinforcement of optimal motor patterns capable of producing auditory inputs that match the auditory template built from the bird's own song [19, 20]. Hence, in a way, variation begets fidelity. Fidelity is fundamental for performance of species-specific song patterns and levels of stereotypy that are adequate to a species' sensory ecology (e.g., in species recognition), without which successful social interactions (e.g., enchanting females) are less likely [21]. On the other hand, the ability to introduce variation creates an openness to innovation and more complex patterns of

cultural transmission, leading to increased song complexity, such as simultaneous learning from multiple tutors [22].

Context-dependent changes in song are correlated with key differences in gene expression in the songbird brain. The earliest indication of this came from the discovery of different patterns of immediate gene expression in AFP between UD and FD, leading to the original proposal of catecholaminergic modulation in Area X in context-dependent changes in song [23]. Subsequent studies show that in contexts associated with increased song stereotypy, as in FD singing, extracellular dopamine (DA) levels in the male basal ganglia song control nucleus called Area X are higher than in contexts associated with increased song variability, as in UD singing [24]. These changes are paralleled by another key molecule in vocal learning, the transcription factor FOXP2, for which Area X gene expression decreases during UD but not FD singing [25, 26]. Rather than coincidental, these concordant changes between FOXP2 gene expression and dopaminergic regulation appear causative: Experimental 'knock-down' of FOXP2 in Area X both disrupts the possibility of context-dependent modulation of vocal variability and decreases levels of dopamine receptor 1 (D1R) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), components in the D1R signaling cascade [27].

A role for endocannabinoids in vocal learning

Darwin noted that although the main function of birdsong during the breeding season is mate attraction, "birds continue singing for their own amusement after the season for courtship is over" [28]. Several studies now provide evidence supporting the hypothesis that song practice is indeed stimulated and maintained by intrinsic reward mechanisms [29]. These systems operate at two integrated levels: one regarding memetic aspects of song learning (i.e., of the reward-related memories) and associated dopaminergic neuromodulation, and the other regarding pleasure (i.e., elicited positive affective states), associated with opioid and endocannabinoid neuromodulation [30]. The role of cannabinoids in song variability and learning is substantiated by the reduction in stereotypy and number of learned notes in the songs of young birds exposed to an

endocannabinoid agonist relative to songs of non-exposed juveniles [31]. Notably, the treatment had no measurable effect on the structure of already-learned song patterns in adults, indicating that the mechanisms underlying critical period learning are more sensitive cannabinoid disruption. Here, we present further evidence supporting the involvement of the endocannabinoid system for vocal learning in humans and songbirds.

In prior work, our group applied an unsupervised weighted gene co-expression network analysis (WGCNA) [32] to songbird brain transcriptomes, and discovered groups of genes whose transcripts exhibit co-expression patterns, which in gene network terminology are referred to as 'modules'. As part of this unbiased approach, modules are assigned arbitrary color names (e.g. darkgrey) [8, 9]. Within the resultant network, some of the modules were unique to Area X and others were unique to the juvenile critical period for learning. Specifically, the authors tested whether the co-expression relationships observed in juvenile Area X were preserved in a non-song control brain region - neighboring basal ganglia termed the ventral striato-pallidum (VSP) - and in adulthood (i.e., region-specific and age-dependent modules). Statistically significant correlations (q.value < 0.05) to song features were also reported including to song production (the number of motifs sung by the bird at the time of the experiment) or learning (the spectral similarity of a pupil's song to that of its tutor's).

In another behavioral measure, the authors leveraged an experimental paradigm validated in several publications from the White Lab and others that detects singing-induced changes in zebra finch song variation, as seen during natural social modulation [33]. Here we refer to this as the 'variability induction' (VI) paradigm [33-36]. In these experiments, the variability of UD song after a period of non-singing is compared to the variability of UD song after an equivalent period of continuous UD singing. This comparison reveals that, on average, continuous UD 'practice' in the morning leads to increased song variability, while singing after a morning period of quiescence is more stereotyped, similar to FD song [33].

In this prior study, our group identified gene modules that were correlated to song-related behavioral features. Briefly, the authors found modules of genes whose co-expression was up- or down-regulated by the amount of song that birds sung on the morning of the experiment. Strikingly these 'song modules' were specific to Area X and observed in both adult and juveniles, speaking to the profound influence of singing behavior on the transcriptome [8, 9]. Excitingly, the analysis shows modules correlated to the amount of tutor song learning by a pupil, and these were restricted to juveniles. The gene co-expression pattern for these learning-related modules disappeared in adults that were beyond the critical period for learning, suggesting that they are important for the neural plasticity underlying critical period learning. Additional modules were correlated to the morning modulation of song variability as measured by the VI paradigm.

Here, we newly integrate those prior findings of behaviorally relevant gene co-expression modules in Area X of juvenile birds [8] with findings published by Pfenning et al., who identified gene expression patterns that are conserved in the brains of humans and song-learning birds [2]. Specifically, the authors analyzed transcriptomes from multiple brain regions from humans and song-learning birds (zebra finch, parrot, and hummingbird) as well as vocal non-learning birds (dove and quail) and a non-learning primate species (macaque). They found evidence for transcriptional convergence in vocal-learners' brains, including between Area X and the human putamen, a dorsal portion of the human striatum.

Our new analysis points to a total of 45 genes (Supp. table 1.) that form behaviorally relevant modules in the juvenile network and exhibit transcriptional convergence between Area X and the human putamen. Among these, monoacylglycerol lipase (MGLL) shows high intramodular connectivity that is specific to the Area X of juvenile birds, and for which expression correlates with a bird's ability to introduce variability in its song (Supp. table 1.). MGLL is responsible for the metabolism of endocannabinoid 2-arachidonoylglycerol (2-AG) [37]. These findings suggest that the regulation of MGLL metabolism of 2-AG in Area X allows for song variability in juvenile birds. Endocannabinoids such as 2-AG mediate activity-dependent changes in synaptic strength via

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activation of type 1 cannabinoid (CB1) receptors in several regions of the mammalian brain, including the striatum [38]. They retrogradely suppress both inhibition and excitation of dopaminergic neurons by inhibiting neurotransmitter release by GABAergic medium spiny neurons and glutamatergic terminals, respectively [39]. Endocannabinoid–DA striatal interactions seem to be conserved in songbirds, as components of both systems are regulated in Area X [24, 40]. Thus, the regulation of MGLL metabolism of 2-AG in Area X during the juvenile critical period likely has consequences for FOXP2-mediated synaptic plasticity (e.g. [41, 42] and dopaminergic neuromodulation.

Sex hormones and related metabolites and enzymes are regulated in a context-dependent fashion and as a function of the developmental trajectory (see below) in several organisms, including songbirds [43] and mammals [44]. In zebra finches, directed FD singing is associated with higher levels of circulating plasma testosterone than during more variable UD singing [45, 46]. Moreover, the plastic period of juvenile song learning closes when zebra finches mature and testosterone levels rise [47, 48]. These endogenous hormones are long recognized to modulate dopamine in mammalian and songbird brains [49, 50], and have more recently been investigated in endocannabinoid-associated reward systems in both animal groups [30, 51-53]. Therefore, context-dependent surges in testosterone levels and changes in the timing of sexual maturation impact the function of intrinsic reward systems in both songbird and mammalian brains.

Slow developmental trajectories and the evolution of vocal learning

Human development is delayed and prolonged relative to other great apes, and this distinction is key to understanding human uniqueness [54]. Songbird development is also delayed relative to non-vocal learning birds [55], suggesting that similar changes in the timing of development (heterochrony) contributed to the emergence of vocal learning in songbirds and humans. Charvet and Striedter argue that changes in the timing of development are a necessary prerequisite for the evolution of learned vocal communication [56]. Developmental timing, though not sufficient, may have been key to the evolution of complex vocal production learning, at least in songbirds

and humans. Slowed development supports the vast metabolic demands of the developing songbird [57] and human brain [58]. This allows the generation of an expanded telencephalon, which is then capable of being adapted for learned vocalization circuitry. With prolonged development, the window of juvenile plasticity also expands, creating an opening for the evolution of complex learned behaviors [59].

In the wild, slow developmental trajectories reflect some of the most varied evolutionary processes, including the effects of limiting environmental conditions [60-62]. However, some cases of delayed developmental trajectories may evolve through a change in selective pressures whereby commonly found environmental sources of selection that work as constraints to behavioral plasticity and flexibility, such as predation, foraging, and species recognition are either superseded or overcome by sources of selection imposed by socialization [63, 64]. This sort of change in evolutionary regime is also observed in processes of domestication [65]. Incidences of domestication thus serve as fruitful models for studying such evolutionary phenomena [66].

Domestication has reduced the time to sexual maturation in a variety of animals, including the domestic chicken. However, another parcel of domestic animals, likely following domestication practices other than selection for earlier reproduction, show slow developmental trajectories and retain in adulthood a suite of behavioral, physiological, and morphological traits typically found at younger ages in the ancestral species (i.e., the domesticated phenotype/syndrome), a form of heterochrony called "neoteny" [66, 67]. This is reflected in a reduction of sexually stereotyped traits (e.g., morphological and behavioral dimorphisms) and extended neuroplasticity and learning, which leads to behavioral flexibility and an openness to cultural transmission [68].

Humans may have undergone a similar process, but one in which we were the solo protagonists of our own domestication (i.e., self-domestication) [69]. Sources of relaxation of selection during human evolution include the development of collective ambushing strategies to hunt for stronger prey; taking part in 'traditional learning' (i.e. the cultural transference of information from generation to generation), and division of labor which allows for the optimization of tool-making,

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hunting-and-gathering as well as long-range trading, among other fundamental human activities. In addition, increases in affiliative behaviors towards the elderly, children, and disabled individuals, minimize the fitness consequences of their vulnerability. In these contexts, human sociality would have entered a feedback loop, reducing common environmental selective pressures, but making itself the main source of selection in our species. Individuals showing more tolerance to social stress, and more cooperative --instead of aggressive-- behaviors would have granted selective advantages. Several unique traits that define our species, including language, could have evolved associated to these selective pressures for more peaceful and cooperative living.

Like language, the evolution of birdsong is challenging to study because song is not directly reflected in non-perishable or fossilizable forms. As Don Kroodsma, a pioneer in the field, has pointed out, "perhaps the best hope for understanding why some birds learn is to examine far more recent evolutionary events [...] in which we can better identify the social circumstances that led to the origins [and] loss of vocal learning" [70]. To that goal, recent studies have added the evolution of more complex patterns of vocal learning, such as learning from multiple tutors and more variable song. A preeminent example of this type of evolutionary change is the domesticated Bengalese finch (BF; *Lonchura striata domestica*) [71].

Beginning ~250 years ago, BFs were caught and domesticated from their wild Chinese ancestor, the white-backed munia (WBM; *Lonchura striata*). According to extensive historical reports, the BF was never bred for its singing ability, yet it evolved a much more complex vocal behavior than that found in WBMs, marked by increased sequence variability and simultaneous learning from multiple tutors. Together with less aggressive and less neophobic (i.e., fear of novelty) behaviors, these features complete a suite of neotenic traits in the BF that enhance exploration. Female choice has been proposed as a major selective force leading to the increase in BF's vocal complexity [72]. Additionally, relaxation of sources of selection present in the wild, such as species recognition and environmental stress [73, 74], may have been crucial in allowing for new or

continuing sources of positive selection to operate toward the evolution of more complex vocal behavior in the BF (i.e., female choice for more complex songs) [75, 76].

To sum up thus far, the delayed developmental trajectories in both humans and songbirds holds parallel implications for reward systems leading to the evolution of vocal learning. Evidence suggests that, rather than the work of a single evolutionary force this was the product of a similar interplay of adaptive pressures relating to the enrichment of social over environmental demands in both groups. This advance holds timely relevance to the current path of our species in meeting modern day socio-political and climate-related challenges on the global scale.

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Appendix I A Sing-Song Way of Vocalizing: Generalization and Specificity in Language and Birdsong.

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Efficient performance of motor skills, including ones of vocal nature, relies on the precise control of movements to 1) generate individual motor gestures and 2) rapidly organize them into sequences. Multiple occurrences of the same motor gesture can exhibit substantial variability, adding complexity to their identification by a potential receiver and their articulation by the sender. One source of variability stems from the influence of sequential context, as when gestures present modifications depending on their interaction with other gestures composing a motor sequence. Co-articulatory effects represent this type of variability, whereby gestures are modified depending on other gestures preceding or following them. Such effects were first described for human speech but have since been expanded to include other complex motor sequences, such as those used to fingerspell American sign language [1].

It has been therefore proposed that learning or performing a complex motor skill requires a balance between generalization and specificity. Generalization is defined as the ability to identify or articulate a motor gesture or modification thereof in multiple sequential contexts. Specificity refers to the ability to do so only in a certain sequential context. An example of generalization is the transfer of modifications of a motor gesture induced by external perturbation to contexts other than the learned one. Generalization can thus be efficient for learning, including by reducing the need to relearn how to produce a vocal gesture correctly in each possible context. This adaptive

quality of motor gestures is important for the motor response to weakening of muscles, as evidenced in neuromuscular disorders and natural aging.

Human speech generalization can be studied by exposing subjects to perturbations of their own speech patterns, using real time feedback of transformed speech signals, and analyzing the transfer of adaptations to the same trained phoneme produced in different phonetic contexts, or across phonemes other than the trained one. In a representative early example of this kind of analysis, Houde and Jordan (1998) show that speakers learn to adjust their production of a vowel to compensate for feedback alterations that change the vowel's formants, and that this effect indeed generalizes to the same vowel produced in different words, and across different vowels [2]. A degree of specificity also applies to the identification and articulation of motor gestures, in which modification of phonemes and their associated movements are acquired in a manner that applies only to specific phonetic contexts. This effect has been shown in speech where subjects receive and efficiently correct for auditory feedback that is altered in opposing ways for the same vowel produced in different words [3].

Tian and Brainard present evidence that generalization and specificity of vocal gestures also occurs in birdsong, an essential model of learned vocalization that offers the ability to mechanistically test the neural bases of such phenomena. Using Bengalese finches (*Lonchura striata domestica*), the authors first investigate the generalization of learned modifications of vocal gestures (song syllables) by employing a negative reinforcement paradigm in which the bird must learn to shift the pitch of a targeted syllable in a single sequential context to escape a burst of white noise. Previously, investigation of vocal generalization by Hoffman and Sober (2014) employed a different experimental setting where Bengalese finches were fitted with miniaturized headphones to selectively perturb auditory feedback of their syllables (Hoffmann and Sober, 2014). Under this training paradigm, the pitch of auditory feedback from a single syllable in each bird's song was perturbed only when it occurred in a specific sequential context. The altered feedback induced the bird to error correct by shifting the pitch of its own syllable in an adaptive

direction, that is, opposite to the auditory feedback. This suggests that songbirds can generate internal error signals specific to vocal gestures, thus paralleling findings in humans who alter the acoustics of single phonemes in response to manipulated auditory feedback [2].

In both songbird studies, generalization was tested by measuring the bird's ability to shift the pitch of syllables of the same type as the targeted syllable in sequential contexts other than trained. Findings from both groups demonstrate the same effect, where learned modifications of the targeted syllable are maximal for the trained sequential context but also partially generalize to other sequential contexts, demonstrating that this phenomenon observed in human behavioral studies applies to birdsong [2]. They both also attribute a significant influence of sequential factors on the degree of generalization, each one uncovering a different sequence-dependent effect.

In Hoffman and Sober's (2014) error-correction paradigm, nontargeted syllables changed in the adaptive direction when they were produced in close sequential proximity to the pitch-shifted syllable, suggesting that error information from one syllable is used to generate adaptive vocal changes in other syllables [4]. This finding has been attributed to an "error credit assignment" mechanism by which the sensory error is partially assigned to similar nearby syllables [5]. Interestingly, the same sequence-dependent effect is not reported here by Tian and Brainard. The different outcome may relate to differences in the experimental paradigm and reflect the influence of available sensory cues in generalization. A possible explanation is that the white noise serves as a highly salient negative reinforcement signal the bird uses to localize vocal changes in time, thus assigning errors more specifically to the targeted syllables. Tian and Brainard's white-noise paradigm present us with a newly reported sequence-dependent effect. They show that the magnitude of generalization for a given non-targeted context can be explained by its sequential similarity to the targeted context which they call "contextual similarity".

Using the same white noise negative reinforcement paradigm as they did for generalization. Tian and Brainard go further to directly interrogate the specificity of the learned modifications: They measure the bird's ability to shift the pitch of a targeted syllable in one direction for a single

sequential context, and in the opposite direction for another sequential context. Again, their findings mirror human behavioral studies, where opposite modifications of a targeted gesture can be learned for different sequential contexts [3]. Tian and Brainard's paradigm thus directly compare, in the same study, generalization and specificity in songbird vocal learning. Their findings endorse birdsong as a tractable model for investigating components of human speech and firmly establish the Bengalese finch in the study of context-dependent vocal motor learning.

The neural mechanisms underlying birdsong have been studied for >100 years, providing us with a well-characterized picture of a system composed of anatomically distinct groups of neurons within an anterior forebrain pathway (AFP) and a posterior motor pathway [6]. The AFP consists of a cortico-basal ganglia loop and intersects with the posterior motor pathway which itself consists of direct projections from cortical neurons onto brainstem motor neurons that control the vocal organs. Tian and Brainard take advantage of this prolific body of work to test key hypotheses regarding the neural mechanisms that operate to support the coding or representation of vocal gestures as it relates to generalization and specification. Specifically, it has been proposed that modifications of targeted syllables are generated by biasing signals from the AFP. This hypothesis is based on a collection of more recent studies, using a variety of experimental methods, including chemical inactivation, electrical stimulation, and single-unit neural recordings, that demonstrate the role that the AFP plays in natural vocal variability and in responding to perceived vocal errors which can be used for learning in juvenile and adult birds [7]. The authors further hypothesize that if those same mechanisms contribute to all adaptive modifications of song, they could expect that the early expression of learning in the target context would also rely on biasing signals from the AFP.

Their results indeed show that chemical inactivation of LMAN, the cortical output nucleus of the AFP, reduces the magnitude of expressed learning that is specific to the trained sequential context; an effect that is reversible upon wash-out of the drug. Tian and Brainard propose a model to integrate their observations. They suggest that the AFP provides context-specific biasing

signals that modify a context-independent syllable representation in the motor circuitry involving RA, another cortical nucleus in the songbird brain that is analogous to the human primary motor cortex (*Figure -1*) [8]. They provide evidence supporting their model's prediction that generalization would be reduced when incongruent modifications of a syllable are reinforced in different contexts. This would not be the case if separate representations of a given syllable in each context were being generated and consolidated.

The overall architecture of speech related areas in human brain finds parallel in the organization of the AFP in the songbird brain (Bolhuis et al., 2010). In both humans and songbirds, basal ganglia circuitry is highly interconnected with widespread areas of the cortex, including areas related to several aspects of vocal behavior, such as vocal motor control, auditory processing, and sensory-motor integration. This suggests that the cortico-basal ganglia circuitry not only influences motor aspects of vocal processes but also plays an important role in other related cognitive functions, including auditory categorization. In speech category learning, this idea is supported by neuroimaging evidence that demonstrates the recruitment of the basal ganglia learning system during auditory categorization tasks that are based on outcome feedback [9]. Learning appropriate behavioral actions to achieve goals in specific environments can be thought of as a reinforcement process in which one builds and updates predictions about receiving future rewards. Based on such predictions, behavior adjusts adaptively to maximize future rewards such that actions leading to rewards are reinforced, whereas incorrect behaviors leading to punishment, or no rewards are modified. This overarching model provides a useful framework for understanding the way by which cortico-basal ganglia circuitry contributes to the generation of context-specific biasing signals necessary also for auditory/vocal learning. While the exact nature of auditory representation remains for the most part unexplained, in general, evidence from both birdsong and human speech agree in which they implicate cortico-basal ganglia circuitry in eliciting biasing signals that are likely related to auditory categorization in goal-oriented contexts and are necessary to guide responses to perceived vocal errors in adaptive ways.
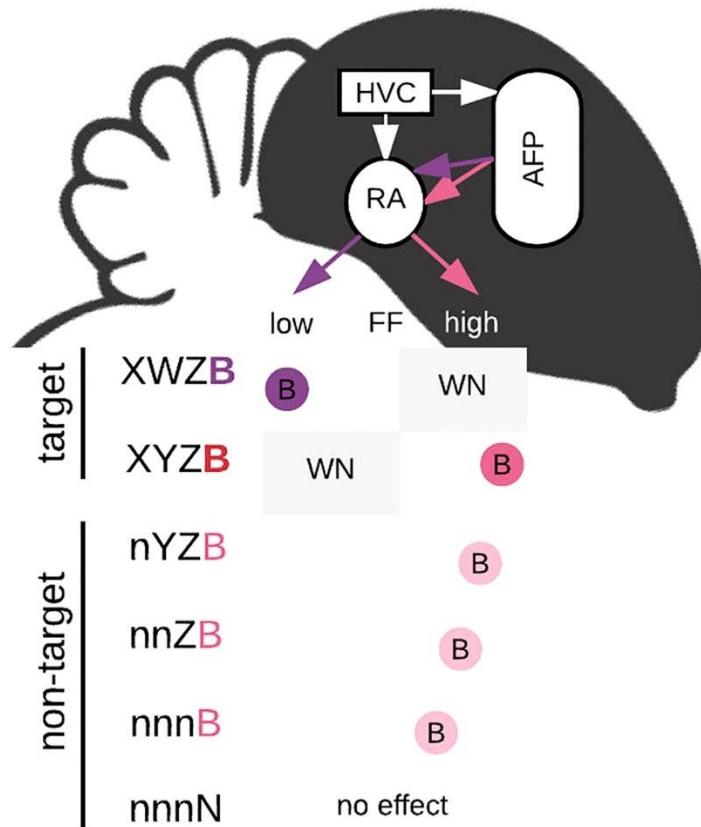
Figure -1 **Biasing signals from the avian cortico-basal ganglia circuitry.** Pitch changes in contexts where white noise (WN hit) is applied to target note B (dark pink) are labeled as "target", whereas contexts other than trained are labeled "non-target". Different contexts include syllables of the same (light pink) or different type (N) as the target syllable and may share syllables in the same position as the targeted context ("X", "Y" and "Z" – "n" denotes any other syllable type). From bottom to top: The pitch of a targeted syllable can be changed in one direction for a certain sequential context, and in the opposite direction for another sequential context (i.e., specificity). The magnitude of generalization of pitch changes to syllables of the same type as the targeted syllable is relative to how many syllables are shared between target and non-target contexts. This process derives from biasing signals from the avian cortico-basal ganglia circuitry.

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Appendix II Cell-type-specific Behavioral Regulation of Gene Expression in Avian Basal Ganglia

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Abstract

Zebra finch (*T. guttata*) courtship song is a complex and highly motivated learned behavior that enables study of neural mechanisms underlying sensorimotor learning. Juvenile male birds learn their songs during two partially overlapping critical periods for learning, during which a song from an adult tutor is first internalized and then imitated with increasing fidelity. Brain regions necessary for song learning, including the song-dedicated portion of the basal ganglia (Area X), exhibit highly convergent gene expression patterns with human forebrain regions linked to speech development. Bulk-sequencing of Area X previously revealed changes in gene expression following undirected singing (a form of vocal practice) and across developmental learning stages. However, it is not known which specific Area X cell type(s) participate in these behavior-linked and age-related changes. Here we use single-cell RNA sequencing to investigate cell-type-specific changes in Area X gene expression between singing and non-singing juvenile and adult male sibling pairs. Cell-type clusters were identified, and differential expression analysis performed within each cell-type across ages and behavioral conditions. These data identify a subset of medium spiny neurons labeled by the cAMP-regulated phosphoprotein ARPP21 that show singing-driven gene regulation across behavioral conditions and provide insight into broad

transcriptional changes across critical period closure. These discoveries aid in better understanding how distinct cell-types within the basal ganglia circuitry contribute to motor learning, and how repeated practice during development contributes to long-term mastery of motor skills.