Hormones and Behavior 129 (2021) 104911

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Estrogen and sex-dependent loss of the vocal learning system in female zebra finches

Ha Na Choe [a,b,*], Jeevan Tewari [a], Kevin W. Zhu [a], Matthew Davenport [c], Hiroaki Matsunami [a,b,*], Erich D. Jarvis [b,c,d,*]

[a] Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA
[b] Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA
[c] Laboratory of Neurogenetics of Language, The Rockefeller University, New York, NY 10065, USA
[d] The Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA

ARTICLE INFO

Keywords:
Specialized gene expression
Transcriptomics
Plumage
Juvenile
Development
Aromatase inhibitor
Sex hormones
Exemestane
Birdsong

ABSTRACT

Sex hormones alter the organization of the brain during early development and coordinate various behaviors throughout life. In zebra finches, song learning is limited to males, with the associated song learning brain pathways only maturing in males and atrophying in females. While this atrophy can be prevented by treating females with exogenous estrogen during early post-hatch development, the requirement of estrogen during normal male song system development is uncertain. For the first time in songbirds, we administered exemestane, a potent third generation estrogen synthesis inhibitor, from the day of hatching until adulthood in order to reassess the role of estrogen in song circuit development. We examined the behavior, brain anatomy, and transcriptomes of individual song nuclei in these pharmacologically manipulated animals. We found that males with long-term exemestane treatment had diminished male-specific plumage and impaired song learning, but minimal effect on song nuclei sizes and their specialized transcriptome. Consistent with prior findings, females with long-term estrogen treatment retained a functional song system with song nuclei that had specialized gene expression similar, but not identical to males. We also observed that different song nuclei responded to estrogen manipulation differently, with Area X in the striatum being the most altered by estrogen modulation. These findings support the hypothesis that song learning is an ancestral trait in both sexes that was subsequently suppressed in females of some species and that estrogen has come to play a critical role in modulating this suppression as well as refinement of song learning.

1. Introduction

Sexually dimorphic behavior is widespread in the animal kingdom, including but not limited to courtship, mate choice, predator avoidance, and parental care; all necessary for a species' survival (Breed and Moore, 2010). These behaviors are often highly stereotypic, suggesting that they utilize "hard-wired" pathways within the brain that are established during early development (Carrer and Cambiasso, 2009; Kurian et al., 2010; McCarthy and Arnold, 2011; Wu et al., 2009). One such behavior is the capacity for vocal learning, the ability to imitate sounds heard. In its advanced form, this rare trait is found in only three groups of birds (songbirds, parrots, and hummingbirds) and five groups of mammals (humans, bats, cetaceans, pinnipeds, and elephants), and is one of the most critical components for spoken language in humans (Jarvis, 2019).

One of the most commonly studied non-human vocal learners is the zebra finch, where the trait is highly sexually dimorphic (Nottebohm and Arnold, 1976). Like many other songbirds, male zebra finches learn their father's/tutor's song and use it to attract female mates. Female zebra finches do not learn to sing, although like most other vertebrates, the females can produce innate vocalizations. While unable to sing, she can form auditory memories of sounds heard, which she uses to evaluate the male's song (Riebel, 2009).

This sex difference in vocal learning behavior is associated with sexually dimorphic circuits in the zebra finch brain. Male zebra finches (and songbirds generally) have a forebrain vocal learning system comprised of seven telencephalic nuclei and one diencephalic nucleus (Fig. 1A) with parallels to brain pathways for spoken language in humans (Jarvis, 2019; Pfenning et al., 2014). These 7 regions can be

* Corresponding authors at: Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA.
E-mail addresses: ha.choe@duke.edu (H.N. Choe), hiroaki.matsunami@duke.edu (H. Matsunami), ejarvis@rockefeller.edu (E.D. Jarvis).

https://doi.org/10.1016/j.yhbeh.2020.104911
Received 14 February 2020; Received in revised form 29 November 2020; Accepted 1 December 2020
Available online 23 January 2021

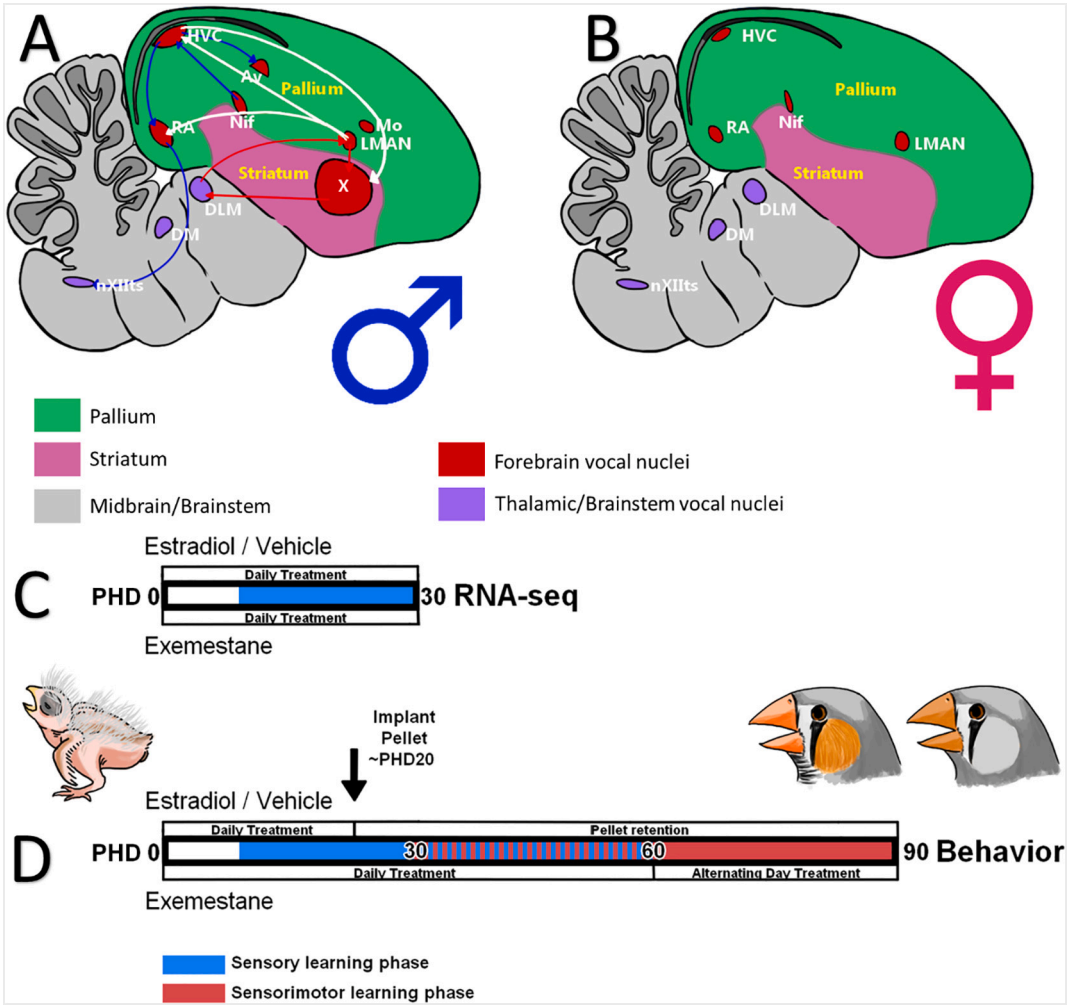

Fig. 1. Brain organization sex differences and experimental paradigm. (A) Adult male zebra finch brain. (B) Adult female zebra finch brain. There are 7 telencephalic song nuclei: HVC, RA, LMAN, Area X, Av, Nif, and MO. Additionally, there are 3 vocal nuclei in the thalamus (DLM) and brainstem (DM and the XIIts motor nucleus). The male brain (A) has all 7 song nuclei, and the female (B) 4 confirmed song nuclei, but is missing Area X, and Av. HVC and RA are also smaller in females. LMAN is not visually different between males and females. Some connections of male song nuclei are shown; medial MAN projects to HVC. (C) PHD30 experimental timeline. Vehicle, estradiol, and exemestane treated animals were dosed daily until PHD30 and taken for brain transcriptome analysis. (D) PHD90 experimental timeline. Estradiol and vehicle animals were dosed daily until PHD20, whereupon they received silastic implants with estradiol or vehicle until sacrifice at PHD90. Exemestane animals were dosed daily until PHD60, and then every other day until behavior collection and sacrifice at PHD90. HVC, proper name; RA, Robust nucleus of the Arcopallium; LMAN, Lateral Magnocellular nucleus of the Anterior Nidopallium; Area X, proper name; Av, Avalanche; Nif, Nucleus Interface of the Nidopallium; MO, Oval nucleus of the Mesopallium; DLM, Dorsal Lateral nucleus of the Medial thalamus; DM, Dorsal Medial nucleus; XIIts, 12th motor nucleus, tracheosyringeal part.

subdivided into 2 pathways, a posterior motor pathway for the production of learned vocalizations and an anterior pathway for the learning of these vocalizations. Using the pathway division as initially clarified in Jarvis et al. (1998), HVC (proper name), RA (Robust nucleus of the Arcopallium), Av (Avalanche) and NIf (Interfacial nucleus of the Nidopallium) make up the posterior motor pathway; Area X (proper name), LMAN (Lateral Magnocellular nucleus of the Anterior Nidopallium), MO (Oval nucleus of the Mesopallium) and aDLM (anterior Medial nucleus of the Dorsolateral Thalamus) make up the anterior motor pathway (Fig. 1A).

During development, both male and female brains start out with the same telencephalic song learning nuclei that are first seen between 5 and 15 days post hatch (PHD5-15). However, after PHD15, the male song nuclei continue to grow while the female nuclei atrophy and become barely visible, if at all, in adulthood at >PHD90 (Fig. 1B) (Bottjer et al., 1985; Garcia-Calero and Scharff, 2013; Konishi and Akutagawa, 1985; Nixdorf-Bergweiler, 1996; Nordeen, 1988; Nottebohm and Arnold, 1976; Shaughnessy et al., 2018). Additionally, around PHD30, a robust axon tract that extends from HVC to RA innervates the RA in males but not the atrophying RA in females (Fig. 1A,B) (Holloway and Clayton, 2001; Konishi and Akutagawa, 1985; Mooney and Rao, 1994).

Remarkably, even brief treatment with estrogen a week prior to PHD30 can cause zebra finch females to retain some of their vocal learning systems into adulthood, allowing these treated females to produce courtship songs similar to males; this suggests that estrogens are potent masculinizing agents in the song system of zebra finches (Gurney, 1982; Simpson and Vicario, 1991a). In contrast, androgens are far less effective at masculinizing the female song system (Grisham and Arnold, 1995).

The reverse scenario with attempts at demasculinizing the male song system by blocking estrogens or androgens has yielded inconclusive results. Prior studies using fadrozole, a drug that inhibits aromatase from converting androgens to estrogens, did not reveal any impacts on male song development either behaviorally or anatomically (Merten and Stocker-Buschina, 1995; Wade and Arnold, 1994). However, fadrozole was later found to effect song behavior and aromatase activity only briefly within 30 min of injection; the effects were lost after 4 h (Alward et al., 2016). It was also determined that fadrozole can stabilize the

aromatase protein, which is then followed by increased estrogen synthesis after drug clearance, causing a "rebound effect" (Harada and Hatano, 1998). Tamoxifen, originally thought to act only as an estrogen receptor antagonist, was paradoxically found to increase the size of certain nuclei in the zebra finch song system rather than diminish them (Mathews and Arnold, 1990, 1991; Mathews et al., 1988). Later it was discovered that when tamoxifen binds to the estrogen receptor in competition with estrogen, it can behave either as an agonist or antagonist. This agonist/antagonist duality depends on the tissue type tamoxifen is acting in (Martinkovich et al., 2014; McDonnell, 2005; Wardell et al., 2012; Wardell et al., 2014), with the drug acting as a "super-estrogenic" agonist in the brain (Mathews and Arnold, 1991). Treatment of males with flutamide, a non-steroidal androgen receptor antagonist, failed to demasculinize any aspect of the song system, and in fact hyper-masculinized RA (Schlinger and Arnold, 1991). Lastly, studies using selective estrogen receptor disruptors (Bender and Veney, 2008) and G-protein coupled estrogen receptor (GPER) antagonists (Tehrani and Veney, 2018) found small decreases in male song nuclei soma, decreases in HVC and Area X sizes, but potential effects on song behavior were not reported. To further complicate the issue, many studies blocked estrogen only during a limited period of development and not across the entire PHD2-PHD45 estrogen-responsive critical period for vocal learning (Gobes et al., 2017; Konishi and Akutagawa, 1988; Pohl-Apel and Sossinka, 1984). In sum, the effects from pharmacologically blocking estrogen during development of the sexually dimorphic song system remains enigmatic.

Prior reports have shown that short term estrogen blockades in juvenile males fail to induce feminization/de-masculinization (Wade and Arnold, 1994), providing the current hypothesis that estrogen is not critical for the development of the song system in males, and is therefore not the primary source of song system sexual dimorphism in songbirds. Subsequent microarray studies further suggested that extra dose of Z chromosome genes in males may play a dominant role over sex hormones for male-typical brain development, which is absent in the hemizygous female (Tomaszycki et al., 2009). Further refining this hypothesis, recent findings from Odom et al. (2014) suggest that vocal learning may have evolved in both sexes in the ancestor of all songbird species, which was then lost in the females of some species due to evolutionary pressure (Jarvis, 2004). These and the above reviewed findings lead us to suggest two alternative hypotheses: 1) That estrogen is required for development of the song learning system in both sexes of zebra finches, but prior studies missed discovering this because they did not sufficiently block it in males; or 2) Post evolution of song learning in both sexes, females of some species lost the trait and reversal of this sex-dependent loss is dependent on the gene-modulatory effects of estrogen signaling.

In the present study, we test these hypotheses by utilizing exemestane, a third generation steroidal aromatase inhibitor, that has been shown to lack the "rebound effect" (Wang and Chen, 2006). Exemestane is the current gold standard for adjuvant therapy in treating estrogen sensitive cancers in the clinic due to its efficacy and specificity (Lonning and Eikesdal, 2013). We treated male and female zebra finch chicks chronically with either exemestane or estradiol from the day of hatching until sacrifice, either at the beginning of the sensorimotor learning period (PHD30) or into adulthood, and confirmed manipulation of estrogen levels in the blood and brain. We then conducted behavioral and anatomical comparisons, and RNA-seq analyses of song nuclei gene expression specializations. We found estrogen exerts un-equal effects in a sex-dependent, brain region-dependent manner, supporting hypothesis 2; we further reveal that estrogen is required for normal male plumage development, normal song learning development, and the gene expression specializations in the Area X of females during early development.

2. Materials and methods

2.1. Husbandry for hormone treated chicks

Zebra finches from our breeding colony at Duke University were kept on a 12:12 light/dark cycle between 23 and 29 °C and 30–70% humidity. Fortified finch seed mixture (Kaytee), enriched grit (Higgins), poultry feed (Purina), cuttlefish bone and water were provided *ad-libetum*. This normal diet was supplemented with hardboiled eggs and oranges given twice weekly. Water was also supplemented with liquid calcium borogluconate (Morning Bird). Baths were given weekly with cage pan changes, and cage enrichments were provided and exchanged during cage changes (every 3 months). Breeding animals were kept in pairs in their own cages, and native or foster offspring were kept with their parents until PHD60 (or sacrifice at PHD30) when the offspring completed their sensory learning period and were able to feed on their own. Nest boxes were cleared out and replenished with fresh nesting material between clutches. Non-breeding animals were kept in single sex cages with no more than 6 animals per cage.

Animals were used from 32 breeding pairs. To synchronize embryo development from these pairs, eggs were collected daily from nest boxes during a 2-week period and placed in developmental stasis at 15 °C with 80% humidity on a 30° angle rotator set to rotate once every 2 h, in a P-008A BIO incubator (Showa Furanki Corp). Eggs were kept in stasis for no longer than 3 weeks. Egg collection from nesting pairs chosen for fostering was ceased 3 days prior to artificially incubating the synchronized eggs, permitted these foster parents to brood a clutch of 3–4 eggs. After synchronizing a cohort of eggs in the low temperature incubator, they were then moved to a higher temperature incubator at 37.5 °C with ~50% humidity on a 30° angle rotator set to rotate once per hour and incubated for up to 14–15 days. When chicks started to pip (crack the eggshell), usually beginning around incubation day 13, all eggs were transferred from the incubator's egg rotators to the hatch plate. Within 16 h of hatching, animals were tagged and transferred to the chosen foster nests in the aviary. We found that this incubation protocol resulted in chicks hatching at approximately the same time as native chicks hatched under their biological parents. To control for potential genetic effects, artificially incubated chicks from the same biological parents were distributed to different unrelated nests and treatment groups to be fostered by non-biological parents, alongside chicks that were hatched by their biological parents in an approximate 1:1 ratio. To tag these non-biological fosters, a distal toe joint of the newly hatched animals were removed with sterile forceps and a scalpel; the removed sample was used to determine the sex of the animal by PCR-genotyping with degenerate P2 (TCTGCATCGCTAAATCCTTT) and P8 (CTCCCAAGGATGAGRAAYTG) primers as previously described by Adam et al. (2014). Chicks born natively under their biological parents had their downy feathers removed in unique patterns for identification and were later sexed using cells collected from buccal swabs during banding (~PHD 10). Nests were limited to no more than 5 chicks to reduce nestling mortality. Occasionally some nests had chicks that were born either long before or long after foster chicks were introduced to nest boxes; these mixed age nests were not treated and served as environmentally exposed controls for the colony.

Animals were separated into two colonies kept in two different rooms: one for animals treated with exemestane or estradiol, and the other treated with vehicle. The exemestane/estradiol colony had 12 breeding pairs and no extra animals of either sex. The vehicle colony had 20 breeding pairs, and 4 cages of single sexed extra animals (~10 males and ~10 females). In general, there were 3× more animals in the vehicle colony as this was also our general colony, however cage density (animals/cage) was the same in both rooms.

To prevent cross contamination, exemestane and estradiol treatments were not done concurrently. Only a single active pharmacological agent was permitted in the estradiol/exemestane room at a time. After a drug cohort had completed treatment and the last treated animal was

removed from the room for sacrifice or behavioral recording, animal husbandry staff was notified and a mandated 48 h wash-out period was instituted to alert staff of changes in the room, allowing for the removal of contaminated equipment and the installation of freshly sanitized replacements. Following this 48-h period, egg-collection and incubation was started again to prepare for the next treatment cohort, either with the same treatment or the opposing treatment. This resulted in 21 to 28 days between treatment cohorts, ensuring that if estradiol or exemestane cross-contamination occurred, the level of cross-contamination was negligible.

2.2. Pharmacological preparations

Exemestane, trademarked by Pfizer as Aromasin (Pfizer, 2018), irreversibly binds to the testosterone binding site of aromatase and alters the confirmation of the protein for ubiquitin mediated degradation, pharmacologically removing the sole enzyme responsible for estradiol and estriol synthesis (Lonning and Eikesdal, 2013). Exemestane has a half-life of 24 h in mammals, and after a single oral dose of radiolabeled exemestane, all drug metabolites are fully eliminated within 1 week (Pfizer, 2018). Exemestane (Sigma PHR1634) was dissolved in DMSO (PanReac Applichem 191,954) at a concentration of 100 mg/mL, which was then suspended in olive oil (Sigma 75,343) for a final concentration of either 10 mg/mL or 20 mg/mL. This vehicle was used to prolong absorption as done previously with sesame oil in quail (Çiftci, 2012) and rat (Theodorsson et al., 2005). Vehicle was the same solution without exemestane.

Estradiol (E2) is the most potent form of estrogen. As with exemestane above, estradiol (Sigma E1024-1G) was also dissolved in DMSO at a concentration of 100 mg/mL, which was further suspended in olive oil to a final concentration of 1 mg/mL. Initially in a pilot experiment, we treated chicks with daily subcutaneous estradiol injections at 20 μg/g body weight, but this resulted in high mortality even after lowering the dose to 5 μg/g body weight. We therefore later transitioned to daily/alternating-daily topical treatments with one drop (~30–50 μL) of the 1 mg/mL solution applied near the flank as this was the easiest and least invasive route of treatment. Cutaneous absorption of sex hormones has been historically well documented (Moore et al., 1938). For subcutaneous implants, these were made by mixing medical grade silicone adhesive (Nusil MED-1037) with estradiol dissolved in DMSO (100 mg/mL) or adhesive mixed with DMSO alone as a vehicle control. The mixture was extruded from syringes into ropes that were cured overnight. The resulting pellets were cut, weighed, and kept in sterile conditions at 4 °C until use (Gurney, 1982; Sahores et al., 2013; Simpson and Vicario, 1991a). Each implant carried approximately 150–200 μg of estradiol, and vehicle implants were size matched. Both exemestane and estradiol are steroidal in structure, and peripherally circulating sex steroids have been historically well documented in being able to readily cross the blood brain barrier (Cornford et al., 1982; Pardridge and Mietus, 1979; Pardridge et al., 1980).

2.2.1. Juvenile treatment timeline

Exemestane or vehicle treatments were given daily via subcutaneous injection with a 28.5-gauge needle from PHD0 until time of sacrifice at PHD30 (Fig. 1C). From PHD0-PHD30, doses were given as close as possible to 10–20 μg/g body weight. Between PHD2-PHD15, doses ranged between 10 and 60 μg/g body weight (between 5 μL and 30 μL) depending on differences in the body weight of animals from the same clutch. Animals typically achieved parity in body size by the time they reached PHD30. We did not exceed 100 μg/g, as doses over 125 μg/g (mg/kg) in mammals have been shown to be toxic (Pfizer, 2018). Estradiol solution was given daily via topical treatment at doses no higher than 50 μg of estradiol (or equivalent vehicle volume) until ~PHD14, and then the solution was given every other day until time of sacrifice at PHD30.

2.2.2. Adult treatment timeline

For animals that were sacrificed at PHD90, exemestane treatments were given subcutaneously every day until ~PHD60, after which the treatments were given every other day until sacrifice at ~PHD90 (Fig. 1D). Estradiol was given topically daily until ~PHD14 before receiving alternating daily treatments until PHD20, after which drug impregnated silastic pellets were implanted. The silastic pellets were surgically placed subcutaneously on the flank under the wing and retained until sacrifice date at ~PHD90. PHD20 was the earliest the birds could reliably hide the surgical site from their parents. We found that this combined approach was less detrimental for animal health, as we found that daily or alternating daily topical application of estradiol past PHD30 resulted in animals with bones too fragile to properly fly or walk. We also found that silastic pellet implantation prior to PHD20 resulted in high mortality from parents pecking at the exposed surgical site or from parental rejection. Vehicle animal cohorts were split between receiving exemestane-style daily/alternating-daily subcutaneous injections and estradiol-style daily/alternating-daily topical application followed with implantation at PHD20.

To carry out the implant surgeries, prior to surgery, the animals were given meloxicam (Metacam NDC 0010-6013-01, 5 mg/mL) intramuscularly at 0.3 mg/kg body weight. They were then initially anesthetized with 3–4% isoflurane (Isothesia NDC 11695-6776-1) in 100% oxygen, and then sustained with 1.5–2% isoflurane for the duration of the procedure. The surgical site was plucked, and the exposed skin was scrubbed with 70% ethanol and 10% povidone-iodine prior to the creation of a shallow incision. A pocket was created under the skin using a blunt hemostat, and the implant was placed in the pocket. The incision was sealed with veterinary adhesive (3M Vetbond, 1469SB or Henry Schein Vetclose 031477), and bupivacaine (Hospira NDC 0409–1159-01, 0.25%) was applied topically afterwards. The animals were observed continuously for the first 2 h, and daily afterwards. The animals were also given additional intramuscular meloxicam 24 h and 48 h after surgery to manage pain. When handling estradiol, in addition to normal standard lab protective clothing, experimenters wore N-95 respirator masks to avoid inhaling any possible estradiol-contaminated airborne particles.

2.2.3. Collection of samples

On the day of sacrifice, all experimental animals were kept in the dark for approximately 1 h to return most brain gene expression activity to baseline levels (Jarvis and Nottebohm, 1997; Whitney et al., 2014). For animals collected at PHD30, they were separated from their parents for 1 h to rest in the dark. The conditions of adults are described below for vocalization analyses. For both adults and juveniles, the animals were rapidly anesthetized via isoflurane inhalation overdose followed by rapid decapitation when the animals were unresponsive, to reduce possible stress-induced brain gene expression. The brains were quickly dissected and embedded in OCT before snap freezing in a slurry of dry ice and ethanol. Trunk blood was collected from the neck at sacrifice and left at room temperature for approximately 20 min to clot before centrifuging at 20,000*g* for 3 min to separate the cells from serum. This serum was kept at −80 °C until use. Gonads were examined post-mortem to confirm sex.

2.3. Vocalization behavior analyses

For animals sacrificed at ~PHD90, vocalization behavior was recorded starting at PHD60, when the animals were weaned and past the sensory learning period of song development (Kojima and Doupe, 2007). They were recorded continuously in sound isolation chambers where they could neither see nor hear other animals. At ~PHD90, experimental animals (males and females) were provided with a novel female to collect directed songs (males and estradiol treated females) and other vocalizations for over 2 h, regardless of sex or treatment. After this period of directed vocalization, the novel females were returned to their

home-cage and the experimental animals were kept in the dark for approximately 1 h to fast and return brain activity to baseline. The animals were then sacrificed.

One estradiol treated PHD90+ female underwent audio/visual recording with a novel control female as a visual stimulus. Both animals were able to hear the other but were separated by an electrochromic glass plate that could be turned opaque or transparent remotely. This estradiol treated female underwent several sessions for 5 h, with 30-min intervals of seeing and 1-h of not seeing the stimulus female. This estradiol treated female was later returned to her sound isolation home cage overnight before conducting recording behavior the same as all other experimental animals.

Recordings were collected using microphones (Earthworks SR69 or SRO) and an Aardvark 24/96 Pro pre-amplifier connected to a computer operating Windows XP sp3. Avisoft recording software was used to gate and record sounds that were due to vocalizations, namely with an energy threshold of >1%, entropy threshold of <70%, and duration of >3 milliseconds. Recordings included 500 milliseconds before and after the triggering event. Avisoft Recorder v4.2.18 (Avisoft Bioacoustics) generated sound files were opened in Raven Lite 2.0 interactive sound analysis software (Cornell lab of Ornithology) and examined manually by blinded personnel to identify sound files with excessive cage noise or sound files with suitable vocalizations for automated thresholding in subsequent analysis. Syllable characterization and quantification was performed in Sound Analysis Pro 2011 (SAP2011) (Tchernichovski et al., 2000). Syllables were segmented and had their feature characteristics tabulated through automated batch analysis functions included in SAP2011. These characteristics were tabulated across development for all individuals. Syllables were then visualized through Nearest Neighbor Hierarchical clustering functions provided in SAP2011, and the discrete clusters separated in Euclidian space were identified manually by 2 blinded evaluators.

2.4. Protein modeling of aromatase binding site

The complete amino acid sequences of human aromatase (UniprotKB: P11511) and zebra finch aromatase (UniprotKB: Q92112) were taken from the UniProtKB database and aligned using the UniProt alignment tool (https://www.uniprot.org/align). The model of zebra finch aromatase was built via homology modeling using Modeller (v9.23) based on a mono template of the human experimental structure bound to exemestane and the heme co-factor (Protein Data Bank (PDB) ID: 3S7S). Visualization of aromatase bound to exemestane and the heme co-factor was performed on the Visual Molecular Dynamics (VMD v1.9.3) program.

2.5. Steroid panel assay with high performance liquid chromatography and tandem mass spectroscopy

To test the efficacy of exemestane in zebra finches, adult animals were subcutaneously injected with exemestane or vehicle (60–40 μg/g body weight: 600–800 μg) for three days, and then sacrificed 24 h after final treatment. Animals were euthanized in the same fashion as outlined above. Serum and whole brain samples were submitted to the metabolomics core facility at Duke university. Tissue homogenization and sterol extraction was performed and these samples were assayed for a complete steroid panel analysis (Cortisol, Cortisone, 11-Deoxycortisol, 17α-Hydroxyprogesterone, Progesterone, Aldosterone, Corticosterone, 11-Deoxycorticosterone, Estradiol, Estrone, Androstenedione, Androsterone, Dehydroepiandrosterone, Dehydroepiandrosterone sulfate, Dihydrotestosterone, Etiocholanolone, and Testosterone), using the AbsoluteIDQ Stero17 kit (BioCrates) on the Xevo TQ-S MS UPLC/MS/MS instrument (Waters Corporation). The full extraction and assay protocol is included in Supplemental Note 1.

2.6. ELISA assays to measure blood estrogen levels

Trunk blood was collected as described above in Section 2.2.3. The collected serum was gently thawed on ice before being serially diluted in ultrapure water (Invitrogen 10977015) and used directly in the estradiol enzyme immunosorbent assay (EIA, Cayman Chemical 582251; newer product 501890) following the manufacturer's protocol (Vedder et al., 2014). A total of 4 dilutions were run in triplicate for each exemestane treated animal (1:5, 1:10, 1:20, 1:40) and for each vehicle or estrogen vehicle treated animal (1:10, 1:20, 1:40, 1:80). Results were obtained using a SpectraMax M3 micro-plate reader (Molecular Devices) with Softmax Pro software v6.2.1 on a computer operating Windows 7 professional.

2.7. Cresyl violet staining histology

The left hemisphere of brains frozen in OCT were sagittally sectioned over 9 serial slides. These sections were cut at 14–16 μm thickness on a microtome cryostat (Leica CM1850), thaw mounted onto charged borosilicate slides (Fisherbrand Superfrost #12-550-15) and stored at −80 °C until use. One series was dehydrated and rehydrated in graded ethanols (0%, 50%, 70%, 95% 100%), stained in 0.3% cresyl violet acetate (Sigma C5042), defatted in mixed xylenes (Fisher X5), coverslipped with permount (Fisher SP15) mounting media and cured for one week in a chemical cabinet prior to imaging on a stereomicroscope (Zeiss Stemi 305) equipped with a colour camera (Zeiss Axiocam 105). Images were obtained on a computer operating Windows 7 using Zeiss Zen Blue 2.0 software.

2.8. Chromogenic in-situ hybridization

Previously cloned *CADPS2* plasmids (Accession: DV955943) were grown from bacterial stock and collected via miniprep columns (Qiagen 27104). *CADPS2* was verified for sequence identity and orientation using Sanger sequencing services provided by Eton Biosciences or GeneWiz. We performed a modified version of the in-situ hybridization protocol as first described by Takatoh et al. (2013). Template DNA was PCR amplified from plasmids using M13 forward and reverse primers and Phusion high-fidelty DNA polymerase (Thermofisher F530S). The target product was gel purified using the NucleoSpin mini-spin columns (Machery-Nagel 740609.50). DIG-labelled (Roche 11277073910) RNA probes were transcribed via the T3 promotor from 1 μg of purified DNA template and cleaned via ethanol-salt purification with GenElute linear polyacrylamide (Sigma 56575-1ML) as a neutral carrier. The probe pellet was rehydrated in 100 μL of 90% formamide, and frozen in 5 μL aliquots at −80 °C. Probes were only freeze-thawed once to prevent RNA degradation.

Slides with fresh-frozen sections were fixed in freshly prepared 4% PFA/1× PBS for 5 min at room temperature. The slides were then washed in 1× PBS and acetylated in 0.1 M triethylamine + acetic acid for 10 min before they were dehydrated in a series of graded ethanols. The opaque tissue sections were outlined with hydrophobic marker (Thermofisher 008899) and then prehybridized (50% formamide [ThermoScientific 15515026], 5× SSC [ThermoScientific AM9763], 1× denhardt's solution [Sigma D2532], 250 μg/mL Brewer's yeast tRNA [Roche 10109495001], 500 μg/mL herring sperm DNA [ThermoScientific 15634017]) with parafilm coverslips at room temperature for 1 h in a humidified chamber. The hybridization-probe solution was made with probe diluted 1:100 (from frozen aliquots outlined above) in hybridization buffer (300 mM NaCl, 20 mM Tris-Hcl pH 8.0, 5 mM EDTA, 10 M Na_2HPO_4 pH 7.2, 10% dextran sulfate, 1× denhardt's solution [Sigma D2532], 500 μg/mL Brewer's yeast tRNA [Roche 10109495001], 200 μg/mL Herring sperm DNA [ThermoScientific 15634017], 50% formamide [ThermoScientific 15515026]) hydrolyzed at 80 °C for 6 min before being chilled on ice before application. After prehybridization was complete, the parafilm coverslips were removed,

and the excess prehybridization buffer was removed with a kimwipe. The prepared hybridization-probe solution was applied and then the slides were coverslipped with glass coverslips (VWR 48393106). The slides were then incubated at 65 °C in a hybridization oven overnight (>16 h) in a humidified chamber. Humidification was done using either RNase free water or a solution of 5× SSC, 50% formamide.

After overnight hybridization, the coverslips were gently removed in room temperature 5× SSC. The hydrophobic pen residues were wiped off using kimwipes. The slides were then washed in 5× SSC at 68 °C for 10 min, and then washed 4 times for 30 min per wash in 0.2× SSC at 68 °C. After the final wash, the container holding the 0.2× SSC and slides were allowed to cool to room temperature. The slides were washed once more in fresh 0.2× SSC at room temperature.

After the high stringency washes, the slides were washed in Buffer B1 (0.1 M Tris pH = 7.5, 0.15 M NaCl) for 5 min at room temperature before being transferred to slide mailers. The slides were then incubated in blocking Buffer B2 (10% Sheep serum [Sigma S3772] in Buffer B1) at room temperature for a minimum of one hour. After blocking, the slides were incubated in an AP-conjugated anti-DIG antibody solution ([Roche 11093274910; Lot. 16646822 & 16646820] diluted 1:2000 in 1% sheep serum in Buffer B1) overnight at 4 °C.

Following antibody incubation, the slides were washed 3 times for 10 min per wash in Buffer B1 at room temperature and then equilibrated in 100 mM Tris-HCl pH = 9.5 for 5 min. The slides were then incubated in a working solution of NBT/BCIP (Vector labs sk-5400), prepared according to the manufacturer's protocol, for 16 h in the dark at room temperature.

When the NBT/BCIP signal was optimal, the reaction was stopped in 1× PBS, and the slides were washed 3 times for 5 min per wash in 1× PBS at room temperature. The slides were then rinsed in diH_2O and counterstained in a 1:3 diluted solution of Nuclear fast red (Vector labs H-3403). Counterstaining was done for no longer than 3 min. The slides were then rinsed with diH_2O and dipped in 100% histology grade ethanol (no more than 10 dips) before being left to airdry. Once the dried sections were opaque, the slides were mounted with Vectamount permanent mounting solution (Vector labs H-5000) and dried overnight at room temperature in a dark, dry location before imaging.

2.9. Imaging and area calculations

Cresyl violet and in-situ hybridized sections were imaged on a stereomicroscope (Zeiss Stemi 305) equipped with a colour camera (Zeiss Axiocam 105). Images were obtained on a computer operating Windows 7 using the Zeiss Zen Blue 2.0 software. Images were saved as .czi files (Zeiss) and area size values were obtained using the "region of interest" tools available in Zen Blue 3.0 (Zeiss). The area of song nuclei and surrounding brain subdivisions were obtained from sections stained with either Cresyl violet (LMAN, RA, striatum, and arcopallium) or *CADPS2* as a marker gene (HVC, mesopallium and Area X). Sections were selected based on anatomical landmarks to compare across individuals. Song nuclei and brain subdivision areas were divided by the area of the whole telencephalon within each respective section. Brain subdivisions were determined according to the online zebra finch histological atlas (ZEBrA, n.d) from the Mello lab (http://www.zebrafinchatlas.org) and molecular markers established by Feenders et al. (2008) and Jarvis et al. (2013).

2.10. RNA-Seq data generation

Following a modified protocol from Whitney et al. (2014), the right hemisphere of brains frozen in OCT were sectioned coronally for regions of interest at 14 μm and thaw mounted onto polyethylene naphthalate (PEN) membrane slides (Applied Biosystems LCM0522). As soon as the sections dried, the slides were promptly stored at −80 °C until further use. A series of adjacent sections on regular glass slides were stained with cresyl violet and used as references to identify sections with song nuclei (Supplemental Fig. 1). Processing one PEN slide at a time, sections containing the target song nucleus and its immediate surrounding area were taken out of -80 °C and submerged at -20 °C in 75% ethanol. The slide then was dehydrated in ice cold graded ethanols: 75%, 95%, 95% (second rinse), 100%, 100% ethanol (second rinse), for 10 dips each, before incubating in fresh xylenes twice for 5 min each incubation. After the second xylene incubation, the slide was air dried and the desired regions were collected one brain region at a time across multiple sections, using laser capture microscopy (LCM) on an ArcturusXT LCM system (Nikon) with CapSure Macro LCM caps (Applied Biosystems LCM0211). These regions were Area X and the medial striatum (MSt) ventromedial to Area X; HVC and the HVC shelf ventral to HVC; LMAN and the anterior nidopallium lateral to LMAN (LANido); RA and the lateral intermediate arcopallium (Lai) lateral to RA. Care was done to protect samples from Rnase degradation by using only Rnase-free materials/reagents and cleaning all reusable equipment with Rnase Zap (Invitrogen AM9780). All collection was done within 35 min upon exposure to open air.

RNA was isolated from the LCM collected tissues using the Arcturus Picopure kit (Applied Biosystems KIT0204) following manufacturer's instructions. RNA quality was determined using an Agilent 2100 bioanalyzer with the high sensitivity RNA 6000 pico kit (Agilent 5067-1513). Only samples with RIN numbers higher than 5 were used for further processing.

cDNA was synthesized using the SMART-Seq v4 Ultra Low input RNA Kit (Takara 634892) following the manufacturer's protocol. The cDNA product was validated using an Advanced Analytical fragment analyzer (Agilent) with the HS NGS 1-6000 fragment kit (Agilent DNF-474). Sequencing libraries were created using the NEBNext Ultra II DNA Library Prep kit for Illumina sequencing (New England Biolabs E7645L). All cDNA and library clean-up was done using SPRIselect beads (Beckman Coulter B23317).

Sequencing services were conducted by Novogene Co., Ltd. On the Novaseq 6000 platform (Illumina) via the s4 flow cell for 150 bp paired-end reads. The resulting reads were aligned to the TaeGut 3.2.4 zebra finch genome assembly (GCA_000151805.2) using two independent approaches in custom designed pipelines: Kallisto (Bray et al., 2016) with reads aligned to cDNA transcripts from Ensembl; and the splice aware STAR (Dobin et al., 2013) to the annotated zebra finch genome in NCBI (Warren et al., 2010). In both analyses, the output of read counts for all genes were called. The read counts were comparable with both methods. For example, of the genes in vehicle males that were differentially expressed (DEG) between the song nucleus and surrounding brain subdivisions (FDR $\leq$ 0.05), 89–96% (depending on brain region) overlapped between the Kallisto and STAR aligning methods (Table 1). We only included in our analyses DEGs found using both methods.

Table 1
Number of mapped DEGs using STAR or Kallisto before IEG removal.

	Area X	HVC	RA	LMAN
STAR | Kallisto	386 | 407	934 | 926	232 | 239	1162 | 1159
Shared	363	860	217	1076
Differences(%) STAR	23(94%)	74(92%)	15(96%)	86(93%)
Differences(%) Kallisto	44(89%)	66(93%)	22(91%)	83(93%)

2.11. Statistics

Estradiol measurements did not fit a normal distribution and were thus analyzed using the non-parametric Kruskal-Wallace tests with post-hoc Steel-Dwass tests for each pair comparisons or Wilcoxon rank sum tests. Effects sizes for differences in measured estradiol levels were obtained using Cliff's delta. Song-syllable cluster data was normally distributed, and thus analyzed using standard least squares models for a 2-way ANOVA with post-hoc Tukey's Honest Significant Difference (HSD) tests for each pair comparisons. Effects sizes for differences in

Table 2
Sample numbers of animals per experiment.

	ELISA (PHD15–60)			ELISA (PHD90)		
	Veh	Exem	E2	Veh	Exem	
Male	8	15	8	7	4	
Female	10	9	8			
	RNA-Seq (PHD30)			LC-MS/MS (Adult)		
	Veh	Exem	E2	Veh	Exem	
Male	3	3	3	5	5	
Female	3	3	3	5	5	
	Histology (PHD30)			Behavior (PHD90)		
	Veh	Exem	E2	Veh	Exem	E2
Male	3	7	4	7	4	4
Female	4	3	4	4	4	6

syllable production in response to hormone modulation were obtained using via omega-squared for whole model effects, and Hedges' g for pairwise comparisons. Song nuclei:surround or song nuclei:telencephalon area ratio measurements were analyzed using Aligned-ranks transformed ANOVA (Wobbrock et al., 2011) with post-hoc Wilcoxon tests for each pair.

For the RNA-seq analyses, to compare expression across all genes in all samples, including those without formal gene names, we normalized the data via variance stabilization transformation and then performed principle component analysis (PCA). To identify significant DEGs between the song nuclei and their surrounding brain subdivisions, we took the Kallisto and STAR generated read counts and applied them to the DESeq2 package (Love et al., 2014) in R and performed two types of DESeq2 analysis using the Wald test: unpaired group comparisons between brain regions for each of the six groups of birds (Area); and paired comparisons between brain regions for each bird (Subject + Area). Genes with FDR ≤ 0.05 were considered significantly DEG. DEG log2-fold change values greater than 2 were binned at $|2|$ for generating heatmaps. We also removed DEG genes that were identified as singing-regulated by Whitney et al. (2014), for each song nucleus within 1 h of singing (40–67 genes), to remove potential differences associated with vocalizing behavior in the hours before sacrifice. Vignette for DESeq2 and downstream analysis are available on the Jarvis lab Github as "Choe_2020_Vignette" (https://github.com/neurogenetics-jarvis). All statistical functions not included in the default DESeq2 software as-is, were carried out using one of two software/programming packages: JMP 15.1 or RStudio with various packages (Supplemental Note 2). Sample sizes for all groups are in Table 2.

3. Results

Our experiments were designed to test two competing hypotheses: 1) That estrogen is required for development of the song learning system in both sexes of zebra finches; or 2) Post evolution of song learning in both sexes, female zebra finches lost the trait and reversal of this sex-dependent loss has become dependent on estrogen signaling. We first determined whether exemestane can inhibit estrogen production in zebra finches and then assessed the potential effects of this inhibition on development of vocal learning behavior, vocal learning brain regions, and associated genes.

3.1. Exemestane is a potent blocker of estrogen synthesis in zebra finch brain and blood

In mammals, exemestane is a well-established inhibitor of aromatase, the sole enzyme responsible for synthesizing estradiol and estrone from androgens, but this drug's efficacy has not been demonstrated in birds. When we compared the human and zebra finch aromatase amino acid sequences, they were 75% identical (Fig. 2A). Structural homology modeling revealed that the binding site of testosterone/exemestane in aromatase was highly conserved between human and zebra finch (Fig. 2B), with 11 of the 12 amino acids in the site being identical (Fig. 2A). The single non-conserved amino acid, at position 373 in the alignment, was a valine in humans and an isoleucine in zebra finches, two of the three branch chain amino acids. The high amount of conservation and nominal discrepancy in the binding site structure of human and finch aromatase strongly suggested that exemestane should bind with and inhibit aromatase similarly in the two species.

To test the effectiveness of exemestane in modulating zebra finch estrogen levels in vivo, we treated healthy adult male and female breeding animals (n = 5 each) for three days with either exemestane or vehicle and examined steroid levels from blood serum and brain tissue using an UHPLC-MS/MS assay, ~24 h after the last dose was given. We found that in all birds treated with exemestane (n = 5 males, 5 females), there was no detectable estradiol levels in the brain (Fig. 3A) and barely detectable levels in serum (Fig. 3B). However, in vehicle treated animals, half of the birds (n = 2 of 5 males, 3 of 5 females) showed moderate to higher estradiol levels. The overall levels in the brain were less than in the serum. Statistically, the differences in the female treated animals were significant or approached significance, whereas in the males they did not, in part because there were fewer males with higher levels in the control group (full statistics and effect sizes in Supplemental Table 1). There were no differences between control adult males versus control females. Together this suggests a bimodal distribution in the controls, that is removed in the exemestane animals with no detectable estradiol. Analyses of seven other steroids showed a statistically significant decrease of aldosterone in female brains and decrease of progesterone in male serum, alongside an increase in cortisone in male serum, but no other significant changes (Supplemental Fig. 2; Supplemental Table 1). These findings indicate that exemestane is a potent inhibitor of estrogen synthesis in zebra finches, with limited off target effects.

In juvenile (~PHD15–60) animals that underwent long-term treatments with exemestane, estradiol, or vehicle, we measured serum estradiol levels by EIA assays. We found that vehicle treated juvenile females (n = 10) had on average 10 times more serum estradiol than vehicle treated juvenile males (n = 8), with overlapping ranges ($p = 0.0144$). Unlike adults, all vehicle juveniles tested had detectable estradiol. There was a bimodal distribution in the exemestane treated juveniles, where 2/3rds of treated males (n = 10/15) had no detectable serum estradiol. A single exemestane treated juvenile female (n = 1/9) had no detectable serum estradiol while the rest (n = 8/9) had levels lower than but overlapping with vehicle controls. When we consider all exemestane treated animals at both ages for both sexes, nearly half of them (46%) had very low to no detectable serum estradiol levels when compared to vehicle treated animals. Statistically, exemestane treatment had large ($d = 0.67$) and significant ($p = 0.02$) decrease in serum estradiol levels in juvenile males; large ($d = 0.58$) and nearly significant ($p = 0.09$) decrease in serum estradiol levels in juvenile females. Estradiol treatment showed the opposite result, a complete uncoupling ($d = -1$) and very significant increase in serum estradiol levels in males ($p = 0.0002$) and females ($p = 0.0013$; Fig. 3C). We believe the greater variability we found in exemestane treated juveniles could be due to differences in their ability to metabolize or otherwise clear the drug, suggesting that exemestane could have a shorter half-life than 24 h in adolescent animals as pediatric pharmacokinetics have not yet been determined (Pfizer, 2018). Nevertheless, despite the varying estradiol levels, findings below support our conclusion that chronic exemestane treatment had systemic effects that induced physiological changes in the animals.

Although purely qualitative, when we examined the gonads of PHD60+ animals on the day of sacrifice, we saw that exemestane treated males had testes somewhat smaller than vehicle treated males and estradiol treated males had testes larger than vehicle treated males. Females treated with exemestane or estradiol had ovaries that were

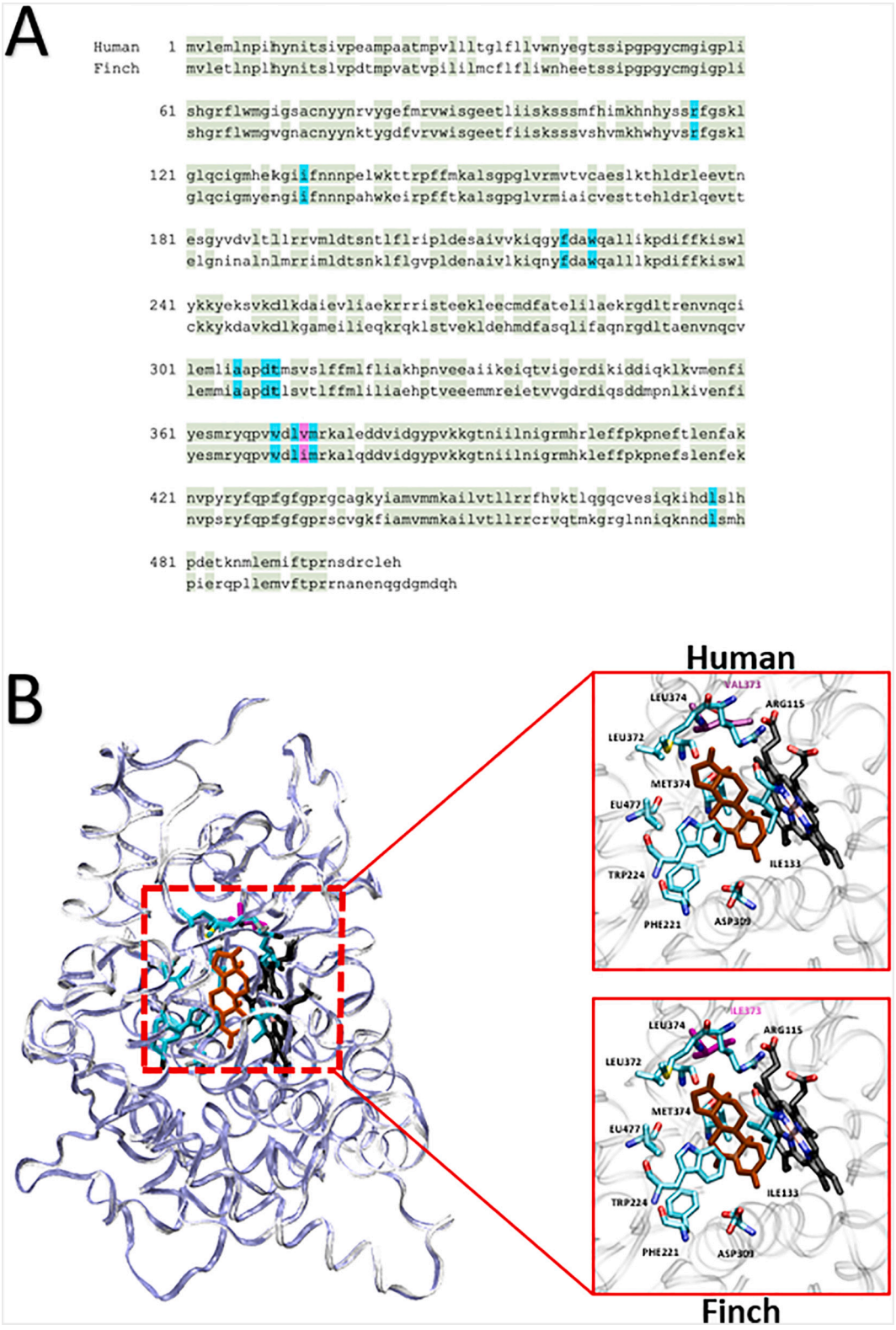

Fig. 2. Exemestane binding site in human and zebra finch aromatase. (A) Primary protein sequence alignment between human and zebra finch aromatase protein (CYP19A1). Light green, conserved amino acids outside the ligand binding site. Cyan, conserved amino acids in the ligand binding site. Magenta, non-conserved amino acid in the binding site. (B) Superimposed tertiary structure of human (PDB ID: 3S7S – white ribbon) and finch (built by homology modeling – iceblue ribbon). Binding sites are magnified in right insets. Cyan, conserved residues between human and finch. Magenta, non-conserved amino acid. Black, the cofactor heme (HEM) group. Orange, exemestane molecule.

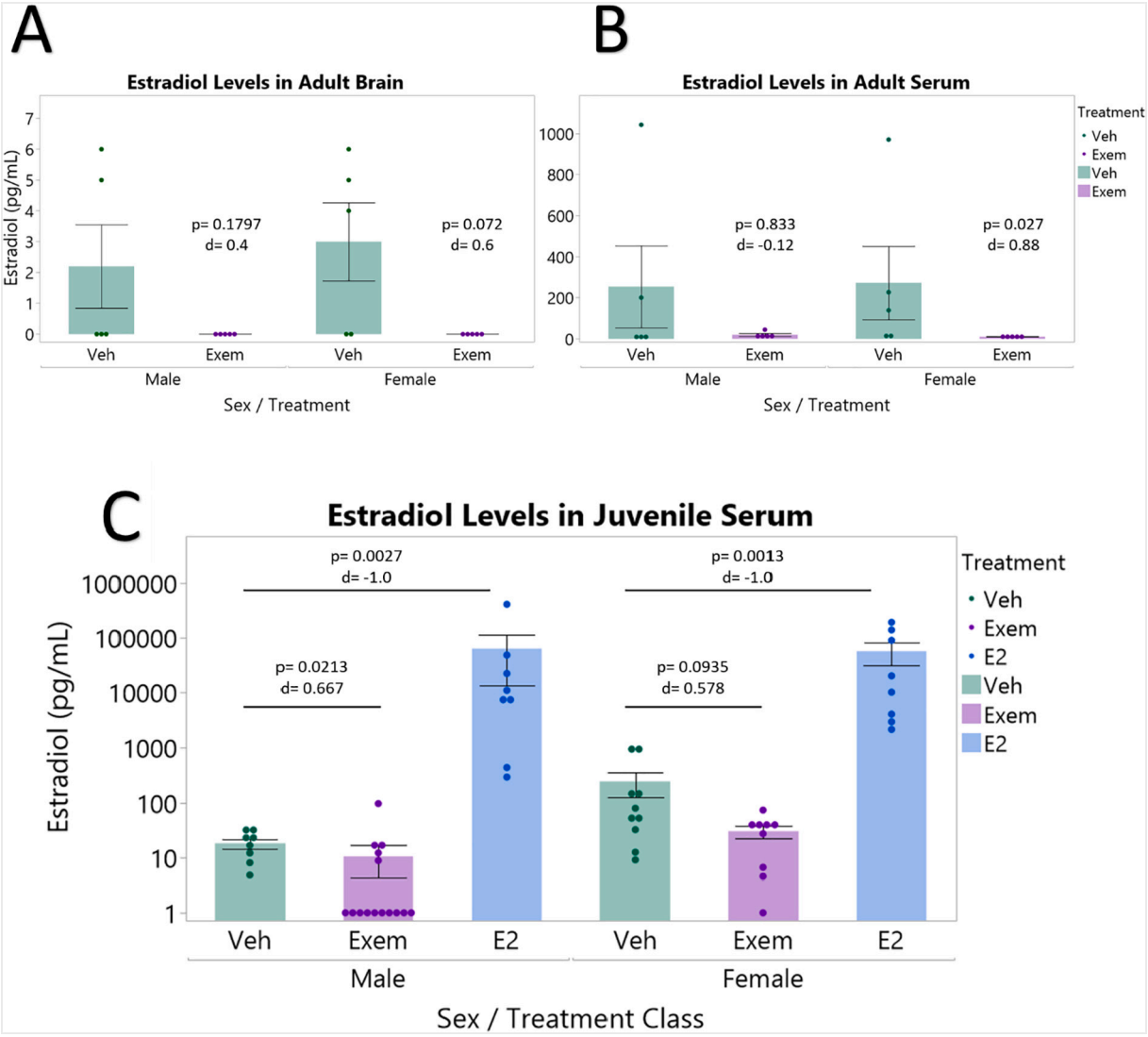

Fig. 3. Brain and blood estradiol levels in treatment groups. (A) Adult brain uHPLC-MS/MS. None of the exemestane treated animals have detectable E2 levels in the brain. (B) Adults serum uHPLC-MS/MS. All of the exemestane treated animals have barely detectable E2 levels in the serum. Differences were tested with Wilcoxon test (n = 5 each group) (C) Juvenile serum EIA. E2 treated animals (n = 8 males; n = 8 females) had significantly higher serum E2 levels when compared to vehicle treated animals (n = 8 males; n = 10 females). Exemestane treated animals (n = 15 males; n = 9 females) had significantly lower serum E2 levels when compared to vehicle treated animals, for all animals (p = 0.0067), in males alone (p = 0.0213), but not in females alone (p = 0.0935). Kruskal-Wallis test with Steel-Dwass post-hoc, effect sizes are reported using Cliff's delta "d". Full statistics in Supplemental Table 1.

similar to that of vehicle treated females. These observations suggest that estradiol manipulation impacted the testes in developing birds.

3.2. Estrogen is necessary for normal male plumage development

We found that all males treated chronically with exemestane showed impaired development of male-specific plumage after their first molt around PHD60, displaying patterns that were intermediate between normal (or vehicle treated) males and females (Fig. 4A–C). This included weak to no orange cheek patches, and minimal zebra striped throat feathers. By PHD90, all long-term exemestane treated males had distinctly less vibrant plumage than either vehicle or estradiol treated counterparts (Fig. 4D–F). To our knowledge, this is the first observation linking estrogen to zebra finch male plumage and is consistent with the hypothesis that estrogen levels can interact with some feather proteins in birds. Prior literature in waterfowl and songbirds have shown that elevation of androgens, or depletion of estrogens typically enhance male plumage vibrancy (Ralph, 1969; Somes and Smyth, 1967). Thus, we were surprised to find that depletion of estrogens suppressed male plumage in our zebra finches. However, this finding increased our confidence that our chronic exemestane dosing protocol exerted a strong endocrine disrupting effect within the zebra finch.

3.3. Estrogen is required for normal song development in males

Under our experimental conditions (Fig. 1D), males treated with vehicle over their 90-day development period produced normal songs with stereotyped syllables arranged into repeating, well-structured motifs (Fig. 5A). However, males treated with exemestane produced fewer unique syllable types, and thus simpler songs (Fig. 5B, Supplemental Audios 1–9). The songs of males treated with estradiol were similar to vehicle treated males (Fig. 5C). Vehicle treated females did not produce song, but only innate calls as expected (Fig. 5D). Interestingly we found that some females treated with exemestane showed repeated production of 1 or 2 apparently innate syllables, similar to the type of song seen in males treated with exemestane (Fig. 5C,E). All females treated with estradiol produced songs that resembled males (Fig. 5F), which they sometimes directed to other females (Supplemental Movie

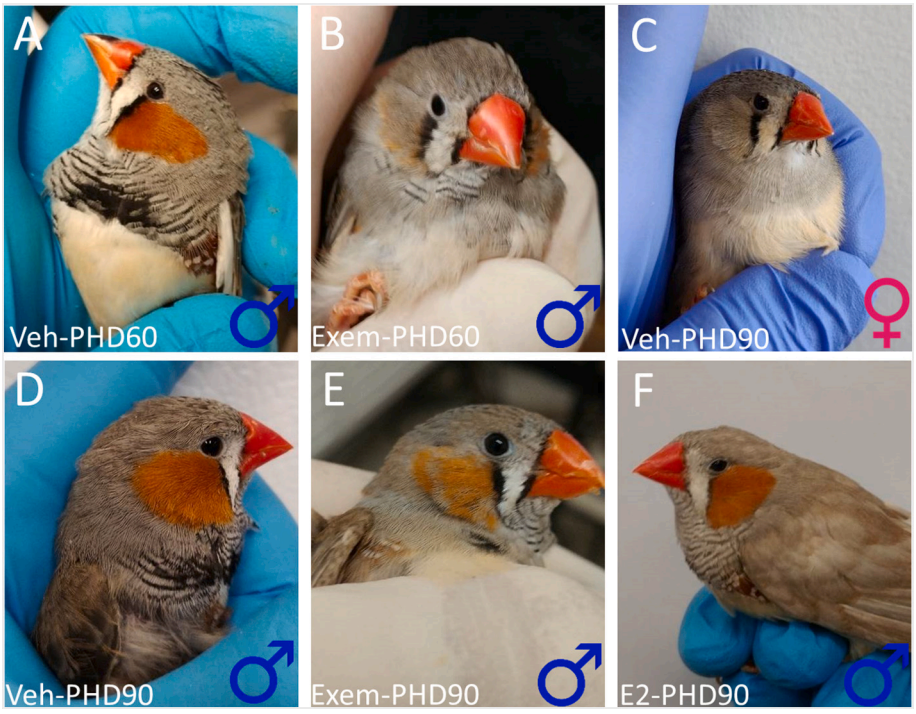

Fig. 4. Estrogen manipulation effects on male plumage. (A) PHD60 vehicle treated male following his first molt with normal plumage, and left-over black juvenile coloration on the beak. (B) PHD60 exemestane treated male showing weak male plumage. (C) PHD90 vehicle treated female. (D) PHD90 vehicle treated male. (E) PHD90 exemestane treated male, still showing weaker male plumage. (F) Estradiol treated male, showing typical male plumage.

1).

To quantify these observations, we performed a nearest neighbor hierarchical clustering of syllables (Sound Analysis Pro 2011) using different acoustic features. Based on these clusters, we found that males chronically treated with exemestane (n = 4) had 40% fewer unique syllable types compared to males treated with vehicle (n = 7) (Fig. 5G,H; g = −1.544, p = 0.049). Males treated with estradiol (n = 4) had no significant difference with vehicle treated males. Estradiol treated females (n = 6) produced songs with comparable unique syllable types as vehicle or estradiol treated males (Fig. 5G,H; Sonograms and cluster plots for all birds are available in Supplemental Fig. 3 and the full statistics are provided in Supplemental Table 2). The number of syllables produced by exemestane treated females (n = 4) was not significantly different from that produced by vehicle treated females (n = 4). These findings suggest that ready availability of estrogen is not required for males to acquire the ability to sing, but it is necessary for males to sing normally. Our findings also validate previous conclusions, that increasing estrogen levels is sufficient for females to develop the ability to sing.

3.4. Estrogen induces early changes in specialized gene expression and size of song nuclei

Differences in behavior presumably reflect differences in the brain. To examine the brain, we focused on PHD30 animals (Fig. 1C), as this is the beginning of the sensorimotor learning stage when song nuclei have established their connections and are beginning to be utilized to produce sub-song (Mooney and Rao, 1994). Also at this stage, clear differences in song nuclei between males and females start to become apparent (Bottjer et al., 1985). We used cresyl violet staining to identify song nuclei. Area X was difficult to identify by cresyl violet at this age, and thus we used in-situ hybridization for mRNA of the Calcium Dependent Secretion Activator 2 gene (*CADPS2*), which identifies Area X and HVC in adults (Jarvis et al., 2013).

We found that in PHD30 vehicle treated controls, there was clear *CADPS2* signal in male Area X and HVC, but only in female HVC (Fig. 6A), supporting the proposal that Area X may not be present in females at this age (Garcia-Calero and Scharff, 2013). Treatment with exemestane did not change this pattern. In contrast, treatment of females with estradiol resulted in the appearance of an Area X with specialized *CADPS2* gene expression, similar to males (Fig. 6A). Adjacent cresyl violet stained sections support these results (Fig. 6B), where in all males, regardless of treatment, we could identify pallial song nuclei (HVC, RA, and LMAN) and Area X, but among females we could identify an Area X-like region only in estradiol-treated animals.

For HVC, RA and Area X, we noted clear visible size differences between PHD30 males and females. We quantified the areas of these song nuclei relative to individual brain subdivisions or to the whole telencephalon in the same sections, using *CADPS2* signal and cresyl violet staining to identify nuclear/regional boundaries. In vehicle controls, besides no detectable Area X in females (Fig. 6C), HVC and RA was larger in males (Fig. 6D,E), while LMAN was the same size in both sexes (Fig. 6F; Supplemental Fig. 1). Exemestane had no effect on male Area X, male or female HVC sizes, but did result in a trend for decreased male RA size that became near significant after an outlier was removed in estradiol treated males (Fig. 6E). In females, estradiol treatment caused a notably enlarged HVC (Fig. 6D) and the appearance of Area X (Fig. 6C) with sizes that approached those of males. There were no effects of sex or treatment on the sizes of the striatum, mesopallium or arcopallium relative to the telencephalon (Supplemental Fig. 4; full statistics are provided in Supplemental Table 3), indicating that the differences seen were specific to the song nuclei and not their respective brain subdivisions.

3.5. Specialized transcriptomes of song nuclei are differentially influenced by sex and hormones

The above findings revealed that one gene, *CADPS2*, which is specialized in juvenile male Area X is also impacted by estrogen manipulation in juvenile females. However, many genes in adults have

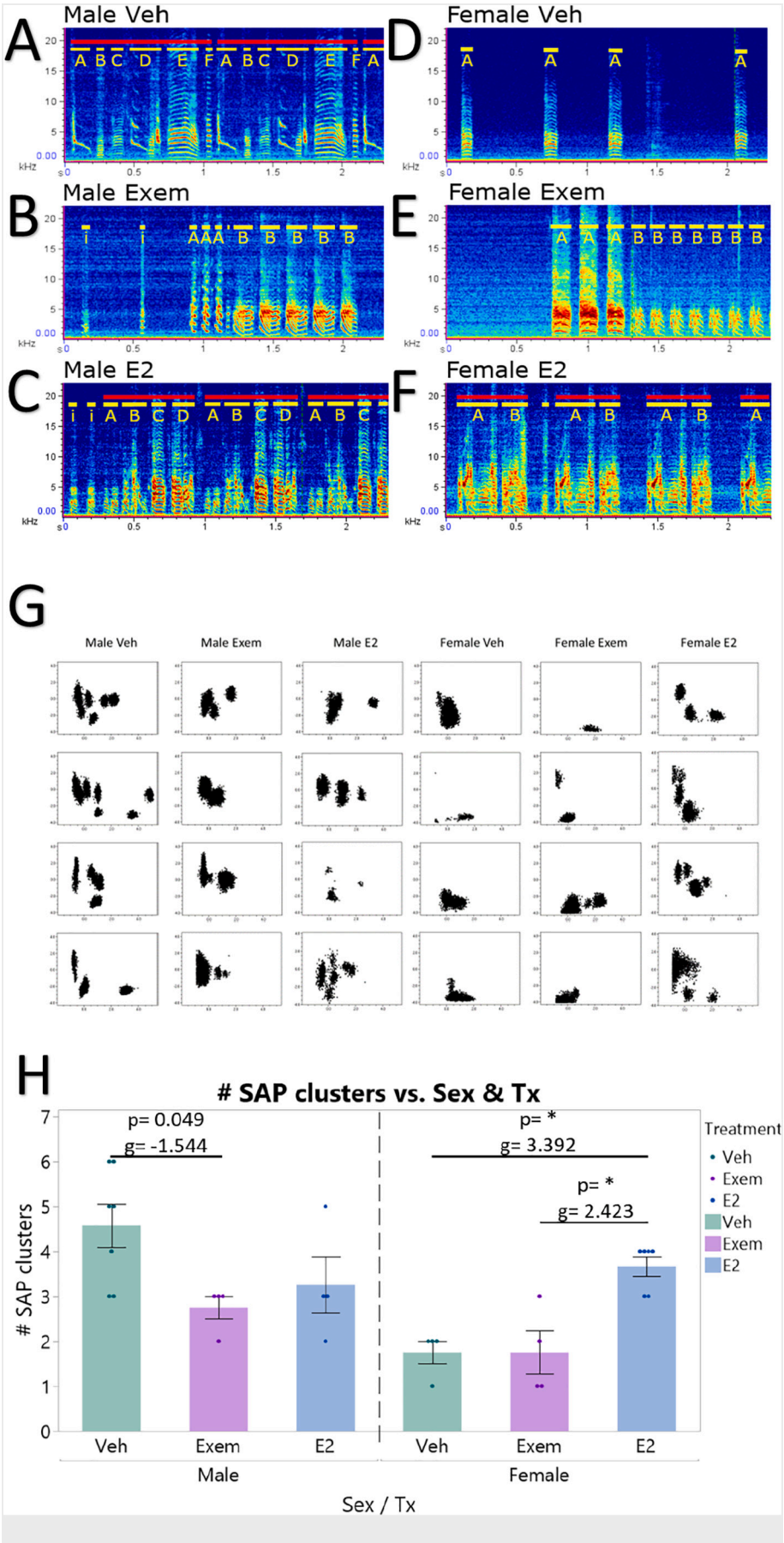

Fig. 5. Estrogen manipulation effects on song behavior. (A) Sonogram of vehicle treated male with structured repeating motifs. Distinct syllable types are highlighted and named in yellow, and motifs are highlighted in red. (B) Exemestane treated male with simpler song. (C) Estrogen treated males with apparent normal song. (D) Vehicle treated female producing innate calls. (E) Exemestane treated females producing a series of simple syllables. (F) Estrogen treated female with song similar to that seen in males. (G) Example clusters showing song syllable repertoires of four birds for each group. Syllables clustered based on k-nearest neighbor hierarchical clustering within SAP2011, plotted by duration x frequency modulation, and converted to a binary image. (H) Cluster number was compared between sex and treatment. 2-way ANOVA and Tukey's HSD post hoc. Sex: df = 1, SS = 7.041667, F = 8.5932, p = 0.0089 Treatment: df = 2, SS = 6.58333, F = 4.0169, p = 0.0361 Sex*Treatment: df = 2, SS = 10.583333, F = 6.4576, p = 0.0077. Effect sizes are reported for exemestane treatment by sex as omega-squared, and pairwise using Hedges' g. Males: SS = 9.719, p = 0.052, $\omega^2 = 0.274$. Females: SS = 12.595, p = 0.001, $\omega^2 = 0.656$. Full statistics are in Supplemental Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

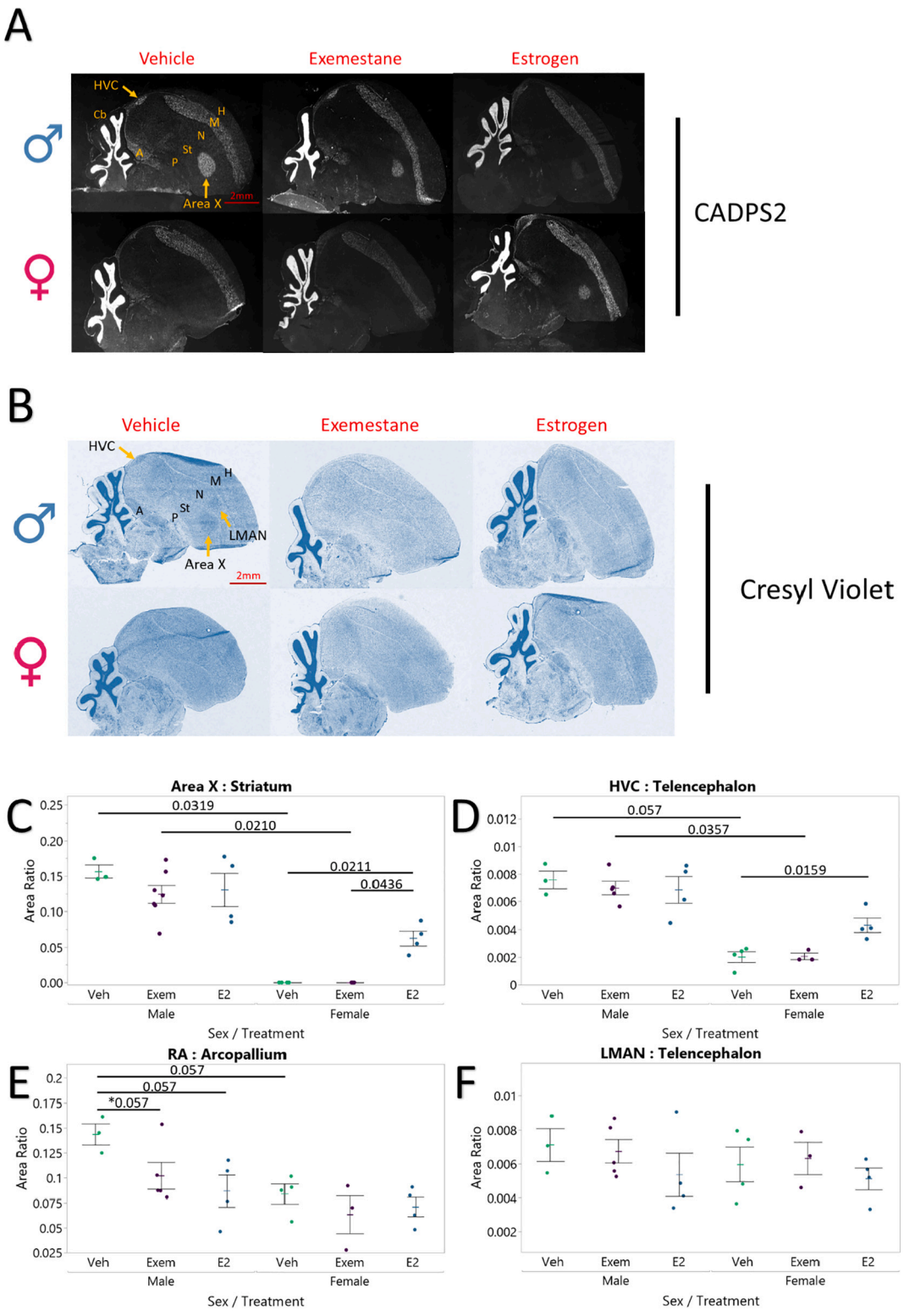

Fig. 6. Effects of estrogen manipulation on *CADPS2* expression and sizes of song nuclei. (A) *CADPS2* mRNA expression (white) in the brains of example animals from each treatment group. Anterior is right, dorsal is up. (B) Adjacent cresyl violet stained sections to A. (C–F) Relative song nuclei sizes for HVC, Area X, RA, and LMAN respectively. Aligned ranks transformation ANOVA with post-hoc Wilcoxon rank sum. Statistics in Supplemental Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specialized expression in different song nuclei (Lovell et al., 2008; Lovell et al., 2018; Olson et al., 2015; Pfenning et al., 2014). Prior work from our lab has shown that *SLIT1,* an axon guidance ligand, has specialized down regulation in RA beginning at PHD20–35 in males and females, but its receptor *ROBO1* has specialized upregulation in RA around PHD35, followed by down regulation in males during adulthood with no changes in females (Wang et al., 2015). We wondered if exemestane or estradiol treatments impacted the expression of many genes, and whether there were differences between males and females, and among song nuclei. We therefore laser micro-dissected HVC, RA, LMAN, Area X

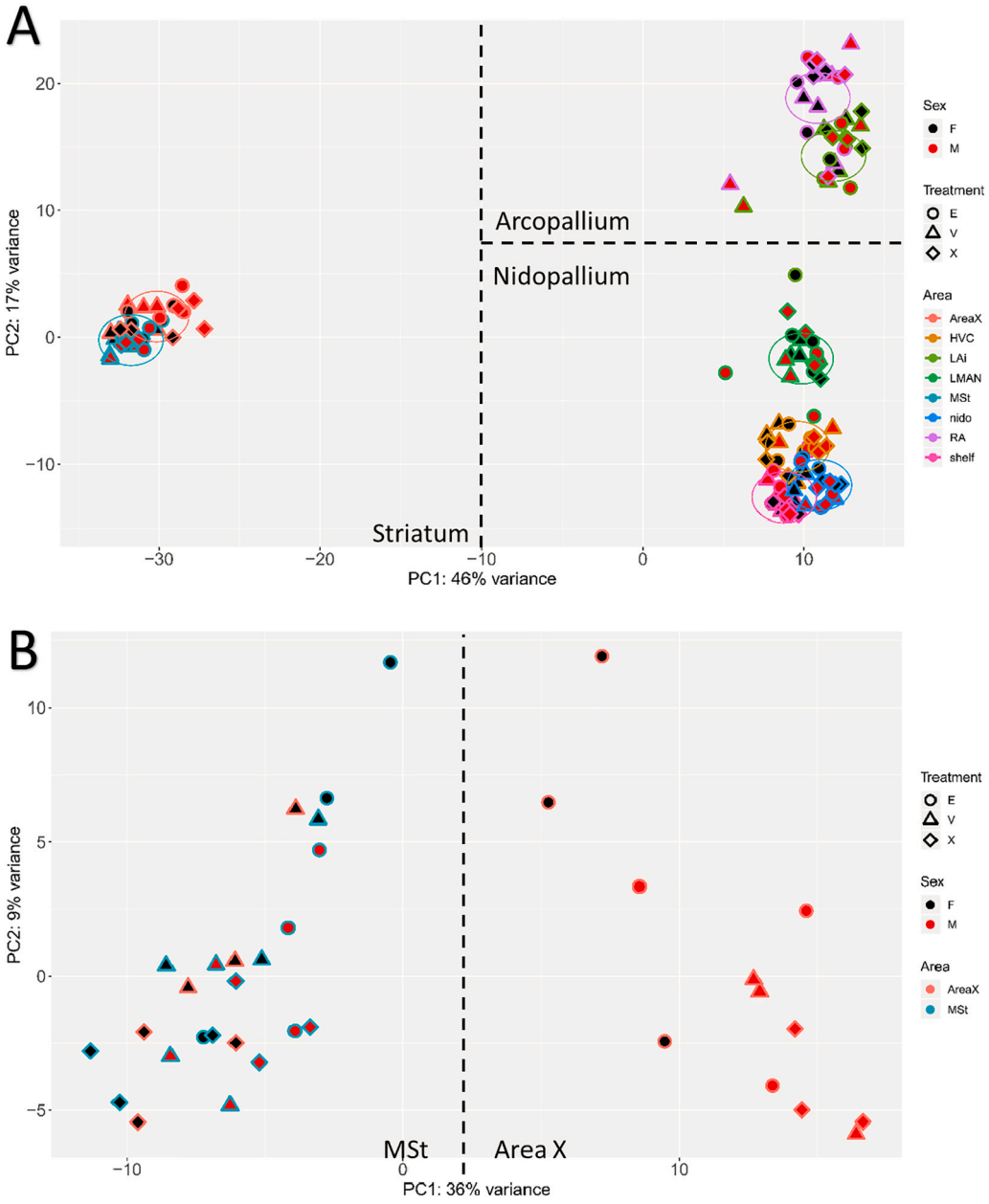

Fig. 7. Differential gene expression in song nuclei in response to estrogen manipulation. (A) PCA plot of all RNA-Seq samples. Sex, fill colour; Treatment, symbol shape; Brain region, symbol outline colour. (B) PCA plot of all MSt and Area X RNA-seq samples. Female estradiol Area X samples (black filled salmon circles) cluster more closely with all male Area X samples to the right of the plot.

(or the region where Area X would be in vehicle and exemestane treated females) and their immediate surrounding areas (Supplemental Fig. 1), and then performed RNA-seq transcriptomic profiling, with three PHD30 animals per group (a total of 144 samples; Methods).

PCA of expression of all 18,618 annotated genes assessed (16,882 expressed with 100 or more reads in at least one brain region), that overlapped in the Kallisto and STAR mapped alignments, showed that samples from all 8 brain regions first clustered closely into 3 main groups that reflected their specific brain subdivision (striatum, nidopallium, and arcopallium), and then by song nucleus and surround (Fig. 7A). This same data set was also plotted as a Euclidian distance matrix plot to assess degree of similarities between all 144 samples. The pairwise sample distance matrix mirrored the PCA results, but it more dramatically showed that LMAN was highly specialized, separating as an outgroup outside of the nidopallium in both sexes at this age (Supplemental Fig. 5). Area X showed the biggest separation by sex and treatment, where samples from males and estradiol treated females separated from all surrounding MSt samples and samples where Area X would have been located in vehicle and exemestane treated females (Fig. 7B; Supplemental Fig. 5).

The ratio of DEG levels between the song nucleus and their respective surrounding regions were also compared between individuals in a Spearman's correlation matrix (Supplemental Table 4). The Spearman's correlations validate the PCA (Fig. 7B), showing that the Area X:MSt DEG ratio is most similar between male and estradiol treated female individuals (animals with Area X; $\rho = \sim 0.7–0.9$; Supplemental Table 4), and dissimilar between these animals and vehicle or exemestane treated females (animals without an Area X; $\rho = \sim 0.1–0.5$); the low correlations of the later are presumably due to lack of DEGs differentiating an Area X analogous regions and the surrounding striatum.

Unpaired statistical analysis for DEGs over- or under-expressed in each song nucleus relative to their surrounding brain subdivision

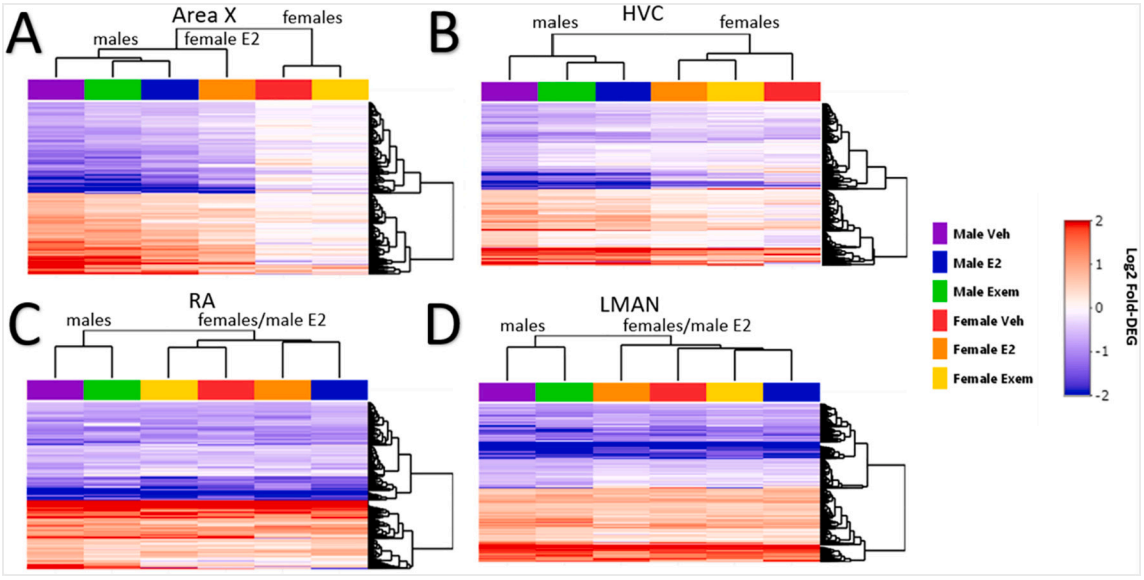

Fig. 8. Heatmap of all song nuclei gene expression specializations for all treatment groups. Shown are genes with significant differential expression in male song nuclei of PHD30 animals, and their profiles in all other groups. (A) Vehicle male Area X had 326 DEGs. Note vehicle and exemestane treated females had hardly any specialized gene expression of the genes found in males. (B) Vehicle male HVC had 818 DEGs. Regardless of treatment, DEG patterns separated based on sex with males showing far greater fold changes for the 818 genes than females. (C) Vehicle male RA had 202 DEGs. Vehicle treated animals clustered with exemestane treated animals within sex, however estradiol treatment animals were most similar to each other and then more similar to females than males. (D) Vehicle male LMAN has 1023 DEGs. Vehicle and exemestane treated males were most similar to each other. Heatmaps show differential expression in log2 fold change (log2FC) values for each gene (row) by experimental group (column). Values with log2FC greater than |2| were binned at |2|. Red, increased expression in song nuclei relative to the surround. Blue, decreased expression.

revealed that, as in adults (Pfenning et al., 2014), vehicle treated males at PHD30 already had hundreds of such DEGs (Fig. 8, purple; heatmap of individuals in Supplemental Fig. 6). This included *CADPS2*, which was among the top 3 genes with the greatest magnitude of specialization in male Area X, but not in the female equivalent region (Fig. 9A–F). When comparing overlap of all DEGs in song nuclei between vehicle control males and females, we noted that using a strict FDR $\leq$ 0.05 resulted in over-estimates of the sex differences as displayed in Venn Diagrams (Fig. 9G–J) relative to the pattern seen with the log2-fold DEG values in heat maps (Fig. 8) and their Pearson correlations (Fig. 10). Thus, taking all these analyses into consideration, we find that for the 326 DEGs in male Area X, there was no appreciable log2-fold correlation with females in the region where Area X would have been located (Figs. 8A, 9G, 10A). Similarly, but less dramatic, of the 818 genes with specialized DEG in male HVC at FDR $\leq$ 0.05, about 30 had a log2-fold change in the same direction in the small but still present female HVC, and these differences were diminished in magnitude compared to males (Figs. 8B, 10C), with only 3 being significant (Fig. 9H). In the inverse comparison, we barely found any (11) DEG with FDR $\leq$ 0.05 in female HVC (Figs. 9H, 10D). In contrast to the findings in Area X and HVC, for the 202 DEGs in male RA and 1023 in male LMAN at FDR $\leq$ 0.05 (Fig. 9I,J), the vast majority also had similar log2-fold DEG in female RA and LMAN, with not much more than 20 showing ~0 difference in females (Figs. 8C,D; 10E–H). Interestingly, a handful of genes (5) had a large downregulation in male RA, but large upregulation in female RA (Fig. 10F). When the same data was subjected to the more sensitive pairwise statistical analysis, even with a greater number of DEG's passing the FDR $<$ 0.05 threshold, the results were similar (Supplemental Fig. 7A–D; Table 3, full list of genes in Supplemental Table 5); however, with thousands of genes as variables, the pairwise analyses was more subject to false positives, and thus we focused on the more stringent unpaired analyses for this study. These findings demonstrate that at PHD30, without estrogen manipulation, the song nuclei DEG profile for RA and LMAN is at a similar molecular state between males and females, whereas for HVC and Area X they are at dramatically different states.

Exemestane treatment had little impact on the level of specialized gene expression in male song nuclei (Figs. 8, green; 9C), indicating that blocking estrogen did not demasculinize male song nuclei gene expression. There were also no notable large changes in females treated with exemestane (Figs. 8, yellow; 9D). Estradiol treatment, however, resulted in a dramatic change of female Area X to have a DEG profile similar to males, albeit at lower magnitude of differential expression for most genes (Fig. 8A, orange) with only the highest magnitude differences (14 genes) passing an unpaired FDR $<$ 0.05 (Fig. 9F). Estradiol only marginally made female HVC to be more male-like in its specialized gene expression profile compared to vehicle control females (Fig. 8B, red). In RA and LMAN, although they began with much fewer differences between control males and control females, estradiol induced male specializations to be more female-like (Fig. 8C,D, blue). These conclusions are also supported by the pairwise statistical analyses of song nuclei and surrounding areas for each bird, which revealed many more of these genes in females that pass a paired FDR $<$0.05 (Supplemental Fig. 8D–F).

We evaluated a subset of genes by in-situ hybridization (36 genes across 4 song nuclei) from PHD30 animals performed in our lab, or from the online zebra finch gene expression atlas (ZEBrA, n.d) on control adult males (http://www.zebrafinchatlas.org) as well as our past adult studies (Jarvis et al., 2013). We found that 100/140 song nuclei patterns (71%) of the top DEGs examined from our RNA-seq data were congruent with the in-situ hybridization data (Table 4). This validation rate is presumably lower than what it really is, as we know differences exist between DEGs in juveniles (this study) and adults (Hayase et al., 2018; Kubikova et al., 2010; Olson et al., 2015; Qi and Wade, 2013; Tang and Wade, 2010; Wang et al., 2015). Overall, these findings indicate that the molecular specializations that define each song learning nucleus are on different developmental trajectories in their growth in males and atrophy in females. This trajectory in females is altered in response to estrogen, but long-term blockage of estrogen synthesis minimally changes these specializations in males.

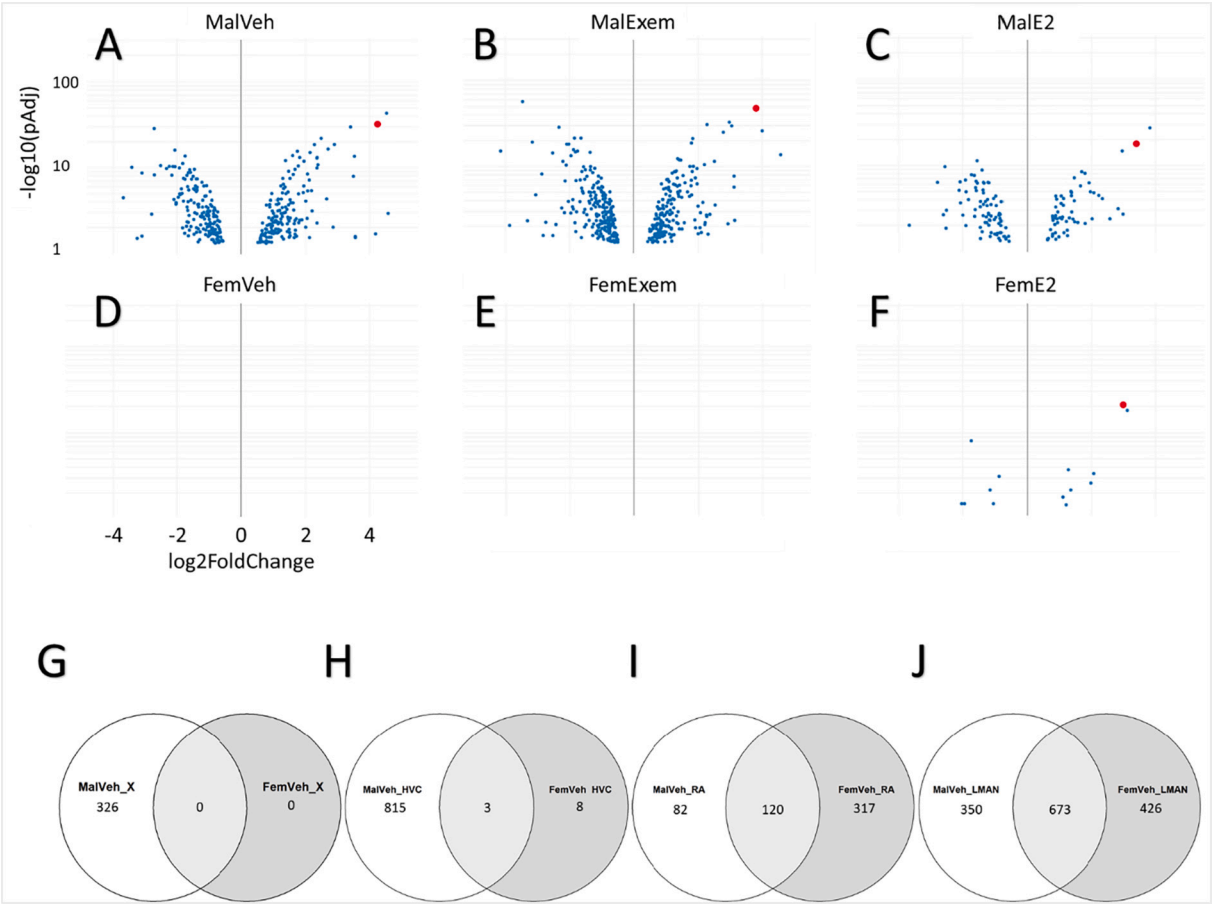

Fig. 9. Fold changes and overlaps in DEGs between groups for Area X. (A-F) Volcano plots of differentially expressed genes in Area X as compared to MSt from male and female finches treated with vehicle, estradiol, or exemestane. Only DEGs with FDR $\leq$ 0.05 were plotted. *CADPS2* is colored in red. X-axis is log2- fold change values with MSt enriched genes appearing to the left, and Area X enriched genes appearing to the right. Y-axis is the -log10 transformed FDR values on a log10 scale. (G–J) Venn diagram of DEGs at unpaired FDR $<$ 0.05 in Area X, HVC, RA and LMAN between vehicle treated males and females. Venn diagrams of paired FDR $<$ 0.05 are shown in Supplemental Fig. 7.

3.6. Each song learning nucleus has specific functional molecular specializations

The DEGs suggest that each song nucleus has different specializations regarding different functions (Pfenning et al., 2014; Whitney et al., 2014). We performed gene ontology (GO) analyses on the specialized DEGs identified in the unpaired test from control males at PHD30, and found that the top biological categories in Area X were associated with synapse transmission and signaling, neurotransmitter release, and neuron development (Fig. 11A); HVC and LMAN were specialized for neuron development, synaptic development and transynaptic signaling (Fig. 11B,D); and RA was specialized also for neurotransmitter release, and synaptic transmission and signaling (Fig. 11C). In terms of specific molecular function specializations, top GO terms were ion and cation channels in Area X, HVC, and RA, but receptor-ligand activity in RA (Fig. 11). Additional genes with lower magnitude differences in the paired analyses did not change these results for the top GO categories, except glycosaminoglycan and calcium-protein binding was more specialized than receptor-ligand activity for RA (Supplemental Fig. 9).

We next examined GO enrichment of genes that changed in response to estrogen or exemestane treatment. To do this, we examined genes that were differentially expressed in estradiol treated females at FDR $<$ 0.05 when compared to vehicle or exemestane treated females, and exemestane treated males when compared to vehicle or estradiol treated males, for each song nucleus, from the paired statistical analyses to include more of the lower magnitude differences (Supplemental Fig. 10). Top biological GO terms in exemestane treated males for Area X DEGs included signaling, action potential regulation/processes, and ion transport (Supplemental Fig. 10A); and RA had terms for cellular respiration (Supplemental Fig. 10C). For estradiol treated females, Area X included ion transport, ATP metabolic processes, and respiratory/oxidation processes (Supplemental Fig. 10E); HVC and RA included cellular differentiation, axonogenesis, and development (Supplemental Fig. 10F,G); and LMAN included synapse organization/regulation (Supplemental Fig. 10H). Overall, the GO analyses indicate that the genes that are most responsive to estrogen manipulation varied between song nuclei, which may lead to diverse functional outcomes among the different song nuclei in the different sexes.

4. Discussion

Through dose-controlled treatment with exemestane or estradiol, we were able to examine the effects of chronic estrogen manipulation on song behavior and the transcriptome of the song system during the onset of the sensorimotor vocal learning period in zebra finches. In males, we found an unexpected interaction between estrogen and male specific plumage development. We also found that male song learning was diminished by estrogen depletion, and with the exception of RA, there was little impact on the gross anatomy of the song learning circuit and its molecular specializations. In contrast, in females, we recapitulated prior studies establishing the need for artificially high levels of estrogen for female song learning and discovered that the development of the different song learning nuclei have different responses to estrogen manipulation. Notably Area X and its molecular specializations were the

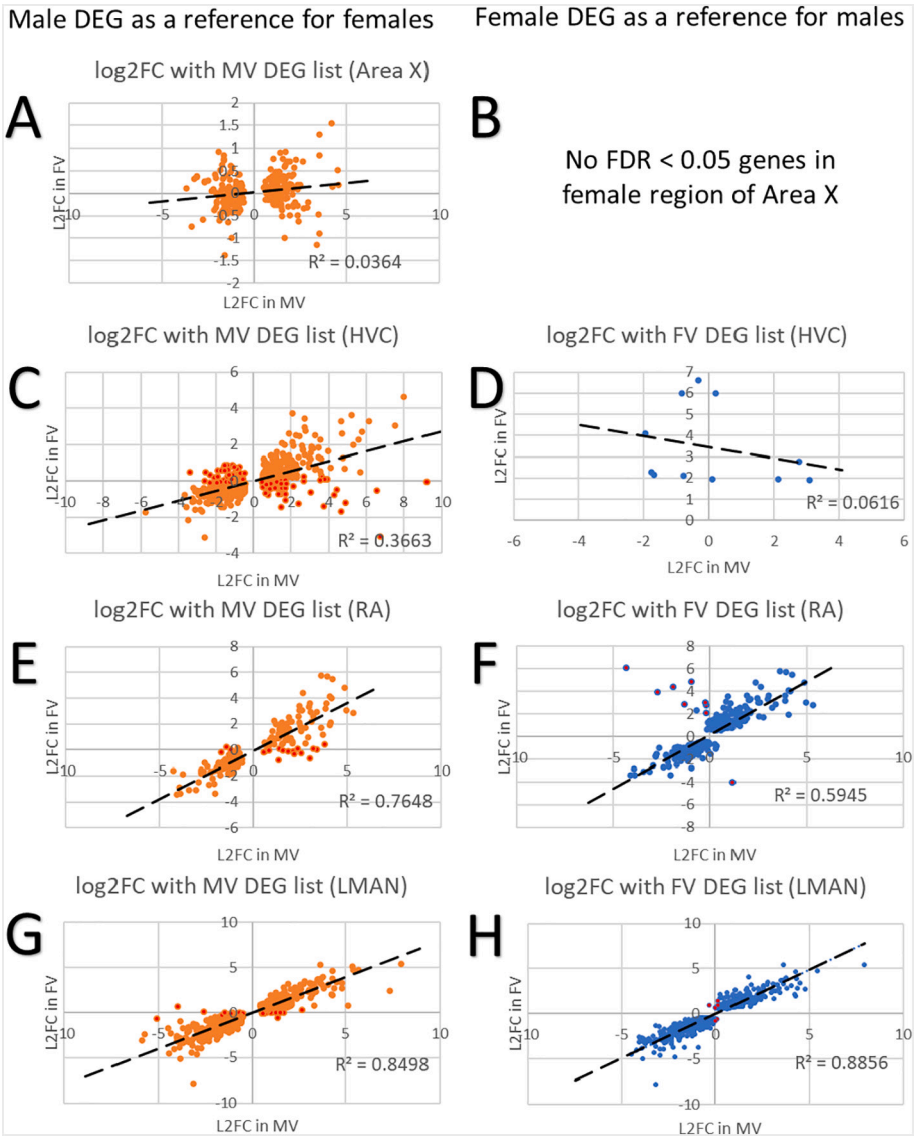

Fig. 10. Comparison of log2-fold changes between vehicle treated males and females. DEG list is from unpaired statistical analysis with genes common to both Kallisto and STAR. Fold change values are derived from DeSEQ2 results using STAR aligned reads. (A) Male vehicle DEG list of Area X. Only 3% of the observed log2-fold changes can be predicted using the male DEG list, which is expected as vehicle treated females do not have DEGs in their Area X region, and no discernable Area X. (B) Female vehicle DEG list of Area X. There were no genes detected at FDR < 0.05. (C) Male vehicle DEG list of HVC. (D) Female vehicle DEG list of HVC. A total of 36% of the observed values log2 fold change values from females can be predicted by the male DEG list. (E) Male vehicle DEG list of RA. (F) Female vehicle DEG list of RA. For RA, 76% (male DEG list) and 59% (female DEG list) of the observed log2-fold change values can be predicted using their respective DEG lists. (G) Male vehicle DEG list of LMAN. (H) Female vehicle DEG list of LMAN. In LMAN, 85% and 88% of the observed log2-fold changes can be predicted using the male and female DEG lists, respectively. Values with red fill are those were male and female show clear quantitative and qualitative differences.

Table 3
Number of DEGs in different Sex/Tx groups without IEGs.

	Non-Pairwise				Pairwise			
	Area X	HVC	RA	LMAN	Area X	HVC	RA	LMAN
Male Veh	326	818	202	1023	448	1516	537	1530
Male E2	137	555	392	1092	382	739	804	1817
Male Exem	406	713	390	1424	814	1084	638	1879
Female Veh	0	11	437	1099	3	73	945	1755
Female E2	229	140	286	690	236	802	881	1460
Female Exem	0	153	695	901	19	599	1213	1398

most dependent on high levels of estrogen in females at the start of the sensorimotor learning period. These findings support the hypothesis that vocal learning loss in female zebra finches evolved alongside a sex specific dependence on estrogen for vocal learning, suggesting that vocal learning evolved in both sexes first independent of estrogen and was later lost in the females. This behavioral loss is associated with an uneven neural loss of the different song nuclei. We suggest that Area X is the first to atrophy or not even appear, followed by HVC, then RA, and finally LMAN with limited atrophy.

Our results with estrogen inhibition agree with some, but not all past publications (Table 5). Some of the differences could be due to the pharmacological agent we used. Exemestane is more specific for aromatase inhibition with limited extra-target effects and without the "rebound effects" seen with other aromatase inhibitors (Harada and Hatano, 1998; Lonning and Eikesdal, 2013). Other differences may be due to our delivering the agents chronically until the time of sacrifice. The requirement of estrogen for sexually dimorphic male plumage was unexpected for us, and not seen in prior studies that we are aware of (Ralph, 1969; Somes and Smyth, 1967). The highly reduced male plumage in the presence of still well-developed song learning nuclei

Table 4
DEGs with localization data from the zebra finch gene expression atlas and other sources.

Gene Name	Area X		HVC		RA		LMAN	
	RNA-Seq	ISH Atlas	RNA-Seq	ISH Atlas	RNA-Seq	ISH Atlas	RNA-Seq	ISH Atlas
KCNT2	UP	✓	-	✓	-	✓	-	✓
TAC1	UP	✓	DOWN	✗	-	✓	DOWN	✓
FLRT2	UP	✓	UP	✓	UP	✗	-	✗
CACNG3	DOWN	✓	DOWN	✗	-	✓	DOWN	✓
ALDH1A2	-	✓	UP	✓	UP	✗	UP	✓
PVALB	-	✓	UP	✓	UP	✓	UP	✓
IGF1	UP	✗	-	✓	UP	✓	DOWN	✗
ADCYAP1	-	✓	DOWN	✓	DOWN	✓	-	✓
GRIN3A	DOWN	✗	-	✓	DOWN	✓	DOWN	✓
CLMN	-	✗	-	✓	DOWN	✗	-	✓
CBLN2	UP	✗	UP	✓	-	✓	UP	✓
SEMA3E	UP	✓	UP	✓	-	✓	UP	✓
ADRA2A	-	✓	-	✓	UP	✗	UP	✓
SCN4B	-	✓	UP	✓	-	✓	UP	✓
PLXNC1	-	✓	DOWN	✓	-	✗	DOWN	✓
HPCAL1	-	✗	DOWN	✓	-	✓	DOWN	✓
SLC6A7	-	✓	-	✓	-	✗	DOWN	✗
CCK	-	✓	-	✓	DOWN	✗	DOWN	✓
CACNG4	DOWN	✗	DOWN	✗	-	✓	DOWN	✗
CPE	-	✓	-	✓	-	✓	DOWN	✓
SEMA3A	DOWN	✗	DOWN	✗	-	✓	DOWN	✗
DACH1	-	✓	DOWN	✗	-	✓	DOWN	✗
NR0B1	-	✓	UP	✓	-	✓	UP	✗
ROBO2	-	✗	UP	✓	-	✓	-	✓
ZEB2	-	✓	UP	✓	-	✓	DOWN	✗
FOSL2	-	✓	UP	✓	-	✓	-	✓
POMC	-	✓	UP	✗	-	✓	-	✓
VIP	DOWN	✓	UP	✗	DOWN	✓	-	✓
GRIA4	DOWN	✗	DOWN	✓	-	✓	DOWN	✗
HPCAL1	-	✗	DOWN	✗	-	✓	DOWN	✓
ADCYAP1	-	✓	DOWN	✓	DOWN	✓	-	✓
GLRA4	-	✗	DOWN	✓	-	✓	DOWN	✗
AMIGO2	DOWN	✗	DOWN	✗	-	✓	DOWN	✗
MAPK1	-	✓	DOWN	✗	-	✓	-	✓
AKR1D1	-	✓	DOWN	✓	-	✓	-	✓

RNA-seq differential analysis data (left subcolumn) from post hatch day 30 vehicle/control males compared to RNA in-situ *hybridization* data (right subcolumn) from adult control males. Congruent DEGs are highlighted in blue and incongruent DEGs are highlighted in salmon.

with generally preserved gene expression specializations in exemestane treated animals suggest that estrogenic influence on these two anatomical systems are likely to be independent.

Our findings suggest that estrogen may still have a modulatory role in song learning, since all males dosed with exemestane had impoverished singing ability. We were surprised to see that chronic exemestane treatment in females caused some females to produce 1–2 repeated syllables with multiple notes each, in a song-like manner, even though they had atrophied song nuclei. Prior studies have shown that acute aromatase inhibition can suppress the rate of singing in zebra finches and canaries (Alward et al., 2016; Vahaba et al., 2019; Walters and Harding, 1988), but no changes in song learning acuity have been reported (Merten and Stocker-Buschina, 1995; Vahaba et al., 2019). Perhaps the more potent and long-term application of the estrogen synthesis inhibitor we used revealed an unknown involvement of estrogen in vocal learning. This could be through estrogenic modulation of a subset of genes with specialized expression in the song nuclei detected here that change with exemestane treatment in males. Alternatively, the mechanism could be through the auditory pathway, as recent work has shown that acute aromatase inhibition with fadrozole alters hearing-induced expression of activity-dependent genes in the zebra finch auditory pallium, and more so in males than in females (Krentzel et al., 2020).

Although the estrogen-induced "masculinization" of the female song system and behavior we observed is considerable, it is still incomplete with regards to song nuclei size and molecular specializations. This indicates that there may be other factors at play besides estrogen in suppressing the development of the song learning system in females, or our estrogen administration protocol was not optimal, which is a possibility as we had to use far less estradiol (200 μg pellets at PHD20 instead of 1500μg) than was published previously (Simpson and Vicario, 1991a; Simpson and Vicario, 1991b). This was due to the toxic effects of long-term elevated estrogen had on bone integrity in birds (Whitehead, 2004). The finding that most HVC gene expression specializations are not sensitive to estrogen treatment in females, whereas specializations in Area X are extremely sensitive, was unexpected as HVC is the only song nucleus known to express the classical NR (nuclear receptor) ESR1 (estrogen receptor α), and this ESR1 expression is not yet sexually dimorphic at ~PHD30 (Gahr, 1996; Gahr and Konishi, 1988; Jacobs et al., 1999). There are also other estrogen receptors, ESR2 (Estrogen receptor β) (Kuiper et al., 1996; Mosselman et al., 1996) and GPER (Maggiolini and Picard, 2010) that maybe involved. Selective receptor agonist/antagonist studies will need to be conducted in the future to determine their individual effects. Additionally, an intact HVC is necessary for RA development in males and estrogen-treated females, and for Area X in estrogen-treated females (Akutagawa and Konishi, 1994; Herrmann and

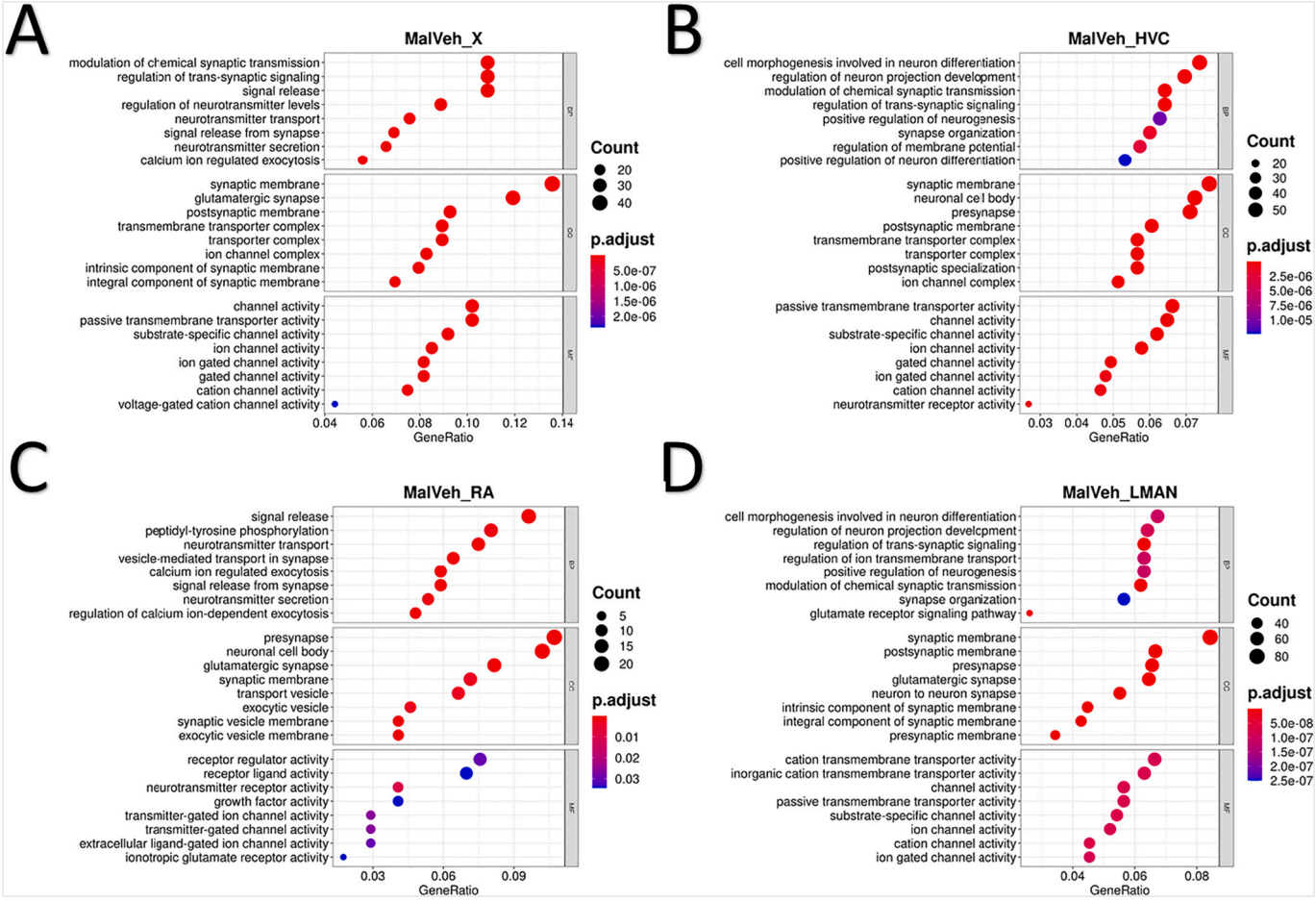

Fig. 11. Top GO terms for DEGs in each song nucleus in vehicle males. (A-D) Area X, HVC, RA, and LMAN respectively. X-axis indicates the ratio of genes with specialized expression out of the total list of genes, which contribute to each value. Count, number of DEGs that contributed to each category. BP, Biological Process; CC, Cell Compartment; MF, Molecular Function. Genelist is from all vehicle male DEGs for each region with FDR <0.05. GO enrichment results include FDR value <0.05. Heatmap scale, level of significance.

Table 5
Summary of prior studies modulating estrogen in zebra finches.

Drug	Class	Timecourse	Study	Results in the song system
LY117018 (raloxifene)	SERM	PHD0-20	Mathews and Arnold, 1990	Hypermasculinization
CI628 (nitromifene)	SERM	PHD0-20	Mathews and Arnold, 1990	Hypermasculinization
Tamoxifen	SERM	PHD0-20	Mathews et al., 1988	Hypermasculinization
ICI182780 (fulvestrant)	SERD	PHD0-25	Bender and Veney, 2008	Decreased cell size & nuclei volume
Fadrozole	2nd gen AI	PHD1-30	Wade and Arnold, 1994	No effect
Fadrozole	2nd gen AI	PHD10-30	Merten and Stocker-Buschina, 1995	Smaller neuron soma, no behavior change
G-15	GPER agonist	PHD0-25	Tehrani and Veney, 2018	Smaller HVC & Area X volume
R76713 (vorozole)	3rd gen AI	PHD0-45	Balthazart et al., 1994	No effect in nuclei size, reduced singing

Arnold, 1991). Combining findings, one interpretation could be that the ESR1 receptor in HVC may lead to specialization of some genes in HVC that influences a cue for Area X development, but only in females. The higher degree of stability for specialized gene expression in RA and LMAN regardless of sex or estrogen manipulation suggest that their specialized functions during development are not strongly estrogen dependent and in the case of RA, may instead be subtly modulated by the actions of estrogenic signaling instead. We were surprised to find that RA size was decreased in males treated with either exemestane or estradiol. This is consistent with our above hypothesis that each song nucleus may be responding differently to changes in estrogen levels, with RA responding in a U-shaped optimal manner in terms of size, where too low or too high E2 levels may inhibit the expansion of RA neuronal size.

Our findings help shed light on the hypotheses of the origin of vocal learning systems in songbirds and their sex differences. The song learning system of songbirds is inferred to have evolved ~30 million years ago with the emergence of the split between oscine and suboscine (songbird) Passeriformes (Jarvis et al., 2014). For many years it was assumed that sex differences in song learning could be an ancestral trait, consistent with the findings that many songbird species use their learned vocalizations for sexually dimorphic behaviors such as mate attraction and territorial defense. This begged the question as to why the female song system appeared to be estrogen dependent, while the male song system was estrogen independent. A fact underappreciated by many scientists in temperate regions, is that in the vast majority of songbirds, both males and females, sing, and they are concentrated more in equatorial regions (Jarvis, 2004). An analysis of many songbird species

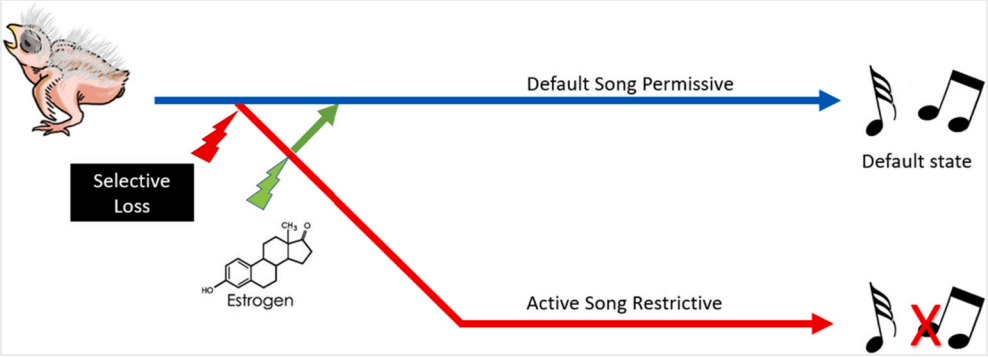

Fig. 12. Alternative hypothesis of female song loss in sexually dimorphic songbirds. The song permissive state is the ancestral trait of songbirds (blue line), the rapid degeneration of the W chromosome either alone or in tandem with rapid evolution of the Z chromosome may contribute to the active restriction of the development of the song system in sexually dimorphic songbirds (red line). Estrogen may work downstream of this induced atrophy to rescue the nascent song system (green line).

globally and phylogenetically suggest that female-selective loss occurred multiple independent times among songbirds (Odom et al., 2014), indicating that there may be positive selection for song repression in females rather than a gain/expansion only in males. One explanation for the geographic difference in sex-specific song learning, is that after songbird song learning evolved ~30 million years ago, species in temperate zones faced a more demanding environment that selected for more division of labor between the sexes and/or increased competition for limited resources (Jarvis, 2004). Here we further propose that sex specific division of labor for vocal learning in zebra finches was selected for by a sex hormone-dependent mechanism in females. This would also allow for evolution to reverse this loss in females should environmental factors reselect for it. These hypotheses can be further tested in future studies using species with and without vocal learning sexual dimorphism.

Our findings are also informative for understanding variations in sex determination on behavior. In mammals, females are homogametic and generally regarded as the default sex. In mammals, genetic signals from the male Y chromosome induce development of male reproductive organs and the second X chromosome in females become silenced to prevent overdosage of X chromosomal genes. In birds, males are the homogametic sex, carrying two Z chromosomes, with elevated Z chromosome gene products due to incomplete dosage compensation (Dementyeva and Zakian, 2010; Itoh et al., 2007). Either signals from reduced Z chromosome dosage or the female specific W chromosome may influence development of female reproductive organs. Whether the brain has a default sex state in birds or mammals is still open to debate, but it is well known that estrogen plays an organizational role in the sexual differentiation of the central nervous system (McCarthy, 2008). Sexually dimorphic structures within the hypothalamus can be found in all vertebrates, indicating that these structures are ancient, present long before the synapsid/sauropsid split >300 million years ago (Bruce, 2009; Godwin and Crews, 1997; Kumar and Hedges, 1998). The sexually dimorphic hypothalamic medial preoptic nucleus in chicken and quail are sensitive to estrogen modulation during pre-hatching in a manner that is opposite to that seen in mammals, where estrogen administration feminizes and aromatase inhibition masculinizes this nucleus and associated behaviors (Balthazart and Ball, 1995; Balthazart et al., 1992; Kurian et al., 2010; Lambeth et al., 2016; McEwen et al., 1977; Panzica et al., 2001; Panzica et al., 1998). Further, in many amniotic vertebrate systems, estrogenic activity during an early critical period sets up the bipotential brain to develop in a masculine or feminine manner (Juntti et al., 2010; Kurian et al., 2010). Taking all this into consideration, we propose that vocal learning in songbirds is the default state, not linked to sex chromosome determination genes nor ancient brain pathways that exist in a sex-specific state (Fig. 12, blue). After vocal learning evolved, we suggest that selective loss/suppression of vocal learning in females of some species became linked to sex determination genes (Fig. 12, red), which can then be overturned or otherwise modulated through the actions of estrogen (Fig. 12, green).

If this hypothesis is correct, it would mean that the molecular specializations of the vocal learning systems, particularly Area X in the striatum, became subsequently linked to the estrogen regulatory pathway and the W chromosome in females. Finding that link may help towards understanding the mechanism of specialized gene regulation in vocal learning systems in songbirds, but also the convergently evolved specializations shared with human speech brain regions (Pfenning et al., 2014). Although humans have very small sex differences in vocal learning and spoken language behaviors (Ross et al., 2015; Weis et al., 2019) relative to sexually dimorphic vocal learning birds, some language deficits and other learning deficits are strongly linked to sex (Ferri et al., 2018; Werling and Geschwind, 2013), and a subset of the specialized genes in songbirds is convergent with those found in human spoken language brain regions (Pfenning et al., 2014). Future comparative studies in genomics, behavior, and sex hormone regulation in humans as well as other vocal learning species should help shed light on these hypotheses.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2020.104911.

Funding

This work was supported by funds from Howard Hughes Medical Institute (USA) and Rockefeller University (USA) start up to E.D.J, and NIH-NIDCD (USA) grant (DC014423) to H.M. K.Z. is supported by NIH-NIDCD fellowship (F31DC017394).

Declaration of competing interest

We have no competing interest.

Acknowledgements

We thank: Matthew Biegler and Lindsey Cantin for optimizing the in situ *hybridization* protocol for use in zebra finch sections. Gregory Gedman and Matthew Davenport for their instruction on laser capture microscopy. Bettina Haase and Olivier Fedrigo for guidance during RNA-Seq library preparation; Gregory Gedman and James Cahill for helpful discussion regarding transcriptomic analysis. Claire de March for her guidance on in silico protein -modeling. We also thank Robyn Donahue for animal husbandry, and members of the Duke metabolomics core for the mass spec steroid assays: Lisa St. John-Williams, Will Thompson, and Arthur Moseley. Lastly, we thank Claire de March, Aashutosh Vihani, Tatjana Abaffy, and Veronica Choe for helpful feedback on the manuscript.

References

Adam, I., Scharff, C., Honarmand, M., 2014. Who is who? Non-invasive methods to individually sex and mark altricial chicks. J Vis Exp 51429.

Akutagawa, E., Konishi, M., 1994. Two separate areas of the brain differentially guide the development of a song control nucleus in the zebra finch. Proc. Natl. Acad. Sci. 91, 12413.

Alward, B.A., de Bournonville, C., Chan, T.T., Balthazart, J., Cornil, C.A., Ball, G.F., 2016. Aromatase inhibition rapidly affects in a reversible manner distinct features of birdsong. Sci. Rep. 6, 32344.

Balthazart, J., Ball, G.F., 1995. Sexual differentiation of brain and behavior in birds. Trends Endocrinol. Metab. 6, 21–29.

Balthazart, J., De Clerck, A., Foidart, A., 1992. Behavioral demasculinization of female quail is induced by estrogens: studies with the new aromatase inhibitor, R76713. Horm. Behav. 26, 179–203.

Balthazart, J., Absil, P., Fiasse, V., Ball, G.F., 1994. Effects of the aromatase inhibitor R76713 on sexual differentiation of brain and behavior in zebra finches. Behaviour 131, 225–259. https://doi.org/10.1163/156853994X00451.

Bender, A.T., Veney, S.L., 2008. Treatment with the specific estrogen receptor antagonist ICI 182,780 demasculinizes neuron soma size in the developing zebra finch brain. Brain Res. 1246, 47–53.

Bottjer, S.W., Glaessner, S.L., Arnold, A.P., 1985. Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J. Neurosci. 5, 1556.

Bray, N.L., Pimentel, H., Melsted, P., Pachter, L., 2016. Near-optimal probabilistic RNA-seq quantification. Nat. Biotechnol. 34, 525–527.

Breed, M.D., Moore, J., 2010. Encyclopedia of Animal Behavior. Elsevier B.V, Amsterdam.

Bruce, L.L., 2009. Evolution of the hypothalamus in amniotes. In: Binder, M.D., Hirokawa, N., Windhorst, U. (Eds.), Encyclopedia of Neuroscience. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp. 1363–1367.

Carrer, H.F., Cambiasso, M.J., 2009. Sexual differentiation of the brain: genetic, hormonal and trophic factors. In: Janigro, D. (Ed.), Mammalian Brain Development, 1ed. Springer, pp. 1–15.

Çiftci, H.B., 2012. Effect of estradiol-17β on follicle-stimulating hormone secretion and egg-laying performance of Japanese quail. Animal 6, 1955–1960.

Cornford, E.M., Braun, L.D., Oldendorf, W.H., Hill, M.A., 1982. Comparison of lipid-mediated blood-brain-barrier penetrability in neonates and adults. Am. J. Phys. Cell Phys. 243, C161–C168.

Dementyeva, E.V., Zakian, S.M., 2010. Dosage compensation of sex chromosome genes in eukaryotes. Acta Nat. 2, 36–43.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21.

Feenders, G., Liedvogel, M., Rivas, M.V., Zapka, M., Horita, H., Hara, E., Wada, K., Mouritsen, H., Jarvis, E.D., 2008. Molecular mapping of movement-associated areas in the avian brain: a motor theory for vocal learning origin. PLoS One 3, e1768.

Ferri, S.L., Abel, T., Brodkin, E.S., 2018. Sex differences in autism spectrum disorder: a review. Current Psychiatry Reports 20, 9.

Gahr, M., 1996. Developmental changes in the distribution of oestrogen receptor mRNA expressing cells in the forebrain of female, male and masculinized female zebra finches. Neuroreport 7, 2469–2473.

Gahr, M., Konishi, M., 1988. Developmental changes in estrogen-sensitive neurons in the forebrain of the zebra finch. Proc. Natl. Acad. Sci. U. S. A. 85, 7380–7383.

Garcia-Calero, E., Scharff, C., 2013. Calbindin expression in developing striatum of zebra finches and its relation to the formation of area X. J. Comp. Neurol. 521, 326–341.

Gobes, S.M.H., Jennings, R.B., Maeda, R.K., 2017. The sensitive period for auditory-vocal learning in the zebra finch: consequences of limited-model availability and multiple-tutor paradigms on song imitation. Behav. Process. S0376-6357 (0317), 30163–30168.

Godwin, J., Crews, D., 1997. Sex differences in the nervous system of reptiles. Cell. Mol. Neurobiol. 17, 649–669.

Grisham, W., Arnold, A.P., 1995. A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. J. Neurobiol. 26, 163–170.

Gurney, M.E., 1982. Behavioral correlates of sexual differentiation in the zebra finch song system. Brain Res. 231, 153–172.

Harada, N., Hatano, O., 1998. Inhibitors of aromatase prevent degradation of the enzyme in cultured human tumour cells. Br. J. Cancer 77, 567–572.

Hayase, S., Wang, H., Ohgushi, E., Kobayashi, M., Mori, C., Horita, H., Mineta, K., Liu, W.-c, Wada, K., 2018. Vocal practice regulates singing activity–dependent genes underlying age-independent vocal learning in songbirds. PLoS Biol. 16, e2006537.

Herrmann, K., Arnold, A.P., 1991. Lesions of HVc block the developmental masculinizing effects of estradiol in the female zebra finch song system. J. Neurobiol. 22, 29–39.

Holloway, C.C., Clayton, D.F., 2001. Estrogen synthesis in the male brain triggers development of the avian song control pathway *in vitro*. Nat. Neurosci. 4, 170–175.

Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., Nas, A.V., Replogle, K., Band, M.R., Clayton, D.F., Schadt, E.E., Lusis, A.J., Arnold, A.P., 2007. Dosage compensation is less effective in birds than in mammals. J. Biol. 6.

Jacobs, E.C., Arnold, A.P., Campagnoni, A.T., 1999. Developmental regulation of the distribution of aromatase- and estrogen-receptor- mRNA-expressing cells in the zebra finch brain. Dev. Neurosci. 21, 453–472.

Jarvis, E.D., 2004. Chapter 8 - brains and birdsong. In: Marler, P., Slabbekoorn, H. (Eds.), Nature's Music. Academic Press, San Diego, pp. 226–271.

Jarvis, E.D., 2019. Evolution of vocal learning and spoken language. Science 366, 50.

Jarvis, E.D., Nottebohm, F., 1997. Motor-driven gene expression. Proc. Natl. Acad. Sci. 94, 4097–4102.

Jarvis, E.D., Scharff, C., Grossman, M.R., Ramos, J.A., Nottebohm, F., 1998. For whom the bird sings: context-dependent gene expression. Neuron 21, 775–788.

Jarvis, E.D., Yu, J., Rivas, M.V., Horita, H., Feenders, G., Whitney, O., Jarvis, S.C., Jarvis, E.R., Kubikova, L., Puck, A.E.P., Siang-Bakshi, C., Martin, S., McElroy, M., Hara, E., Howard, J., Pfenning, A., Mouritsen, H., Chen, C.-C., Wada, K., 2013. Global view of the functional molecular organization of the avian cerebrum: mirror images and functional columns. J. Comp. Neurol. 521, 3614–3665.

Jarvis, E.D., Mirarab, S., Aberer, A.J., Li, B., Houde, P., Li, C., Ho, S.Y.W., Faircloth, B.C., Nabholz, B., Howard, J.T., Suh, A., Weber, C.C., da Fonseca, R.R., Li, J., Zhang, F., Li, H., Zhou, L., Narula, N., Liu, L., Ganapathy, G., Boussau, B., Bayzid, M.S., Zavidovych, V., Subramanian, S., Gabaldón, T., Capella-Gutiérrez, S., Huerta-Cepas, J., Rekepalli, B., Munch, K., Schierup, M., Lindow, B., Warren, W.C., Ray, D., Green, R.E., Bruford, M.W., Zhan, X., Dixon, A., Li, S., Li, N., Huang, Y., Derryberry, E.P., Bertelsen, M.F., Sheldon, F.H., Brumfield, R.T., Mello, C.V., Lovell, P.V., Wirthlin, M., Schneider, M.P.C., Prosdocimi, F., Samaniego, J.A., Velazquez, A.M.V., Alfaro-Núñez, A., Campos, P.F., Petersen, B., Sicheritz-Ponten, T., Pas, A., Bailey, T., Scofield, P., Bunce, M., Lambert, D.M., Zhou, Q., Perelman, P., Driskell, A.C., Shapiro, B., Xiong, Z., Zeng, Y., Liu, S., Li, Z., Liu, B., Wu, K., Xiao, J., Yinqi, X., Zheng, Q., Zhang, Y., Yang, H., Wang, J., Smeds, L., Rheindt, F.E., Braun, M., Fjeldsa, J., Orlando, L., Barker, F.K., Jønsson, K.A., Johnson, W., Koepfli, K.-P., O'Brien, S., Haussler, D., Ryder, O.A., Rahbek, C., Willerslev, E., Graves, G.R., Glenn, T.C., McCormack, J., Burt, D., Ellegren, H., Alström, P., Edwards, S.V., Stamatakis, A., Mindell, D.P., Cracraft, J., Braun, E.L., Warnow, T., Jun, W., Gilbert, M.T.P., Zhang, G., 2014. Whole-genome analyses resolve early branches in the tree of life of modern birds. Science 346, 1320.

Juntti, S.A., Tollkuhn, J., Wu, M.V., Fraser, E.J., Soderborg, T., Tan, S., Honda, S.-I., Harada, N., Shah, N.M., 2010. The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. Neuron 66, 260–272.

Kojima, S., Doupe, A.J., 2007. Song selectivity in the pallial-basal ganglia song circuit of zebra finches raised without tutor song exposure. J. Neurophysiol. 98, 2099–2109.

Konishi, M., Akutagawa, E., 1985. Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. Nature 315, 145–147.

Konishi, M., Akutagawa, E., 1988. A critical period for estrogen action on neurons of the song control system in the zebra finch. Proc. Natl. Acad. Sci. U. S. A. 85, 7006–7007.

Krentzel, A.A., Ikeda, M.Z., Oliver, T.J., Koroveshi, E., Remage-Healey, L., 2020. Acute neuroestrogen blockade attenuates song-induced immediate early gene expression in auditory regions of male and female zebra finches. Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology 206, 15–31. https://doi.org/10.1007/s00359-019-01382-w.

Kubikova, L., Wada, K., Jarvis, E.D., 2010. Dopamine receptors in a songbird brain. J. Comp. Neurol. 518, 741–769.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.A., 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. 93, 5925.

Kumar, S., Hedges, S.B., 1998. A molecular timescale for vertebrate evolution. Nature 392, 917–920.

Kurian, J.R., Olesen, K.M., Auger, A.P., 2010. Sex differences in epigenetic regulation of the estrogen receptor-alpha promoter within the developing Preoptic area. Endocrinology 151, 2297–2305.

Lambeth, L.S., Morris, K.R., Wise, T.G., Cummins, D.M., O'Neil, T.E., Cao, Y., Sinclair, A. H., Doran, T.J., Smith, C.A., 2016. Transgenic chickens overexpressing aromatase have high estrogen levels but maintain a predominantly male phenotype. Endocrinology 157, 83–90.

Lonning, P.E., Eikesdal, H.P., 2013. Aromatase inhibition 2013: clinical state of the art and questions that remain to be solved. Endocr. Relat. Cancer 20, R183–R201.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Lovell, P.V., Clayton, D.F., Replogle, K.L., Mello, C.V., 2008. Birdsong "Transcriptomics": neurochemical specializations of the oscine song system. PLoS One 3, e3440.

Lovell, P.V., Huizinga, N.A., Friedrich, S.R., Wirthlin, M., Mello, C.V., 2018. The constitutive differential transcriptome of a brain circuit for vocal learning. BMC Genomics 19, 231.

Maggiolini, M., Picard, D., 2010. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J. Endocrinol. 204, 104–114.

Martinkovich, S., Shah, D., Planey, S.L., 2014. Selective estrogen receptor modulators: tissue specificity and clinical utility. Clin. Interv. Aging 9, 1437–1452.

Mathews, G.A., Arnold, A.P., 1990. Antiestrogens fail to prevent the masculine ontogeny of the zebra finch song system. Gen. Comp. Endocrinol. 80, 48–58.

Mathews, G.A., Arnold, A.P., 1991. Tamoxifen's effects on the zebra finch song system are estrogenic, not antiestrogenic. J. Neurobiol. 22, 957–969.

Mathews, G.A., Brenowitz, E.A., Arnold, A.P., 1988. Paradoxical hypermasculinization of the zebra finch song system by an antiestrogen. Horm. Behav. 22, 540–551.

McCarthy, M.M., 2008. Estradiol and the developing brain. Physiol. Rev. 88, 91–124.

McCarthy, M.M., Arnold, A.P., 2011. Reframing sexual differentiation of the brain. Nat. Neurosci. 14, 677–683.

McDonnell, D.P., 2005. The molecular pharmacology of estrogen receptor modulators: implications for the treatment of breast cancer. Clin. Cancer Res. 11, 871–877.

McEwen, B.S., Lieberburg, I., Chaptal, C., Krey, L.C., 1977. Aromatization: important for sexual differentiation of the neonatal rat brain. Horm. Behav. 9, 249–263.

Merten, M.D.P., Stocker-Buschina, S., 1995. Fadrozole induces delayed effects on neurons in the zebra finch song system. Brain Res. 671, 317–320.

Mooney, R., Rao, M., 1994. Waiting periods versus early innervation: the development of axonal connections in the zebra finch song system. J. Neurosci. 14, 6532.

Moore, C.R., Lamar, J.K., Beck, N., 1938. Cutaneous absorption of sex hormones. J. Am. Med. Assoc. 111, 11–14.

Mosselman, S., Polman, J., Dijkema, R., 1996. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49–53.
Nixdorf-Bergweiler, B.E., 1996. Divergent and parallel development in volume sizes of telencephalic song nuclei in and female zebra finches. J. Comp. Neurol. 375, 445–456.
Nordeen, E.J., Nordeen, K.W., 1988. Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J. Neurosci. 8, 2869.
Nottebohm, F., Arnold, A.P., 1976. Sexual dimorphism in vocal control areas of the songbird brain. Science 194, 211.
Odom, K.J., Hall, M.L., Riebel, K., Omland, K.E., Langmore, N.E., 2014. Female song is widespread and ancestral in songbirds. Nat. Commun. 5, 3379.
Olson, C.R., Hodges, L.K., Mello, C.V., 2015. Dynamic gene expression in the song system of zebra finches during the song learning period. Developmental Neurobiology 75, 1315–1338.
Panzica, G.C., Castagna, C., Viglietti-Panzica, C., Russo, C., Tlemçani, O., Balthazart, J., 1998. Organizational effects of estrogens on brain vasotocin and sexual behavior in quail. J. Neurobiol. 37, 684–699.
Panzica, G., Viglietti-Panzica, C., Balthazart, J., 2001. Sexual dimorphism in the neuronal circuits of the quail preoptic and limbic regions. Microsc. Res. Tech. 54, 364–374.
Pardridge, W.M., Mietus, L.J., 1979. Transport of steroid hormones through the rat blood-brain barrier: primary role of albumin-bound hormone. J. Clin. Invest. 64, 145–154.
Pardridge, W.M., Mietus, L.J., Frumar, A.M., Davidson, B.J., Judd, H.L., 1980. Effects of human serum on transport of testosterone and estradiol into rat brain. American Journal of Physiology-Endocrinology and Metabolism 239, E103–E108.
Pfenning, A.R., Hara, E., Whitney, O., Rivas, M.V., Wang, R., Roulhac, P.L., Howard, J.T., Wirthlin, M., Lovell, P.V., Ganapathy, G., Mouncastle, J., Moseley, M.A., Thompson, J.W., Soderblom, E.J., Iriki, A., Kato, M., Gilbert, M.T.P., Zhang, G., Bakken, T., Bongaarts, A., Bernard, A., Lein, E., Mello, C.V., Hartemink, A.J., Jarvis, E.D., 2014. Convergent transcriptional specializations in the brains of humans and song-learning birds. Science 346, 1256846.
Pfizer, 2018. AROMASIN (Exemestane) Product Monograph Kirkland, Quebec.
Pohl-Apel, G., Sossinka, R., 1984. Hormonal determination of song capacity in females of the Zebra finch: critical phase of Treatment1. Z. Tierpsychol. 64, 330–336.
Qi, L.M., Wade, J., 2013. Sexually dimorphic and developmentally regulated expression of tubulin-specific chaperone protein A in the LMAN of zebra finches. Neuroscience 247, 182–190.
Ralph, C.L., 1969. The control of color in birds. Am. Zool. 9, 521–530.
Riebel, K., 2009. Chapter 6 Song and Female Mate Choice in Zebra Finches: A Review, Advances in the Study of Behavior. Academic Press, pp. 197–238.
Ross, L.A., Del Bene, V.A., Molholm, S., Frey, H.P., Foxe, J.J., 2015. Sex differences in multisensory speech processing in both typically developing children and those on the autism spectrum. Front. Neurosci. 9, 185.
Sahores, A., Luque, G.M., Wargon, V., May, M., Molinolo, A., Becu-Villalobos, D., Lanari, C., Lamb, C.A., 2013. Novel, low cost, highly effective, handmade steroid pellets for experimental studies. PLoS One 8, e64049.
Schlinger, B.A., Arnold, A.P., 1991. Androgen effects on the development of the zebra finch song system. Brain Res. 561, 99–105.
Shaughnessy, D., Hyson, R., Bertram, R., Wu, W., Johnson, F., 2018. Female zebra finches do not sing yet share neural pathways necessary for singing in males. J. Comp. Neurol. 527.
Simpson, H.B., Vicario, D.S., 1991a. Early estrogen treatment alone causes female zebra finches to produce learned, male-like vocalizations. J. Neurobiol. 22, 755–776.
Simpson, H.B., Vicario, D.S., 1991b. Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations. J. Neurobiol. 22, 777–793.
Somes, R.G., Smyth, J.R., 1967. Effects of estrogen on feather phaeomelanin intensity in the Fowl1,2. Poult. Sci. 46, 26–32.
Takatoh, J., Nelson, A., Zhou, X., Bolton, M.M., Ehlers, M.D., Arenkiel, B.R., Mooney, R., Wang, F., 2013. New modules are added to vibrissal premotor circuitry with the emergence of exploratory whisking. Neuron 77, 346–360.
Tang, Y.P., Wade, J., 2010. Sex- and age-related differences in ribosomal proteins L17 and L37, as well as androgen receptor protein, in the song control system of zebra finches. Neuroscience 171, 1131–1140.
Tchernichovski, O., Nottebohm, F., Ho, C.E., Pesaran, B., Mitra, P.P., 2000. A procedure for an automated measurement of song similarity. Anim. Behav. 59, 1167–1176.
Tehrani, M.A., Veney, S.L., 2018. Intracranial administration of the G-protein coupled estrogen receptor 1 antagonist, G-15, selectively affects dimorphic characteristics of the song system in zebra finches (Taeniopygia guttata). Developmental Neurobiology 78, 775–784.
Theodorsson, A., Hilke, S., Rugarn, O., Linghammar, D., Theodorsson, E., 2005. Serum concentrations of 17β-estradiol in ovariectomized rats during two times six weeks crossover treatment by daily injections in comparison with slow-release pellets. Scand. J. Clin. Lab. Invest. 65, 699–706.
Tomaszycki, M.L., Peabody, C., Replogle, K., Clayton, D.F., Tempelman, R.J., Wade, J., 2009. Sexual differentiation of the zebra finch song system: potential roles for sex chromosome genes. BMC Neurosci. 10, 24.
Vahaba, D.M., Hecsh, A., Remage-Healey, L., 2019. Blocking neuroestrogen synthesis modifies neural representations of learned song without altering vocal imitation accuracy in developing songbirds. bioRxiv 702704.
Vedder, L.C., Bredemann, T.M., McMahon, L.L., 2014. Estradiol replacement extends the window of opportunity for hippocampal function. Neurobiol. Aging 35, 2183–2192.
Wade, J., Arnold, A.P., 1994. Post-hatching inhibition of aromatase activity does not alter sexual differentiation of the zebra finch song system. Brain Res. 639, 347–350.
Walters, M.J., Harding, C.F., 1988. The effects of an aromatization inhibitor on the reproductive behavior of male zebra finches. Horm. Behav. 22, 207–218.
Wang, X., Chen, S., 2006. Aromatase destabilizer: novel action of exemestane, a food and drug administration–approved aromatase inhibitor. Cancer Res. 66, 10281.
Wang, R., Chen, C.-C., Hara, E., Rivas, M.V., Roulhac, P.L., Howard, J.T., Chakraborty, M., Audet, J.-N., Jarvis, E.D., 2015. Convergent differential regulation of SLIT-ROBO axon guidance genes in the brains of vocal learners. J. Comp. Neurol. 523, 892–906.
Wardell, S.E., Kazmin, D., McDonnell, D.P., 2012. Research resource: transcriptional profiling in a cellular model of breast cancer reveals functional and mechanistic differences between clinically relevant SERM and between SERM/estrogen complexes. Mol. Endocrinol. 26, 1235–1248.
Wardell, S.E., Nelson, E.R., McDonnell, D.P., 2014. From empirical to mechanism-based discovery of clinically useful Selective Estrogen Receptor Modulators (SERMs). Steroids 90, 30–38.
Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Kunstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., Heger, A., Kong, L., Ponting, C.P., Jarvis, E.D., Mello, C.V., Minx, P., Lovell, P., Velho, T.A., Ferris, M., Balakrishnan, C. N., Sinha, S., Blatti, C., London, S.E., Li, Y., Lin, Y.C., George, J., Sweedler, J., Southey, B., Gunaratne, P., Watson, M., Nam, K., Backstrom, N., Smeds, L., Nabholz, B., Itoh, Y., Whitney, O., Pfenning, A.R., Howard, J., Volker, M., Skinner, B. M., Griffin, D.K., Ye, L., McLaren, W.M., Flicek, P., Quesada, V., Velasco, G., Lopez-Otin, C., Puente, X.S., Olender, T., Lancet, D., Smit, A.F., Hubley, R., Konkel, M.K., Walker, J.A., Batzer, M.A., Gu, W., Pollock, D.D., Chen, L., Cheng, Z., Eichler, E.E., Stapley, J., Slate, J., Ekblom, R., Birkhead, T., Burke, T., Burt, D., Scharff, C., Adam, I., Richard, H., Sultan, M., Soldatov, A., Lehrach, H., Edwards, S.V., Yang, S. P., Li, X., Graves, T., Fulton, L., Nelson, J., Chinwalla, A., Hou, S., Mardis, E.R., Wilson, R.K., 2010. The genome of a songbird. Nature 464, 757–762.
Weis, S., Patil, K., Hoffstaedter, F., Nostro, A., Yeo, B.T.T., Eickhoff, S.B., 2019. Sex classification by resting state brain connectivity. bioRxiv 627711.
Werling, D.M., Geschwind, D.H., 2013. Sex differences in autism spectrum disorders. Curr. Opin. Neurol. 26, 146–153.
Whitehead, C.C., 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83, 193–199.
Whitney, O., Pfenning, A.R., Howard, J.T., Blatti, C.A., Liu, F., Ward, J.M., Wang, R., Audet, J.-N., Kellis, M., Mukherjee, S., Sinha, S., Hartemink, A.J., West, A.E., Jarvis, E.D., 2014. Core and region-enriched networks of behaviorally regulated genes and the singing genome. Science 346, 1256780.
Wobbrock, J., Findlater, L., Gergle, D., Higgins, J., 2011. The Aligned Rank Transform for Nonparametric Factorial Analyses Using Only ANOVA Procedures.
Wu, M.V., Manoli, D.S., Fraser, E.J., Coats, J.K., Tollkuhn, J., Honda, S.-I., Harada, N., Shah, N.M., 2009. Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 139, 61–72.

ZEBrA. Oregon Health & Science University, Portland, OR 97239.