Supporting Information

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SI Results

Alternative Promoter Use Does Not Vary by Haplotype. To determine whether estrogen receptor α (ERα) mRNA is processed differently in the two morphs, we looked for evidence of alternative splice isoforms from each haplotype using recently generated RNA-seq data from white-throated sparrow brain (*SI Materials and Methods*). Two alternative first exons for estrogen receptor 1 (*ESR1*) were observed on the basis of reads spanning splice junctions, suggesting that transcription of the *ESR1* gene can be regulated by up to three distinct promoters (Fig. S1). Both of these alternative first exons spliced into the first exon of the third transcript, resulting in three isoforms that differed only at the 5′ end. One of the extended transcripts (containing exon 1b) is predicted to result in a protein that is 34 amino acids longer than the other two transcripts. No evidence for additional splice isoforms of the gene was revealed by either the alignment or the assembly of the RNA-seq data. PCR of transcripts in ZAL2/2 and $2^m/2^m$ homozygous individuals confirmed that all three isoforms were transcribed from both chromosomes. Three SNPs were identified in the first exon shared by all three isoforms, two of which were used to phase sequences. One SNP was identified in exon 1b, within the predicted translated region, that would not affect the translated protein sequence. No genomic information was available for exon 1c on the $ZAL2^m$ chromosome.

Multiple isoform-specific promoters have been identified for *ESR1* in human, rat, mouse, and chicken (1–3). In each of these species, isoform-specific promoters initiate transcription from exon 1a or upstream exons, which are spliced into a conserved acceptor splice site located in exon 1a. On the basis of sequence homology between sparrow and chicken, we determined that the two alternate exons identified in white-throated sparrow splice into exon 1a at this conserved splice site but do not align to any of the alternative first exons identified in chicken (2).

The ZAL2 and $ZAL2^m$ ERα Alleles Encode Alternative Protein Isoforms. Alignment of the translated ERα alleles revealed no differences between ZAL2 and $ZAL2^m$ in the DNA and ligand-binding domains, which are typically highly conserved across species. For example, ZAL2 and $ZAL2^m$ show 77% sequence identity with human ERα across the entire protein and 100% and 94% sequence identity in the DNA and ligand-binding domains, respectively. The ZAL2 and $ZAL2^m$ alleles code for 597 amino acids, with two fixed differences driving a Val73Ile and Ala552Thr polymorphism in $ZAL2^m$. To analyze the potential impact of these fixed sequence differences between the ZAL2 and $ZAL2^m$ alleles, we generated an alignment containing a reduced set of 22 ERα sequences sampling diverse taxa (Fig. S2). The first polymorphism was observed within the unstructured N-terminal activation function 1 region (AF-1), which confers ligand-independent transactivation via direct interactions with TATA box binding protein and plays a role in ligand-induced receptor degradation (4–6). This region of the protein is unstructured in solution, and folding is induced upon interaction with basal transcription factors (7), thus little is known about the structural and sequence requirement for AF-1–mediated transactivation. Although Val73Ile is located within a highly conserved region within the AF-1, Ile is present in the salamander, newt, and lungfish (Fig. S2). Valine, leucine, and isoleucine are similar hydrophobic amino acids that differ by a single methyl group, making them nearly isosteric. These three amino acids are classified as branched chain hydrophobic amino acids and are often functionally interchangeable with a protein sequence or structure. This, in combination with the observation that either a valine, leucine, or isoleucine is tolerated at the equivalent position in orthologs, strongly suggests that the Val73Ile replacement has little or no functional consequence on the derived function of the $ZAL2^m$ ERα.

We also observed an Ala552Thr polymorphism located at the extreme C terminus of the ligand-binding domain, just past the activation function helix. This residue is typically disordered in crystal structures and does not pack against the body of the protein (8, 9). Alanine and threonine are in different classes of amino acids, on the basis of their chemical parameters. Because they are small amino acids, however, and because this substitution is located on a disordered stretch of protein, it is not likely to have significant functional impact. Our alignment reveals that this position tolerates alanine, threonine, serine, arginine, phenylalanine, or asparagine in ERα orthologs (Fig. S2). For example, whereas an alanine at this position is conserved among birds, reptiles, and most mammals, a threonine is present in hamsters and amphibians. Given its location and the ability of ERα to tolerate a threonine at this position in other species, we do not expect Ala552Thr to impact either ligand binding or interaction with transcriptional coactivators.

SI Materials and Methods

Analysis of Promoter Sequence. To analyze differentiation of the *ESR1* promoter region, we aligned a 2-kb sequence (GenBank ID DP001174; 3710343–3712276) upstream of the most proximal exon 1 and confirmed all polymorphisms by sequencing the region in 5 white-striped (WS) and 5 tan-striped (TS) individuals. For all of the SNPs, all of the WS birds showed evidence of heterozygosity, whereas none of the TS birds did. ZAL2 and $ZAL2^m$ sequences were then submitted to TFSEARCH for prediction of transcription factor binding sites (10). For prediction, the vertebrate transcription factor matrix was used with a threshold of 85.0. To assess which regions of the *ESR1* promoter may be readily available for transcription factor binding, we used DNase hypersensitivity data from the ENCODE project (11), accessed through the University of California, Santa Cruz genome browser (12).

Luciferase Reporter Assays. To assess whether the cis-acting polymorphisms we identified have the potential to modify ERα gene expression, we performed luciferase reporter assays in HeLa cells. A 2-kb sequence in the *ESR1* promoter (of the most proximal exon 1; see below) was isolated from a $ZAL2/2^m$ heterozygote and cloned upstream of firefly luciferase in the pGL3 basic vector. Constructs were prepared from both the ZAL2 and $ZAL2^m$ alleles. Twenty-five or 50 ng (experiment 1) or 25, 50, 100, or 200 ng (experiments 2 and 3) of ZAL2 or $ZAL2^m$ construct and 5 (experiment 1) or 10 ng (experiments 2 and 3) of constitutively active *Renilla* luciferase were transfected with FuGene HD (Promega) in OptiMEM (Invitrogen) into HeLa cells cultured in AlphaMEM (Invitrogen) supplemented with 10% charcoal stripped FBS (Atlanta Biologicals). For each experiment, $n = 6$ wells per construct at each concentration. After 18 h, firefly and *Renilla* luciferase activities were measured with the Dual-Glo assay system (Promega) on a Biotek Synergy plate reader. Firefly luciferase activity was normalized to that of *Renilla* luciferase for each well, and the resulting ratios for the $ZAL2^m$ construct were normalized to that for cells transfected with the ZAL2 construct within each concentration and experiment (13). Data were analyzed in an ANOVA (SPSS v.20) to assess the effects of construct, construct concentration, and experiment (13).

To test whether estradiol (E2) affects transcription efficiency, we repeated the luciferase experiments in the presence or absence of E2. Cells were transfected and cultured as described above; after 24 h, the cells were treated with 1 μM 17-β estradiol (Calbiochem) or media. After an additional 24 h, firefly and *Renilla* luciferase activities were measured as above. The ratios of luciferase to *Renilla* activity were normalized to that for cells transfected with the ZAL2 construct and not treated with E2, within each concentration and experiment. Data were analyzed in an ANOVA as above to assess the effects of construct, E2, and the interactions between them.

Field Study. To test whether ERα expression in the brain differs between the morphs and correlates with territorial or parental behavior, we conducted a field study in a natural population of breeding white-throated sparrows near Argyle, Maine. The sample consisted entirely of WS male × TS female or WS female × TS male pairs (no same-morph pair types). Territories of each pair type were evenly dispersed throughout the site. We quantified territorial aggression during the early stages of breeding to coincide with the peak of territorial behavior. During the nestling stage, at a different location on the site, we quantified the rate at which the parents provisioned the nestlings (number of trips made by the parents to the nest to feed nestlings). The methods for each type of behavioral test have been previously described (14–16) and are summarized below.

Simulated territorial intrusions. Early during the breeding seasons of 2010 and 2011, we captured and banded territory-holding males. Their female mates were also captured when possible. Thirteen pairs were WS-male × TS female, and 12 were the opposite pair type. Each bird received an aluminum US Geological Survey identification band and a unique combination of colored leg bands, and was released at the site of capture. Between 3 and 11 d later, during the peak fertile period, we conducted simulated territorial intrusions (STIs), a standard paradigm for eliciting territorial responses (16, 17). A live male decoy in a cage was placed in the center of a pair's territory, and conspecific male song played from a portable speaker next to the cage. Each recording consisted of the same male singing once every 15 s. The recording was played until the resident male was detected in the area, and for 10 min after that. In most cases the female was also present. During the 10-min behavioral observation, we recorded the number of soft songs, partial songs, and full songs given by the residents (for descriptions of each type of song, see ref. 18). Both male and female white-throated sparrows can perform all of these vocalizations in response to STI (16, 18, 19), so we scored all of the vocalizations for both members of each pair. Females may also perform a trill that accompanies copulation solicitation displays (18), but only one female in this study solicited a male decoy. Thus, female responses in this study, like those of males, were largely aggressive in nature. Two females that did not respond to playback during the STI were discovered to be incubating eggs and were excluded from behavioral analyses.

Because the aggressive responses of residents can vary according to the morph of the intruder (16), we conducted two STIs per pair, one with a TS decoy and one with a WS decoy, in counterbalanced order, on consecutive days. We used the same decoys ($n = 10$ WS, 10 TS) for no more than two pairs within a pair type. Each decoy was randomly assigned a single song recording, which was used only for that decoy in a given year.

On the day after the second STI, we captured both members of each pair by luring them into a mist net with conspecific song playback. Most birds were captured rapidly (mean 4.5 ± 0.4 min). Within 8.5 min of capture, a blood sample (150–350 μL) was collected from the brachial vein for RIA of sex steroid hormones. Each bird was then deeply anesthetized with isoflurane and decapitated. Brains were rapidly harvested from the skulls and frozen in powdered dry ice. Brains were stored at −80 °C at the University of Maine, then shipped frozen to Emory University, where they were again stored at −80° until cryosectioning.

Measurement of parental behavior. We used a different area of our study site to record parental behavior later in the breeding season, during June and July of each year. Nests were found either by flushing incubating or brooding females or by observing nesting behavior. Once located, nests were monitored every other day until hatching. Each parent was captured and fitted with aluminum and plastic leg bands as described above. The identity of the parents was confirmed by observations of their parental behavior at the nest.

The morphs have been shown to differ in the rate at which they provision young, but not in the number or size of items they deliver per trip (20). We therefore recorded on video the number of feeding trips per hour made by each parent. Sanyo Xacti HD video cameras were concealed among vegetation 3–5 m from the nest, and parental behavior was recorded for 3 continuous hours, before 11:30 AM, on 2 d when the nestlings were 5 and 6 d old (fledging normally occurs on day 9; see ref. 4). Rates of provisioning were later scored by trained observers, who also counted the spontaneous songs of the male when he could be heard in the recording (parental females rarely sing). In most cases the observer was able distinguish the song of the focal male from that of his neighbors; song data were excluded if the songs were indistinguishable.

When nestlings were 7 d old, we placed mist nets near the nest to capture the parents. Blood samples and brain tissue were collected and processed as described above. We also collected the nestlings as part of a different study.

Radioimmunoassay. We assayed plasma testosterone (T), E2, and 5-α dihydrotestosterone (DHT) according to the procedures of Stevenson et al. (21). Briefly, plasma samples were fractionated by column chromatography to separate gonadal steroids and analyzed by RIA. Hormone concentrations were corrected for individual extraction efficiencies; average recoveries were 70% for T, 30% for DHT, and 48% for E2. Samples were run in two assays, and samples from 2010 and 2011 were randomly allocated to each assay. Interassay variation was 10.2% for T, 9.1% for DHT, and 5.5% for E2. Intraassay variations were 13.4% and 14.2% for T, 9.4% and 9.1% for DHT, and 4.1% and 13.1% for E2. Lower detectable limits were 0.11 ng/mL for T, 0.30 ng/mL for DHT, and 0.11 ng/mL for E2.

Riboprobe preparation. To perform a quantitative analysis of ERα mRNA expression in a number of small brain regions, we opted to use a ^{35}S-labeled riboprobe. The riboprobe was prepared according to a standard protocol (22). Briefly, we amplified a 501-bp sequence spanning exons 4–8 (GenBank ID DP001174; 2646051–26889054) and cloned it into pCRII using a TOPO TA Cloning Kit (Invitrogen). The sequence of the insert was confirmed by Retrogen and contained no ZAL2/2^{m} polymorphisms. Linearized plasmids were used to generate antisense or sense riboprobes with a T7/SP6 Riboprobe In Vitro Transcription Kit (Promega) and ^{35}S-UTP (PerkinElmer). The resulting riboprobes were purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare) and diluted in hybridization buffer to a concentration of 10^7 cpm/mL.

In situ hybridization. Our in situ hybridization (ISH) protocol has been previously described (22). Briefly, brains were sectioned at 20 μm onto Superfrost Plus microscope slides (Fisher). Seven series were cut, one of which was used in this study. After sections were defrosted, delipidated, acetylated, and dehydrated (23), 100 μL of riboprobe in hybridization buffer was applied to each slide. Slides were then coverslipped and incubated at 56 °C overnight in a mineral oil bath (24). The following day, the slides were washed, treated with RNase, washed again at 55 °C, and dehydrated. Dry slides were placed into film cassettes against Kodak BioMax maximum-resolution film and protected from light for 3 d. The film was then developed in a Konica SRX101-A

developer and scanned at 2,500 dpi using a digital Epson V700 scanner. The slides were then Nissl-stained in toluidine blue and scanned at the same resolution.

Tissue from each sex and behavioral study was run separately. In other words, we performed separate ISH runs on tissue from STI males, STI females, parental males, and parental females. With the exception of the STI females, each of these groups was further divided into two separate ISH runs with year and morph balanced across them. Thus, the tissue was processed in a total of seven ISH runs.

Image analysis. We quantified ERα mRNA labeling in regions of interest (ROIs) that were previously implicated in territorial aggression or parenting in songbirds. Most of these regions are part of an estrogen-sensitive social behavior network (25, 26). ERα mRNA signal was quantified in each ROI in two to four consecutive sections, which in this series were 140 μm apart. Our ROIs were as follows: the medial preoptic area (POM; at the level of the split in the septomesencephalic tract), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), the paraventricular nucleus (PVN), the medial portion of the bed nucleus of the stria terminalis (BSTm), the ventrolateral portion of the caudal lateral septum (LSc.vl), and the medial amygdala (MeA). We also quantified signal in the song nucleus HVC (used as a proper name) and the intercollicular nucleus (ICo), which are vocal control regions that express ERα.

Digital images of the films were opened in ImageJ and converted to 8-bit. ROIs were located using landmarks in the Nissl-stained sections. When the ERα signal had clear boundaries (e.g., HVC, ICo), we traced it using the lasso tool. For VMH, a rectangle (~2–4 mm^2) was placed across the midline that encompassed the ERα signal on both sides. For other ROIs, we sampled the label by placing a circle (~0.05 to ~0.3 mm^2, depending on the region) inside it. The average gray value of each selection was recorded. For each selection we obtained a background measurement by sampling a nearby region without obvious label. The corrected gray value of the ROI was then expressed as the absolute value of the difference. Corrected gray values were averaged across the consecutive sections to arrive at a single corrected gray value for each ROI in each bird.

Statistics. Our goals for the field study were first to test whether ERα expression differs between the morphs, and second to test whether that expression may mediate the effect of morph on behavior. To test the first hypothesis, we used analysis of covariance (ANCOVA) to test for effects of morph while controlling for plasma T and E2 level. To minimize type I error due to testing for effects in nine different brain regions, we first entered the corrected gray values for each region of interest in all birds into a single multiple analysis of covariance (MANCOVA) for each sex, with morph and ISH run as between-subjects variables and plasma T and E2 as covariates. Because MANCOVAs exclude all of the data from a bird with one missing data point, any missing data were replaced by the series mean (<2% of data). We found highly significant effects of morph for both sexes (*Results*) and no interactions between ISH run and morph. These tests were thus followed by univariate tests for effects of morph on ERα expression within each ROI. No missing values were replaced for the univariate tests. As we did for the MANCOVAs, we entered ISH run as a fixed effect and plasma T and E2 as covariates for each ANCOVA. All analyses were carried out in SPSS v.20. Because tissue from the two sexes and the two behavioral studies was processed in separate ISH runs, we did not test for effects of sex or breeding stage.

To test our second hypothesis, whether ERα expression may mediate the effects of morph on behavior, we performed mediation analysis with bootstrapping and bias-corrected confidence estimates (27, 28). This method tests whether the effect of morph on behavior (*c* path, Fig. 3) could be mediated by ERα (*a* and *b* paths). This analysis requires that the *a*, *b*, and *c* paths be significant; in other words, a significant correlation between morph and ERα (*a* path), between ERα and behavior (*b* path), and between morph and behavior (*c* path). For ERα to qualify as a mediator, the direct effect of morph on behavior (*c'* path) must be reduced when the *ab* path, which includes ERα, is considered (27). We restricted these analysis to the behavioral studies in which we clearly demonstrated the *a*, *b*, and *c* paths using appropriate corrections for multiple tests. In the males, there was a significant morph difference in both STI-induced song rate ($P = 0.021$) and provisioning rate for the first nest of the season ($P = 0.041$) in this sample. Thus, we used a sample of males in which there was a clear, significant effect of morph on behavior (*c* path) to test whether ERα expression mediates that effect. The number of females for which we had data on song was relatively small ($n = 5$ of each morph), yet within that sample we were able to detect a morph difference in the rate of STI-induced song ($P = 0.037$). Consistent with other studies of parental care in this species, however, we have detected no morph difference in female provisioning rate at this or nearby sites (14). We thus proceeded with behavioral analysis for female song only.

In addition to an effect of morph on behavior (*c* path), a mediation model requires that morph predicts the mediator (*a* path, Fig. 3) and the mediator predicts behavior (*b* path). To reduce the number of ROIs for which we performed the bootstrapping analysis, we included only ROIs in which ERα expression met these requirements as a mediator. For all ROIs in which there was an effect of morph (Fig. 3), thus satisfying the *a* path requirement, we ran partial Pearson correlations between ERα expression and either songs in response to STI or nestling provisioning trips per hour (*b* path), controlling for plasma T and E2 (Table S1). Alpha was adjusted for the number of tests in each group of birds using a sequential Bonferroni correction. We then performed bootstrapping analyses for those ROIs for which there was a significant correlation between ERα expression and behavior (two regions for the STI males and one for the parental males). In each model we included plasma T and plasma E2 as possible alternative mediators. We obtained 95% confidence intervals for each indirect effect with 5,000 bootstrap samples. All analyses were carried out in SPSS v.20 with the INDIRECT custom dialogue (28).

Alternative Splicing and Promoter Use. To look for evidence that haplotype affects mRNA structure, we used recently generated RNA-seq data from three white-throated sparrows of the genotypes ZAL2/2, ZAL2/2^m, and a rare "superwhite" ZAL2^m/2^m (29). The paired-end RNA-seq reads from the three individuals (205–216 million pairs of 101-nt reads) were aligned to an assembly of the white-throated sparrow genome (GenBank Assembly ID GCA_000385455.1; data generated by The Genome Institute, Washington University School of Medicine) using a splicing aware aligner, STAR (30). All of the reads mapping to the *ESR1* locus and upstream and downstream regions were visualized using the IGV viewer (31) to identify potential alternative splicing events. Uniquely mapped individual reads or read pairs that spanned introns were visually identified and evaluated in the context of all uniquely mapped reads in the region to determine the location of all detectable *ESR1* exons and corresponding splice sites. Note that the *ESR1* gene is completely contained within a single scaffold in the genome assembly. The physical spacing of the exons and exon/intron boundaries was determined by aligning the inferred sparrow transcripts to the zebra finch genomic region containing *ESR1* using Spidey (32), an mRNA-to-genomic aligner. To confirm differential promoter use, we designed primers to amplify the alternative exons and inspected the sequence of bands produced by PCR. To determine which allele was expressed, we identified SNPs between the alleles located on ZAL2 chromosome vs. ZAL2^m by aligning transcripts to sparrow BAC assemblies for ZAL2 and ZAL2^m (DP001173.2, DP001174.2). A common reverse primer

(R1: CTGGACATCCTCTCCCGGAT) and three unique forward primers (F1A: TGTTCCTTGCTCACTGCCAT; F1B: GAGGGAATGGCAACAGCCT; F1C: GTGCAAAGAAGCCACCCAAA) were designed to amplify each splice isoform of *ESR1*. A sequencing primer (S1: AGCTGCCCTGATCGTTTTCA) was designed downstream of three SNPs located in the region shared by all three isoforms. PCR products were sequenced, and transcripts were phased according to the SNPs in the products. Sequencing was done in cDNA generated from the brains of one ZAL2/2 homozygote, the $ZAL2^m/2^m$ individual, and three $ZAL2/2^m$ heterozygotes.

Analysis of Protein Sequence. We identified SNPs and insertion/deletion polymorphisms in the *ESR1* gene by comparing sequence from a BAC clone library constructed from a WS female (http://bacpac.chori.org/library.php?id=469). The *ESR1* sequence is located on target 213 (ZAL2 allele: DP001173; $ZAL2^m$ allele: DP001174). We translated the coding region and compared the protein sequences in ClustalW using Geneious Pro-6.1.6 (Biomatters). To predict the functional impact of observed differences, the ZAL2 and $ZAL2^m$ protein sequences were aligned in Geneious Pro with 128 other ERα sequences identified either through a BLAST search or retrieved directly from the Ensembl database. This alignment was reduced to 22 ERα sequences sampling diverse taxa (Fig. S2). For differences located with the ERα ligand binding domain, structural analyses were performed using Pymol (Schödenger).

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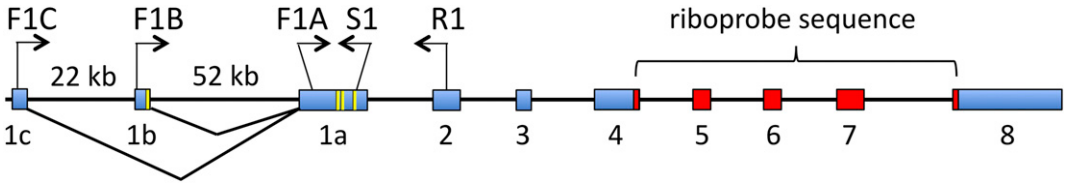

Fig. S1. Alternative promoter use in white-throated sparrow *ESR1*. Two alternative first exons were observed. All three isoforms were transcribed from both alleles. No evidence for additional splice isoforms was detected, nor were any other alternative transcripts observed. PCR was used to amplify alternate transcripts are indicated by arrows. R1: common reverse primer. F1A, F1B, F1C: forward primers used to amplify transcripts containing exons 1a, 1b, and 1c, respectively. S1: primer used for sequencing and phasing. Locations of SNPs are indicated by yellow lines. The riboprobe for the in situ hybridization (shown in red) contained no SNPs or alternatively spliced regions.

	69 72 80	540 552 556
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ZAL2^{m}	...-----TAP**I**YSSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
ZAL2	...-----TAP**V**YSSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
Collard flycatcher	...-----TAP**V**YSSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
Zebra finch	...-----TAP**V**YSSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
Budgerigar	...-----TAP**V**YSSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
Peregrine falcon	...-----TAP**V**YSSTTLSY...	...LLLEMLDAHRLH**A**PGAR...
Chicken	...-----TAP**V**YGSTTLSY...	...LLLEMLDAHRLH**A**PAAR...
Crocodile	...-----AAP**V**YSSTSLSY...	...LLLEMLDAHRLH**A**PAAR...
Sea turtle	...-----AAP**V**YSSTSLSY...	...LLLEMLDAHRLH**A**PAAK...
Cow	...AAPASAP**V**YGQSGLPY...	...LLLEMLDAHRLH**A**PANF...
Human	...AAAANAQ**V**YGQTGLPY...	...LLLEMLDAHRLH**A**PSTR...
Hamster	...AT-APAP**V**YGQTGIAY...	...LLLEMLDAHRLH**T**PVSR...
Rat	...AAGASAP**V**YGQSSITY...	...LLLEMLDAHRLH**A**PASR...
Giant Salamander	...---AAAP**I**YSSASLNY...	...LLLEMLDAHRIH**T**PRDK...
Newt	...---AAAP**I**YSSASLSY...	...LLLEMLDAHRIH**T**PRDK...
African clawed frog	...----TAAP**V**YSSASLSY...	...LLLEMLDAHRIH**T**PRDK...
Xenopus	...----AAAP**V**YSSASLSY...	...LLLEMLDAHRIH**T**PRDK...
African lungfish	...SVPATGP**I**YNSPGVSY...	...LLLEMLDAHRLH**R**LGKK...
Sturgeon	...AASAAAP**L**YNPSNLGF...	...LLLEMLDAHRLH**F**LGDT...
Lamprey	...-SGASQP**L**PPSCTEFS...	...LLLELLDAHSLQ**N**TGLR...
Zebra fish	...PTGTSSP**A**EPASV-GY...	...LLLEMLDAQRFQ**S**SGKV...
Rainbow Trout	...PAQGPAP**L**YYSTT---...	...LLLEMLDGHRLQ**S**PGKV...

Fig. S2. Alignment of ZAL2 and ZAL2^{m} *ESR1* alleles with those of other species. We noted two sequence polymorphisms that alter protein sequence. Val73Ile is located within a highly conserved region within the AF-1; because Ile also occurs in the salamander, newt, and lungfish, Val73Ile is unlikely to significantly impact ERα function. Similarly, an Ala552Thr polymorphism located within the ligand-binding domain is present also in hamster and amphibians and is unlikely to impact either ligand binding or interaction with transcriptional coactivators.

Table S1. Results of partial Pearson correlations between ERα mRNA expression and morph-dependent behaviors in males and females

	Songs			Trips		
Sex	R^2	*P*	df	R^2	*P*	df
Males						
POM	0.015	0.618	17	0.812	<0.001*	8
VMH	0.182	0.054	19	0.289	0.109	8
PVN	0.332	0.006*	19	0.468	0.031	8
BSTm	0.105	0.151	19	0.010	0.788	8
LSc.vl	0.013	0.621	19	0.181	0.220	8
MeA	0.340	0.006*	19	0.084	0.419	8
HVC	0.142	0.101	18	0.123	0.322	8
Females						
AH	0.146	0.350	6			
VMH	<0.001	0.991	6			
PVN	0.650	0.016	6			
BSTm	0.052	0.589	6			
LSc.vl	0.310	0.152	6			
MeA	0.403	0.091	6			

Tests were performed only for brain regions in which we found a significant effect of morph (Fig. 3); in other words, for which the *a* path for mediation analysis (Fig. 4) was satisfied. No tests were performed for female provisioning trips because that behavior did not differ between the morphs in this study (*c* path not satisfied). Plasma concentrations of T and E2 were controlled in each model. Sequential Bonferroni adjustments were performed to account for seven tests in each group of males or six tests in females. The *b* path for mediation analysis was satisfied for male song for PVN and MeA, and for male trips for POM.