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Rapid effects of estradiol on aggression depend on genotype in the white-throated sparrow, a species with an estrogen receptor polymorphism

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# Rapid effects of estradiol on aggression depend on genotype in the white-throated sparrow, a species with an estrogen receptor polymorphism

By

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An abstract of

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**Abstract**

Rapid effects of estradiol on aggression depend on genotype in the white-throated sparrow, a species with an estrogen receptor polymorphism

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The white-throated sparrow represents a powerful model in behavioral neuroendocrinology because it occurs in two plumage morphs that differ with respect to steroid-dependent social behaviors. Birds of the white-striped (WS) morph engage in more territorial aggression than birds of the tan-striped (TS) morph. This behavioral polymorphism is caused by a chromosomal inversion that has captured many genes, including estrogen receptor alpha (ERα). ERα expression depends on morph in a number of brain regions implicated in social behavior, including the rostral medial preoptic area (rPOM) and nucleus taeniae of the amygdala (TnA), suggesting that the behavioral polymorphism might be explained by differential sensitivity to sex steroids. In this study, we tested whether exogenous estradiol (E2) administration produces differential effects on behavior and the brain in the two morphs, as predicted by the ERα polymorphism. We administered a bolus dose of E2 and quantified aggression toward a conspecific 10 min later—a time point at which E2 is known to increase aggression in song sparrows (Experiment 1). E2 increased aggression in WS birds, but not TS birds. Thus, in this study we found that the rapid effects of E2 depended on morph. To map neural responses to E2, we administered an identical dose of E2 and quantified Egr-1 Expression in regions with known differential expression of ERα (Experiment 2). E2 treatment decreased Egr-1 immunoreactivity (IR) in both rPOM and TnA, but this effect did not depend on morph. We then tested whether morph differences in Egr-1-IR emerge after birds are treated with E2 for much longer (7 days; Experiment 3). We found an interaction between morph and treatment; E2 treatment increased Egr-1 in the TnA of WS birds, but decreased it in TS birds. Overall, our results suggest that the ERα polymorphism may contribute to morph differences in aggression *via* both nongenomic and genomic mechanisms.

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## Introduction

Polymorphic behavior can be caused by disruptive selection pressure on genes with pleiotropic effects, such as those involved in sex steroid signaling (Ketterson & Nolan, 1992; Horton, et al., 2014a, Horton et al., 2014b; Tuttle et al., 2016). For example, social behaviors such as parental care and territorial aggression are negatively and positively correlated, respectively, with plasma sex steroids in the white-throated sparrow (*Zonotrichia albicollis*). In many vertebrates, high levels of sex steroids such as testosterone (T) are associated with increased competition for territories or mates, and low T corresponds with increased parental effort (Archer, 2006; Ketterson & Nolan, 1994). Model organisms that exhibit polymorphic, hormone-dependent behavior, such as the white-throated sparrow, can be used for dissecting the mechanisms underlying those behaviors. Thus, the white-throated sparrow is a powerful model for understanding hormone-behavior relationships in vertebrates because it exhibits tradeoffs between territorial and parental behavior mediated by genetic divergence (Horton et al., 2014).

The cause of polymorphic behavior in the white-throated sparrow is a series of nested inversions on chromosome 2 has captured ≈1,100 genes (Thomas et al., 2008). The inversion has suppressed recombination, resulting in the divergence of captured genes (Thomas et al., 2008; Davis et al., 2011) such that the inverted and non-inverted haplotypes, ZAL2$^{m}$ and ZAL2, are now 1% different from each other (Huynh, Maney, & Thomas, 2011). Furthermore, ZAL2$^{m}$ segregates with plumage and behavior (Thomas et al., 2008; Throneycroft, 1975). White-striped birds (WS) are heterozygous for the ZAL2$^{m}$ rearrangement (Throneycroft, 1966; Throneycroft, 1975) and respond to territorial threats with higher levels of vocal aggression than tan-striped (TS) birds, which are ZAL2

homozygotes (Falls, 1969; Horton et al., 2014a; Lowther, 1961; Tuttle, 2003; Tuttle et al., 2016). Therefore, genes on chromosome 2 that contribute to aggression are likely responsible for the morph differences in territorial singing.

The captured genes that are likely candidates for explaining morph differences in aggression can be discovered by investigating the biology of territorial singing, which has a known endocrine basis. Territorial singing is positively correlated with sex steroids in many species of songbirds (Hau, 2007; Ketterson & Nolan, 1992; Sandell, 2007; Soma & Wingfield, 2001; reviewed by Soma, 2006), including the white-throated sparrow (Horton et al., 2014a). WS birds of both sexes sing more and have higher circulating levels of testosterone (T) and estradiol (E2) than do TS birds (Horton et al., 2014a; Horton et al., 2014b; Swett & Breuner, 2008). Morph differences in levels of plasma steroids, however, do not account for the polymorphism in territorial singing. When T was experimentally equalized, morph differences in singing persisted (Maney, Lange, Raees, Reid, & Sanford, 2009), suggesting that morph differences in plasma levels of steroids do not completely explain morph differences in singing. Given that singing in songbirds depends on sex steroids and the morphs differ in both song rates and plasma steroid levels, morph differences in song rates could be caused by polymorphisms in genes involved in the steroid signaling pathway (Horton et al., 2014b; Maney, Horton, & Zinzow-Kramer, 2015).

The morphs may differ in sensitivity to sex steroids because ESR1, the gene for estrogen receptor alpha (ERα), has been captured by the inversion (Horton et al., 2014b). ESR1 contains haplotype-specific sequences that may affect the transcription of ERα (Horton et al., 2014b). E2 acts through ERα to modulate territorial aggression in other

species. In resident-intruder tests, male mice with the ERα gene knocked out are less aggressive than wild-type littermates (Ogawa, Lubahn, Korach, & Pfaff, 1997; Scordalakes & Rissman, 2004), which increase attacking upon administration of ERα-specific agonists (Clipperton-Allen, Almey, Melichercik, Allen, & Choleris, 2011). Additionally, in mice and songbirds, individual variation in ERα expression in a number of brain nuclei predicts agonistic behavior and territorial singing (Trainor, Greiwe, & Nelson, 2006; Rosvall, Burns, Barske, Goodson, Schlinger, Sengelaub, & Ketterson, 2012). In the white-throated sparrow, differential expression of ERα is associated with morph differences in aggressive behavior (Horton et al., 2014b; Maney et al., 2015). Quantification of ERα mRNA in the brains of white-throated sparrows revealed that expression differs according to morph in at least eight brain regions (Horton et al., 2014b). WS birds have more ERα mRNA in the nucleus taeniae of the amygdala (TnA), the paraventricular nucleus (PVN), and HVC (used as a proper name). In contrast, TS birds have more ERα mRNA in the rostral medial preoptic area (rPOM), anterior hypothalamus (AH), medial ventromedial hypothalamus (VMH-m), bed nucleus of the stria terminalis (BSTm), and ventrolateral portion of the caudal lateral septum (LSc.vl) (Horton et al., 2014b). In these regions, morph differences in ERα expression may confer differential sensitivity to E2.

In the white-throated sparrow, ERα expression differs between the morphs throughout the brain, but only in TnA and PVN does it predict singing (Horton et al., 2014b; Zinzow-Kramer et al., 2015). Expression in TnA and PVN predicts singing even when morph and sex steroids are controlled in regression analyses (Horton et al., 2014b). Mediation analyses demonstrated that ERα expression in TnA and PVN explains

territorial singing better than morph itself. Considering these associations between ERα expression in TnA and PVN and territorial singing (Horton et al., 2014b), ERα expressed in these regions may mediate the morph differences in aggression. These analyses provide correlative evidence that ERα expression in these regions predicts aggression; however, a causal role for the ERα expression polymorphism in the behavioral polymorphism has not been firmly established experimentally. Thus, there is a need to assess the effects of experimental manipulation of E2 on behavior. Here, we tested whether E2 stimulates more aggression in WS birds than TS birds, as predicted by the ERα polymorphism.

Recently, Heimovics, Ferris, and Soma (2015) showed that E2 rapidly increased aggression in song sparrows (*Melospiza melodia*). A bolus dose of E2 stimulated attacking and other agonistic behaviors just 10 minutes later, suggesting that E2 can rapidly affect aggression in a confamilial sparrow. Here, we applied the behavioral paradigm of Heimovics et al. (2015) to white-throated sparrows to test the hypothesis that E2 would induce aggression more efficiently in one morph than the other. We used birds in non-breeding condition so that endogenous sex steroids would be naturally low and ERα unlikely to be saturated. Our hypothesis was testable in non-breeding birds because morph differences in ERα expression persist year-round in TnA and PVN, brain regions in which expression predicts territorial aggression (Maney et al., 2015; D.L. Maney, unpublished).

We hypothesized that morph differences in aggression are due in part to morph differences in sensitivity to E2 in the brain. E2 induces rapid changes in the brain through intracellular signaling; E2 binds to membrane-associated estrogen receptors and

stimulates the MAPK signaling pathway (reviewed in Micevych & Dominguez, 2009). Recently, Heimovics, Prior, Maddison, and Soma (2012) reported that E2 rapidly affected the phosphorylation of ERK (pERK), a molecule in the MAPK signaling cascade, in TnA of song sparrows 15 minutes after treatment with E2 (Heimovics et al., 2012). pERK serves as a transcription factor to induce the transcription of many genes, including Egr-1, an immediate early gene (Shi, Kishore, McMullen, & Nagy, 2002). Therefore, we quantified immunoreactivity (IR) for Egr-1, the downstream target of the rapid E2 signaling cascade, as a measure of the magnitude of the response to E2 to determine which regions were responding to E2 and whether that response depended on morph.

## Methods

### Experimental Design

In Experiment 1, we tested whether a bolus dose of E2 can rapidly induce aggression, as previously observed in song sparrows (Heimovics et al., 2015), and if so, whether that effect depends on morph. In Experiment 2, to map the neural responses to this dose of E2, we administered a dose of E2 identical to Experiment 1 and quantified the expression of Egr-1 in five brain regions in which ERα expression has been shown to depend on morph in this species (Horton et al., 2014b). Then, as a follow-up to Experiment 2, in Experiment 3 we did the same mapping study in birds that were treated with E2 for one week.

### Experiment 1

**Animals.** All procedures involving animals were approved by the Emory University Institutional Animal Care and Use Committee, were in keeping with all

federal, state, and local laws, and adhered to guidelines set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* WS (male *n=3*, female *n=12*) and TS (male *n=6*, female *n=15*) white-throated sparrows (*Zonotrichia albicollis*) were collected in mist nets on the campus of Emory University in Atlanta, GA during November and December of the 2014 fall migration. Sex was confirmed by PCR analysis of a small blood sample (Griffiths, Double, Orr, & Dawson, 1998). Assessments of morph were made using a PCR assay (Horton et al., 2013) and by visual inspection of plumage (Michopoulos, Maney, Morehouse, & Thomas, 2007; Piper & Wiley, 1989). Assessments of age were determined by the shape of the primary coverts and outer rectrices and the degree of skull ossification (Pyle, 1997). Birds were housed in the Emory animal care facility in walk-in flight cages (4' x 7' x 6'), supplied with *ad libitum* seed, Mazuri chow, and water, and supplemented with parsley or cilantro mix twice per week. The day length was kept constant at 10L:14D, which corresponds to the shortest day the birds would experience on their wintering grounds in Georgia. Prior to behavioral assays, birds were transferred to individual cages (15" x 15" x 17") inside walk-in sound-attenuating booths (Industrial Acoustics, Bronx, NY). They were housed two to six birds per booth until the behavioral trials. All booths were identical and the day length remained at 10L:14D throughout the experiment. In laboratory-housed birds kept on short days, morph is not related to dominance rank or aggression (Dearborn & Wiley, 1993; Harrington, 1973; Maney & Goodson, 2011; Piper & Wiley, 1989; Schlinger, 1987; Schwabl, Ramenofsky, Schwabl-Benzinger, Farner, & Wingfield, 1988; Watt, Ralph, & Atkinson, 1984; Wiley, Steadman, Chadwick, & Wollerman, 1999), so any dominance

relationships formed among the birds prior to the onset of the study are unlikely to have influenced their behavior in a morph-specific way during the behavioral test.

**Pre-screening for social dominance.** The goal of this study was to determine whether the effect of E2 on aggression depends on morph. Therefore, we used a protocol that has previously been used to demonstrate that E2 facilitates aggression in songbirds (Heimovics et al., 2014). Following Heimovics et al. (2014), we first determined the focal bird was dominant to the subordinate opponent during a pre-screening trial, because E2 treatment is expected to affect aggression in dominant, but not subordinate, birds (Piper & Wiley, 1988; Dearborn & Wiley, 1993; Wiley, Steadman, Chadwick, & Wollerman, 1999).

We paired birds into same-sex, same-morph dyads and observed them to determine which bird was dominant. In order to prevent habituation to the other bird, each bird was housed such that it was visually isolated from its partner. During the pre-screening trial to assess dominance, we placed the cages of the two birds adjacent to one another in an empty booth. The birds were then allowed to interact vocally and visually for 30 minutes. The interactions were recorded using a camcorder placed on a tripod ~1 meter away so that both cages were completely visible in the video. After the trial, the cages were returned to the home booth in their original positions. An observer blind to treatment scored two aggressive behaviors in the videos. First, this observer quantified attacks, defined as the bird making contact with both feet on the wall of its cage facing the opponent (Heimovics et al., 2015). Second, every 30 seconds the observer scored the bird's position with respect to the opponent's cage. Approaching the opponent is considered to be an aggressive behavior that is expressed similarly in captivity and the

field (Sperry et al., 2003) and been used to approximate aggression in captive songbirds previously (Heimovics et al., 2015; Goodson et al., 2005; Sperry et al., 2003). If the focal bird was located in the third of its cage closest to the opponent, it was considered to be in proximity to the opponent and received a score of "1". If it was located in the other two-thirds of its cage, in other words not in proximity to the opponent, it received a score of "0". The zone of the cage defined as proximal to the opponent was marked by a perch. The member of the dyad that made more attacks and was more often in proximity to the opponent was deemed dominant. If neither bird in the dyad dominated in terms of both behaviors, the dyad was dissolved and each bird was tested again with a new bird. The dominant bird within each dyad was designated as the focal bird and the subordinate as the opponent for the behavioral tests described below.

**Hormone manipulation.** In order to minimize any stress associated with delivery of E2, we administered E2 non-invasively. Non-invasive administration has been used previously to examine the rapid effects of steroids on behavior in birds (Breuner, Greenberg, & Wingfield, 1998; Breuner & Wingfield, 2000; Heimovics et al., 2015; Hodgson, Meddle, Christians, Sperry, & Healy, 2008; Saldanha, Schlinger, & Clayton, 2000). In male song sparrows, which are in the same family and similar in size to white-throated sparrows, 300 μg of E2 delivered orally induced aggression 10 minutes later (Heimovics et al., 2015). Therefore, we used the same dose of E2. Larvae of the wax moth (*Achroia grisella*), were prepared as described by Heimovics et al., (2015). A Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) was used to inject each larva with 20 μl of water containing 300 μg of cyclodextrin-encapsulated 17β-estradiol (E2; Sigma-Aldrich, cat. no. E4389) or cyclodextrin alone, as a control (CON; Sigma-

Aldrich, cat no. C0926). In order to habituate the birds to the presentation of the larva, each bird received one larva in a weigh boat on the floor of its cage throughout the habituation period (each day for at least 1 month). Only birds that always consumed the larvae within one minute were included in Experiment 1. Using this criterion, four birds were not tested during behavioral trials.

**Rapid effects of E2 on behavior.** Each bird was treated with E2 and CON in a counterbalanced order. This experimental design allowed us to control for individual differences in aggression at 'baseline', as measured during the CON trial. Birds received both treatments in an order that was balanced according to age, sex, and morph, with a 48 hour washout period between each trial. We chose this washout period between trials because in song sparrows, plasma E2 is non-detectable 48 hours after the same dose of E2 (Heimovics et al., 2015).

Behavioral testing occurred within 3 days of determining which bird was dominant in a dyad. Before a behavioral trial, the focal (dominant) bird was placed in an empty sound-attenuating booth for 1 hour to acclimate it to that environment (Goodson, Evans, Lindberg, & Allen, 2005). One hour later, the opponent was placed immediately adjacent to the focal bird's cage with an opaque partition visually isolating the birds. Then, video recording began. Behavior was recorded on a camcorder placed on a tripod ~1 m away from the cages such that the entire cage of each bird was visible. A larva injected with E2 or CON was placed in a weigh boat on the floor of the focal bird's cage. When the focal bird consumed the larva, the trial began (T0; Fig. 1) and the dyad was left undisturbed for 10 minutes. Ten minutes later, at T10, the experimenter quietly entered the booth and quickly removed the opaque partition, then immediately left. The dyad was

then allowed to interact vocally and visually (T10-T20). Ten minutes later (T20), the trial ended and the experimenter quietly entered the booth and replaced the opaque partition, then immediately left. The number of attacks, the position of the bird, and full and partial songs were scored by an experimenter blind to treatment as described above.

**Analysis of behavior.** Attacks were counted in one-minute bins. Data were tested for homogeneity of variance using Levene's test and normal distribution using the Shapiro-Wilk test, but the data violated the assumptions of parametric tests. Therefore, we performed a GLM with a Gaussian distribution to test for an interaction between treatment and morph, and to test whether the number of attacks changed over the course of the trial. The fixed effects included morph, treatment, minute, age, sex, trial, and an interaction of morph and treatment. The birds' identity was treated as a random effect to control for repeated measures. After running the full model, fixed factors with p values that were non-significant were dropped, then the remaining, significant fixed factors were included in the reduced model. When significant interactions were found, post-hoc tests were run by splitting the data by morph and data were analyzed within morph using the significant factors from the reduced model with fixed effects of treatment, order of treatment, and minute and a random effect of the birds' identity.

We used the position of the bird to test whether E2-treated birds were more likely to be close to their opponents and whether this effect depended on morph. To test for these effects, we used a GLM fit to a binomial distribution. The purpose of this analysis was to test for an interaction between treatment and morph, and to test whether the behavior changed over the course of the trial. The analysis was performed as above. Singing occurred too infrequently during the trials for statistical analysis. All data were

analyzed by GLM in R version 3.2.4 using the *glmer* function of the *lme4* package. The $\alpha$ level was set at $p \leq 0.05$.

**Experiment 2**

Our goal in Experiment 2 was to determine whether E2 administration has rapid effects on Egr-1 expression in the brain, and if so, whether that effect depends on morph in regions that exhibit morph differences in ERα expression (Horton et al., 2014b).

**Hormone treatment and tissue collection.** The same birds used in Experiment 1 were used in Experiment 2. In order to minimize the possibility that any morph-dependent Egr-1 expression could be caused by morph differences in social behavior, birds were isolated starting two hours before the trial. One hour after being transferred to an empty sound-attenuating booth, each bird was presented with a larva injected with a solution containing 300 μg E2 or vehicle only (CON), the same dose as Experiment 1. Exactly one hour after consuming the larva, each bird was deeply anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL). Brains were rapidly harvested and fixed in 5% acrolein as previously described (Maney et al., 2003). Ovaries and testes were inspected to verify a regressed state.

**Histology.** Immunohistochemistry was performed on brain tissue as previously described (Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003). Briefly, every third 50-μm section was incubated with a 1:16,000 dilution of polyclonal antibody (anti-Egr-1; cat. #Sc-189, lot #L0104, Santa Cruz Biotechnology, Santa Cruz, CA) raised against the C-terminus of Egr-1 of human origin (sequence STGLSDMTATFSPRTIEIC). This antibody has been used in published investigations of Egr-1-IR (also called ZENK) in the avian brain. Mello & Ribeiro (1998) demonstrated

that in zebra finches, incubating the working dilution with a 10-fold excess of Egr-1 peptide results in a complete loss of nuclear staining. In our study, Egr-1 was labeled by using a biotinylated secondary antibody and the ABC method (Vector, Burlingame, CA). Labeling was visualized by using diaminobenzidine enhanced with nickel (Maney et al., 2003; Shu, Ju, & Fan, 1988). Sections were mounted onto microscope slides and coverslipped in DPX (Sigma, St. Louis, MO).

**Quantification of Egr-1 expression.** Because we were primarily interested in responses to E2 that could explain the behavioral polymorphism, we quantified Egr-1-IR in regions where ERα mRNA differs by morph (Horton et al., 2014b). ImageJ software (version 1.44 K; NIH, Bethesda, MD) was used to count the labeled cell nuclei inside a selected area (Supplemental Table 1) within the region of interest (ROI) (Fig. 2). Sections were photographed with a Leica DFC480 camera attached to a Zeiss Axioskop microscope. The 4× objective (total magnification 40×) was used to acquire images. Egr-1-IR was quantified in each bird in 5 ROIs in which ERα expression differs between the morphs (Horton et al., 2014): TnA, PVN, rPOM, VMH-m, and BSTm. The number of labeled cells was determined as previously described (Maney, Cho, & Goode, 2006). Briefly, each image was opened in ImageJ (Bethesda, MD) and the number of particles with an optical density higher than a threshold value was counted within a predetermined area in each ROI (Fig. 2). Because of variability in background staining among brains, this threshold was set manually for each image such that clusters of pixels highlighted by the computer program agreed with what the observer considered to be labeled nuclei. In order to correct for overlapping labeled nuclei, the average size of the individual nuclei was calculated using an image with little overlap, and the total area covered by labeled

nuclei was then divided by the average size to approximate the number of labeled nuclei. The corrected number of nuclei was then divided by the total area within which nuclei were counted, to arrive at a measure of labeled nuclei per unit area (Supplemental Table 1), and expressed as cells per unit area. If a section containing an ROI was damaged, that section was skipped.

**Analysis of Egr-1 expression.** Homogeneity of variance was tested using Levene's test and the distribution of the data was tested using the Shapiro-Wilk test. Then, for each brain region, average Egr-1-IR cell counts per unit area were entered into an ANOVA with morph (WS or TS) and treatment (E2 or CON) as factors. Data were analyzed in R version 3.2.4 using the *aov* function of the *stats* package. The $\alpha$ level was set at $p < 0.05$.

**Experiment 3**

**Animals.** In a previous study, our group showed that seven days of E2 administration increased song rates in WS but not TS females (Maney et al., 2009). Therefore, we wanted to test for a morph difference in Egr-1 expression after the same duration of E2 treatment. Here, as a follow-up to Experiment 2, we tested whether seven days of E2 treatment leads to differential Egr-1 expression in the brains of WS and TS females (WS $n = 7$, TS $n = 7$) that were part of a larger study (Sanford, Lange, & Maney, 2010). These birds were housed under the conditions described in Experiment 1.

**Hormone Manipulation.** Hormone manipulations were previously described by Sanford et al. (2010) in accordance with Maney, et al. (2006). Briefly, birds were collected during fall migration and kept under a winter-like photoperiod. Each bird was implanted with one subcutaneous silastic capsule (length 12 mm, ID 1.47 mm, OD 1.96

mm, Dow Corning, Midland, MI) sealed at the ends with A-100-S Type A medical adhesive (Factor 2, Lakeside, AZ). Seven birds (WS *n* = 4, TS *n* = 3) received an empty capsule and 7 birds (WS *n* =3, TS *n* = 4) received a capsule packed with 17-beta-estradiol (Steraloids, Newport, RI). This dose of E2 brings plasma levels to breeding-typical levels within a few days (Maney et al., 2006). After receiving the implants, the birds were housed in individual cages in groups of four per sound-attenuating booth, which included birds from each hormone treatment.

**Tissue Collection.** The evening before tissue collection, birds were isolated in a sound-attenuating booth and the tissue was collected the next morning. Two hours after lights-on, each bird was deeply anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and a blood sample (≥ 200 µL) was taken from the jugular vein. After isoflurane overdose, brains were harvested and immersion-fixed in 5% acrolein (Maney et al., 2003). Ovaries were inspected to verify a regressed state.

**Histology.** Egr-1-IR was labeled by performing immunohistochemistry on brain tissue as described above (see also Sanford et al., 2010). For that study, brains had been cut in the parasagittal plane. One hemisphere from each brain was labeled for Egr-1. Whether the left or right hemisphere was chosen was balanced across morph and treatment. Every other 50-µm parasagittal section was immunolabeled for Egr-1 protein.

**Quantification of Egr-1 expression.** Egr-1-IR was quantified in four ROIs: TnA, rPOM, VMH-m, and BSTm (Fig.3), as described above in Experiment 2 (Supplemental Table 1). The PVN was not sampled in parasagittal sections because it is located so close to the midline that it was not present/identifiable in the available sections for many of the birds.

**Analysis of Egr-1 expression.** Homogeneity of variance was tested using Levene's test and the distribution of the data was tested using the Shapiro-Wilk test. Then, average Egr-1-IR cell counts per unit area for each brain region were entered into an ANOVA with morph (WS or TS) and treatment (E2 or CON) as between-subjects factors. When main effects were found, Tukey tests were conducted to make pairwise comparisons between groups. Data were analyzed in R version 3.2.4 using the *aov* and *TukeyHSD* functions of the *stats* package. The $\alpha$ level was set at $p \leq 0.05$.

## Results

### Experiment 1

**Attacks.** When we analyzed attacks in the full GLM, the effects of morph ($z = -0.284$; $p = 0.776$), treatment ($z = -1.111$; $p = 0.267$) , sex ($z = -0.655$; $p = 0.512$), and age ($z = 0.115$; $p = 0.908$) were not significant, so these factors were dropped for the reduced model (Supplemental Table 2). The reduced GLM yielded a significant interaction between morph and treatment for the number of attacks ($z = -3.143$; $p = 0.001$; $r = -0.61$). Post-hoc tests revealed that E2 increased the number of attacks in WS birds ($z = 3.127$; $p = 0.001$; $r = 0.90$), but the effect of E2 was not significant in TS birds ($z = -1.157$; $p = 0.25$; $r = -0.30$) (Fig. 4A). The effect of trial was a significant fixed factor ($z = -2.537$; $p= 0.011$; $r = -0.488$); there were more attacks during trial 1 than trial 2.

**Cage Position.** In the full model GLM for cage position, the effects of morph ($z = -1.294$; $p = 0.196$), treatment ($z = -0.681$; $p = 0.496$), sex ($z = -0.723$; $p = 0.460$), and age ($z = 0.935$; $p = 0.350$) were not significant, so these factors were dropped for the reduced model (Supplemental Table 3). The reduced GLM yielded a significant interaction between morph and treatment ($z = -3.205$; $p < 0.01$; $r = -0.62$) (Supplemental Table 3).

Post-hoc tests revealed that E2-treated WS birds were closer to the opponent than WS-treated CON birds ($z = 3.238$; $p = 0.001$; $r = 0.93$), but the effect of E2 was not significant in TS birds ($z = -0.731$; $p = 0.47$; $r = -0.19$) (Fig. 4B). The effect of trial was a significant fixed factor ($z = -2.121$; $p= 0.034$; $r= - 0.408$); birds were closer to the opponent during trial 1 than trial 2.

**Experiment 2**

**Egr-1 immunoreactivity.** E2 decreased Egr-1 in TnA, as shown by a main effect of treatment ($p = 0.01$; Cohen's $d = 0.90$) (Fig. 5). Similarly, E2 treatment tended to reduce Egr-1 expression in rPOM, as shown by a trend for a main effect of treatment ($p = 0.0747$; Cohen's $d = 0.68$) (Fig. 5). No other effects of treatment, morph, or interactions between treatment and morph were significant for any brain region (all $p > 0.25$; Supplemental Table 4).

**Experiment 3**

**Egr-1 immunoreactivity.** As a follow-up to Experiment 2, we measured Egr-1 expression in birds treated with E2 for one week. A two-way ANOVA yielded a significant interaction between morph and treatment ($F = 29.7$; $p < 0.001$), but no main effect of morph ($F = 0.068$; $p = 0.79$) or treatment ($F = 0.63$; $p = 0.44$) in TnA. E2 treatment increased Egr-1-IR in TnA of WS birds, ($p = 0.03$; Cohen's $d = 2.05$), whereas E2 treatment decreased Egr-1-IR in TnA of TS birds ($p < 0.01$; Cohen's $d = 4.76$) (Fig. 6). No effects of treatment, morph, or interactions between treatment and morph were significant for any other brain region (all $p > 0.25$; Supplemental Table 5).

## Discussion

### Rapid Effects of E2 on Aggression

WS and TS birds may be differentially sensitive to exogenous administration of androgens and estrogens; when plasma levels of T or E2 were experimentally equalized, WS birds nonetheless sang more than TS birds (Maney et al., 2009). Recently, our group demonstrated that morph differences in behavior might be attributable to morph differences in ERα expression, which are especially pronounced in TnA and PVN (Horton et al., 2014b). In the current study, we tested whether E2 has a different effect in the two morphs, as predicted by the ERα polymorphism.

In order to test the effects of E2 on behavior, we used the behavioral paradigm of Heimovics et al. (2015), who found that in song sparrows, E2 treatment rapidly induced physical aggression. Using the same dose, we found that the effect of E2 on aggressive behavior depended on morph, as demonstrated by a significant interaction between treatment and morph (Fig. 4A, 4B). The increase in aggression in WS birds occurred just 10-20 minutes after E2 administration. This time course is inconsistent with a genomic mechanism of action (reviewed by Charlier, Cornil, Ball, & Balthazart, 2010). Thus, the effects we report here, that the effects of E2 on aggression depend on morph, are likely due to nongenomic mechanisms of E2.

In this study, we predicted that WS birds would respond to E2 administration with more aggression than TS birds. WS birds have higher ERα expression in TnA; this difference has been hypothesized to mediate the morph difference in behavior (Horton et al., 2014; Maney et al., 2015). Aggression is positively correlated with ERα expression in the TnA of juncos (Rosvall et al., 2011), white-throated sparrows (Horton et al., 2014b)

and California mice (Trainor et al., 2006). Additionally, mice with the ERα gene knocked out are less aggressive than wild-type littermates (Ogawa, Lubahn, Korach, & Pfaff, 1997; Scordalakes & Rissman, 2004). Our prediction of differential action of E2 behavior in the two morphs was supported; E2 treatment increased attacks and nearness to the intruder in WS birds, but not TS birds (Fig. 4A, 4B). Our behavioral results are consistent with the hypothesis that morph differences in physical aggression are attributable to morph differences in sensitivity to E2, which may be due to the ERα expression polymorphism. Whether morph differences in aggression are caused by rapid, differential expression of ERα will be the focus of future studies.

The best-known morph difference in behavior is in vocal aggression (Horton et al., 2014; Maney et al., 2009). Levels of vocal aggression were very low in the current study; therefore, we focused on physical aggression. In a field study of free-living white-throated sparrows, Horton et al. (2014a) showed that breeding WS females were more physically aggressive than TS in that they spent more time near the intruder during a simulated territorial intrusion. In the current study, our sample consisted mostly of females. Thus, our finding of greater physical aggression is consistent with the field study by Horton et al. (2014a).

In the field, WS birds of both sexes displayed greater amounts of territorial singing, an aggressive behavior, than TS birds during STIs performed during the breeding season (Horton et al., 2014a; Horton et al., 2014b; Collins & Houtman 1999; Kopachena & Falls, 1993). In the present study, E2 did not rapidly induce singing. This observation is consistent with other studies that have shown that captive white-throated sparrows in non-breeding condition sing infrequently (Grozhik, Horoszko, Horton, Hu, Voisin, &

Maney, 2014; Maney et al., 2009; Wiley, Piper, Archawaranon, & Thompson, 1993). They will sing at higher rates, however, if treated with T or E2 for several days (Maney et al., 2009), which is consistent with the time course reported for other songbirds (reviewed in Soma, 2006). For example, male canaries (*Serinus canaria*) require continuous exposure to T for four days before they begin to sing, and reach singing rates typical of the breeding season only after 11 days of T treatment (Sartor, Balthazart, & Ball, 2005). Considering our results in the context of the previous literature, we may have observed few songs because singing depends on longer-term effects of sex steroids, and not the rapid, nongenomic effects of E2.

**Rapid Effects of E2 on Egr-1 Expression**

Given that ERα expression differs between morphs (Horton et al., 2014b), we hypothesized that the neural response to E2 may differ as well. By treating birds with E2 and measuring the Egr-1 response, we were able to map the response to E2 and test whether that response depends on morph. We found that E2 rapidly affected Egr-1 expression in TnA and rPOM (Fig. 5). The findings of the current study are consistent with those of Heimovics et al. (2012), who found that in non-breeding song sparrows, TnA and POM responded rapidly to E2. Those authors found that E2 modulated the activity of two transcription factors in the MAPK signaling cascade, ERK and CREB, which target Egr-1 (Shi et al., 2002; Mayer & Thiel, 2009). In white-throated sparrows, ERα mRNA expression in TnA and rPOM is thought to facilitate E2-sensitive behaviors (Horton et al., 2014b). In vertebrates, these regions are components of the social behavior network (Newman, 1999; Goodson, 2005; Maney et al., 2008) and are implicated in many forms of E2-dependent social behavior, including aggression (Ogawa et al., 1997;

Rosvall et al., 2012; Horton et al., 2014b; Heimovics et al., 2015; Balthazart & Ball, 2007; Patil & Brid, 2010). In the current study, E2 rapidly modulated Egr-1 expression in TnA and rPOM.

Our findings that E2 treatment decreased Egr-IR in TnA and rPOM (Fig. 5) were consistent with those of Heimovics et al. (2012), who reported inhibitory effects of E2 administration on pERK-IR and pCREB-IR. pERK, which directly targets Egr-1, was downregulated in the TnA of song sparrows 15 minutes after treatment with E2 (Heimovics et al., 2012). The authors also reported that E2 rapidly downregulated the phosphorylation of molecules in the MAPK pathway in TnA and POM. Like Heimovics et al., we demonstrated a rapid, inhibitory effect of E2 on the MAPK cascade in TnA and rPOM (Fig. 5). In addition to *in vivo* studies, studies *in vitro* have demonstrated inhibitory effects of E2 on intracellular signaling. In rat neocortical cultures, E2 decreased pERK after 30 minutes in wildtype, but not ERα knockout cultures, suggesting ERα could have an inhibitory role in this pathway (Toran-Allerand et al., 2002). Additionally, adding the ERα-specific agonist propyl pyrazole triol (PPT) to wildtype neocortical cultures inhibited pERK (Singh, Sétáló, Guan, Frail, & Toran-Allerand, 2000; Toran-Allerand, 2002 et al.; reviewed by Toran-Allerand, 2005).

We did not detect an interaction between morph and treatment in any of the ROIs measured (Fig. 5), despite previous findings of morph differences in ERα expression in these ROIs. During the breeding season, the expression of ERα in TnA and the PVN predicts territorial singing (Horton et al., 2014b). In this study, birds were kept in non-breeding condition by housing them under a winter-like photoperiod and sex steroids were naturally low. Morph differences in ERα have been shown in TnA of non-breeding

males (Maney et al., 2015); however, we did not detect an interaction between morph and E2 on Egr-1 expression in that region.

There are at least three possible explanations for not detecting an interaction between the effects of morph and treatment on Egr-1 expression in any of the regions we investigated. First, we collected tissue at only one time point after E2 administration, so it is possible that we missed the time point at which a morph difference would have been detectable. Heimovics et al. (2012), found an effect of E2 on immunoreactivity for phosphorylated molecules in the MAPK signaling cascade 15 minutes after treatment. We chose to investigate one downstream target of the MAPK signaling cascade, Egr-1. We collected tissue 60 minutes after birds consumed E2 or CON, a time point at which Egr-1 is maximally expressed after stimulus onset in songbirds (Mello & Ribeiro, 1998; Clayton, 2000); however, we do not know whether maximum expression of Egr-1 is the timepoint at which we might expect a morph and/or treatment effect of maximal Egr-1 expression in this paradigm.

Second, we may not have detected an interaction between morph and treatment because our manipulation did not specifically target ERα. E2 binds not only to ERα, but also to other estrogen receptors (ERs), including GPR30 and ERβ (reviewed in Cornil, Ball, & Balthazart, 2006; McCarthy, 2009). GPR30 rapidly activates the MAPK signaling cascade (Kelly & Rønnekleiv, 2008). Similar to other ERs, ERβ has rapid effects on cellular signaling (Ábrahám, Todman, Korach, & Herbison, 2004; Szegő, Barabás, Balog, Szilágyi, Korach, Juhász, & Ábrahám, 2006; Ábrahám, Han, Todman, Korach, & Herbison, 2003). In addition, ERβ is also expressed in many of the regions we investigated in this study, in some cases at higher levels than ERα (Metzdorf, Gahr, &

Fusani, 1999; Gahr, 2001; Gahr, Guttinger, & Kroodsma, 1993). For example, in European starlings, ERβ is expressed at much higher levels than ERα in TnA (Bernard et al., 1999), a region in which we hypothesized we would detect an interaction between morph and treatment in Egr-1 expression. Neither GPR30 nor ERβ are captured in the inversion on chromosome 2 and the expression of these ERs does not differ according to morph in the TnA or hypothalamus (Zinzow-Kramer et al., 2015). Thus, the possibility remains that stimulation of other ERs swamped out the Erg-1 expression caused by ERα signaling. In future studies, ERα-specific agonists, such as PPT, will be used in order to tease apart the contributions of ERα from those of other ERs.

Third, perhaps the most important possible explanation for the lack of an interaction between morph and E2 is that in this study we measured Egr-1 expression in birds that had been treated with E2, but not exposed to social challenge. Since morph differences in behavior are observed during social encounters (e.g., Horton et al., 2014a; Horton et al., 2014b), social interaction might be necessary to see a morph difference in Erg-1 expression. In other words, E2 could modulate the response to social stimulation in a morph-specific way. Local concentrations of hormones in the brain and an animal's behavior reciprocally affect one another (reviewed in Remage-Healey, Saldanha, & Schlinger, 2011). We measured Egr-1 expression in the absence of social stimulation in order to map sensitivity to E2 without introducing confounds associated with engaging in aggressive behavior, which differed by morph in this study (Fig. 4A, B). Thus, our experimental design intentionally ruled out possible effects of social interactions on Egr-1 expression, yet morph-typical neural responses in response to social stimulation may be required to observe morph differences in E2-induced Egr-1 expression.

**Slow-Acting Effects of E2 on Egr-1 Expression**

We have shown here that the effects of seven days of administration of E2 on Egr-1 expression depend on morph. In a previous study, WS birds treated with E2 for seven days sang more in response to playback than E2-treated TS birds (Maney et al., 2009). Thus, we asked whether the effect of E2 on Egr-1 expression depends on morph in birds treated for the same amount of time. After seven days of E2 treatment, white-throated sparrows exhibited morph differences in Egr-1-IR in TnA; E2 increased Egr-1 in WS birds, but decreased it in TS birds (Fig. 6). This pattern is unlike the results of Experiment 2, in which there was no interaction between E2 treatment and morph one hour after treatment with E2 (Fig. 5). Together, Experiments 2 and 3 suggest that the morph-specific effects of E2 on Egr-1 expression in TnA do not depend, at least entirely, on nongenomic mechanisms. In birds treated for 7 days, E2 may act at a variety of structures in the brain to alter both cell signaling and behavior, thus altering Egr-1 expression in TnA.

In Experiment 3 we detected a morph difference in Egr-1 expression in TnA in the control group (Fig. 6), suggesting differences in MAPK signaling even during the non-breeding season. If that is the case, then we should have detected a morph difference in our control group also in Experiment 2; however we detected no such difference (Fig. 5).There are a few experimental factors, such as method of hormones administration, that differed between the two studies and thus may have contributed to this discrepancy. Additionally, because the tissue in Experiment 3 was part of a larger study (see Sanford et al., 2010), the sample size was limited (n = 3 or 4 per group). Therefore, Experiment 3 should be replicated with a larger sample size, and the morph difference we detected in

CON birds should be considered preliminary. Future studies should directly test whether morph differences in Egr-1 expression depend on the duration of exposure to E2 in order to separate the effects of non-genomic and genomic E2 signaling.

## Summary

In this study, we investigated the rapid effects of E2 on aggressive behavior and Egr-1 expression in the white-throated sparrow, a species with an ERα polymorphism. Our results show that E2 rapidly induced aggression in WS, but not TS birds. Effects were observed just 10 minutes after treatment, suggesting that E2 may induce aggression through nongenomic mechanisms. In the brain, E2 treatment rapidly downregulated Egr-1 expression in the TnA and rPOM but this effect did not depend on morph. In contrast, the effect of E2 on Egr-1 expression depended on morph in birds treated with E2 for seven days, demonstrating that morph differences in Egr-1 expression emerge upon exposure to E2 for a longer duration. Our behavioral results are consistent with the hypothesis that morph differences in physical aggression could be attributable to morph differences in sensitivity to E2. Whether morph differences in aggression are caused by rapid, differential expression of ERα in free-living, breeding birds will be the focus of future studies.

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## Figures

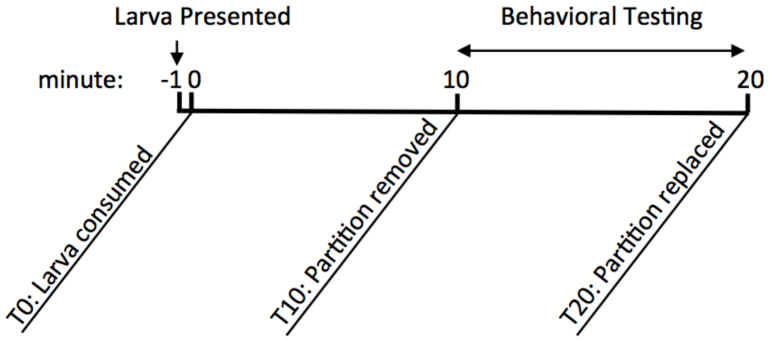


Figure 1.

Time course of behavioral testing. A focal bird was presented with a larva injected with E2 or CON immediately before the onset of the trial, which began upon larva consumption (T0). Ten minutes later (T10), a visual barrier between the focal animal and the opponent was removed, and the birds could interact through adjacent cage walls (T10-T20). At T20, the visual barrier was replaced.

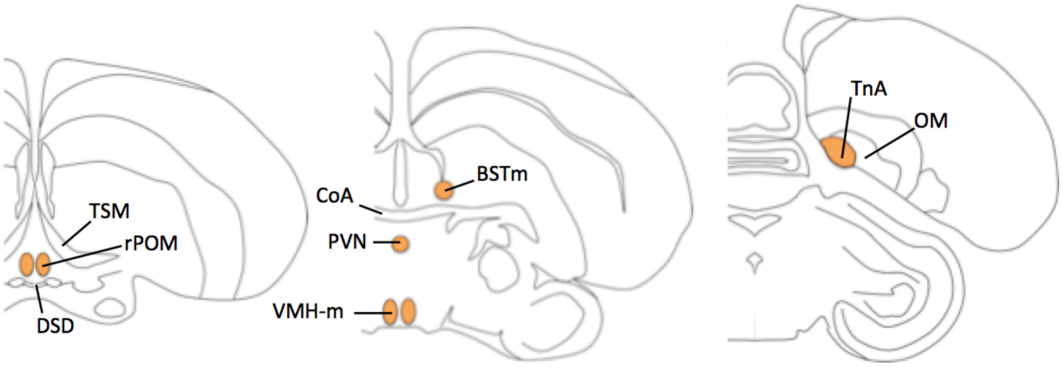


Figure 2.

Regions of interest in Experiment 2. Egr-1 was quantified in the regions shaded in orange. BSTm, medial portion of the bed nucleus of the stria terminalis; CoA, anterior commissure; DSD, supraoptic decussation; TnA, nucleus taeniae of the amygdala; OM, occipito-mesencephalic tract; PVN, paraventricular nucleus; rPOM, rostral medial preoptic area; TSM, septo-mesencephalic tract; VMH-m, medial portion of the ventromedial hypothalamus.

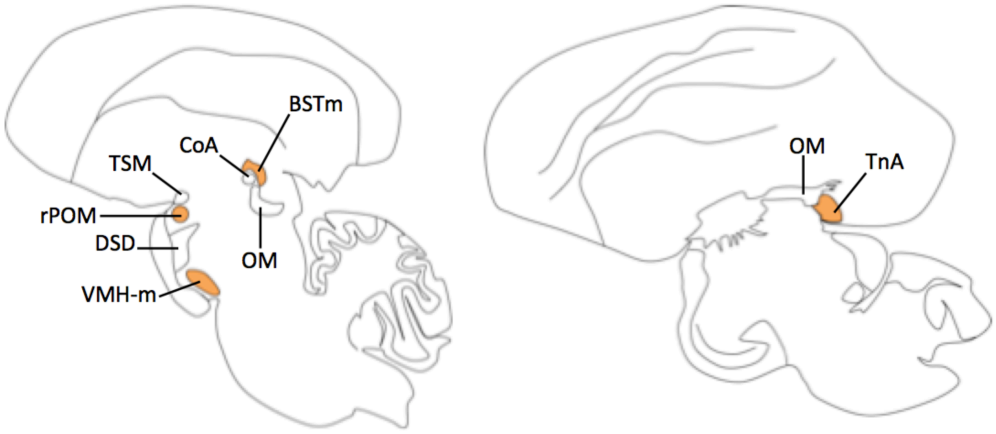


Figure 3.

Regions of interest in Experiment 3. Egr-1 was quantified in the regions shaded in orange. BSTm, medial portion of the bed nucleus of the stria terminalis; CoA, anterior commissure; DSD, supraoptic decussation; TnA, nucleus taeniae of the amygdala; OM, occipito-mesencephalic tract; rPOM, rostral medial preoptic area; TSM, septo-mesencephalic tract; VMH-m, medial portion of the ventromedial hypothalamus.

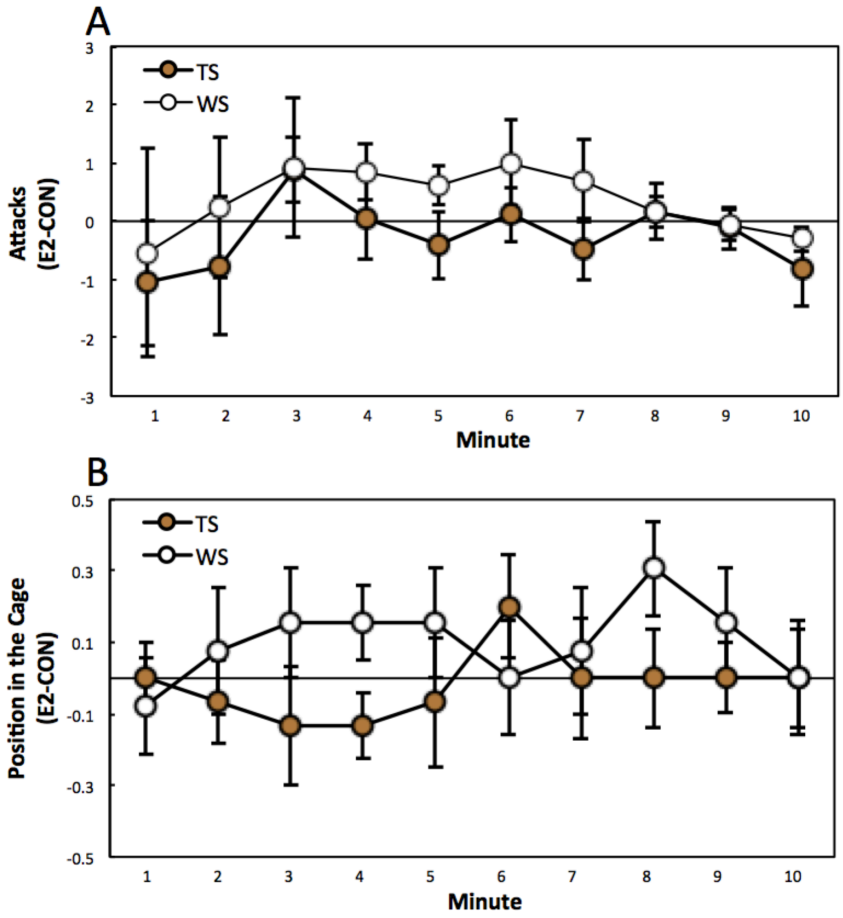


Figure 4.

The rapid effects of E2 treatment on behavior. All scores were normalized by subtracting the baseline values (behavior during the CON trial) from experimental values (behavior during the E2 trial), in order to visualize the results in terms of the main hypothesis that E2 affects the morphs differently. (A) E2 increased the number of attacks in WS birds ($p = 0.001$), but this effect was not significant in TS birds ($p = 0.25$). (B) WS birds treated with E2 moved closer to the opponent ($p = 0.001$) but this effect was not significant in TS birds ($p = 0.47$). On the Y-axis, "Position in the Cage" refers to the position of the focal bird inside its cage during behavioral testing (see text). A higher score indicates a position closer to the opponent.

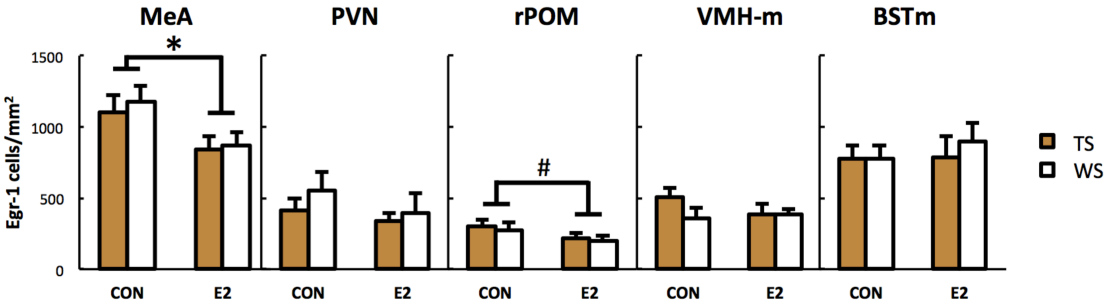


Figure 5.

Rapid Egr-1 expression in response to E2 treatment. Egr-1-IR cells were counted in TnA, PVN, rPOM, VMH-m, and BSTm. A main effect of treatment was found in TnA ($p = 0.01$) and a trend for an effect of treatment was observed in the rPOM ($p = 0.07$). No other effects were noted. * $p < 0.05$. # $p < 0.10$. Abbreviations, see caption of Fig. 2.

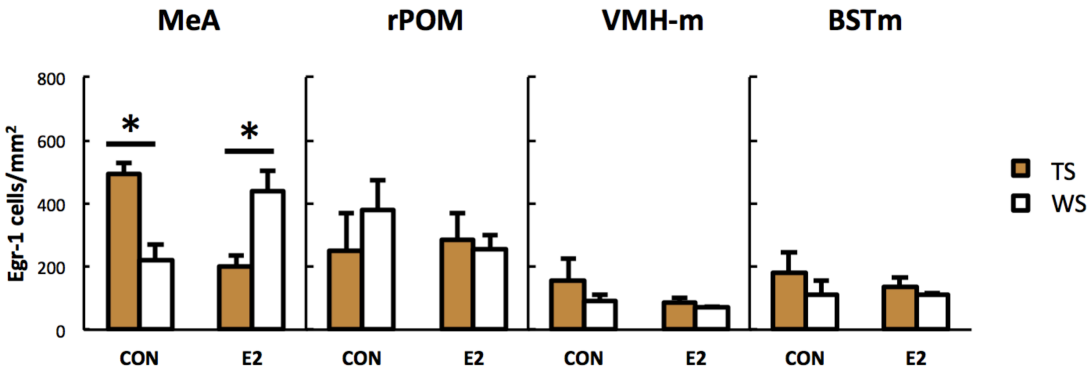


Figure 6.

Egr-1 expression after seven days of E2 treatment. Egr-1 cells were counted in TnA, rPOM, VMH-m, and BSTm. A significant interaction between morph and treatment was detected in TnA ($p < 0.001$). E2 increased the number of Egr-1 cells in WS birds ($p = 0.03$) and decreased the number of Egr-1 cells in TS birds ($p < 0.01$). No other effects were significant. * significant effect of treatment, $p < 0.05$. Abbreviations, see caption of Fig. 2.

Supplementary Table 1: Regions of Interest (ROIs)

| Experiment | Plane | Nucleus | ROI shape | ROI area ($mm^2$) | No. Sections‡ |
|---|---|---|---|---|---|
| 2 | Coronal | rPOM | Oval† | 0.23 | 2 |
| 2 | Coronal | TnA | Tracing† | NA | 5 |
| 2 | Coronal | VMH-m | Oval† | 0.05 | 6 |
| 2 | Coronal | BSTm | Circle† | 0.10 | 5 |
| 2 | Coronal | PVN | Circle | 0.05 | 6 |
| 3 | Parasagittal | rPOM | Circle | 0.03 | 3 |
| 3 | Parasagittal | TnA | Tracing | NA | 3 |
| 3 | Parasagittal | VMH-m | Oval | 0.06 | 3 |
| 3 | Parasagittal | BSTm | Tracing | NA | 3 |

Notes
†Egr-1 was quantified bilaterally in these ROIs.
‡Sections with extensive tissue damage were excluded.

Supplemental Table 2: Analysis of attacks during behavioral testing

| | | Full Model | | | | Reduced Model | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Estimate | Std. Error | z value | p value | Estimate | Std. Error | z value | p value |
| Intercept | | 0.325 | 0.568 | 0.571 | 0.568 | 0.428 | 0.540 | 0.792 | 0.428 |
| Morph | | -0.201 | 0.708 | -0.284 | 0.776 | | | | |
| Treatment | | -0.115 | 0.103 | -1.111 | 0.267 | | | | |
| Morph x Treatment | | 0.507 | 0.157 | 3.222 | 0.001 ** | -0.393 | 0.125 | -3.143 | 0.001 ** |
| Minute† | *1* | -0.565 | 0.123 | -4.602 | < 0.001 *** | -0.565 | 0.123 | -4.602 | < 0.001 *** |
| | *2* | -0.721 | 0.129 | -5.578 | < 0.001 *** | -0.721 | 0.129 | -5.578 | < 0.001 *** |
| | *3* | -1.184 | 0.153 | -7.754 | < 0.001 *** | -1.184 | 0.153 | -7.754 | < 0.001 *** |
| | *4* | -1.202 | 0.154 | -7.818 | < 0.001 *** | -1.202 | 0.154 | -7.818 | < 0.001 *** |
| | *5* | -1.239 | 0.156 | -7.944 | < 0.001 *** | -1.239 | 0.156 | -7.944 | < 0.001 *** |
| | *6* | -1.202 | 0.154 | -7.818 | < 0.001 *** | -1.202 | 0.154 | -7.818 | < 0.001 *** |
| | *7* | -1.546 | 0.176 | -8.765 | < 0.001 *** | -1.546 | 0.176 | -8.765 | < 0.001 *** |
| | *8* | -1.713 | 0.189 | -9.057 | < 0.001 *** | -1.713 | 0.189 | -9.057 | < 0.001 *** |
| | *9* | -1.808 | 0.197 | -9.180 | < 0.001 *** | -1.808 | 0.197 | -9.180 | < 0.001 *** |
| Sex | | -0.519 | 0.792 | -0.655 | 0.512 | | | | |
| Age | | 0.098 | 0.855 | 0.115 | 0.908 | | | | |
| Trial ($1^{st}$ vs. $2^{nd}$) | | -0.208 | 0.082 | -2.535 | 0.011 * | -0.208 | 0.082 | -2.537 | 0.011 * |
| Observations | | 600 | | | | 600 | | | |
| Log Likelihood | | -554.3 | | | | -554.5 | | | |
| AIC | | 1142.6 | | | | 1139 | | | |
| BIC | | 1217.3 | | | | 1205 | | | |
| Residual d.f. | | 583 | | | | 585 | | | |
| Deviance | | 1108.6 | | | | 1109 | | | |

Notes

*p < 0.05, ** p < 0.01, *** p < 0.001

†p values for Minute compared to time point 0

Run in R version 3.2.4, *glmer* function of package *lme4*

Supplemental Table 3: Analysis of position near the opponent during behavioral testing

| | | Full model | | | | Reduced model | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Estimate | Std. Error | z value | p value | Estimate | Std. Error | z value | p value |
| Intercept | | 4.414 | 1.063 | 4.154 | < 0.001 *** | 4.170 | 0.864 | 4.824 | < 0.001 *** |
| Morph | | -0.995 | 0.769 | -1.294 | 0.196 | | | | |
| Treatment | | -0.180 | 0.264 | -0.681 | 0.496 | | | | |
| Morph x Treatment | | 1.006 | 0.376 | 2.678 | < 0.01 ** | -0.8249 | 0.2574 | -3.205 | < 0.01 ** |
| Minute† | *0.5* | -0.266 | 0.730 | -0.364 | 0.716 | -0.272 | 0.739 | -0.368 | 0.713 |
| | *1* | -1.070 | 0.676 | -1.582 | 0.114 | -1.093 | 0.684 | -1.599 | 0.110 |
| | *1.5* | -0.799 | 0.695 | -1.149 | 0.250 | -0.915 | 0.693 | -1.320 | 0.187 |
| | *2* | -1.293 | 0.671 | -1.928 | 0.053 # | -1.416 | 0.6702 | -2.113 | 0.035 * |
| | *2.5* | -1.439 | 0.665 | -2.163 | 0.031 * | -1.5649 | 0.6653 | -2.352 | 0.019 * |
| | *3* | -0.975 | 0.685 | -1.422 | 0.155 | -1.093 | 0.684 | -1.599 | 0.110 |
| | *3.5* | -1.712 | 0.657 | -2.607 | < 0.001 *** | -1.844 | 0.658 | -2.803 | < 0.01 ** |
| | *4* | -1.712 | 0.657 | -2.607 | < 0.001 *** | -1.844 | 0.658 | -2.803 | < 0.01 ** |
| | *4.5* | -1.293 | 0.671 | -1.928 | 0.053 # | -1.416 | 0.670 | -2.113 | 0.035 * |
| | *5* | -1.139 | 0.677 | -1.681 | 0.093 # | -1.259 | 0.676 | -1.862 | 0.063 # |
| | *5.5* | -1.841 | 0.653 | -2.818 | < 0.01 ** | -1.977 | 0.655 | -3.017 | < 0.01 ** |
| | *6* | -1.967 | 0.651 | -3.023 | < 0.01 ** | -2.106 | 0.653 | -3.224 | < 0.01 ** |
| | *6.5* | -1.139 | 0.677 | -1.681 | 0.093 # | -1.259 | 0.676 | -1.862 | 0.063 # |
| | *7* | -1.578 | 0.660 | -2.389 | 0.017 * | -1.707 | 0.661 | -2.582 | < 0.01 ** |
| | *7.5* | -1.841 | 0.653 | -2.818 | < 0.01 ** | -1.977 | 0.655 | -3.017 | < 0.01 ** |
| | *8* | -1.293 | 0.671 | -1.928 | 0.054 # | -1.416 | 0.670 | -2.113 | 0.035 * |
| | *8.5* | -1.139 | 0.677 | -1.681 | 0.093 # | -1.259 | 0.676 | -1.862 | 0.063 # |
| | *9* | -1.841 | 0.653 | -2.818 | < 0.01 ** | -1.977 | 0.655 | -3.017 | < 0.01 ** |
| | *9.5* | -1.578 | 0.660 | -2.389 | 0.017 * | -1.707 | 0.661 | -2.582 | < 0.01 ** |
| Sex | | -0.594 | 0.822 | -0.723 | 0.469 | | | | |
| Age | | 0.935 | 1.000 | 0.935 | 0.350 | | | | |
| Trial ($1^{st}$ vs. $2^{nd}$) | | -0.418 | 0.198 | -2.113 | 0.035 * | -0.405 | 0.191 | -2.121 | 0.034 * |
| Observations | | 1120 | | | | 1120 | | | |
| Log Likelihood | | -440.2 | | | | -441.7 | | | |
| AIC | | 936.5 | | | | 933.3 | | | |
| BIC | | 1077.1 | | | | 1058.9 | | | |
| Residual d.f. | | 1092 | | | | 1095 | | | |
| Deviance | | 880.5 | | | | 883.3 | | | |

Notes

#p < 0.1, *p < 0.05, ** p < 0.01, *** p < 0.001

†p values for Minute compared to time point 0

Run in R version 3.2.4, *glmer* function of package *lmer4*

Supplemental Table 4: Rapid effects of E2 on Egr-1 expression

| | Morph | | Treatment | | Morph x Treatment | |
|---|---|---|---|---|---|---|
| Region | F | p value | F | p value | F | p value |
| TnA | 0.125 | 0.726 | 6.896 | 0.0133 * | 0.034 | 0.854 |
| rPOM | 0.377 | 0.544 | 3.830 | 0.059 # | 0.038 | 0.846 |
| PVN | 0.964 | 0.334 | 1.283 | 0.266 | 0.131 | 0.720 |
| VMH-m | 0.964 | 0.334 | 1.283 | 0.266 | 0.131 | 0.720 |
| BSTm | 0.246 | 0.623 | 0.229 | 0.636 | 0.121 | 0.731 |

Notes #p < 0.1, *p < 0.05

Run in R version 3.2.4, *aov* function of package *stats*

Supplemental Table 5: Long-term effects of E2 on Egr-1 expression

Two-way ANOVAs

| | Morph | | Treatment | | Morph x Treatment | |
|---|---|---|---|---|---|---|
| Region | F | p value | F | p value | F | p value |
| TnA | 0.068 | 0.799 | 0.632 | 0.445 | 29.679 | <0.001 *** |
| rPOM | 0.468 | 0.511 | 0.005 | 0.947 | 1.514 | 0.250 |
| VMH-m | 0.015 | 0.907 | 0.821 | 0.391 | 0.000 | 0.995 |
| BSTm | 0.000 | 1.000 | 0.158 | 0.701 | 0.276 | 0.614 |

TnA Post-Hoc Tests

| Comparisons within group | | CI† (lower) | CI† (upper) | adjusted p value |
|---|---|---|---|---|
| E2 x CON | WS | 15.518 | 426.809 | 0.035 * |
| | TS | -502.363 | -91.071 | <0.01 ** |
| TS x WS | E2 | 35.613 | 446.904 | 0.022 * |
| | CON | -482.268 | -70.977 | <0.01 ** |

Notes

*p < 0.05, **p < 0.01, ***p < 0.001

†CI represent 95% family-wise confidence interval

ANOVAs performed in R version 3.2.4, *aov* function of package *stats*

Tukey tests performed in R version 3.2.4, *TukeyHSD* function of package *stats*