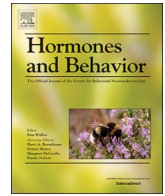




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# Hormones and Behavior

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## Rapid effects of estradiol on aggression depend on genotype in a species with an estrogen receptor polymorphism

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### A B S T R A C T

The white-throated sparrow (*Zonotrichia albicollis*) 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 do birds of the tan-striped (TS) morph, and the TS birds engage in more parenting behavior. This behavioral polymorphism is caused by a chromosomal inversion that has captured many genes, including estrogen receptor alpha (ER $\alpha$ ). In this study, we tested the hypothesis that morph differences in aggression might be explained by differential sensitivity to estradiol (E2). We administered E2 non-invasively to non-breeding white-throated sparrows and quantified aggression toward a conspecific 10 min later. E2 administration rapidly increased aggression in WS birds but not TS birds, consistent with our hypothesis that differential sensitivity to E2 may at least partially explain morph differences in aggression. To query the site of E2 action in the brain, we administered E2 and quantified Egr-1 expression in brain regions in which expression of ER $\alpha$  is known to differ between the morphs. E2 treatment decreased Egr-1 immunoreactivity in nucleus taeniae of the amygdala, but this effect did not depend on morph. Overall, our results support a role for differential effects of E2 on aggression in the two morphs, but more research will be needed to determine the neuroanatomical site of action.

### 1. Introduction

Most, if not all, alternative behavioral phenotypes are linked to variation in sex steroid hormones (Oliveira et al., 2008). Species exhibiting alternative phenotypes therefore represent an opportunity to investigate the mechanisms underlying hormone-dependent behaviors and their evolution. One such species is the white-throated sparrow (*Zonotrichia albicollis*), which exhibits alternative plumage phenotypes associated with a polymorphism in sex steroid-dependent behaviors. White-throated sparrows occur in two plumage morphs, white-striped (WS) or tan-striped (TS). WS birds respond to territorial threats with higher levels of vocal and physical aggression than do TS birds, which invest more in provisioning nestlings (Falls, 1969; Horton et al., 2014a; Lowther, 1961; Tuttle, 2003; Tuttle et al., 2016). This behavioral polymorphism segregates with the presence or absence of a rearrangement on chromosome 2 called ZAL2<sup>m</sup> (Thomas et al., 2008; Thorneycroft, 1966). All birds with the ZAL2<sup>m</sup> arrangement exhibit the WS plumage coloration, whereas TS birds lack the rearrangement, having two copies of the standard ZAL2. Suppression of recombination

between ZAL2 and ZAL2<sup>m</sup> has resulted in the divergence of genes inside the rearrangement (Davis et al., 2011; Thomas et al., 2008) such that the two haplotypes are now 1% different from each other (Huynh et al., 2011). Only the WS birds carry a copy of ZAL2<sup>m</sup>; the genes captured by the rearrangement are thus likely responsible for the majority of morph differences in this species, including those in territorial aggression and parenting behavior.

In songbirds, territorial aggression and parental provisioning are known to be affected by sex steroids (Goodson et al., 2005; Hegner and Wingfield, 1987; Schlinger and Callard, 1990; Wingfield, 1985; Wingfield et al., 1987). Therefore, some of the top candidate genes that could explain the morph difference in behavior in white-throated sparrows are those in the sex steroid pathway. The gene for estrogen receptor alpha (ER $\alpha$ ), known as ESR1, has been captured by the rearrangement (Thomas et al., 2008) and is differentiating between ZAL2 and ZAL2<sup>m</sup>. This divergence is thought to cause differential expression of the receptor, and in turn, to affect behavior (see Horton et al., 2014b; Maney, 2017; Maney et al., 2015). In CD-1 mice (*Mus musculus*) and dark-eyed juncos (*Junco hyemalis*), individual variation in ER $\alpha$

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expression in a number of brain regions predicts agonistic behavior (Rosvall et al., 2012; Trainor et al., 2006). ER $\alpha$  is well-known to mediate aggression in rodents; in male mice, for example, specific ER $\alpha$  agonists increase attacking behavior (Clipperton-Allen et al., 2011), and knocking out ESR1 reduces aggression (Ogawa et al., 1997; Scordalakes and Rissman, 2004). The clear role for ER $\alpha$  in aggression in other vertebrates makes it a top candidate for mediating morph differences in aggression in white-throated sparrows.

In the white-throated sparrow, ER $\alpha$  expression in at least eight brain regions depends on morph (Horton et al., 2014b). WS birds have more ER $\alpha$  mRNA in nucleus taeniae of the amygdala (TnA), the paraventricular nucleus (PVN), and HVC (used as a proper name). In contrast, TS birds have more ER $\alpha$  mRNA in the rostral medial preoptic area (rPOM), anterior hypothalamus, ventromedial hypothalamus (VMH), bed nucleus of the stria terminalis (BSTm), and ventrolateral portion of the caudal lateral septum (Horton et al., 2014b). Expression in TnA and PVN predicts territorial singing even when morph and sex steroids are controlled in regression analyses; in fact, expression in these areas predicts singing better than morph itself (Horton et al., 2014b).

The tight correlation between ER $\alpha$  expression and territorial aggression suggests that morph-dependent behavior may be caused, in part, by differential actions of estradiol (E2). To test this hypothesis, we need to assess the effects of exogenous E2 on behavior. Previously, we showed that when plasma sex steroids are experimentally equalized between the morphs, the morph difference in aggression persists (Maney et al., 2009). Treating non-breeding males with testosterone for seven days increased aggressive responses to song playback more in WS birds than TS birds. In non-breeding females, seven days of E2 treatment increased spontaneous aggressive vocalizations in WS females only. These results suggest that the neural circuits involved in aggression are more sensitive to E2 in WS than TS birds.

Maney et al. (2009) measured behavior at one time point, after seven days of treatment, which did not provide much information about underlying mechanisms. The steroid hormones administered in that study could have acted *via* genomic or nongenomic mechanisms, or both. When E2 acts *via* nongenomic mechanisms, behavior can be affected rapidly, within minutes. Heimovics et al. (2015a) showed that in non-breeding song sparrows (*Melospiza melodia*), E2 treatment rapidly increased aggression within 20 min. The time course of this result suggested a nongenomic mechanism of E2 action. In the present study, we tested whether E2 treatment increases aggression on the same time scale in white-throated sparrows and if so, whether that effect depends on morph. We administered E2 to birds in non-breeding condition, in which endogenous plasma E2 is very low and thus receptors would not be saturated; nonetheless expression of ER $\alpha$  is higher in WS than TS birds in both TnA and PVN (Maney et al., 2015; B. M. Horton, unpublished). Greater behavioral responses to E2 in WS than TS birds would be consistent with the hypothesis that ER $\alpha$  mediates the behavioral polymorphism in this species.

Finding differential effects of systemic E2 treatment on behavior would not indicate the neuroanatomical site of action. Therefore, we mapped induction of the immediate early gene Egr-1 after E2 administration. Our methods were similar to those of Heimovics et al. (2012), who showed that in song sparrows, exogenous E2 rapidly affected the phosphorylation of molecules in the MAPK signaling cascade, ERK and CREB, in TnA and POM, respectively. Both ERK and CREB serve as transcription factors to induce the transcription of many genes, including Egr-1 (Shi et al., 2002). We hypothesized that if morph differences in E2-induced aggression in white-throated sparrows result from differential expression of ER $\alpha$ , then E2 administration should induce differential Egr-1 expression in TnA or other regions with differential expression of ER $\alpha$ .

## 2. Methods

### 2.1. 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., 2015a), and if so, whether that effect depends on morph. In Experiment 2, to assess the neural responses to this dose of E2, we administered a dose of E2 identical to that used in Experiment 1 and quantified the expression of Egr-1 in five brain regions in which ER $\alpha$  expression has been shown to depend on morph in this species (Horton et al., 2014b).

### 2.2. Experiment 1

#### 2.2.1. 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*. White-throated sparrows of both sexes and morphs were collected in mist nets on the campus of Emory University in Atlanta, GA during fall migration. Sex was confirmed by PCR analysis of a small blood sample (Griffiths et al., 1998). Assessments of morph were made using a PCR assay (Horton et al., 2013; Michopoulos et al., 2007) and by visual inspection of plumage (Michopoulos et al., 2007; Piper and Wiley, 1989a). 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'  $\times$  7'  $\times$  6'), supplied with *ad libitum* seed and water. 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. At least one month prior to behavioral assays, birds were transferred to individual cages (15"  $\times$  15"  $\times$  17") inside walk-in sound-attenuating booths (Industrial Acoustics, Bronx, NY). They were housed two to six birds per booth, with opaque barriers between the cages to prevent visual contact, until the behavioral trials. All booths were identical and the day length remained at 10L:14D throughout the experiment.

#### 2.2.2. Pre-screening for social dominance

The goal of Experiment 1 was to determine whether E2 administration affects aggression differently in the two morphs. Like many songbirds, white-throated sparrows establish dominance hierarchies such that subordinate birds rarely show aggression toward dominant ones (Archwaranon et al., 1991). Therefore, because we were interested in measuring the effects of E2 on aggression, we limited our investigation to dominant birds (see Heimovics et al., 2015a). Dominance rank does not depend on plumage morph in non-breeding white-throated sparrows (Archwaranon et al., 1991; Maney and Goodson, 2011; Piper and Wiley, 1989b) and administration of sex steroids does not affect established dominance relationships (Wiley et al., 1993). In order to identify pairs of birds with clear dominance relationships, we performed prescreening trials on same-morph, same-sex pairs. Within these pairs, the dominant bird would become the focal bird and the subordinate the "opponent". During prescreening trials, the cages of the two birds were placed adjacent to one another in an empty booth. The birds were then allowed to interact vocally and visually for 30 min. The interactions were recorded using a camcorder placed on a tripod ~1 m away so that both cages were completely visible in the video. After the trial, the birds in their cages were returned to the home booth.

To determine which bird in the dyad was dominant, we scored two aggressive behaviors in the videos. Aggression was operationalized according to Heimovics et al. (2015a), who demonstrated a rapid effect of E2 on aggression in a related songbird. First, we quantified attempted attacks, defined as the bird making contact with both feet on the wall of its cage facing the opponent and flapping its wings. We considered this behavior to be clearly aggressive and we never saw a bird perform this behavior on another wall of the cage. Second, every 30 s the observer scored the bird's position with respect to the opponent's cage. If the focal bird was located in the third of its cage closest to the opponent, it

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