



Published in final edited form as:

*Horm Behav.* 2018 August ; 104: 41–51. doi:10.1016/j.yhbeh.2018.03.010.

## Rapid effects of 17 $\beta$ -estradiol on aggressive behavior in songbirds: environmental and genetic influences

Sarah A. Heimovics<sup>1</sup>, Jennifer R. Merritt<sup>2</sup>, Cecilia Jalabert<sup>3</sup>, Chunqi Ma<sup>4</sup>, Donna L. Maney<sup>2</sup>, and Kiran K. Soma<sup>3,4</sup>

<sup>1</sup>University of St. Thomas, Department of Biology, St. Paul, MN USA

<sup>2</sup>Emory University, Department of Psychology, Atlanta, GA USA

<sup>3</sup>University of British Columbia, Department of Zoology, Vancouver, BC CANADA

<sup>4</sup>University of British Columbia, Department of Psychology, Vancouver, BC CANADA

### Abstract

17 $\beta$ -estradiol (E<sub>2</sub>) has numerous rapid effects on the brain and behavior. This review focuses on the rapid effects of E<sub>2</sub> on aggression, an important social behavior, in songbirds. First, we highlight the contributions of studies on song sparrows, which reveal that seasonal changes in the environment profoundly influence the capacity of E<sub>2</sub> to rapidly alter aggressive behavior. E<sub>2</sub> administration to male song sparrows increases aggression within 20 min in the non-breeding season, but not in the breeding season. Furthermore, E<sub>2</sub> rapidly modulates several phosphoproteins in the song sparrow brain. In particular, E<sub>2</sub> rapidly affects pCREB in the medial preoptic nucleus, in the non-breeding season only. Second, we describe studies of the white-throated sparrow, which reveal how a genetic polymorphism may influence the rapid effects of E<sub>2</sub> on aggression. In this species, a chromosomal rearrangement that includes ESR1, which encodes estrogen receptor  $\alpha$  (ER $\alpha$ ), affects ER $\alpha$  expression in the brain and the ability of E<sub>2</sub> to rapidly promote aggression. Third, we summarize studies showing that aggressive interactions rapidly affect levels of E<sub>2</sub> and other steroids, both in the blood and in specific brain regions, and the emerging potential for steroid profiling by liquid chromatography tandem mass spectrometry (LC-MS/MS). Such studies of songbirds demonstrate the value of an ethologically informed approach, in order to reveal how steroids act rapidly on the brain to alter naturally-occurring behavior.

### Keywords

aggression; aromatase; estrogen; neurosteroids; non-genomic; season; testosterone

### INTRODUCTION

Many studies have shown the critical roles that steroid hormones play in the proximate regulation of agonistic behavior, particularly within the context of reproduction (Wingfield et al., 2006). Studies of birds have been at the forefront of this topic, starting with Berthold's pioneering research that showed testicular secretions are necessary for the full expression of aggressive behavior in roosters (Quiring, 1944). Wingfield's pioneering work on free-living song sparrows (*Melospiza melodia*) revealed the context-dependent effects of testosterone

(T) on aggression, particularly during periods of social instability (“Challenge Hypothesis”) (Wingfield et al., 1990). Subsequent studies of Japanese quail (*Coturnix japonica*) demonstrated that aromatization of T to 17 $\beta$ -estradiol (E<sub>2</sub>) within the brain regulates aggressive behavior in males (Schlinger and Callard, 1989, 1990a, b; Ubuka and Tsutsui, 2014).

Historically, the effects of T and E<sub>2</sub> on aggression were thought to be mediated by the classical genomic mechanism of steroid action. That is, steroids bind to receptors located in the cytoplasm which dimerize, translocate to the nucleus, bind to response elements in DNA, and alter gene transcription (Adkins-Regan, 2005). The time course of this mechanism of action is relatively slow (from hours to days), but consistent with the seasonal regulation of aggressive behavior by gonadal steroids.

More recently, effects of steroids on behavior are evident over time courses that are too short to be attributed to changes in gene expression (Wendler et al., 2010). Transcription and translation often take hours (Barnea and Gorski, 1970; Zangenehpour and Chaudhuri, 2002), but effects of steroids on neuronal physiology and behavior can be seen in some cases within seconds or minutes (Kelly et al., 1976, 1977). In general, effects of steroids seen within 30 min of administration are likely mediated by non-genomic mechanisms (Cornil et al., 2006). However, given that non-genomic signaling can potentiate genomic signaling (and vice versa) (Wilkenfeld et al., 2017), we will hereafter refer to these as “rapid” effects of E<sub>2</sub>. Research on avian model systems has been at the forefront of our understanding of the ethological relevance of these non-classical mechanisms of steroid action. This review focuses on rapid effects of E<sub>2</sub> on aggressive behavior in songbirds.

### **Proximate and ultimate causes of songbird aggression.**

Aggressive behavior can occur when two or more individuals compete for access to limited resources such as food, territories, and mates. Among birds that breed in the temperate zone, territorial aggression and reproductive behavior co-occur during the breeding season. Males aggressively defend breeding territories against intruders and intensely guard their mates to prevent cuckoldry. Circulating levels of T are elevated above baseline during the breeding season, and neural aromatization of plasma T into E<sub>2</sub> is critical for the expression of aggressive behavior (Figure 1B) (Schlinger and Callard, 1990b; Silverin et al., 2004).

During the non-breeding season, the gonads regress and plasma T is basal (Ball et al., 2004; Soma et al., 1999; Soma and Wingfield, 2001; Wingfield and Hahn, 1994b). In many avian species, this decline in plasma T is associated with territory abandonment and migration to lower latitudes (Garamszegi et al., 2008). Upon arrival at the wintering grounds, some migratory species form flocks with dominance hierarchies while others establish and defend non-breeding territories (Wingfield et al., 2006). Notably, some temperate-zone breeding songbirds do not migrate, but rather remain in the same general area throughout the year. These non-migratory (also known as sedentary) species form flocks or defend territories during the non-breeding season (Wingfield and Monk, 1992).

Regardless of their overwintering behavioral strategy, songbirds that breed in the temperate zone have basal plasma T in autumn and winter (Garamszegi et al., 2008). Therefore,

researchers initially believed that agonistic behavior outside of the breeding season was not regulated by sex steroids. However, in non-migratory male song sparrows, both chronic (10 day) and acute (1 day) inhibition of aromatase reduce territorial aggression during the non-breeding season (Soma et al., 1999; Soma et al., 2000a; Soma et al., 2000b). Taken together, these data suggested that sex steroids, particularly estrogens, can regulate aggressive behavior during the non-breeding season.

During the non-breeding season, secretion of aromatizable androgens from the gonads is dramatically reduced. Multiple lines of evidence suggest that the inactive prohormone dehydroepiandrosterone (DHEA) serves as an alternative source of such steroids (Figure 1C). In song sparrows, DHEA is detectable in the circulation and brain during the non-breeding season (Newman et al., 2008; Newman and Soma, 2009; Soma and Wingfield, 2001). Further, the enzyme that catalyzes the conversion of DHEA into androstenedione, 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase (3 $\beta$ -HSD), is present in the song sparrow brain and upregulated during the non-breeding season (Pradhan and Soma, 2012). There is some evidence that androgens derived from DHEA may play a role in aggression. For example, seasonal changes in plasma DHEA levels match seasonal changes in aggression: both are elevated in the breeding and non-breeding seasons and reduced during molt (Newman et al., 2008; Soma and Wingfield, 2001). Whereas this correlation does not indicate that DHEA directly drives aggression, causal relationships between DHEA and aggression have been shown. DHEA treatment during the autumn increases territorial singing (Soma et al., 2002; Wacker et al., 2016). Also, a simulated territorial intrusion (STI) during the non-breeding season rapidly upregulates 3 $\beta$ -HSD activity in the forebrain (Pradhan et al., 2010b), suggesting that aggressive interactions affect local metabolism of DHEA to androstenedione.

In addition to 3 $\beta$ -HSD, aromatase is present in the song sparrow brain in the breeding and non-breeding seasons, including in two brain regions that regulate aggressive behavior: nucleus taeniae of the amygdala (TnA) and the ventromedial hypothalamus (VMH) (Soma et al., 2003; Wacker et al., 2010). Aromatase activity in the ventromedial telencephalon, which contains TnA, is high in both the breeding and non-breeding seasons but reduced at molt, which parallels seasonal changes in aggressive behavior (Soma et al., 2003). Given that acute administration of an aromatase inhibitor reduces non-breeding aggression in song sparrows (Soma et al., 2000a), these data suggest that aggressive behavior in the non-breeding season is activated by estrogens synthesized locally within the brain (“neuroestrogens”).

### **Mechanisms mediating the effects of E<sub>2</sub> on behavior**

The mechanisms by which estrogens and other steroids influence target cells can be broadly categorized as occurring via either genomic (“nuclear-initiated”) or non-genomic (“membrane-initiated”) (Losel and Wehling, 2003; Roepke et al., 2009; Thomas, 2012; Trainor et al., 2007; Vasudevan and Pfaff, 2007, 2008). As early as 1941, Hans Selye demonstrated that intraperitoneal injection of steroids (e.g., deoxycorticosterone acetate, progesterone) produced deep anesthesia in rats within 15 min (Seyle, 1941). Because effects of steroids within 30 min are widely considered incompatible with a genomic mechanism of action (Cornil et al., 2006), this was the first study to show that steroids can affect

physiology and behavior within minutes via (putatively) non-genomic mechanisms. Today, rapid effects of all major classes of steroids are well-established (Wendler et al., 2010) and known to occur in a variety of taxa, including plants, invertebrates, and vertebrates (Belkhadir and Chory, 2006; Losel and Wehling, 2003; Srivastava et al., 2005).

While all classes of steroids have rapid effects in the brain, the effects of estrogens are the best characterized. E<sub>2</sub> has numerous and widespread effects on the brain and behavior within 30 min. Some of these rapid effects of E<sub>2</sub> might be mediated by intracellular estrogen receptors (ERs), but most rapid effects of E<sub>2</sub> are thought to be initiated at the cell surface, either via ER $\alpha$  or ER $\beta$  that have been trafficked to the plasma membrane or via G-protein-coupled estrogen receptor 1 (GPER1, previously known as GPR30). These membrane-associated ERs (mERs) have myriad downstream effects, including rapid modulation of neurotransmitter release, neuronal firing, and intracellular calcium flux. The mERs also rapidly affect kinases such as extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase, and protein kinases A and C (Heimovics et al., 2012; Mermelstein and Micevych, 2008; Micevych and Dominguez, 2009; Micevych and Mermelstein, 2008; Moss and Gu, 1999). Importantly, in some contexts, the non-genomic effects of E<sub>2</sub> potentiate subsequent genomic effects of E<sub>2</sub> (Vasudevan and Pfaff, 2008; Wilkenfeld et al., 2017). While rapid effects of E<sub>2</sub> have become widely accepted within the past 20 years, much of this work has been done *in vitro* with dissociated neurons, brain slices, or cell lines. Much less work has been done *in vivo*, with a focus on behavior. Some of the gaps in our understanding of the ethological relevance of rapid effects of E<sub>2</sub> have begun to be addressed through research utilizing avian model systems (Remage-Healey et al., 2017). We describe some of these advances in the sections that follow, and we highlight the impact of environmental and genetic variation on the rapid effects of E<sub>2</sub> on aggression in sparrows.

## RAPID EFFECTS OF E<sub>2</sub> ON AGGRESSION: IMPACT OF SEASONAL CHANGES IN THE ENVIRONMENT

Among male song sparrows living on the Pacific coast of the United States and Canada, the nature and the intensity of aggressive behavior during a territorial challenge are similar across seasons. That is, during an STI, male song sparrows sing and show a variety of aggressive behaviors with similar frequencies during the breeding and non-breeding seasons (Figure 2A) (Mukai et al., 2009; Newman and Soma, 2011; Wingfield and Hahn, 1994a). Note that these responses can be robust even during the non-breeding season, when the testes are fully regressed and plasma sex steroid levels are basal. Notably, while aggressive behavior *during* an STI is consistent across seasons, aggressive behavior *after* an STI ends is seasonally variable. Breeding males show elevated territorial singing behavior for hours or even days, whereas non-breeding males significantly reduce singing behavior almost immediately (Figure 2B) (Wingfield, 1994). In other words, once activated, territorial aggression is persistently expressed in the breeding season but transiently expressed in the non-breeding season. This seasonal plasticity in the persistence of aggression could be adaptive. That is, persistent aggression in the non-breeding season might be disadvantageous because it would greatly reduce the amount of time available for foraging, which is already

challenging because of shorter day lengths (~8 hr of light), lower ambient temperatures, increased precipitation, and decreased food availability (Heimovics et al., 2013).

Brain-derived estrogens regulate non-breeding aggression in song sparrows (Soma et al., 1999; Soma et al., 2000a; Soma et al., 2000b). Neurosteroids such as brain-derived estrogens often have rapid and transient effects mediated by non-genomic mechanisms (Pradhan et al., 2010a; Pradhan et al., 2008; Schmidt et al., 2008; Woolley, 2007). The transient nature of non-breeding aggression in this species (Wingfield, 1994) suggested that it is regulated via non-genomic mechanisms. This phenomenon may extend to other taxa as well; groundbreaking work in California mice (*Peromyscus californicus*) revealed that rapid effects of E<sub>2</sub> regulate aggression during short photoperiods (Heimovics et al., 2015b; Laredo et al., 2013; Laredo et al., 2014; Trainor et al., 2008; Trainor et al., 2007).

To test the hypothesis that non-breeding aggression is regulated by the rapid effects of E<sub>2</sub>, we performed a laboratory experiment with male song sparrows that we caught in the field (Heimovics et al., 2015a). Subjects were captured in both the breeding and non-breeding seasons, and then housed singly in cages in outdoor aviaries to ensure that they were exposed to the natural photoperiod, temperature, and humidity. Using a within-subjects design, song sparrows were fed wax moth larva injected with E<sub>2</sub> or vehicle control. Then 10 min after this non-invasive treatment, they were tested using a laboratory STI (L-STI). A live male conspecific in a cage (the opponent) was placed next to the subject's cage, and the birds were allowed to interact for 10 min. The number of times subjects made contact with the cage wall adjacent to the opponent (barrier contacts) as well as the amount of time subjects spent in the third of their cage adjacent to the opponent (proximity time) were used as measures of aggression (Goodson et al., 2005). Acute administration of E<sub>2</sub> had no effect on aggressive behavior in the breeding season. In contrast, E<sub>2</sub> administration rapidly increased the number of barrier contacts in the non-breeding season (Heimovics et al., 2015a) (Figure 2C). These effects of E<sub>2</sub> on a high-intensity aggressive behavior (barrier contacts) were seen within 20 min, suggesting a non-genomic mechanism. Moreover, these data indicate that environmental cues (e.g., short photoperiod, cool temperature) fundamentally alter the mechanisms by which E<sub>2</sub> regulates behavior in song sparrows. Given the similar results in mice (Trainor et al., 2008; Trainor et al., 2007), this phenomenon may be common to many species.

In a related experiment, we explored the rapid effects of E<sub>2</sub> on intracellular signaling cascades in the male song sparrow brain (Heimovics et al., 2012). In this study, we took advantage of the fact that some mERs are coupled to the mitogen-activated protein kinase (MAPK) pathway. We assessed phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK), one of the best characterized members of the MAPK pathway (Abraham et al., 2004; Bryant et al., 2005; Kuroki et al., 2000; Singer et al., 1999). When ERK is phosphorylated, it can in turn phosphorylate cAMP response element binding protein (CREB) (Xing et al., 1998) and tyrosine hydroxylase (TH). TH is the rate-limiting enzyme in dopamine (DA) synthesis (Lindgren et al., 2002), and DA regulates highly motivated, goal-directed behaviors, including aggressive behavior (de Almeida et al., 2005; O'Connell and Hofmann, 2011; van Erp and Miczek, 2000). Thus, we used immunohistochemistry to visualize the phosphorylated forms of these proteins (pERK, pCREB, and pTH) in multiple

brain regions in breeding and non-breeding male song sparrows. As before, subjects were housed singly in cages in outdoor aviaries and thus experienced seasonally-appropriate environmental cues. In this study, subjects were pre-treated with an aromatase inhibitor to reduce endogenous E<sub>2</sub> synthesis. Then E<sub>2</sub> or vehicle was administered, and subjects were euthanized 15min later.

We found that E<sub>2</sub> administration rapidly reduced immunoreactivity (-ir) for pERK, pCREB, and pTH in multiple regions that comprise the 'social decision-making network' (SDMN) (O'Connell and Hofmann, 2011, 2012), including the preoptic area (POA), VMH, lateral septum (LS), TnA, midbrain central gray (GCT), and ventral tegmental area (VTA) (Heimovics et al., 2012). Rapid reduction by E<sub>2</sub> of brain phosphoproteins has been reported elsewhere. For example, Trainor et al. reported a significant reduction in pCREB-ir in the anterior hypothalamus (AH) of mice following an aggressive encounter (Trainor et al., 2010). Also, ER $\alpha$  agonists rapidly lower pERK in neocortical explants (Singh et al., 2000; Toran-Allerand et al., 2002). Acute E<sub>2</sub> administration causes oscillating patterns of pERK levels in rat pituitary cell culture (Watson et al., 2008) and can have bidirectional effects on CREB phosphorylation in hippocampal and striatal cell cultures (Boulware et al., 2005; Grove-Strawser et al., 2010). Inhibitory effects of acute E<sub>2</sub> administration have also been described in another songbird; E<sub>2</sub> treatment decreases expression of a downstream transcriptional target of CREB, Egr-1 (also known as ZENK), in TnA of white-throated sparrows (Merritt et al., 2017). In male song sparrows, the inhibitory effects of E<sub>2</sub> on pERK, pCREB, and pTH were region-specific, but the overall patterns for a given region were generally similar in both seasons. These results suggested that E<sub>2</sub> can rapidly influence intracellular signaling cascades in the male song sparrow brain both during and outside of the breeding season.

In contrast to this general pattern, pCREB-ir in the medial preoptic nucleus (POM), which is located within the POA, was rapidly reduced by E<sub>2</sub> in non-breeding subjects but unaffected in breeding subjects (Heimovics et al., 2012) (Figure 2D). This result is interesting because the POM is a key node in the SDMN and modulates social behavior so that it is seasonally-appropriate (Alger and Ritters, 2006; Heimovics et al., 2011; Ritters, 2012). The observation that acute E<sub>2</sub> administration significantly altered POM pCREB in the non-breeding season only, taken together with the observation that acute E<sub>2</sub> administration increased aggressive behavior in the non-breeding season only, suggested that E<sub>2</sub> regulates non-breeding aggression via non-genomic mechanisms in the POM. In support of that hypothesis are studies in male Japanese quail showing that T must be aromatized in the POM to activate male reproductive behavior (reviewed in Balthazart et al., 2004).

In our studies in song sparrows, we examined a natural social behavior and combined field approaches under natural conditions with laboratory approaches under controlled conditions. These complementary approaches are powerful in combination, and together can elucidate both the molecular mechanisms of rapid estrogen action as well as the adaptive significance of rapid steroid actions. Most research on steroid action employs subjects housed under constant environmental conditions (e.g., lights on from 0700 to 1900). However, studies of song sparrows and mice reveal that environmental cues such as photoperiod (Heimovics et al., 2015b) can profoundly affect the ways in which E<sub>2</sub> affects the brain and behavior.

## RAPID EFFECTS OF E<sub>2</sub> ON AGGRESSION: IMPACT OF GENETIC VARIATION WITHIN A SPECIES

Another common North American songbird, the white-throated sparrow (*Zonotrichia albicollis*), provides a second context in which to understand the ethological significance of rapid effects of E<sub>2</sub> on aggressive behavior. Like many other New World sparrows, birds of this species defend territories during the breeding season. During the non-breeding season, they migrate to wintering grounds and form dominance hierarchies within flocks. In other ways, they are unlike any other sparrow; a plumage polymorphism segregates with a behavioral phenotype. Birds of the white-striped (WS) morph, which have striking black and white crown stripes, respond to territorial threats with higher levels of aggression than do birds of the tan-striped (TS) morph, which have duller brown and tan crown stripes (Figure 3A) (Collins and Houtman, 1999; Horton et al., 2014b; Knapton and Falls, 1983; Kopachena and Falls, 1993a, b; Tuttle, 2003; Tuttle et al., 2016). This species thus provides a natural experiment with which to investigate the neuroendocrine mechanisms underlying aggression in songbirds.

During the early breeding season, plasma T is higher in WS than TS males, and both plasma E<sub>2</sub> and T are higher in WS than TS females (Horton et al., 2014b). Plasma sex steroids are correlated with the behavioral response to STI in both sexes (Horton et al., 2014b), suggesting that the morph difference in aggression might be explained completely by circulating sex steroids. Studies done on captive birds in a controlled setting, however, show otherwise. When plasma sex steroids were experimentally equalized between the morphs via subcutaneous silastic capsules containing T or E<sub>2</sub>, the morph difference in aggressive vocalizations persisted (Maney et al., 2009). This result suggested that the morph difference in behavior cannot be explained solely by morph differences in plasma levels of sex steroids.

The solution to this puzzle lies in the unique genetics of this species. Whether a bird is WS or TS depends on the presence or absence of a chromosomal rearrangement, called ZAL2<sup>m</sup>, on the second chromosome. Birds of the more aggressive WS morph are heterozygous for the rearrangement (ZAL2<sup>m</sup>/ZAL2), whereas birds of the TS morph are homozygous for the standard arrangement (ZAL2/ZAL2) (Figure 3A). ZAL2<sup>m</sup> is maintained in populations by disassortative mating, meaning that almost all breeding pairs consist of one WS bird and one TS bird (Tuttle et al., 2016). Because of this unusual mating system, the ZAL2<sup>m</sup> chromosomal arrangement is in a near-constant state of heterozygosity (Horton et al., 2013; Tuttle et al., 2016) and recombination between ZAL2 and ZAL2<sup>m</sup> is profoundly suppressed (Davis et al., 2011; Thomas et al., 2008). This recombination suppression has led to the divergence of genes on ZAL2<sup>m</sup>, compared with ZAL2 (Thomas et al., 2008). All of the differences between the morphs, including the behavioral differences, can ultimately be traced to those genes.

Because aggression in songbirds is strongly linked to sex steroid signaling, the genes involved in steroid signaling pathways are strong candidates for explaining morph differences in aggression in white-throated sparrows. The gene that encodes ER $\alpha$ , known as ESR1, is inside the ZAL2<sup>m</sup> rearrangement (Thomas et al., 2008). As is the case for many such genes, the ZAL2 and ZAL2<sup>m</sup> alleles of ESR1 are differentiating from each other.

Changes in the genetic sequence of a receptor can affect the ligand's potency in two major ways (Maney, 2017). First, alterations of the coding region of the gene can impact protein sequence and therefore *function*. In other words, the receptor itself could be structurally altered such that it no longer binds E<sub>2</sub>, coactivators, or estrogen response elements as effectively as before. Second, changes in regulatory, non-coding regions of the gene can have profound effects on the levels of expression and therefore receptor *abundance*. In our work, we have investigated the likelihood that either mechanism has contributed to the behavioral polymorphism in white-throated sparrows.

Although there are two changes in the coding sequences of ESR1, neither is predicted to affect receptor function (Horton et al., 2014a). Instead, most of the genetic variation between the ZAL2 and ZAL2<sup>m</sup> alleles of ESR1 has occurred in non-coding regions of the gene. In one of the ESR1 promoter regions, for example, stable polymorphisms between ZAL2 and ZAL2<sup>m</sup> occur in putatively functional sequences in the promoters, such as transcription factor binding sites (Horton et al., 2014a) (Figure 3B). These sites likely contribute to differential expression of ER $\alpha$  mRNA. In cell culture, the promoters of the ZAL2 and ZAL2<sup>m</sup> alleles differ in their ability to drive expression of a reporter gene (Horton et al., 2014a), suggesting that polymorphisms in the promoter sequence may cause morph differences in expression in the brain.

Comparisons of WS and TS brain tissue confirm that levels of ER $\alpha$  mRNA differ according to morph. Particularly in TnA, WS birds have nearly three times more ER $\alpha$  mRNA than do TS birds (Horton et al., 2014a) (Figure 3C). Moreover, the level of ER $\alpha$  mRNA in some regions predicts the vocal response to STI (Figure 3D), even when plasma sex steroids and morph are controlled in statistical models. In fact, levels of ER $\alpha$  mRNA expression in both TnA and the paraventricular nucleus predict aggressive responses even better than morph itself (Horton et al., 2014a). Thus, we have strong correlational evidence that ER $\alpha$  expression in the brain could be an important mechanism underlying the behavioral polymorphism in this species. Perhaps WS birds are more aggressive than TS birds in part because of the greater abundance of ER $\alpha$  expression in key brain areas.

The behavioral paradigm developed by Heimovics et al. (2015), reviewed above, allowed a careful test of whether E<sub>2</sub> elicits a greater behavioral response in WS than TS birds, and whether it does so via rapid mechanisms (Merritt et al., 2017). ER $\alpha$  is well-known to mediate some of the rapid effects of E<sub>2</sub> (Abraham et al., 2004; Bulayeva and Watson, 2004; Dewing et al., 2007; Milner et al., 2001). The ZAL2<sup>m</sup> rearrangement has captured not only ESR1, but also other genes known to play a role in rapid ER $\alpha$  signaling, such as the gene encoding the metabotropic glutamate receptor (GRM1) and genes involved in the MAPK pathway (Davis et al., 2011; Zinzow-Kramer et al., 2015).

We therefore set out to test whether acute administration of exogenous E<sub>2</sub> is more effective at stimulating aggression in WS than in TS birds. To do so, we manipulated E<sub>2</sub> in laboratory-housed male and female white-throated sparrows and quantified their aggressive behavior according to the protocol of Heimovics et al. (2015). We took advantage of the fact that morph differences in ER $\alpha$  expression persist year-round in several brain regions (Maney et al., 2015), consistent with the result that neural expression of ER $\alpha$  does not vary across

seasons in song sparrows (Wacker et al., 2010). Brain levels of sex steroids, on the other hand, are lower during the non-breeding season relative to the breeding season (Heimovics et al., 2016). We therefore reasoned that in non-breeding white-throated sparrows, ER $\alpha$  would be differentially expressed but not fully occupied, allowing us to test our hypothesis that WS birds are more sensitive to E<sub>2</sub>.

A bolus dose of E<sub>2</sub>, administered orally via a wax moth larva, rapidly elevated plasma E<sub>2</sub> similarly in birds of both morphs (Merritt et al., 2017). Nonetheless, E<sub>2</sub> treatment rapidly elevated aggression over baseline levels only in WS birds. Birds of the WS morph, but not the TS morph, exhibited more barrier contacts directed towards an opponent and spent more time near that opponent (Figure 3E). These effects were detected by 20 min after the birds consumed E<sub>2</sub>. At that point in the trial, we placed a visual barrier between the focal bird and the opponent and recorded behavior for 10 more min. Even when they could no longer see the opponent, WS birds, but not TS birds, spent more time on the side of the cage closest to the opponent (Figure 3E). Overall, these results demonstrate that the morphs are in fact differentially sensitive to the rapid effects of E<sub>2</sub>, suggesting that the known polymorphism in ER $\alpha$  or related genes could at least partly explain why WS birds are more aggressive than TS birds.

Our work on white-throated sparrows expands our knowledge about steroids and behavior beyond this single species. Through this model, we can demonstrate how small changes in genetic sequence ultimately lead to changes in behavior; in other words, we can understand how steroid-dependent behaviors evolve. We know that changes in the promoter sequence of ESR1 have led to changes in its expression, but we do not know which changes are the most important. Our future work will pinpoint the exact genetic polymorphisms that are most likely to contribute to variation in expression. Moreover, we know that the expression of ER $\alpha$  mRNA differs between the morphs, but we do not yet have definitive evidence that this variation in expression causes behavioral variation. By manipulating the abundance of ER $\alpha$  in specific brain regions, we will be able to test that hypothesis directly. This sparrow thus represents one of the only vertebrate models in which the effects of polymorphisms in genetic sequence can be traced, through multiple levels of biological organization, to measure their effects on behavior.

## RAPID EFFECTS OF E<sub>2</sub> ON PHYSICAL VS. VOCAL AGGRESSION

Territorial aggression in songbirds is characterized by several distinct behaviors that include both physical aggression (e.g. attacks) and vocal aggression (e.g. songs). Although acute administration of E<sub>2</sub> rapidly increased physical aggression in song sparrows and white-throated sparrows (Heimovics et al., 2015a; Merritt et al., 2017), the number of songs was unaffected. The lack of a rapid effect of E<sub>2</sub> on song stands in contrast to studies showing that vocal aggression can be induced by T or E<sub>2</sub> administered chronically (7 days) in male and female white-throated sparrows, respectively (Maney et al., 2009).

Collectively, these findings are consistent with other studies showing that stimulating song via steroid treatment takes longer than can be explained by non-genomic mechanisms. Sartor et al. (2005) reported that canaries required 4 days of continuous T treatment before

beginning to sing, and they did not sing at breeding-typical levels until 11 days. Similarly, treatment with E<sub>2</sub> has sometimes been reported to increase singing (Harding et al., 1988; Sartor et al., 2005; Soma et al., 2000b; Tramontin et al., 2003) but it does not do so rapidly. Overall, the literature suggests that the brain—presumably the song system and connected structures—must be exposed to sex steroids for several days to see effects on song rate and stereotypy. After exposure to gonadal T for a few days, however, acute inhibition of aromatase can rapidly inhibit singing (Alward et al., 2016). Thus, E<sub>2</sub> may be required for the moment-to-moment control of singing but cannot, alone, stimulate singing without days of priming. It therefore seems unlikely that all steroid-dependent components of territorial aggression in songbirds are completely mediated by rapid effects.

One caveat is that the studies examining the rapid effects of E<sub>2</sub> on aggression have been done in the lab, on birds presented with an opponent at close range (Heimovics et al., 2015a; Merritt et al., 2017). During STIs in the field, males sometimes do not sing when in close proximity to the decoy; they use physical aggression rather than vocal aggression at close range (Heimovics and Soma, *personal observations*). In the lab, presentation of a female at close range inhibits singing in white-throated sparrows (Grozhiik et al., 2014), Cassin's finches (Sockman et al., 2005) and great tits (Krebs et al., 1981). Clearly, rapid effects of E<sub>2</sub> on singing must be tested in behavioral contexts in which singing normally occurs. Ultimately, mechanisms underlying aggression may be quite diverse among species. For example, the extent to which song is used in aggressive contexts varies tremendously among species, whereas other behaviors, such as flights and attacks, are more consistently used in aggressive contexts. Thus, the neuroendocrine mechanisms underlying physical and vocal aggression may be under different selection pressures and therefore at least partially dissociable.

## **RAPID EFFECTS OF AGGRESSIVE BEHAVIOR ON BRAIN E<sub>2</sub>: METHODOLOGICAL CONSIDERATIONS**

For at least 30 years, we have known that social interactions can dramatically alter circulating levels of sex steroids in a variety of vertebrate taxa (Goymann, 2009; Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990). In many songbirds, an STI during the breeding season induces an increase in plasma T levels in males 10 to 30 min after stimulus onset (Wingfield et al., 1990; Wingfield et al., 2006). Moreover, the *neighbors* of highly aggressive males also have increased circulating T, likely driven by exposure to their neighbor's increased singing and aggression (Wingfield, 1984). Further, when male song sparrows are experimentally removed from their breeding territories, floater males take over the now-vacant territories, and an increase in hypothalamic-pituitary-gonadal (HPG) axis activity is seen in both the replacement males and their neighbors (Wingfield, 1985). This result suggests that social interactions associated with the reorganization of territory boundaries elevate plasma T. In all, these data show that during periods of social instability, aggressive social interactions lead to activation of the HPG axis and increased circulating levels of T (Wingfield et al., 2001).

In recent years, the effects of social behavior on brain levels of sex steroids in birds have begun to be characterized (Remage-Healey et al., 2017). The avian brain expresses all the enzymes required to synthesize sex steroids either from circulating inactive precursors, such as DHEA, or *de novo* from cholesterol (Figure 1D) (London et al., 2006; Soma et al., 2004; Soma et al., 2003). Importantly, the activity of steroidogenic enzymes in the song sparrow brain can vary depending on endocrine state. For example, the activity of 3 $\beta$ -HSD in several brain regions is higher in the non-breeding season than in the breeding season, perhaps to compensate for low systemic T levels (Soma, 2006; Soma et al., 2008; Soma and Wingfield, 1999). In non-breeding song sparrows, 3 $\beta$ -HSD activity in the telencephalon is rapidly increased even further in response to an STI (Pradhan et al., 2010b). Thus, an aggressive interaction can rapidly stimulate DHEA conversion to androstenedione, an active and aromatizable androgen, in the forebrain of non-breeding song sparrows.

In order to understand the effects of aggressive encounters on neurosteroid production, we need to be able to accurately measure neurosteroid levels. One of the most powerful tools for this purpose is *in vivo* microdialysis. Using this technique, Remage-Healey et al. (2012; 2008) showed that hearing conspecific song playback causes a rapid increase in E<sub>2</sub> levels in the auditory forebrain in male zebra finches. This technique is thus sensitive enough to detect rapid changes in steroid levels; however, sampling is often limited to only one brain region at a time and is hindered by low recovery of steroids across the dialysis membrane. Additionally, the sampling period (e.g. 30 min) determines the temporal resolution, which is crucial for evaluating rapid changes in local steroid concentrations. Shorter sampling periods increase the temporal resolution but also reduce sample volume, and therefore it is especially important to develop sensitive techniques to measure steroids.

A complementary approach to *in vivo* microdialysis is the Palkovits punch technique (Palkovits, 1973), which has been used in several avian species (Charlier et al., 2011; Charlier et al., 2010; Heimovics et al., 2016; Schumacher and Balthazart, 1987; Vockel et al., 1990). Thick (e.g. 300  $\mu$ m) sections of brain tissue are cut on a cryostat, and cannulae with inner diameters ranging from 250  $\mu$ m to 2 mm are used to microdissect tissue from multiple brain regions. The quantities of tissue collected in these “punches” are very small. For example, one punch that is 300  $\mu$ m thick and 1 mm in diameter weighs 0.245 mg (Taves et al., 2011). Multiple punches from the same region can be pooled to obtain sufficient tissue to measure steroids present at low concentrations, such as E<sub>2</sub>. Because the brain contains many lipids that can interfere with steroid assays, liquid-liquid or solid phase extraction should be used to separate steroids from interfering substances (Appelblad and Irgum, 2002; Taves et al., 2011).

We recently used this approach to examine the rapid effects of a short L-STI on levels of DHEA, T, and E<sub>2</sub> in the brains of breeding and non-breeding male song sparrows (Heimovics et al., 2016). Birds were housed singly in cages placed in outdoor aviaries and thus experienced seasonally-appropriate environmental conditions. Subjects included males that were socially dominant or subordinate during the L-STI, as well as control males that interacted with an empty cage. Brains were collected from subjects immediately following the 5 min L-STI, and steroids were measured in medial striatum, POA, AH, TnA, and midbrain punches using solid phase extraction followed by sensitive radioimmunoassays.

In general, levels of DHEA, T, and E<sub>2</sub> were higher in brain tissue than in plasma. Levels of T and E<sub>2</sub> in several SDMN nuclei were affected by season but, unexpectedly, not affected by the L-STI. For example, T and E<sub>2</sub> levels in the AH were significantly elevated in the breeding season relative to the non-breeding season, but unaffected by either winning or losing an aggressive encounter (Figure 4B, 4C) (Heimovics et al., 2016). In contrast, levels of DHEA in several nuclei were significantly affected by the L-STI. For example, in both seasons, dominant males had lower levels of DHEA in AH relative to subordinate and/or control animals (Figure 4A) (Heimovics et al., 2016). Also, dominant males had lower levels of DHEA in TnA than both subordinate and control animals in the breeding season only. It was surprising that L-STI did not increase brain levels of T or E<sub>2</sub> in this study. It is possible that the L-STI was too short (5 min) to detect such effects. It is also possible that very localized changes in T and E<sub>2</sub> concentrations (e.g. at specific synapses) are difficult to detect and require even greater spatial resolution and assay sensitivity. Regardless, a significant decrease in brain levels of DHEA following the L-STI was consistent with a separate study that showed that a territorial challenge rapidly increases 3 $\beta$ -HSD activity in the brain (Pradhan et al., 2010b) and suggests that metabolites of DHEA regulate aggressive behavior throughout the year. To date, however, the specific DHEA metabolites involved in modulating behavior during an aggressive challenge remain unknown. Resolving this ambiguity is important because steroids that are synthesized locally in the brain are more likely to act via non-genomic mechanisms than those produced in the periphery (Schmidt et al., 2008).

Detecting rapid changes in neuroestrogens and neuroandrogens in punches, particularly with even greater spatial resolution, might require even greater sensitivity than is possible with radioimmunoassays. Moreover, because levels of multiple steroids can change concomitantly within the same brain region, an ideal assay would allow measurement of other active metabolites of DHEA, addition to T and E<sub>2</sub>, such as androstenedione, androstenediol, estrone (E<sub>1</sub>), and 17 $\alpha$ -E<sub>2</sub>. To address this need, we are currently developing novel protocols for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Tobiansky et al., 2018), an ultrasensitive method that allows the measurement of multiple analytes with great specificity (Vogeser and Parhofer, 2007). In a preliminary LC-MS/MS study, we quantified E<sub>2</sub> levels in ovary and punches of male POA from zebra finches (Figure 5). We are now developing protocols to quantify other estrogens, such as E<sub>1</sub> and 17 $\alpha$ -E<sub>2</sub> (Toran-Allerand et al., 2005) as well as E<sub>2</sub> metabolites.

LC-MS/MS sensitivity can be improved even further by derivatization (Barreiros et al., 2016), which increases ionization efficiency of analytes (Faqehi et al., 2016). In another preliminary study, we compared the efficacy of three different reagents to derivatize E<sub>2</sub>: 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS) (Keski-Rahkonen et al., 2015), 1-methylimidazole-2-sulfonyl chloride (ISCl) (Li and Franke, 2015), and dansyl chloride (DSCL) (Anari et al., 2002). All three reagents improved sensitivity, especially DSCL (Figure 6) and might facilitate the profiling of estrogens in brain punches. These methodological advances will enable us to test whether an aggressive interaction rapidly modulates levels of E<sub>2</sub> and other estrogens in a region-specific manner in songbirds, and they will also be useful for studies of estrogens in rodents and humans (Sato and Woolley, 2016a, b; Wang et al., 2016).

## CONCLUSIONS

Studies of avian species have contributed immensely to our understanding of how hormones act in the brain to regulate complex social behaviors. For example, recent studies utilizing songbird model systems have provided several important and novel insights into the rapid effects of E<sub>2</sub> on aggressive behavior. Work in song sparrows highlights the influences of environmental variables, such as photoperiod, on the mechanisms by which E<sub>2</sub> rapidly affects aggression and intracellular signaling cascades. Work in white-throated sparrows highlights the influence of variation in estrogen-related gene sequences on the rapid behavioral effects of E<sub>2</sub>. Songbirds express high levels of aromatase in the brain, facilitating the measurement of aromatase and E<sub>2</sub> in microdissected tissue. Studies in wild songbirds allow examination of natural social behaviors in non-domesticated species, which complements studies of domesticated rodents in the laboratory. There are >6,000 species of songbirds (Ricklefs, 2012), which vary widely in their natural history, physiology, and behavior. Future work on the rapid effects of E<sub>2</sub> on songbird aggression will provide additional insights into the ethological relevance of this non-classical mechanism of E<sub>2</sub> action, and these insights will be broadly relevant to other species, other behaviors, and other steroids.

## ACKNOWLEDGMENTS

We thank Profs. Jacques Balthazart, Elena Choleris, and Luke Ramage-Healey for the invitation to contribute to this Special Issue. The work described herein was supported by Canadian Institutes of Health Research Operating Grant 133606 (to KKS), Canada Foundation for Innovation Grant (to KKS), BC Knowledge Development Fund Grant (to KKS), Uruguay Graduate Scholarship from the Agencia Nacional de Investigación e Innovación (to CJ), NIH R01MH082833 (to DLM), and NIH R21MH102677 (to DLM).

## REFERENCES

- Abraham IM, Todman MG, Korach KS, Herbison AE, 2004 Critical in vivo roles for classical estrogen receptors in rapid estrogen actions on intracellular signaling in mouse brain. *Endocrinology* 145, 3055–3061. [PubMed: 14976146]
- Adkins-Regan E, 2005 *Hormones and animal social behavior* Princeton University Press, Princeton, NJ.
- Alger SJ, Riters LV, 2006 Lesions to the medial preoptic nucleus differentially affect singing and nest box-directed behaviors within and outside of the breeding season in European starlings (*Sturnus vulgaris*). *Behav Neurosci* 120, 1326–1336. [PubMed: 17201478]
- Alward BA, de Bournonville C, Chan TT, Balthazart J, Cornil CA, Ball GF, 2016 Aromatase inhibition rapidly affects in a reversible manner distinct features of birdsong. *Sci Rep* 6, 32344. [PubMed: 27573712]
- Anari MR, Bakhtiar R, Zhu B, Huskey S, Franklin RB, Evans DC, 2002 Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: application in trace analysis of ethinylestradiol in rhesus monkey plasma. *Anal Chem* 74, 4136–4144. [PubMed: 12199585]
- Appelblad P, Irgum K, 2002 Separation and detection of neuroactive steroids from biological matrices. *J Chromatogr A* 955, 151–182. [PubMed: 12075920]
- Ball GF, Auger CJ, Bernard DJ, Charlier TD, Sartor JJ, Riters LV, Balthazart J, 2004 Seasonal plasticity in the song control system: multiple brain sites of steroid hormone action and the importance of variation in song behavior. *Ann N Y Acad Sci* 1016, 586–610. [PubMed: 15313796]
- Balthazart J, Baillien M, Cornil CA, Ball GF, 2004 Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. *Physiol Behav* 83, 247–270. [PubMed: 15488543]

- Barnea A, Gorski J, 1970 Estrogen-induced protein. Time course of synthesis. *Biochemistry* 9, 1899–1904. [PubMed: 5442157]
- Barreiros L, Queiroz JF, Magalhães LM, Silva AMT, Segundo MA, 2016 Analysis of 17- $\beta$ -estradiol and 17- $\alpha$ -ethinylestradiol in biological and environmental matrices — A review. *Microchemical Journal* 126, 243–262.
- Belkhadir Y, Chory J, 2006 Brassinosteroid signaling: a paradigm for steroid hormone signaling from the cell surface. *Science* 314, 1410–1411. [PubMed: 17138891]
- Boulware MI, Weick JP, Becklund BR, Kuo SP, Groth RD, Mermelstein PG, 2005 Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. *J Neurosci* 25, 5066–5078. [PubMed: 15901789]
- Bryant DN, Bosch MA, Ronnekleiv OK, Dorsa DM, 2005 17-Beta estradiol rapidly enhances extracellular signal-regulated kinase 2 phosphorylation in the rat brain. *Neuroscience* 133, 343–352. [PubMed: 15893655]
- Bulayeva NN, Watson CS, 2004 Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. *Environ Health Perspect* 112, 1481–1487. [PubMed: 15531431]
- Charlier TD, Newman AE, Heimovics SA, Po KW, Saldanha CJ, Soma KK, 2011 Rapid effects of aggressive interactions on aromatase activity and oestradiol in discrete brain regions of wild male white-crowned sparrows. *J Neuroendocrinol* 23, 742–753. [PubMed: 21623961]
- Charlier TD, Po KW, Newman AE, Shah AH, Saldanha CJ, Soma KK, 2010 17beta-Estradiol levels in male zebra finch brain: combining Palkovits punch and an ultrasensitive radioimmunoassay. *Gen Comp Endocrinol* 167, 18–26. [PubMed: 20144613]
- Collins C, Houtman A, 1999 Tan and white color morphs of white-throated sparrows differ in their non-song vocal responses to territorial intrusion. *Condor*, 842–845.
- Cornil CA, Ball GF, Balthazart J, 2006 Functional significance of the rapid regulation of brain estrogen action: where do the estrogens come from? *Brain Res* 1126, 2–26. [PubMed: 16978590]
- Davis JK, Mittel LB, Lowman JJ, Thomas PJ, Maney DL, Martin CL, Thomas JW, 2011 Haplotype-based genomic sequencing of a chromosomal polymorphism in the white-throated sparrow (*Zonotrichia albicollis*). *J Hered* 102, 380–390. [PubMed: 21613376]
- de Almeida RM, Ferrari PF, Parmigiani S, Miczek KA, 2005 Escalated aggressive behavior: dopamine, serotonin and GABA. *Eur J Pharmacol* 526, 51–64. [PubMed: 16325649]
- Dewing P, Boulware MI, Sinchak K, Christensen A, Mermelstein PG, Micevych P, 2007 Membrane estrogen receptor-alpha interactions with metabotropic glutamate receptor 1a modulate female sexual receptivity in rats. *J Neurosci* 27, 9294–9300. [PubMed: 17728443]
- Faqehi AM, Cobice DF, Naredo G, Mak TC, Upreti R, Gibb FW, Beckett GJ, Walker BR, Homer NZ, Andrew R, 2016 Derivatization of estrogens enhances specificity and sensitivity of analysis of human plasma and serum by liquid chromatography tandem mass spectrometry. *Talanta* 151, 148–156. [PubMed: 26946022]
- Garamszegi LZ, Hirschenhauser K, Bokony V, Eens M, Hurtrez-Bousses S, Moller AP, Oliveira RF, Wingfield JC, 2008 Latitudinal distribution, migration, and testosterone levels in birds. *Am Nat* 172, 533–546. [PubMed: 18729776]
- Goodson JL, Evans AK, Soma KK, 2005 Neural responses to aggressive challenge correlate with behavior in nonbreeding sparrows. *Neuroreport* 16, 1719–1723. [PubMed: 16189485]
- Goymann W, 2009 Social modulation of androgens in male birds. *Gen Comp Endocrinol* 163, 149–157. [PubMed: 19100740]
- Grove-Strawser D, Boulware MI, Mermelstein PG, 2010 Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons. *Neuroscience* 170, 1045–1055. [PubMed: 20709161]
- Grozhhik AV, Horoszko CP, Horton BM, Hu Y, Voisin DA, Maney DL, 2014 Hormonal regulation of vasotocin receptor mRNA in a seasonally breeding songbird. *Horm Behav* 65, 254–263. [PubMed: 24333848]
- Harding CF, Walters MJ, Collado D, Sheridan K, 1988 Hormonal specificity and activation of social behavior in male red-winged blackbirds. *Horm Behav* 22, 402–418. [PubMed: 3169703]

- Heimovics SA, Cornil CA, Ellis JM, Ball GF, Ritters LV, 2011 Seasonal and individual variation in singing behavior correlates with alpha 2-noradrenergic receptor density in brain regions implicated in song, sexual, and social behavior. *Neuroscience*.
- Heimovics SA, Ferris JK, Soma KK, 2015a Non-invasive administration of 17beta-estradiol rapidly increases aggressive behavior in non-breeding, but not breeding, male song sparrows. *Horm Behav* 69, 31–38. [PubMed: 25483754]
- Heimovics SA, Fokidis HB, Soma KK, 2013 Brain Aromatase and Territorial Aggression Across the Seasons in Male Song Sparrows, in: Balthazart J, Ball GF (Eds.), *Brain Aromatase, Estrogens, and Behavior* Oxford University Press, Oxford; New York.
- Heimovics SA, Prior NH, Ma C, Soma KK, 2016 Rapid Effects of an Aggressive Interaction on Dehydroepiandrosterone, Testosterone and Oestradiol Levels in the Male Song Sparrow Brain: a Seasonal Comparison. *J Neuroendocrinol* 28, 12345. [PubMed: 26648568]
- Heimovics SA, Prior NH, Maddison CJ, Soma KK, 2012 Rapid and widespread effects of 17beta-estradiol on intracellular signaling in the male songbird brain: a seasonal comparison. *Endocrinology* 153, 1364–1376. [PubMed: 22294743]
- Heimovics SA, Trainor BC, Soma KK, 2015b Rapid Effects of Estradiol on Aggression in Birds and Mice: The Fast and the Furious. *Integrative and Comparative Biology* 55, 281–293. [PubMed: 25980562]
- Hirschenhauser K, Oliveira R, 2006 Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. *Animal Behaviour* 71, 265–277.
- Horton BM, Hu Y, Martin CL, Bunke BP, Matthews BS, Moore IT, Thomas JW, Maney DL, 2013 Behavioral characterization of a white-throated sparrow homozygous for the ZAL2(m) chromosomal rearrangement. *Behav Genet* 43, 60–70. [PubMed: 23264208]
- Horton BM, Hudson WH, Ortlund EA, Shirk S, Thomas JW, Young ER, Zinzow-Kramer WM, Maney DL, 2014a Estrogen receptor alpha polymorphism in a species with alternative behavioral phenotypes. *Proc Natl Acad Sci U S A* 111, 1443–1448. [PubMed: 24474771]
- Horton BM, Moore IT, Maney DL, 2014b New insights into the hormonal and behavioural correlates of polymorphism in white-throated sparrows, *Zonotrichia albicollis*. *Anim Behav* 93, 207–219. [PubMed: 25045171]
- Kelly MJ, Moss RL, Dudley CA, 1976 Differential sensitivity of preoptic-septal neurons to microelectrophoresed estrogen during the estrous cycle. *Brain Res* 114, 152–157. [PubMed: 986858]
- Kelly MJ, Moss RL, Dudley CA, 1977 The effects of microelectrophoretically applied estrogen, cortisol and acetylcholine on medial preoptic-septal unit activity throughout the estrous cycle of the female rat. *Exp Brain Res* 30, 53–64. [PubMed: 563341]
- Keski-Rahkonen P, Desai R, Jimenez M, Harwood DT, Handelsman DJ, 2015 Measurement of Estradiol in Human Serum by LC-MS/MS Using a Novel Estrogen-Specific Derivatization Reagent. *Anal Chem* 87, 7180–7186. [PubMed: 26090565]
- Knapton RW, Falls JB, 1983 Differences in parental contribution among pair types in the polymorphic White-throated Sparrow. *Canadian Journal of Zoology* 61, 1288–1292.
- Kopachena JG, Falls JB, 1993a Aggressive Performance as a Behavioral Correlate of Plumage Polymorphism in the White-Throated Sparrow (*Zonotrichia albicollis*). *Behaviour* 124, 249–266.
- Kopachena JG, Falls JB, 1993b Postfledging parental care in the White-throated Sparrow (*Zonotrichia albicollis*). *Canadian Journal of Zoology* 71, 227–232.
- Krebs J, Avery M, Cowie R, 1981 Effect of removal of mate on the singing behaviour of great tits. *Anim Behav* 29, 635–637.
- Kuroki Y, Fukushima K, Kanda Y, Mizuno K, Watanabe Y, 2000 Putative membrane-bound estrogen receptors possibly stimulate mitogen-activated protein kinase in the rat hippocampus. *Eur J Pharmacol* 400, 205–209. [PubMed: 10988335]
- Laredo SA, Villalon Landeros R, Dooley JC, Steinman MQ, Orr V, Silva AL, Crean KK, Robles CF, Trainor BC, 2013 Nongenomic effects of estradiol on aggression under short day photoperiods. *Horm Behav* 64, 557–565. [PubMed: 23763907]

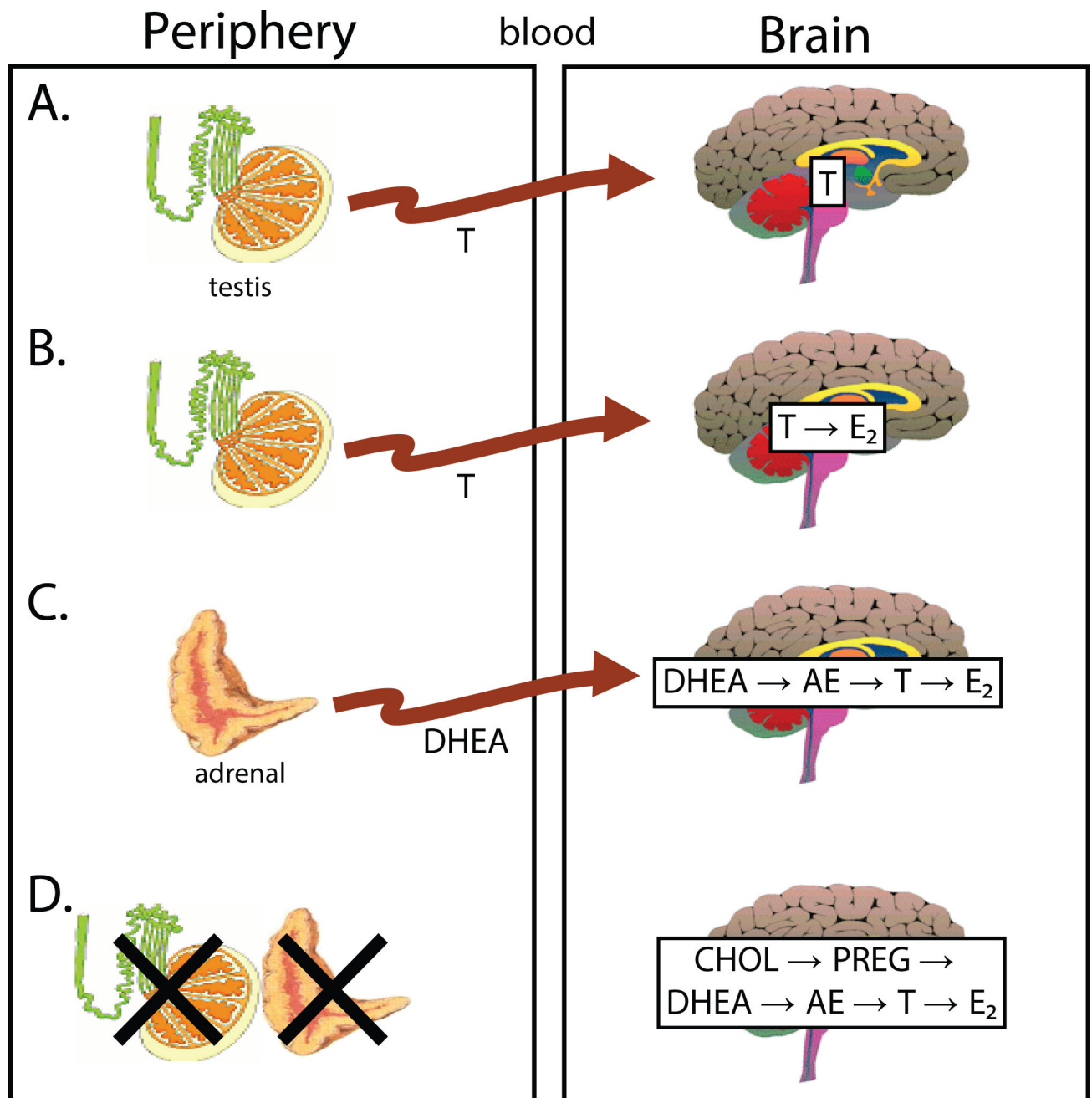
- Laredo SA, Villalon Landeros R, Trainor BC, 2014 Rapid effects of estrogens on behavior: environmental modulation and molecular mechanisms. *Front Neuroendocrinol* 35, 447–458. [PubMed: 24685383]
- Li X, Franke AA, 2015 Improved profiling of estrogen metabolites by orbitrap LC/MS. *Steroids* 99, 84–90. [PubMed: 25543003]
- Lindgren N, Goiny M, Herrera-Marschitz M, Haycock JW, Hokfelt T, Fisone G, 2002 Activation of extracellular signal-regulated kinases 1 and 2 by depolarization stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat brain. *Eur J Neurosci* 15, 769–773. [PubMed: 11886455]
- London SE, Monks DA, Wade J, Schlinger BA, 2006 Widespread capacity for steroid synthesis in the avian brain and song system. *Endocrinology* 147, 5975–5987. [PubMed: 16935847]
- Losel R, Wehling M, 2003 Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* 4, 46–56. [PubMed: 12511868]
- Maney DL, 2017 Polymorphisms in sex steroid receptors: From gene sequence to behavior. *Front Neuroendocrinol* 47, 47–65. [PubMed: 28705582]
- Maney DL, Horton BM, Zinzow-Kramer WM, 2015 Estrogen Receptor Alpha as a Mediator of Life-History Trade-offs. *Integr Comp Biol* 55, 323–331. [PubMed: 25855477]
- Maney DL, Lange HS, Raees MQ, Reid AE, Sanford SE, 2009 Behavioral phenotypes persist after gonadal steroid manipulation in white-throated sparrows. *Horm Behav* 55, 113–120. [PubMed: 18848562]
- Mermelstein PG, Micevych PE, 2008 Nervous system physiology regulated by membrane estrogen receptors. *Rev Neurosci* 19, 413–424. [PubMed: 19317180]
- Merritt J, Davis M, Jalabert C, Libecap T, Williams D, Soma K, Maney D, 2017 Rapid effects of estradiol on aggression depend on genotype in a species with an estrogen receptor polymorphism. *Horm Behav* 98, 210–218.
- Micevych P, Dominguez R, 2009 Membrane estradiol signaling in the brain. *Front Neuroendocrinol* 30, 315–327. [PubMed: 19416735]
- Micevych PE, Mermelstein PG, 2008 Membrane estrogen receptors acting through metabotropic glutamate receptors: an emerging mechanism of estrogen action in brain. *Mol Neurobiol* 38, 66–77. [PubMed: 18670908]
- Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, Alves SE, 2001 Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J Comp Neurol* 429, 355–371. [PubMed: 11116225]
- Moss RL, Gu Q, 1999 Estrogen: mechanisms for a rapid action in CA1 hippocampal neurons. *Steroids* 64, 14–21. [PubMed: 10323668]
- Mukai M, Replogle K, Drnevich J, Wang G, Wacker D, Band M, Clayton DF, Wingfield JC, 2009 Seasonal differences of gene expression profiles in song sparrow (*Melospiza melodia*) hypothalamus in relation to territorial aggression. *PLoS One* 4, e8182. [PubMed: 19997634]
- Newman AE, Pradhan DS, Soma KK, 2008 Dehydroepiandrosterone and corticosterone are regulated by season and acute stress in a wild songbird: jugular versus brachial plasma. *Endocrinology* 149, 2537–2545. [PubMed: 18276756]
- Newman AE, Soma KK, 2009 Corticosterone and dehydroepiandrosterone in songbird plasma and brain: effects of season and acute stress. *Eur J Neurosci* 29, 1905–1914. [PubMed: 19473242]
- Newman AE, Soma KK, 2011 Aggressive interactions differentially modulate local and systemic levels of corticosterone and DHEA in a wild songbird. *Horm Behav* 60, 389–396. [PubMed: 21784076]
- O’Connell LA, Hofmann HA, 2011 The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J Comp Neurol* 519, 3599–3639. [PubMed: 21800319]
- O’Connell LA, Hofmann HA, 2012 Evolution of a vertebrate social decision-making network. *Science* 336, 1154–1157. [PubMed: 22654056]
- Palkovits M, 1973 Isolated removal of hypothalamic or other brain nuclei of the rat. *Brain Res* 59, 449–450. [PubMed: 4747772]
- Pradhan D, Soma K, 2012 Regulation of 3b-HSD activity in the songbird brain. *Journal of Ornithology* 153, 227–234.

- Pradhan DS, Lau LY, Schmidt KL, Soma KK, 2010a 3beta-HSD in songbird brain: subcellular localization and rapid regulation by estradiol. *J Neurochem* 115, 667–675. [PubMed: 20722973]
- Pradhan DS, Newman AE, Wacker DW, Wingfield JC, Schlinger BA, Soma KK, 2010b Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. *Horm Behav* 57, 381–389. [PubMed: 20116379]
- Pradhan DS, Yu Y, Soma KK, 2008 Rapid estrogen regulation of DHEA metabolism in the male and female songbird brain. *J Neurochem* 104, 244–253. [PubMed: 17949414]
- Quiring DP, 1944 Transplantation of testes (by A.A. Berthold). *Bulletin of the History of Medicine* 16, 399–401.
- Remage-Healey L, Dong SM, Chao A, Schlinger BA, 2012 Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. *J Neurophysiol* 107, 1621–1631. [PubMed: 22190616]
- Remage-Healey L, Heimovics SA, Soma KK, Cornil CA, 2017 Rapid Effects of Estrogens on Avian Brain and Social Behavior, in: Pfaff DW, Joëls M (Eds.), *Hormones, Brain, and Behavior* 3rd edition. Oxford: Academic Press.
- Remage-Healey L, Maidment NT, Schlinger BA, 2008 Forebrain steroid levels fluctuate rapidly during social interactions. *Nat Neurosci* 11, 1327–1334. [PubMed: 18820691]
- Ricklefs RE, 2012 Species richness and morphological diversity of passerine birds. *Proc Natl Acad Sci U S A* 109, 14482–14487. [PubMed: 22908271]
- Riters LV, 2012 The role of motivation and reward neural systems in vocal communication in songbirds. *Front Neuroendocrinol* 33, 194–209. [PubMed: 22569510]
- Roepke TA, Qiu J, Bosch MA, Ronnekleiv OK, Kelly MJ, 2009 Cross-talk between membrane-initiated and nuclear-initiated oestrogen signalling in the hypothalamus. *J Neuroendocrinol* 21, 263–270. [PubMed: 19187465]
- Sartor JJ, Balthazart J, Ball GF, 2005 Coordinated and dissociated effects of testosterone on singing behavior and song control nuclei in canaries (*Serinus canaria*). *Horm Behav* 47, 467–476. [PubMed: 15777813]
- Sato SM, Woolley CS, 2016a Acute inhibition of neurosteroid estrogen synthesis suppresses status epilepticus in an animal model. *Elife* 5.
- Sato SM, Woolley CS, 2016b Correction: Acute inhibition of neurosteroid estrogen synthesis suppresses status epilepticus in an animal model. *Elife* 5.
- Schlinger BA, Callard GV, 1989 Aromatase activity in quail brain: correlation with aggressiveness. *Endocrinology* 124, 437–443. [PubMed: 2909376]
- Schlinger BA, Callard GV, 1990a Aggressive behavior in birds: an experimental model for studies of brain-steroid interactions. *Comp Biochem Physiol A Comp Physiol* 97, 307–316. [PubMed: 1979529]
- Schlinger BA, Callard GV, 1990b Aromatization mediates aggressive behavior in quail. *Gen Comp Endocrinol* 79, 39–53. [PubMed: 2191894]
- Schmidt KL, Pradhan DS, Shah AH, Charlier TD, Chin EH, Soma KK, 2008 Neurosteroids, immunosteroids, and the Balkanization of endocrinology. *Gen Comp Endocrinol* 157, 266–274. [PubMed: 18486132]
- Schumacher M, Balthazart J, 1987 Neuroanatomical distribution of testosterone-metabolizing enzymes in the Japanese quail. *Brain Res* 422, 137–148. [PubMed: 3676776]
- Seyle H, 1941 Anesthetic Effect of Steroid Hormones. *Experimental Biology and Medicine* 46, 26–39.
- Silverin B, Baillien M, Balthazart J, 2004 Territorial aggression, circulating levels of testosterone, and brain aromatase activity in free-living pied flycatchers. *Horm Behav* 45, 225–234. [PubMed: 15053938]
- Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM, 1999 The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J Neurosci* 19, 2455–2463. [PubMed: 10087060]
- Singh M, Setalo G, Jr., Guan X, Frail DE, Toran-Allerand CD, 2000 Estrogen-induced activation of the mitogen-activated protein kinase cascade in the cerebral cortex of estrogen receptor-alpha knock-out mice. *J Neurosci* 20, 1694–1700. [PubMed: 10684871]

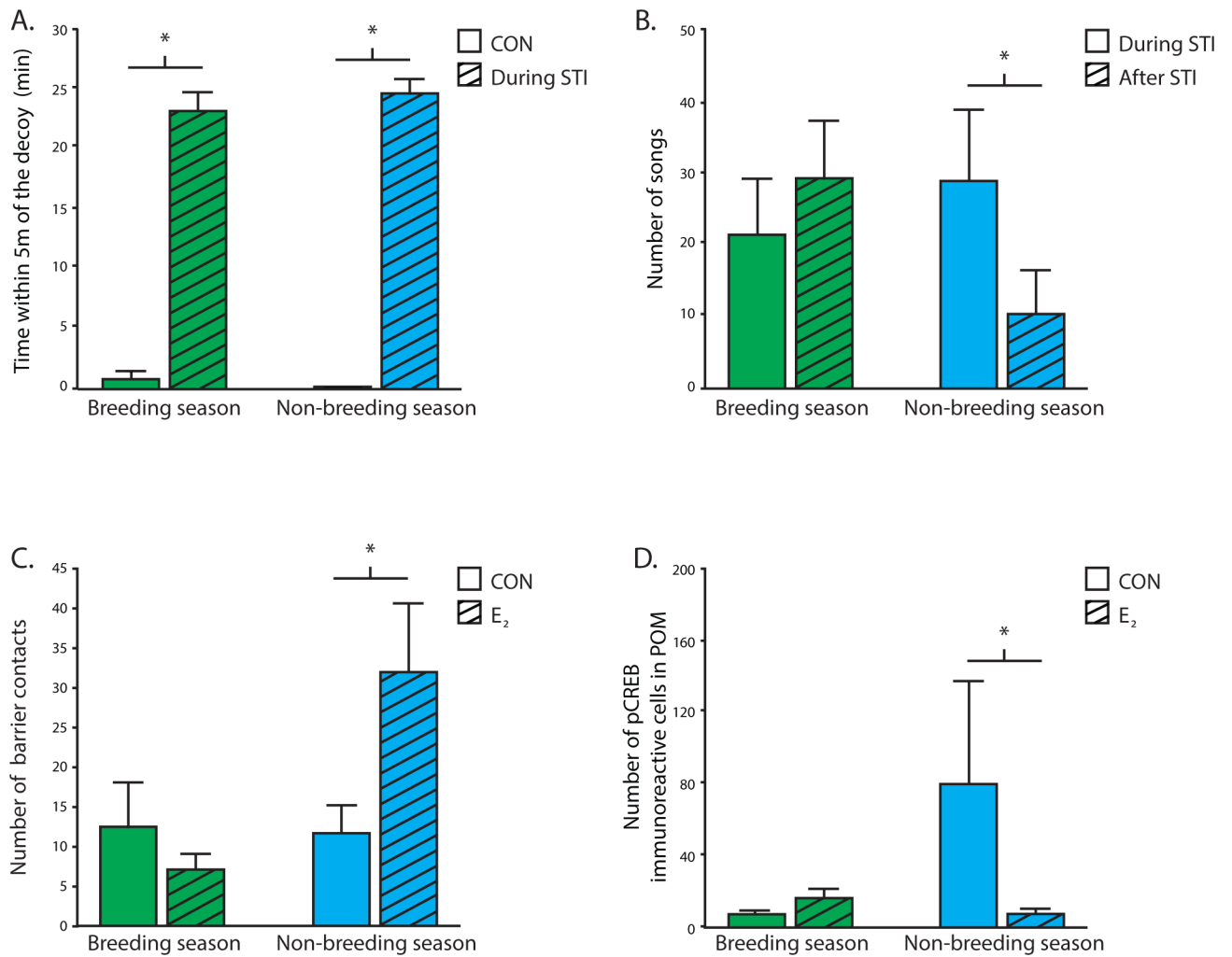
- Sockman KW, Sewall KB, Ball GF, Hahn TP, 2005 Economy of mate attraction in the Cassin's finch. *Biol Lett* 1, 34–37. [PubMed: 17148122]
- Soma KK, 2006 Testosterone and aggression: Berthold, birds and beyond. *J Neuroendocrinol* 18, 543–551. [PubMed: 16774503]
- Soma KK, Alday NA, Hau M, Schlinger BA, 2004 Dehydroepiandrosterone metabolism by 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase in adult zebra finch brain: sex difference and rapid effect of stress. *Endocrinology* 145, 1668–1677. [PubMed: 14670998]
- Soma KK, Schlinger BA, Wingfield JC, Saldanha CJ, 2003 Brain aromatase, 5 alpha-reductase, and 5 beta-reductase change seasonally in wild male song sparrows: relationship to aggressive and sexual behavior. *J Neurobiol* 56, 209–221. [PubMed: 12884261]
- Soma KK, Scotti MA, Newman AE, Charlier TD, Demas GE, 2008 Novel mechanisms for neuroendocrine regulation of aggression. *Front Neuroendocrinol* 29, 476–489. [PubMed: 18280561]
- Soma KK, Sullivan K, Wingfield J, 1999 Combined aromatase inhibitor and antiandrogen treatment decreases territorial aggression in a wild songbird during the nonbreeding season. *Gen Comp Endocrinol* 115, 442–453. [PubMed: 10480996]
- Soma KK, Sullivan KA, Tramontin AD, Saldanha CJ, Schlinger BA, Wingfield JC, 2000a Acute and chronic effects of an aromatase inhibitor on territorial aggression in breeding and nonbreeding male song sparrows. *J Comp Physiol A* 186, 759–769. [PubMed: 11016791]
- Soma KK, Tramontin AD, Wingfield JC, 2000b Oestrogen regulates male aggression in the non-breeding season. *Proc Biol Sci* 267, 1089–1096. [PubMed: 10885513]
- Soma KK, Wingfield J, 1999 Endocrinology of aggression in the nonbreeding season. *Proceedings of the 22nd International Ornithological Congress* (Adams N and Slotow R eds.), 1606–1620.
- Soma KK, Wingfield JC, 2001 Dehydroepiandrosterone in songbird plasma: seasonal regulation and relationship to territorial aggression. *Gen Comp Endocrinol* 123, 144–155. [PubMed: 11482935]
- Soma KK, Wissman AM, Brenowitz EA, Wingfield JC, 2002 Dehydroepiandrosterone (DHEA) increases territorial song and the size of an associated brain region in a male songbird. *Horm Behav* 41, 203–212. [PubMed: 11855905]
- Srivastava DP, Yu EJ, Kennedy K, Chatwin H, Reale V, Hamon M, Smith T, Evans PD, 2005 Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *J Neurosci* 25, 6145–6155. [PubMed: 15987944]
- Taves MD, Ma C, Heimovics SA, Saldanha CJ, Soma KK, 2011 Measurement of steroid concentrations in brain tissue: methodological considerations. *Front Endocrinol* 2, 1–13.
- Thomas JW, Caceres M, Lowman JJ, Morehouse CB, Short ME, Baldwin EL, Maney DL, Martin CL, 2008 The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. *Genetics* 179, 1455–1468. [PubMed: 18562641]
- Thomas P, 2012 Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *Gen Comp Endocrinol* 175, 367–383. [PubMed: 22154643]
- Tobiansky DJ, Korol AM, Ma C, Hamden JE, Jalabert C, Tomm RJ, Soma KK, 2018 Testosterone and Corticosterone in the Mesocorticolimbic System of Male Rats: Effects of Gonadectomy and Caloric Restriction. *Endocrinology* 159, 450–464. [PubMed: 29069423]
- Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr., Nethrapalli IS, Tinnikov AA, 2002 ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J Neurosci* 22, 8391–8401. [PubMed: 12351713]
- Toran-Allerand CD, Tinnikov AA, Singh RJ, Nethrapalli IS, 2005 17alpha-estradiol: a brain-active estrogen? *Endocrinology* 146, 3843–3850. [PubMed: 15947006]
- Trainor BC, Crean KK, Fry WH, Sweeney C, 2010 Activation of extracellular signal-regulated kinases in social behavior circuits during resident-intruder aggression tests. *Neuroscience* 165, 325–336. [PubMed: 19874872]

- Trainor BC, Finy MS, Nelson RJ, 2008 Rapid effects of estradiol on male aggression depend on photoperiod in reproductively non-responsive mice. *Horm Behav* 53, 192–199. [PubMed: 17976598]
- Trainor BC, Lin S, Finy MS, Rowland MR, Nelson RJ, 2007 Photoperiod reverses the effects of estrogens on male aggression via genomic and nongenomic pathways. *Proc Natl Acad Sci U S A* 104, 9840–9845. [PubMed: 17525148]
- Tramontin AD, Wingfield JC, Brenowitz EA, 2003 Androgens and estrogens induce seasonal-like growth of song nuclei in the adult songbird brain. *J Neurobiol* 57, 130–140. [PubMed: 14556279]
- Tuttle EM, 2003 Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. *Behavioral Ecology* 14, 425–432.
- Tuttle EM, Bergland AO, Korody ML, Brewer MS, Newhouse DJ, Minx P, Stager M, Betuel A, Cheviron ZA, Warren WC, Gonser RA, Balakrishnan CN, 2016 Divergence and Functional Degradation of a Sex Chromosome-like Supergene. *Curr Biol* 26, 344–350. [PubMed: 26804558]
- Ubuka T, Tsutsui K, 2014 Review: neuroestrogen regulation of socio-sexual behavior of males. *Front Neurosci* 8, 323. [PubMed: 25352775]
- van Erp AM, Miczek KA, 2000 Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *J Neurosci* 20, 9320–9325. [PubMed: 11125011]
- Vasudevan N, Pfaff DW, 2007 Membrane-initiated actions of estrogens in neuroendocrinology: emerging principles. *Endocr Rev* 28, 1–19. [PubMed: 17018839]
- Vasudevan N, Pfaff DW, 2008 Non-genomic actions of estrogens and their interaction with genomic actions in the brain. *Front Neuroendocrinol* 29, 238–257. [PubMed: 18083219]
- Vockel A, Prove E, Balthazart J, 1990 Sex- and age-related differences in the activity of testosterone-metabolizing enzymes in microdissected nuclei of the zebra finch brain. *Brain Res* 511, 291–302. [PubMed: 2334847]
- Vogeser M, Parhofer KG, 2007 Liquid chromatography tandem-mass spectrometry (LC-MS/MS)--technique and applications in endocrinology. *Exp Clin Endocrinol Diabetes* 115, 559–570. [PubMed: 17943689]
- Wacker DW, Khalaj S, Jones LJ, Champion TL, Davis JE, Meddle SL, Wingfield JC, 2016 Dehydroepiandrosterone Heightens Aggression and Increases Androgen Receptor and Aromatase mRNA Expression in the Brain of a Male Songbird. *J Neuroendocrinol* 28.
- Wacker DW, Wingfield JC, Davis JE, Meddle SL, 2010 Seasonal changes in aromatase and androgen receptor, but not estrogen receptor mRNA expression in the brain of the free-living male song sparrow, *Melospiza melodia morphna*. *J Comp Neurol* 518, 3819–3835. [PubMed: 20653036]
- Wang Q, Mesaros C, Blair IA, 2016 Ultra-high sensitivity analysis of estrogens for special populations in serum and plasma by liquid chromatography-mass spectrometry: Assay considerations and suggested practices. *J Steroid Biochem Mol Biol* 162, 70–79. [PubMed: 26767303]
- Watson CS, Jeng YJ, Kochukov MY, 2008 Nongenomic actions of estradiol compared with estrone and estriol in pituitary tumor cell signaling and proliferation. *FASEB J* 22, 3328–3336. [PubMed: 18541692]
- Wendler A, Baldi E, Harvey BJ, Nadal A, Norman A, Wehling M, 2010 Position paper: Rapid responses to steroids: current status and future prospects. *Eur J Endocrinol* 162, 825–830. [PubMed: 20194525]
- Wilkenfeld SR, Lin C, Frigo DE, 2017 Communication between genomic and non-genomic signaling events coordinate steroid hormone actions. *Steroids*
- Wingfield JC, 1984 Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. II. Agonistic interactions as environmental information stimulating secretion of testosterone. *Gen Comp Endocrinol* 56, 417–424. [PubMed: 6542537]
- Wingfield JC, 1985 Short-Term Changes in Plasma-Levels of Hormones during Establishment and Defense of a Breeding Territory in Male Song Sparrows, *Melospiza-Melodia*. *Hormones and behavior* 19, 174–187. [PubMed: 4040115]
- Wingfield JC, 1994 Regulation of territorial behavior in the sedentary song sparrow, *Melospiza melodia morphna*. *Horm Behav* 28, 1–15. [PubMed: 8034278]
- Wingfield JC, Hahn TP, 1994a Testosterone and Territorial Behavior in Sedentary and Migratory Sparrows. *Animal Behaviour* 47, 77–89.

- Wingfield JC, Hahn TP, 1994b Testosterone and territorial behaviour in sedentary and migratory sparrows. *Animal Behaviour* 47, 77–89.
- Wingfield JC, Hegner RE, Dufty AM, Ball GF, 1990 The Challenge Hypothesis - Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *American Naturalist* 136, 829–846.
- Wingfield JC, Lynn S, Soma KK, 2001 Avoiding the ‘costs’ of testosterone: ecological bases of hormone-behavior interactions. *Brain Behav Evol* 57, 239–251. [PubMed: 11641561]
- Wingfield JC, Monk D, 1992 Control and Context of Year-Round Territorial Aggression in the Nonmigratory Song Sparrow *Zonotrichia-Melodia-Morphna*. *Ornis Scandinavica* 23, 298–303.
- Wingfield JC, Moore IT, Goymann W, Wacker DW, Sperry T, 2006 Contexts and Ethology of Vertebrate Aggression: Implications for the Evolution of Hormone-Behavior Interactions, in: Nelson RJ (Ed.), *Biology Of Aggression* Oxford University Press.
- Woolley CS, 2007 Acute effects of estrogen on neuronal physiology. *Annu Rev Pharmacol Toxicol* 47, 657–680. [PubMed: 16918306]
- Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME, 1998 Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol Cell Biol* 18, 1946–1955. [PubMed: 9528766]
- Zangenehpour S, Chaudhuri A, 2002 Differential induction and decay curves of c-fos and zif268 revealed through dual activity maps. *Brain Res Mol Brain Res* 109, 221–225. [PubMed: 12531532]
- Zinzow-Kramer WM, Horton BM, McKee CD, Michaud JM, Tharp GK, Thomas JW, Tuttle EM, Yi S, Maney DL, 2015 Genes located in a chromosomal inversion are correlated with territorial song in white-throated sparrows. *Genes Brain Behav* 14, 641–654. [PubMed: 26463687]

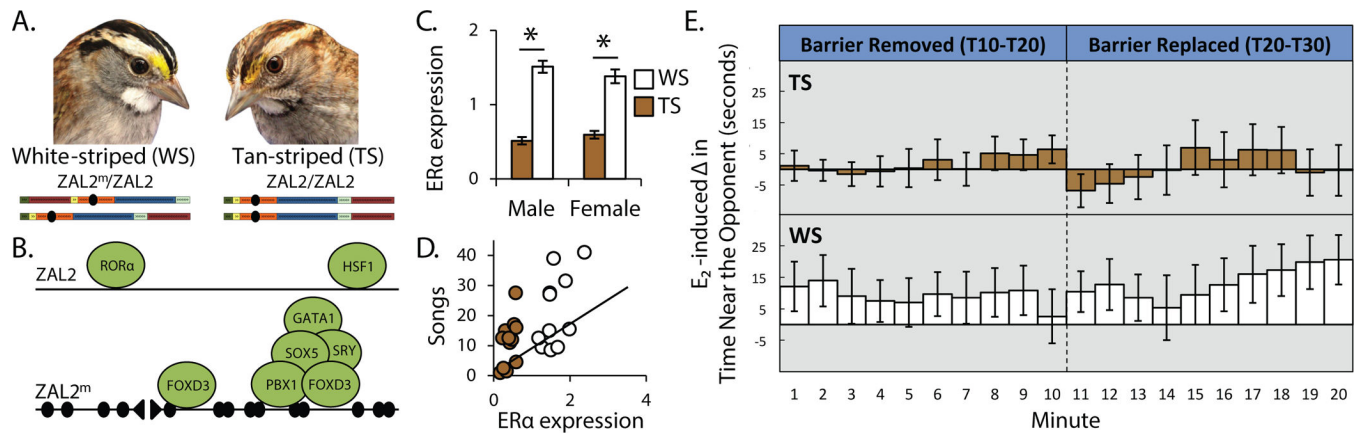


**Figure 1.** Pathways by which sex steroids could affect aggressive behavior in males. (A) Gonadal T acts directly on the brain. (B) Gonadal T is converted locally in the brain to  $E_2$ . (C) Adrenal DHEA is converted locally in the brain to T and  $E_2$ . (D) T and  $E_2$  are produced locally in the brain de novo from cholesterol. A shift from systemic steroid signaling (A and B) to more local steroid signaling (C and D) might be accompanied by a change in steroid signaling mechanisms (i.e., from primarily slow genomic mechanisms in A and B to primarily fast non-genomic mechanisms in C and D). (note: figure is redrawn from Schmidt et al. (2008)).

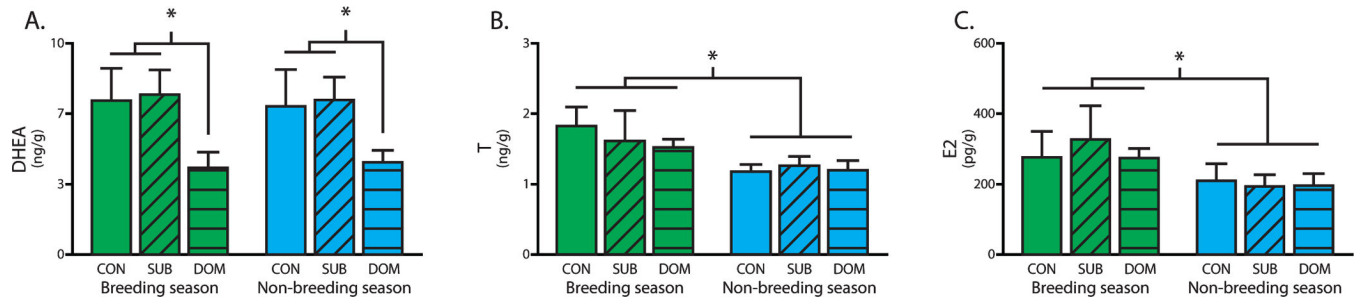


**Figure 2.**

In song sparrows, seasonal plasticity in the persistence of aggression may be related to seasonal differences in the rapid effects of E<sub>2</sub>. (A) Aggressive behavior *during* an STI (the amount of time subjects spend within 5m of a decoy) is quantitatively similar during the breeding and non-breeding seasons. (B) In contrast, aggressive behavior *after* an STI is terminated is seasonally plastic. Males in breeding condition continue to sing at high rates after the decoy is removed. In contrast, non-breeding males significantly reduce their singing by at least 50%. (C) Non-invasive E<sub>2</sub> administration rapidly increases aggressive behavior during an L-STI in non-breeding subjects only. (D) E<sub>2</sub> administration (s.c.) rapidly decreases the number of pCREB immunoreactive cells in the POM in non-breeding subjects only. \* p 0.05 (note: figures are redrawn from (Heimovics et al., 2015a; Heimovics et al., 2013))

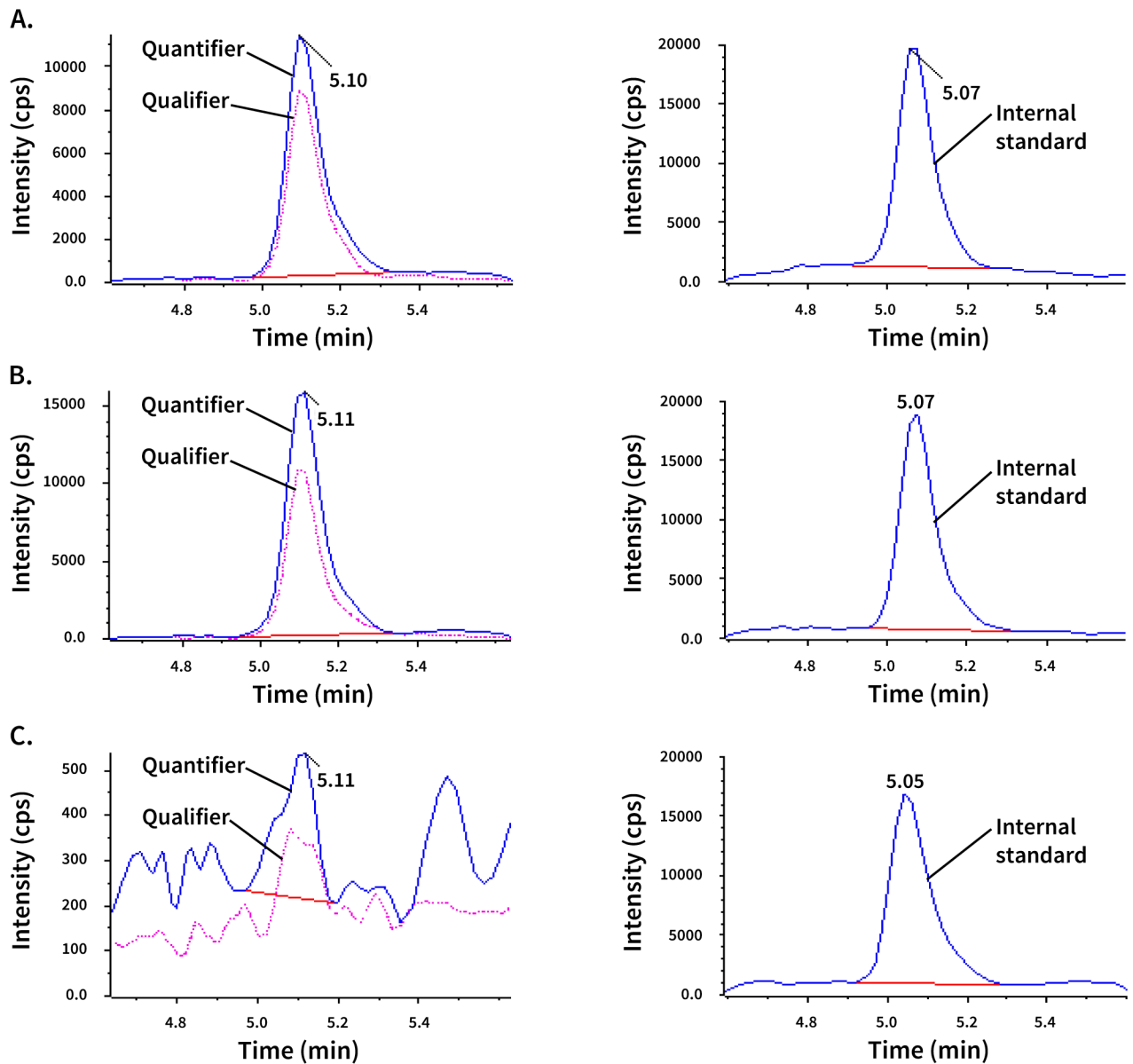
**Figure 3.**

In white-throated sparrows, morph differences in ER $\alpha$  may underlie morph differences in aggression. (A) Birds of the white-striped (WS) morph have one copy of a chromosomal rearrangement called  $ZAL2^m$ . Tan-striped (TS) birds do not. (B) The  $ZAL2$  and  $ZAL2^m$  alleles of the ESR1 promoter are differentiating. SNPs are indicated by circles and a deletion is flanked by triangles. This differentiation has disrupted putative transcription factor binding sites, shown by green ovals. (C) WS birds of both sexes have higher expression of ER $\alpha$  in nucleus taeniae of the amygdala (TnA) than TS birds. (D) Expression of ER $\alpha$  in TnA predicts aggressive singing in response to song playback. (E) A bolus dose of E $_2$  rapidly increases the time spent near a caged opponent in WS birds, but not TS birds, after only 10 min. This effect persists in WS birds, but not TS birds, after the barrier is replaced. (*note: figures are redrawn from (Horton et al., 2014a)*)



**Figure 4.**

Aggressive behavior is associated with the metabolism of dehydroepiandrosterone (DHEA) in the anterior hypothalamus (AH) throughout the year. Specifically, both breeding and non-breeding condition birds that were socially dominant during a 5min L-STI (DOM) had significantly lower levels of (A) DHEA in AH relative to birds that were socially subordinate (SUB) and birds that interacted with an empty cage (CON). In contrast, levels of (B) testosterone (T) and (C) 17 $\beta$ -estradiol (E<sub>2</sub>) were modulated by season (breeding > non-breeding) but appeared to be unaffected by L-STI. \*  $p < 0.05$  (note: figures are redrawn from (Heimovics et al., 2016))



**Figure 5.**

Measurement of  $17\beta$ -estradiol ( $E_2$ ) by LC-MS/MS, without derivatization. We used 2 multiple reaction monitoring (MRM) transitions for  $E_2$  (quantifier  $m/z$  271  $\rightarrow$  145 and qualifier  $m/z$  271  $\rightarrow$  143) (left panels) and one MRM transition for the deuterated internal standard ( $E_2$ -d4) ( $m/z$  275  $\rightarrow$  147) (right panels). (A) Certified  $E_2$  standard (10pg), (B) zebra finch ovary (2mg), and (C) male zebra finch POA punches (1.96mg). After adding 1 mL HPLC-grade acetonitrile (ACN), tissues were homogenized using a bead mill homogenizer. Then samples were centrifuged, and 950  $\mu$ L supernatant was transferred to a glass culture tube. Then 500  $\mu$ L hexane was added, and samples were vortexed and centrifuged. The hexane was discarded, and the ACN was dried in a vacuum centrifuge. Pellets were reconstituted with 50  $\mu$ L 25% methanol. Samples were processed along with a calibration curve. Data were acquired on a Sciex QTRAP 6500 UHPLC-MS/MS System in

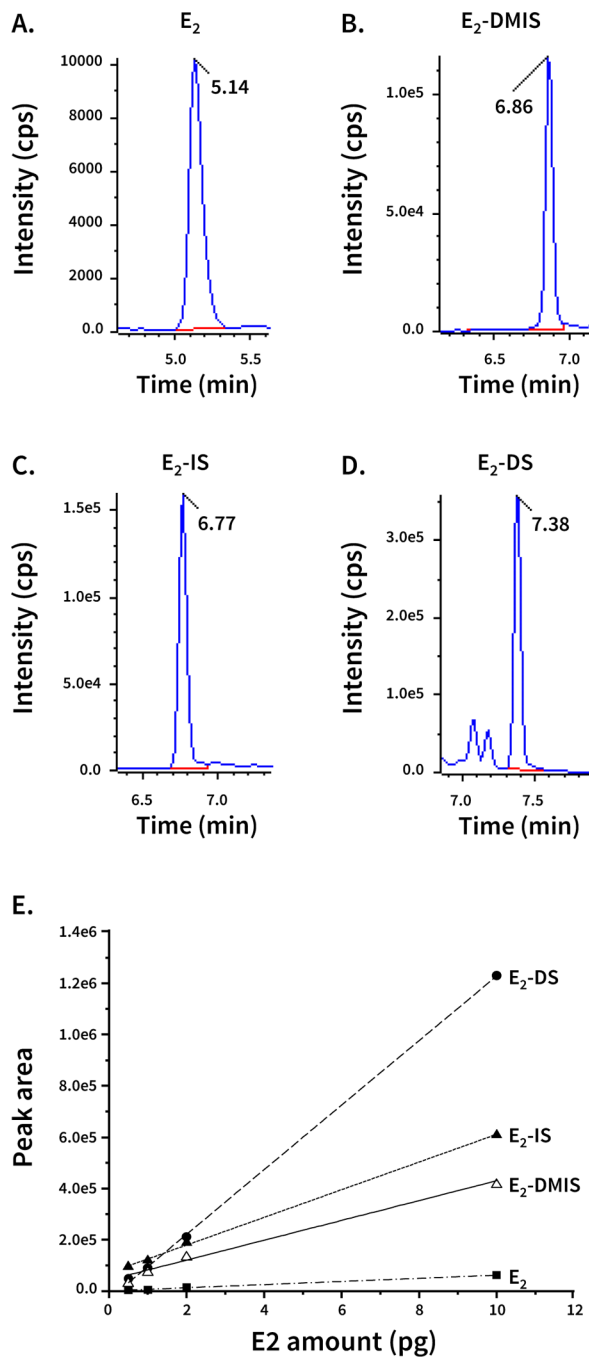
negative electrospray ionization mode (Tobiansky et al., 2018). For ovary and POA, quantifier and qualifier peaks have identical retention times and ion ratios to the E<sub>2</sub> standard. E<sub>2</sub> concentrations in ovary and male POA were 6.89 and 0.23 ng/g, respectively (30-fold difference). cps, counts per second.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 6.** Measurement of E<sub>2</sub> in neat solution by LC-MS/MS, with and without derivatization. (A) No derivatization. (B) E<sub>2</sub> was derivatized with 1,2-dimethylimidazole-5-sulfonyl chloride (E<sub>2</sub>-DMIS). (C) E<sub>2</sub> was derivatized with 1-methylimidazole-2-sulfonyl chloride (E<sub>2</sub>-IS). (D) E<sub>2</sub> was derivatized with dansyl chloride (E<sub>2</sub>-DS). Note the differences for E<sub>2</sub> and E<sub>2</sub> derivatives in intensity (y axis). (E) Calibration curves of E<sub>2</sub> and E<sub>2</sub> derivatives. Calibration curve range was 0.5–10 pg of E<sub>2</sub>. For all calibration curves, r<sup>2</sup>>0.98. Derivatization was carried out in the same fashion for the 3 reagents (following Li and Franke, 2015), with slight

modifications. Estradiol standards were dried and reconstituted in derivatization reagent (25  $\mu\text{L}$  of 1 mg/mL reagent in acetone) and 25  $\mu\text{L}$  of sodium bicarbonate buffer (100 mM, pH 9). Samples were capped and incubated at 65  $^{\circ}\text{C}$  for 15 min. Then samples were dried, resuspended in 50  $\mu\text{L}$  50% methanol, and subjected to LC-MS/MS analysis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript