

# Low Sex Steroids, High Steroid Receptors: Increasing the Sensitivity of the Nonreproductive Brain

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**ABSTRACT:** Male aggressive behavior is generally regulated by testosterone (T). In most temperate breeding males, aggressive behavior is only expressed during the reproductive period. At this time circulating T concentrations, brain steroid receptors, and steroid metabolic enzymes are elevated in many species relative to the nonreproductive period. Many tropical birds, however, display aggressive behavior both during the breeding and the nonbreeding season, but plasma levels of T can remain low throughout the year and show little seasonal fluctuation. Studies on the year-round territorial spotted antbird (*Hylophylax n. naevioides*) suggest that T nevertheless regulates aggressive behavior in both the breeding and nonbreeding season. We hypothesize that to regulate aggressive behaviors during the nonbreeding season, when T is at its minimum, male spotted antbirds increase brain sensitivity to steroids. This can be achieved by locally up-regulating androgen receptors (ARs), estrogen

receptors (ERs), or the enzyme aromatase (AROM) that converts T into estradiol. We therefore compared mRNA expression of AR, ER $\alpha$ , and AROM in free-living male spotted antbirds across reproductive and nonreproductive seasons in two brain regions known to regulate both reproductive and aggressive behaviors. mRNA expression of ER $\alpha$  in the preoptic area and AR in the nucleus taeniae were elevated in male spotted antbirds during the nonbreeding season when circulating T concentrations were low. This unusual seasonal receptor regulation may represent a means for the year-round regulation of vertebrate aggressive behavior via steroids by increasing the brain's sensitivity to sex steroids during the nonbreeding season. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 67: 57–67, 2007

**Keywords:** seasonality; tropical bird; aggressive behavior; nucleus taeniae; preoptic area

## INTRODUCTION

In male vertebrates testosterone (T) has a wide range of effects on the nervous system and on peripheral tissues that are important for the regulation of reproductive physiology and behavior. T can act either directly

via binding to androgen receptors (ARs), or indirectly via binding to estrogen receptors (ERs) after aromatization into 17 $\beta$ -estradiol (E2) within the brain. Indeed, numerous studies have shown the importance of T, its estrogenic metabolite E2, and the enzyme aromatase (AROM) for the regulation of sexual and aggressive behaviors during the reproductive season (Hutchison, 1971; Schlinger and Callard, 1990; Monaghan and Glickman, 1992; Compaan et al., 1994; Balthazart et al., 1997; Ball and Balthazart, 2004; Scordalakes and Rissman, 2004). Birds have proven to be excellent systems to study seasonal changes in physiology and behavior both in the field and in the laboratory (Ball and Balthazart, 2004). Male birds

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breeding in temperate zones typically show great seasonal fluctuations in gonadal growth and circulating T levels (Wingfield and Silverin, 2002). In these males, reproductive and aggressive behaviors are strongly associated with seasonal fluctuations in circulating T levels with the result that both behavior and hormone levels are increased during the breeding season (Balthazart, 1983; Wingfield et al., 1990; Ball and Balthazart, 2004). Outside the breeding season plasma levels of T are low (or even nondetectable) and territorial behavior is usually not expressed. In those species that are territorial during the nonbreeding season, the control mechanism of aggressive behavior is still not understood. Some work suggests that aggressive behavior outside the breeding season is not regulated by T or its estrogenic metabolite (Schwabl and Kriner, 1991; Pinxten et al., 2000; Canoine and Gwinner, 2002). This is a reasonable conclusion because the activation of aggressive behavior by T during the nonbreeding season would inappropriately also activate reproductive behaviors (e.g., Wingfield et al., 2001). In male Western song sparrows (*Melospiza m. melodia*), however, aggressive behavior during the nonbreeding season was reduced after blocking aromatase, the enzyme that converts T into E2. These experiments suggest that E2 might be involved in the regulation of nonbreeding aggression (Soma et al., 1999c, 2000). Differences in the regulatory mechanisms of aggressive behavior during the nonbreeding season might be due to different life styles, for example, migratory versus sedentary habits in different bird species.

In the tropics, seasonal environmental changes are less pronounced than in temperate regions. There is increasing evidence that male tropical birds have generally lower androgen levels than temperate birds during the breeding season (summaries in Levin and Wingfield, 1992; Wikelski et al., 2003; Goymann et al., 2004). Many tropical male birds have low T levels that show only little seasonal fluctuation throughout the year (Gwinner and Dittami, 1990; Levin and Wingfield, 1992; Goymann et al., 2004; but see Moore et al., 2002; Wiley and Goldizen, 2003). Nevertheless, many tropical birds breed seasonally and aggressively defend their territory year-round (Levin and Wingfield, 1992; Hau, 2001; Wiley and Goldizen, 2003). This raises the possibility that tropical birds might have evolved neuroendocrine mechanisms for the regulation of aggressive and/or reproductive behaviors that differ from the pattern known from temperate birds.

To our knowledge, only one year-round territorial tropical bird, the spotted antbird, has been studied in detail with regard to the endocrine regulation of territorial aggression (Hau, 2001). Spotted antbirds breed

seasonally and aggressively defend their territories year-round (Willis, 1972; Wikelski et al., 2000; Hau et al., 2004). In males, circulating T levels are generally low but increase slightly during the breeding season (Wikelski et al., 2000). T appears to modulate aggressive behavior both during the breeding and the nonbreeding season (Wikelski et al., 1999; Hau et al., 2000). Blocking AR and AROM simultaneously reduces aggressive displays in male spotted antbirds during the breeding season, and T administration increases them during the nonbreeding season (Hau et al., 2000). These results suggest that T might regulate reproductive behavior during the breeding season, and territorial behavior year-round. We hypothesize that nonbreeding aggressive behavior is regulated by increased sensitivity of the brain to T, such as by up-regulation of steroid receptors or formation of active steroids within the brain. It is known that T and its estrogenic metabolites are involved in the regulation of aggressive and reproductive behavior particularly in brain areas such as in the preoptic area (POM) and/or nucleus taeniae (nT) (Schlinger and Callard, 1990; Panzica et al., 1996; Thompson et al., 1998; Ball and Balthazart, 2002). We therefore compared seasonal AR-, ER $\alpha$ - and AROM-mRNA expression in POM and nT in relation to territorial response, gonadal status, and circulating T concentrations in free-living male spotted antbirds.

## MATERIALS AND METHODS

The experiments were conducted in Soberania National Park, central Republic of Panama (9° N, 79° W), in areas of secondary forest along the Panama Canal. For seasonal comparisons, experiments were performed at the beginning of the breeding season (June, 2002) and in the middle of the nonbreeding season (end of Dec. 2002 into Jan. 2003). Male spotted antbirds were caught in mist nets and were attracted to the nets with playback of male territorial song broadcast using a Sony tape recorder. The latency between the onset of the playback song and the appearance of the territorial male was recorded as a measure of aggressive behavior (Wikelski et al., 1999; Hau et al., 2004).

### Sampling

Immediately after capture (<10 min), a blood sample was collected from the wing vein in heparinized capillary tubes and stored on ice. Birds were then transferred to cloth bags and transported back to the field station. Within 4 h after capture, birds were deeply anesthetized with an overdose of isoflurane and immediately perfused through the heart with 30 mL 0.9% saline, followed by ice-cold 4% neutral buffered formaldehyde (Sigma) using a peristaltic pump. Brain, testes, and adrenals were dissected out and stored at

−70°C. We recorded the length and width of both testes and adrenals. Plasma was separated after centrifugation and stored at −70°C. All procedures were approved by the UCLA Chancellor's Animal Research Committee and the Smithsonian Tropical Research Institute in Panama.

### Radioimmunoassay (RIA)

T was measured using an indirect RIA after separation on chromatography columns (for methods see Wingfield and Famer, 1975). Trace label (20  $\mu$ L) was added to all samples to determine recoveries ( $46 \pm 0.01\%$ ; mean  $\pm$  SE). Water blanks were used as controls and were below detection limit. The intra-assay coefficient variation was 2.6% and the lower detection limit was at 0.15 ng/mL. Samples below the lower detection limit were assigned to 0.15 ng/mL as the highest possible value.

### In Situ Hybridization

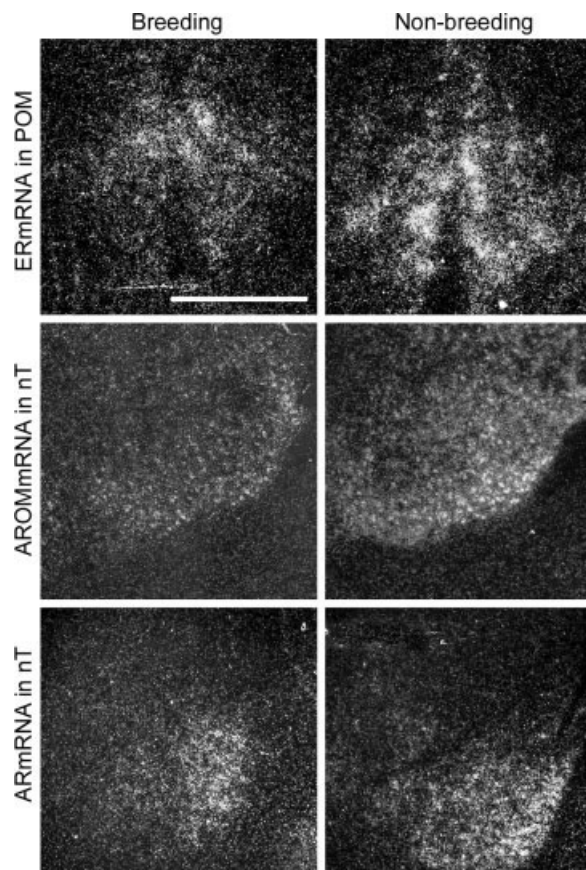
After dissection, the brain was postfixed for 2 h in 4% formalin, and then cryoprotected by immersion at 4°C overnight first in 20% sucrose in phosphate buffer (PB) and then in 30% sucrose in PB. The brains were then frozen on dry ice and stored first in dry ice, later at −80°C until sectioning. They were then sectioned at −21°C using a cryostat (Reikert-Jung) at 16  $\mu$ m and thaw-mounted in five-slide series onto Superfrost Plus slides (Fisher). Sections were air-dried and stored at −80°C until further processing. Our *in situ* hybridization protocol is a modification of protocols described previously (Jacobs et al., 1996; Metzdorf et al., 1999). Probes for AR were prepared from the suboscine golden-collared manakin (*Manacus vitellinus*) pmanAR sequence described in Fusani et al. (in prep.) (GenBank no. DQ020206) after linearization with NotI of two different clones to produce antisense or sense probes (T7 polymerase; Promega, Madison, WI). Probes for ER $\alpha$  were synthesized by linearizing the plasmid containing the 2792 bp zebra finch ER sequence (EJZER1; see Jacobs et al., 1996; GenBank no. L79911.1) with MluI or EcoRI to obtain the antisense (T7) and sense (SP6) probes, respectively. Probes for AROM were synthesized by linearizing the plasmid containing the 1.3 kb zebra finch AROM sequence (ZF1A-10; see Shen et al., 1994; GenBank no. L81143.1) with KpnI or XbaI to obtain the antisense (T7) and sense (SP6) probes, respectively.

<sup>33</sup>P-labeled sense and antisense probes were prepared by *in vitro* transcription in a 10  $\mu$ L solution with 5X transcription buffer (Promega), 40 units RNasin (Promega), 10 mM DTT, 500  $\mu$ M each of ATP, CTP, GTP, approximately 100 ng of linearized template cDNA, 10  $\mu$ L <sup>33</sup>P-UTP (2000 Ci/mole; New England Nuclear, Boston, MA), and 1  $\mu$ L of the appropriate RNA polymerase (SP6 or T7; Promega). Transcription reactions were completed at 37°C for 90 min, after which the cDNA template was removed by a 10 min incubation at 37°C with 1 unit/ $\mu$ L

RNase-free DNase (Promega), and 85  $\mu$ L of 10 mM Tris-HCl / DTT (pH 7.5) was added to each reaction before quenching on ice. Unincorporated nucleotides were then removed by centrifugation through a G-50 sephadex column (Boehringer Mannheim, Indianapolis, IN). Before hybridization, frozen tissue sections were air-dried for at least 1 h. They were then washed for 5 min in PBS. To reduce nonspecific hybridization, basic residues were acetylated by treating the slides with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. Following a brief rinse in PBS, sections were dehydrated through a graded series of ethanols and air-dried for 30 min. The hybridization buffer (600 mM NaCl, 4 mM EDTA, 80 mM Tris-HCl, pH 7.8, Denhardt's medium, 0.2% SHS, 250  $\mu$ g/mL tRNA, 25  $\mu$ g/mL of polyA RNA), containing sense or antisense riboprobe (at a concentration of approximately  $1-10 \times 10^6$  cpm/ $\mu$ L), was mixed with 10% dextran-sulphate and 50% formamide, and placed on the tissue sections. The slides were then covered with coverslips (PGC Scientific, Frederick, MD) and incubated overnight at 55°C in a wet chamber. The subsequent day, slides were washed in the following post-hybridization solutions: 4X SSC for 10 min at 50°C to remove coverslips, 2X SSC for 30 min, RNase A (20  $\mu$ g/mL) for 30 min at 37°C, 2X SSC for 30 min at 37°C, and finally 0.2X SSC for 1 h at 60°C. Slides were dehydrated in a series of washes of increasing ethanol concentrations containing 300 mM ammonium acetate. After drying the slides at room temperature for at least 2 h, the tissue sections were exposed to film (Kodak BioMax) for 2–3 days to obtain whole-body autoradiograms and to estimate the length of exposure needed by subsequent emulsion autoradiography. Hybridization specificity was confirmed for each probe in initial experiments by the absence of label over brain sections hybridized with sense probes. In addition, the overall neural patterns of hybridization with antisense probes closely matched patterns predicted from studies of the corresponding mRNA or protein in other suboscine and oscine species (Balthazart et al., 1998; Foidart et al., 1998; Metzdorf et al., 1999; Fusani et al., 2000; Gahr, 2001).

### Analysis of Optical Density (OD)

The *in situ* hybridization images from Kodak Biomax MR films were acquired with a Zeiss Stemi 2000-C dissecting scope mounted with an AxioCam MRc digital camera, and captured into MRGrab 1.0 (Carl Zeiss Inc., Thornwood, NY). Hybridization intensity was quantified using Scion Image (Scion Corporation, Frederick, MD). The ODs of nucleus taeniae and the medial preoptic area were measured in an ellipsoid covering most of the labeled area within the borders of the nuclei. Background OD was measured in an unlabeled brain region of the same section with an ellipsoid of the same size. Final OD values were obtained by subtracting background OD from the OD of the target area. These measurements were repeated for all sections in which the nucleus was visible and the average was used for the analyses.



**Figure 1** Seasonal comparison of ER $\alpha$ -mRNA expression in POM, and AROM-mRNA and AR-mRNA in nT. *In situ* hybridization images captured under darkfield conditions were black-white inverted in Photoshop. Scale bar = 500  $\mu$ m.

## Statistics

For the seasonal comparison of the expression of mRNA of AR, ER $\alpha$ , and AROM we used a matched-pair experimental design, in which each matched pair was composed of two individuals from the two different seasons that had been processed in the same *in situ* hybridization run (see the section *In Situ* Hybridization above). Data were normally distributed and therefore we used paired *t* tests. Data for adrenal volumes were also normally distributed and an independent *t* test was used. Data for plasma concentrations of T, testis volumes, and response latencies were not normally distributed and were analyzed with a Mann-Whitney U test. All nonbehavioral data are based on a sample size of six animals each for the breeding and the nonbreeding season. As we could not obtain behavioral data for all sacrificed individuals, we included some data for response latency from other males tested at the same time (resulting total sample sizes: breeding season  $n = 9$ , nonbreeding season  $n = 11$ ). The statistical significance was set  $\alpha < 0.05$ . All data are represented as mean  $\pm$  SE.

## RESULTS

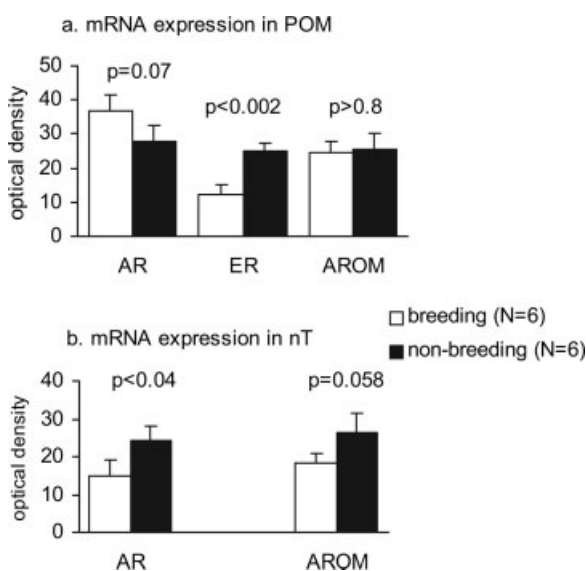
### Expression Levels of AR-, ER $\alpha$ ,- and AROM-mRNA (Fig. 1)

**POM [Fig. 2(a)].** AR-mRNA in the POM tended to be higher during the breeding season (OD:  $36.6 \pm 4.8$ ) compared to the nonbreeding season (OD:  $27.8 \pm 4.7$ ;  $t = -2.3$ ,  $df = 5$ ,  $p < 0.073$ ). AROM-mRNA expression did not differ seasonally ( $t = 0.23$ ,  $df = 5$ ,  $p > 0.8$ ). Contrary to the pattern of AR-mRNA, ER $\alpha$ -mRNA expression was higher during the nonbreeding (OD:  $24.8 \pm 2.5$ ) compared to the breeding season (OD:  $12.5 \pm 2.4$ ;  $t = 18.5$ ,  $df = 5$ ,  $p < 0.002$ ; Fig. 1).

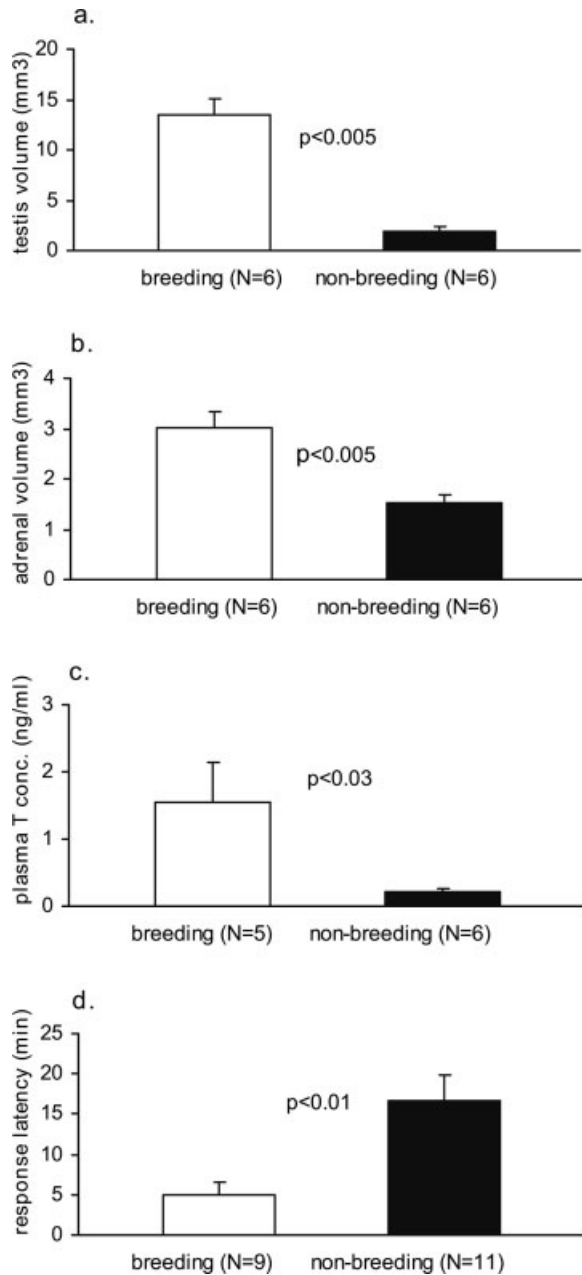
**nT [Fig. 2(b)].** The expression of AR-mRNA in nT was higher during the nonbreeding season (OD:  $20.79 \pm 5.56$ ) than during the breeding season (OD:  $15.87 \pm 5.45$ ;  $t = 3$ ,  $df = 5$ ,  $p < 0.04$ ; Fig. 1). There was also a tendency for AROM-mRNA to be higher during the nonbreeding season (OD:  $26.5 \pm 5.2$ ) compared to the breeding season (OD:  $18.4 \pm 2.3$ ;  $t = 2.5$ ,  $df = 5$ ,  $p = 0.058$ ; Fig. 1). The expression of ER $\alpha$ -mRNA in the nT was very weak in both seasons ( $< 5$  times the background) and therefore could not be quantified.

### Behavioral and Physiological Measurements

Birds differed significantly between the breeding and nonbreeding seasons in several behavioral and physi-



**Figure 2** Seasonal comparison of the optical density of mRNA expression of AR, ER $\alpha$ , and AROM in the POM (a) and nT (b).



**Figure 3** Seasonal comparison of testis volume (a), adrenal volume (b), plasma levels of testosterone (c), and the latency to respond to the playback of a conspecific male song (d). Data in (c) excludes one outlier during the breeding season, lowering the sample size to  $n = 5$ . Data in (d) include additional individuals (see text).

ological measures. Testis volume of breeding birds was nearly six times ( $13.5 \pm 1.6 \text{ mm}^3$ ) the size of nonbreeding birds [ $1.9 \pm 0.5 \text{ mm}^3$ ;  $U = 0.0$ ,  $Z = -2.89$ ,  $p < 0.005$ ; Fig. 3(a)]. Adrenal volume of males caught during the breeding season was two times larger ( $3.0 \pm 0.3 \text{ mm}^3$ ) than that of nonbreeding males [ $1.5 \pm 0.17 \text{ mm}^3$ ;  $df = 10$ ,  $t = -4.25$ ,  $p <$

$0.005$ ; Fig. 3(b)]. Plasma concentrations of T were also higher in breeding ( $2.9 \pm 1.5 \text{ ng/mL}$ ) compared to nonbreeding birds ( $0.2 \pm 0.06 \text{ ng/mL}$ ;  $U = 3.5$ ,  $Z = -2.5$ ,  $p < 0.02$ ). During the breeding season one male had an unusually high concentration of T ( $9.4 \text{ ng/mL}$ ). Excluding this individual from the analysis brought the mean plasma concentrations of T during the breeding season down to  $1.6 \pm 0.6 \text{ ng/mL}$ , but the seasonal difference remained significant [ $U = 3.5$ ,  $Z = -2.3$ ,  $p < 0.03$ ; Fig. 3(c)]. When presented with the playback of conspecific male song, breeding males responded significantly faster ( $5.56 \pm 1.64 \text{ min}$ ) compared to nonbreeding males [ $16.74 \pm 3.22 \text{ min}$ ;  $U = -2.65$ ,  $Z = -2.65$ ,  $p < 0.01$ ; Fig. 3(d)].

## DISCUSSION

Although free-living tropical male spotted antbirds display aggressive territorial behavior year-round, in the present study aggressive responses to conspecific playback and plasma concentrations of T were reduced during the nonbreeding season. Further, we found significant seasonal changes in the expression of AR and ER $\alpha$  in two brain areas thought to control aggressive and reproductive behaviors. Expression of ER $\alpha$  in the POM and AR in nT were elevated in the nonbreeding season, suggesting increased brain sensitivity to sex steroids at this time of year. Such an unusual seasonal steroid receptor modulation may contribute to the regulation of aggression in the nonbreeding season.

### AR, ER $\alpha$ , and AROM Expression in POM

The POM is an area in the diencephalon that responds to sex steroids for the regulation of reproductive and aggressive behaviors (Hutchison, 1976; Panzica et al., 1996; Ball and Balthazart, 2002). In the present study the largest seasonal change was found in the expression of ER $\alpha$ -mRNA in the POM. In this area, ER $\alpha$ -mRNA was more abundant during the nonbreeding than during the breeding season [Fig. 2(a)]. This result suggests that the POM is more sensitive to estrogens during the nonbreeding season. Indeed, E2 has been related to the activation of aggressive behavior in birds and mammals (Harding, 1983; Walters and Harding, 1988; Schlinger and Callard, 1990; Ogawa et al., 1998; Scordalakes and Rissman, 2004). In particular, several studies in mammals have shown that aggressive behavior is modulated by the activation of ER $\alpha$ , but not ER $\beta$  (Ogawa et al., 1998; Scordalakes and Rissman, 2004). In Western song sparrows, E2 appears

to be the main hormone regulating territorial aggression during the nonbreeding season (Soma et al., 1999c; Soma et al., 2000). Therefore, it is plausible that in spotted antbirds the increased expression of ER $\alpha$  in POM during the nonbreeding season is related to an involvement of E2 in the control of aggressive behavior. Interestingly, our data match previous findings of elevated ER $\alpha$  expression during the nonbreeding season in song control nuclei of canaries (*Serinus canaria*), which might also function to increase estrogenic effects on song behavior at this time of year (Gahr and Metzdorf, 1997; Fusani et al., 2000).

In males E2 is mainly produced via aromatization of T in the brain, and brain AROM indeed plays a role in modulating reproductive and aggressive behaviors in male birds (Schlinger and Callard, 1989; Balthazart and Foidart, 1993; Balthazart et al., 1997). In the present study we did not find seasonal changes in AROM expression in the POM (Fig. 2), suggesting that in this brain area AROM does not undergo seasonal regulation. In most temperate birds, however, AROM activity or mRNA expression is positively correlated with seasonal variations in the display of sexual behaviors (in European starlings, *Sturnus vulgaris*, pied flycatchers, *Ficedula hypoleuca*, and Lapland longspurs, *Calcaricus lapponicus*; Foidart et al., 1998; Soma et al., 1999b; Riters et al., 2000, 2001; Silverin et al., 2000). In Western song sparrows, AROM activity is also higher during the breeding season as compared to different periods of the nonbreeding season, even though these birds are aggressive in both seasons (Soma et al., 2003). Seasonal changes in AROM expression or activity in temperate birds might be modulated by the strong seasonal fluctuations in gonadal T secretion. In several bird species it has been shown that T up-regulates AROM activity and its expression in the preoptic area and anterior hypothalamus (Steimer and Hutchison, 1981; Schumacher and Balthazart, 1986; Vockel et al., 1990). The lack of seasonal changes in AROM expression despite the seasonal fluctuations in plasma T concentrations in male spotted antbirds suggests that in this species, AROM expression might be dissociated from circulating T. This might be an adaptive mechanism because it differs from what has been found in most studies on temperate birds. Hence, even though in most temperate birds AROM expression/activity appears to be T-dependent, it seems that it is not always seasonally correlated with circulating androgen concentrations. For example, in the ring dove (*Streptopelia risoria*) AROM activity in the preoptic area is T-dependent (Steimer and Hutchison, 1981), but seasonal changes in T production do

not correlate with AROM activity in this area (Fusani et al., 2003).

Opposite to the pattern of ER $\alpha$ -mRNA, AR-mRNA expression in the POM tended to be higher during the breeding season ( $p = 0.07$ ). These contrasting seasonal patterns in the expression of ER $\alpha$ - and AR-mRNA in POM suggest differential roles for androgens and estrogens in the regulation of aggressive versus reproductive behavior. A separate seasonal control of these two behaviors would be useful in species such as spotted antbirds that reproduce seasonally but defend their territories year-round. The present data raise the possibility that during the breeding season, androgens are involved in regulating behaviors related to reproduction, possibly including certain aggressive behaviors such as mate-guarding. Outside the breeding season, androgenic activation might have to be restricted because it could affect reproductive processes that would be inappropriate during that time (Wingfield et al., 2001). Therefore, during the nonbreeding season estrogens may be primarily used to regulate territorial aggression. Different functions of E2 and T in the regulation of behavior have already been proposed not only with respect to aggressive behavior, but also song (Gahr and Metzdorf, 1997). The up-regulation of AR-mRNA expression in the POM fits with the observed increases in circulating T levels and testis sizes during the breeding season in male spotted antbirds. In the canary it has been shown that AR-mRNA is up-regulated by circulating T levels (Nastiuk and Clayton, 1995).

### AR and AROM Expression in nT

The nT in birds is homologous to parts of the mammalian amygdala and previous work has shown that this region plays an important role in regulating sexual and aggressive behaviors in mammals and birds (Thompson et al., 1998; Delville et al., 2000; Davis and Marler, 2004; Reiner et al., 2004). Interestingly, in nT AR-mRNA expression was increased during the nonbreeding season. Thus, in addition to estrogens, androgens might also be involved in the regulation of aggressive behavior at this time of year, as proposed in a study by Hau and colleagues (2000; see also Wikelski et al., 1999). Elevated AR-mRNA in nT during the nonbreeding season indicates that in this brain area AR expression is not modulated by seasonal changes in gonadal T, but might depend on other factors. For instance, AR expression could be modulated by steroids produced within the brain (*neurosteroids*) (Schlinger and Arnold, 1993, 1995). Neurosteroids might have a regulatory function that

is as important as that of gonadal steroids, as they are produced locally in specific brain areas. Most of the enzymes that are needed to synthesize steroids are found in the brain, suggesting that the brain itself is an important site of steroid production (Baulieu et al., 2001; Schlinger et al., 2001; London et al., 2003). The substrate for the production of neurosteroids in the brain could be of peripheral origin such as dehydroepiandrosterone (DHEA) from the adrenals, which has been found at elevated concentrations in the plasma of spotted antbirds and song sparrows during the nonbreeding season, when both species exhibit aggressive behavior (Soma and Wingfield, 2001; Hau et al., 2004; Hau and Beebe, in prep.; see below).

The lack of ER $\alpha$  expression in nT is surprising and differs from what has been observed in other avian species (Bernard et al., 1999; Gahr, 2001). Despite the lack of ER $\alpha$  mRNA in nT, we found a strong tendency for increased AROM expression in nT during the nonbreeding season ( $p = 0.058$ ). It is possible that E2 produced within this nucleus might act locally on another type of estrogen receptor—ER $\beta$ . Although we did not study the expression of this receptor type in spotted antbirds, ER $\beta$  has been found in nT of European starlings and Japanese quail (*Coturnix c. japonicus*), and is thought to be involved in the regulation of sexual behavior (Ball et al., 2002). Alternatively E2 produced by AROM in nT might act on a different nucleus, for example, POM. Indeed, nT has an important fiber projection to the POM (Panzica et al., 1996; Thompson et al., 1998), where we found an increased ER $\alpha$  expression during the nonbreeding season. Thus, the E2 formed in nT may act transynaptically on other nuclei such as the POM, which is involved in the regulation of song behavior (Balthazart et al., 1996; Riters and Ball, 1999; Saldanha et al., 2000; Riters and Alger, 2004). The strong tendency for increased AROM expression in nT in male spotted antbirds occurred during the nonbreeding season when they have low plasma levels of T. This is in contrast with results on temperate birds in which AROM expression (and/or activity) was increased by T-treatment (Fusani et al., 2001) or positively correlated with territorial behavior (Soma et al., 1999a, 2003). Hence, circulating T levels do not correlate with telencephalic aromatase activity (Fusani et al., 2000; Silverin et al., 2000).

### Gonad Sizes, Aggressive Response, and Plasma T Concentrations

Birds captured in the breeding season had fully enlarged testes, whereas birds captured in the non-

breeding season had regressed gonads (Fig. 3), confirming that males in this study were indeed in different reproductive states (see Wikelski et al., 2000). Similarly, adrenal volume was larger during the breeding season than during the nonbreeding season. Other studies in rodents and birds have reported similar seasonal differences in adrenal size (Kriegsfeld and Nelson, 1996; Ribes et al., 1999). Although the adrenals are larger during the breeding season, plasma concentrations of DHEA are higher in spotted antbirds in the nonbreeding compared to the breeding season (Hau and Beebe, in prep.). This pattern might indicate a role for this adrenal steroid precursor in the regulation of aggressive behavior during the nonbreeding season, as suggested by studies on song sparrows (Soma and Wingfield, 2001). Indeed, as in song sparrows, DHEA concentrations correlate positively with some measures of aggressive behavior in free-living and captive male spotted antbirds (Hau et al., 2004). Hence, more studies are needed to understand the involvement of DHEA in the regulation of aggressive behavior.

Our previous studies have shown that spotted antbirds express robust aggressive behavior year-round and have generally low circulating concentrations of T that increase only slightly during the breeding season (Wikelski et al., 1999, 2000; Hau et al., 2000; Hau and Beebe, in prep.). The present data confirm that male spotted antbirds are aggressive year-round, even though they responded faster to conspecific playback in the breeding season than in the nonbreeding season. In parallel we found seasonal changes in T plasma concentrations (Fig. 2), and higher T concentrations during the breeding season than reported previously. The presentation of conspecific playback can induce an elevation in T concentrations (Wingfield and Wada, 1989), but in spotted antbirds this increase has been shown to occur only when playback durations exceeded 120 min (Wikelski et al., 1999). In the present study males were exposed to the playback song maximally for 35 min. Therefore it is unlikely that increased plasma T concentrations are caused by playback presentation. It is possible that increased T levels in the breeding season in the year of our study were the consequence of a long dry season that resulted in delayed and thus synchronous breeding activity compared to previous years. This synchrony might have led to increased aggressive and reproductive behavior within the population, leading to increased plasma T concentrations. Alternatively, the present experiment was conducted at the very beginning of the rainy season, when breeding activity had just started. At this time, birds might show a stronger response in both aggressive behavior

and T secretion that has been missed by previous investigations. This might also explain the faster response latencies at the beginning of the breeding season in the current study. It will be interesting to see if future studies will confirm this interpretation.

## CONCLUSION

This is the first study on a truly year-round, free-living tropical bird that simultaneously determined brain expression of AR-, ER $\alpha$ -, and AROM-mRNA, gonadal activity, sex steroid levels, and aggressive behavior in the reproductive and nonreproductive seasons. Our results show a higher mRNA expression of AR and ER $\alpha$  in the nonbreeding season in brain regions that are involved in controlling aggressive behavior. Furthermore, we also found opposite seasonal changes of AR- and ER $\alpha$ -mRNA within the same nucleus, the POM, indicating a possible dissociation between the endocrine control of sexual and aggressive behavior. When compared across sex, season, or species, increased expression of steroid receptors is often coupled to increased action of steroids on the brain (Gahr and Metzdorf, 1997; Soma et al., 1999b; Cushing et al., 2004; Shah et al., 2004; Tetel et al., 2004). We therefore propose that AR and ER $\alpha$  are elevated in male spotted antbirds during the nonbreeding season to increase brain sensitivity to the actions of low concentrations of sex steroids.

Our study focused on the regulation of the mRNAs of AR, ER, and AROM and we cannot exclude the possibility that post-transcriptional mechanisms may play a role in regulating brain steroid sensitivity. Nevertheless, the *in situ* hybridization technique allowed us to identify clear patterns in the regulation of brain steroid action in the same individuals with good anatomical resolution. This is particularly desirable in a field study such as the present one where the number of individuals is limited by ethical and conservation issues (see discussion in Fusani et al., 2005).

In many temperate bird species behavior, hormones, brain sex steroid receptors, and brain enzymes are temporally linked and elevated at the same time of year (Soma et al., 1999b, 2003; Riters et al., 2000, 2001). In contrast, in spotted antbirds the mRNA expression of AR, ER $\alpha$ , and AROM appears to be at least partly independent of circulating T concentrations. These results strongly suggest the existence of separate seasonal regulatory mechanisms for aggressive and sexual behaviors. Even though the present data are mainly correlational, we have previously obtained strong experimental evidence in spotted antbirds that T is indeed involved in regulating aggres-

sion both during the breeding and nonbreeding season (Hau et al., 2000). Taken together, our findings therefore suggest that territorial aggression in male spotted antbirds is regulated by sex steroids year-round: the actions of elevated plasma T levels during the breeding season are compensated during the nonbreeding season by an increase in brain sensitivity. The neuroendocrine mechanisms of the control of nonbreeding aggression need further experimental investigation, but at present in spotted antbirds the available evidence suggests that sex steroids affect aggressive behavior mainly via ER $\alpha$  in POM and via AR in nT during the nonbreeding season. Conversely, the upregulation of AR in POM during the breeding season might be related to the regulation of reproductive behaviors that are known to be T-dependent in many species (e.g., Panzica et al., 1996). Studies on wild species will remain important to test existing generalizations, and to discover new solutions in the endocrine control of behavior.

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