

Testosterone and Year-Round Territorial Aggression in a Tropical Bird

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Testosterone (T) regulates avian behaviors such as song and aggression during the breeding season. However, the role of T in year-round territorial birds is still enigmatic, especially in tropical birds. Spotted antbirds (*Hylophylax n. naevioides*) defend territories in the Panamanian rainforest year-round but have low plasma T levels (0.1–0.2 ng/ml), except during brief periods of social challenges. We manipulated T action in captive male Spotted antbirds to test whether this hormone is involved in the regulation of song and aggression. T-implants increased plasma androgen levels (T and dihydrotestosterone) and enhanced song in nonbreeding males. During a staged male–male encounter, T-implanted males sang more and were more aggressive than controls. In a second experiment, we blocked the two known T actions: its binding to androgen receptors and its conversion into estradiol by the enzyme aromatase. For this, we administered the androgen receptor antagonist flutamide (Flut) in combination with the aromatase inhibitor 1-4-6 androstatrien-3,17-dione (ATD) to birds in breeding condition. ATD + Flut treatment significantly elevated plasma levels of luteinizing hormone, presumably via the lack of T feedback from its receptors. ATD + Flut-treated birds gave fewer spontaneous songs than control-implanted males. During staged male–male encounters, ATD + Flut-treated males did not sing at all and showed reduced aggressive behavior. Our data indicate that T can regulate aggressive behavior in these tropical birds. Although plasma T levels can be low

year-round, Spotted antbirds may use T either by secreting it briefly during social challenges, by having a high sensitivity to T action, or by enzymatically converting circulating T precursors directly at the site of action. We hypothesize that plasma T levels are kept low in these year-round territorial birds to avoid potentially detrimental effects of tonically elevated T. Future treatment of nonbreeding birds with ATD + Flut will reveal whether T is indeed involved year-round in regulating aggressive behavior. © 2000 Academic Press

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During the period of reproduction, many birds occupy territories which they defend aggressively against conspecifics. In temperate birds, reproduction and territoriality usually co-occur, and outside the reproductive period territorial aggression and song are uncommon. In most male mid-latitude birds, territorial behavior, such as song and aggressive displays, are regulated by the hormone testosterone (T; overviews in, e.g., Balthazart, 1983; Wingfield and Ramenofsky, 1985; Wingfield and Moore, 1987; Wingfield *et al.*, 1987, 1990, 1997; Silver and Ball, 1989; Ketterson and Nolan, 1992; Wingfield, 1994; Hunt *et al.*, 1995). In general, circulating plasma levels of T increase in early spring from baseline levels (about 0.1–0.2 ng/ml) to about 2–10 ng/ml and then remain elevated throughout most of the reproductive period (e.g., Murton and Westwood, 1977; Farner and Wingfield, 1980; Follett, 1984; Wing-

field and Farner, 1993). Apart from regulating territorial aggression, increased plasma levels of T are important for the expression of secondary sexual characters, sperm production, and sexual behavior in male birds (e.g., Murton and Westwood, 1977; Balthazart, 1983; Ketterson and Nolan, 1994). However, elevated T titers may also incur physiological costs, such as a depressed immune system and a decreased survival probability (e.g., Dufty, 1989; Ketterson and Nolan, 1992; Zuk, 1994; Hillgarth and Wingfield, 1997).

Some bird species extend their territoriality into the nonbreeding season. This applies to some winter-territorial birds from higher latitudes as well as to the majority of tropical birds (Morton, 1996). How is aggressive behavior and song regulated in these species?

In a few birds, in which winter territoriality is associated with reproduction in the following spring (through maintenance of the winter territory for breeding in spring or establishment of a pair-bond for the spring), plasma levels of T are elevated in autumn and winter (Paulke and Haase, 1978; Lincoln *et al.*, 1980; Dawson, 1983; cf. Wingfield *et al.*, 1999; but see Logan, 1992). Most other winter-territorial species studied so far have baseline or low plasma levels of T (and other steroid hormones, Burger and Millar, 1980; Logan and Wingfield, 1990; Schwabl, 1992; Gwinner *et al.*, 1994; Wingfield and Hahn, 1994; but see Silverin *et al.*, 1989), and castration or administration of T or androgen receptor blockers does not change aggressive behavior (Silverin *et al.*, 1989; Logan and Carlin, 1991; Schwabl and Kriner, 1991; Wingfield, 1994; Wingfield and Hahn, 1994). Hence, it was suggested that T is involved in territorial aggression only when territoriality occurs in a reproductive context (e.g., Wingfield *et al.*, 1999).

However, recent detailed experimental studies using new pharmacological methods discovered that T does play a role in winter territoriality in a mid-latitude bird, the Song sparrow (*Melospiza m. morphna*). First, exogenous administration of T to males resulting in springtime plasma titers did enhance territorial aggression in winter (Wingfield, 1994). Second, there are two pathways by which T can influence behavior: (i) by binding to androgen receptors (e.g., Neri and Peets, 1975; Searcy and Wingfield, 1980; Simard *et al.*, 1986; Balthazart and Alexandre, 1987; Schwabl and Kriner, 1991; Labrie, 1993) or (ii) by being converted in

the brain into 17- β estradiol (E_2) by the enzyme aromatase (e.g., Tsutsui and Ishii, 1981; Balthazart and Alexandre, 1987; Archawaranon and Wiley, 1988; Walters and Harding, 1988; Schlinger and Callard, 1990; Schlinger and Arnold, 1991; Wade *et al.*, 1994). Administration of aromatase inhibitors such as ATD (in combination with the androgen receptor blocker flutamide; Soma *et al.*, 1999) or fadrozole (Soma *et al.*, 1998) decreased aggressive behavior in winter-territorial Song sparrows. These results suggest that T is involved in the regulation of winter territoriality in these birds, maybe through conversion into E_2 (Soma and Wingfield, 1999).

Currently, we lack detailed studies of the role of T in aggressive behavior of truly year-round territorial tropical birds. Existing correlational studies yielded mixed results: in some year-round territorial Afrotropical birds (Dittami and Gwinner, 1985, 1990; Dittami, 1986, 1987) and in subtropical Rose-ringed parakeets (*Psittacula krameri*; Krishnapradasan *et al.*, 1988) plasma T levels are continuously elevated (but usually remain below 2 ng/ml), perhaps indicating a coupling of T and territorial behavior. In contrast, in many other tropical species which maintain territories year-round, males have baseline T levels throughout the year, even during the breeding season (Levin and Wingfield, 1992; Wingfield *et al.*, 1992; Wikelski *et al.*, 1999a,b,c). Moreover, in Afrotropical White-browed sparrow weavers (*Plocepasser mahali*) aggressive territorial behavior and song can be induced without concomitant increases in plasma T levels from baseline values (Wingfield *et al.*, 1992; Wingfield and Lewis, 1993). Is aggressive behavior in tropical year-round territorial birds regulated independently of T? This question in fact entails two parts: despite naturally low plasma levels, is T involved in the control of aggression of tropical birds (i) during the breeding season and (ii) during the nonbreeding season?

Using pharmacological tools similar to those used in recent studies of winter territoriality (e.g., Soma and Wingfield, 1999), we investigated the role of T in year-round territorial neotropical Spotted antbirds from central Panama (9°N). Spotted antbirds breed only during the rainy season in Panama, from about May to October (Willis, 1972; Sieving, 1992; Hau *et al.*, 1998, 1999; Robinson *et al.*, 1999; Wikelski *et al.*, 1999b) but occupy all-purpose territories in the understory of

humid forests throughout the year. Both male and female defend the territory with song and aggressive displays (Willis, 1972; Wikelski *et al.*, 1999a,b). Plasma T levels of individual Spotted antbirds are baseline or undetectable (0.1–0.2 ng/ml for both sexes) during most of the year (Wikelski *et al.*, 1999a,b,c). However, circulating T titers can be elevated in males that experience periods of social instability, irrespective of time of year and gonad size (up to 1.5 ng/ml; Wikelski *et al.*, 1999a).

To test for a causal relationship between T and aggressive behaviors in male Spotted antbirds, we conducted two experiments. In captive nonbreeding birds, we first examined whether continuous elevation of T levels through implantation of a T-filled silastic capsule affects (i) spontaneous song activity and (ii) song and aggressive behavior during a staged male–male encounter. Spotted antbirds sing throughout the year (Wikelski *et al.*, 1999b) and song almost always accompanies aggressive territorial encounters (Willis, 1972; our own observations). Hence, for the purpose of the present experiments we consider song as a part of territorial behavior.

In a second experiment, in birds in breeding state we blocked T actions in two ways: first, we continuously administered flutamide to block androgen receptors (Searcy and Wingfield, 1980; Balthazart and Alexandre, 1987; Beletsky *et al.*, 1990; Schwabl and Kriner, 1991; Johnson and Bottjer, 1993); second, at the same time we also gave the aromatase inhibitor ATD to the birds (Balthazart and Alexandre, 1987; Archawaranon and Wiley, 1988; Walters and Harding, 1988; Beletsky *et al.*, 1990; Wozniak *et al.*, 1992; Johnson and Bottjer, 1993). In order to survey the birds' general behavior and body condition, we continuously monitored locomotor activity and determined body mass, fat stores, and hematocrit before and after implantation in both experiments.

MATERIAL AND METHODS

Animal Subjects

Spotted antbirds were caught in Soberania National Park, Republic of Panama, along the road from Gamboa to Panama City. Birds were housed in individual

cages with food and water *ad libitum* in a room at the Smithsonian Tropical Research Institute field station in Gamboa. In both experiments, birds in each group were visually isolated from each other. Females were present in all experimental groups since previous experiments had shown that song activity of male Spotted antbirds was higher when females were present (our own unpublished data). However, apart from group song (see below), which included females, female behavior was not investigated in any of the experiments because of small sample sizes in both experiments. Birds were offered a diet of freshly prepared egg-food mix (after Gwinner *et al.*, 1987; see also Hau *et al.*, 1998), live mealworms (*Tenebrio spp.*), and live crickets (*Acheta domestica*). Food and water were changed twice a day and cages were cleaned every day. Body mass of the birds was determined in about 5-day intervals, in the morning before receiving fresh food.

General Manipulations

Blood samples (ca. 200 μ l) were taken from the wing vein and the state of the gonads was determined by unilateral laparotomy under Isoflurane (Abott) anesthesia without knowledge of treatment (for standard procedures of blood sampling and laparotomy, see Wingfield and Farner, 1976; Hau *et al.*, 1998; Wikelski *et al.*, 1999b). Samples were kept cool at 4°C until centrifugation and plasma was stored at –20°C until analysis. A 3% solution of β -propiolactone was added according to USDA regulations. β -propiolactone is an antiviral substance that does not interfere with the assay (see Wingfield and Lewis, 1993). Samples were transported to the University of Washington on dry ice. Silastic implants (Dow Corning, Midland, MI; inner diameter = 1.47 mm, outer diameter = 1.96 mm), either filled with T, ATD, or flutamide or left empty, were sealed on both ends with liquid silicone and were implanted subcutaneously under Isoflurane anesthesia between the wings and near the thighs (two implants at each site). The implants caused no infections and wounds healed entirely within 3 to 5 days. Implants were removed again under Isoflurane anesthesia. Implants were checked each time the birds were weighed.

General Measurements

Spontaneous song activity of birds was recording in the morning for 30 min. Female Spotted antbirds sing in natural situations and did so during our experiments. Female song could not be distinguished from male song in our studies and is therefore included in group song. However, since females usually sing less frequently and less persistently than males (own unpublished observations) and since group sizes and male/female ratios were comparable between groups, it is unlikely that female song explains much of the effects of endocrine manipulations on group song. Locomotor activity was monitored automatically using microswitches mounted underneath the upper of two perches of each cage. Using the University of Groningen (The Netherlands) registration system (ERS 3), these data were continuously recorded and events were counted and stored in 2-min bins on-line on a computer.

Aggressive Behaviors

Only males were used during the staged encounters. Classifications of Spotted antbird calls and aggressive behavior were made according to Willis (1972) and our own unpublished observations on free-living birds. During staged male–male encounters, we counted numbers of songs, “chips,” “snarls,” “hops from the perch,” and the latency to display the white back patch. “Chip” calls are probably a general expression of excitement, whereas songs and “snarls” have more specific aggressive functions. “Snarls” are hissing calls that are used only during highly aggressive displays. They are directed at a close opponent and accompany the display of the white chest and the white back patch. Counter-singing often occurs during aggressive encounters when the opponents are at medium distance or far away. We used “hops from the perch” as an indication of the dominance of a bird, i.e., we interpreted increased hopping activity during an encounter as a tendency to escape the opponent. This notion was supported by the fact that all birds which increased locomotor activity during the encounter hopped toward the back of the cage rather than toward the front. The white back patch, a plumage pattern specific for antbirds, is exposed only during aggressive displays.

T-Implant Experiment

For the T-implant experiment, 21 birds (15 males and 6 females) were caught in the nonbreeding season, between November 10 and 20, 1996. Birds were assigned to either control (8 males, 2 females) or T-implant group (7 males, 4 females) at random. All birds were housed in one large room, which was partitioned into smaller compartments for each group by a row of cardboard boxes. Natural daylight entered through two windows and additionally a fluorescent light bulb was turned on each day between 7:30 a.m. and 4:00 p.m., illuminating the room at about 100 lux. This resembled light levels in the birds' natural habitat (Wikelski *et al.*, 1999b). Temperature in the room averaged 27°C, also similar to natural conditions. Blood samples were taken on November 25 and on December 15 or 16. On December 2, all birds received either one empty or one T-filled silastic tube with an effective length of 10 mm. In this experiment, females from the T-implant group were also treated with T for another preliminary study (Hau *et al.*, unpubl. data). However, apart from group song, females are not included in any other behavioral data.

The total number of songs produced by all birds in each group was counted every morning for 30 min after the birds received fresh food and water by an observer sitting outside the room at the window. Due to the partitioning of the room, songs coming from each group could easily be distinguished from outside the room. To measure the aggression of birds, we conducted staged encounters between two males in the morning hours (8 a.m. until 10:30 a.m.) between December 11 and 13. Birds were tested in their home cages, whereby two males were placed at 30 cm distance facing each other (within the experimental room). All T-implanted males were tested against a control male. Birds did not have visual contact with their opponents before the staged encounter. During the staged encounter, the two opponents were visually isolated from the other birds in the room and initially separated by a cardboard shield. One of the birds (the focal bird) was monitored using a Hi-8 video camera while the experimenter left the room. After 20 min, visual contact was enabled by removing the cardboard and the behavior of the focal bird was monitored for another 20 min. We analyzed the first 15 min after removal of the cardboard. Implants were removed on

December 15 and birds were released 3 days later at their original capture site.

ATD-Flutamide Experiment

We caught 16 birds (14 males, 2 females) during the prebreeding season, between May 1 and 6, 1997. Birds were housed in individual cages inside two climate chambers (Perceival Scientific, Boone, IA) in the Gamboa field station of STRI and assigned at random to one of the two groups. The doors of the climate chambers remained open, permitting natural daylight to enter. Each chamber was additionally illuminated by one fluorescent light bulb during the daylight hours. Temperature in the chambers averaged 28°C. One bird of the control group died on June 20, reducing the sample size during the male–male encounters for this group to 6.

Between May 3 and 8, blood samples were taken and birds were laparotomized. On June 12, each bird received two silastic tubes (each of 10 mm effective length) filled with the androgen receptor blocker flutamide (or Flut, Schering, Bloomfield, NJ) plus two silastic tubes (each of 6 mm effective length) filled with the aromatase inhibitor ATD (Research Plus Steroid Labs, Bayonne, NJ). Control birds received four empty implants at the same sizes as ATD + Flut-treated birds. One bird lost two of the implants, which were replaced within 24 h. Each implant initially contained about 14.4 mg Flut or 9.3 mg ATD and lost on average 2.25 mg each Flut or 1.3 mg each ATD. Hence, each bird received a total dose of about 25 mg Flut/kg/day and about 14 mg ATD/kg/day.

The total number of songs produced by each group was monitored every morning from June 14 onward for 30 min after feeding, using Dictaphones set up in front of each group. We could not obtain group song data for the preimplantation period because birds were in captivity prior to this experiment, but in different groups. For the present experiment, birds were randomly assigned to one of the two new groups. However, as in the T-implant experiment, we did not expect the two groups to differ before implantation.

On June 10 and again on June 17, the female of each group was placed outside of the chambers. To measure the males' song activity, each male was then placed opposite the female within 30 cm distance to allow acoustic and visual interaction of male and female. The

observer sat about 2 m away, but could not be seen by the subjects. We recorded total number of songs and snarls produced by the male during 30 min but used only the first 15 min for the final analysis (to provide the same time frame as during the preceding T-implant experiment). To measure the aggression of birds on June 20, we conducted staged encounters between two males as described before. The experimental protocol differed from that of the T-implant experiment only in that the two males were immediately allowed visual contact with each other and that we observed the behavior of the focal bird directly from behind a blind. Implants were removed on June 21 and the birds were released.

Hormonal Measurements

Plasma samples from the T-implant study were analyzed in a single radioimmunoassay (RIA) with column separation (Wingfield and Farner, 1976; Wikelski *et al.*, 1999a,c). Prior to RIAs, samples were extracted with dichloromethane. Steroid hormones were then separated on columns packed with diatomaceous earth, the stationary phase consisting of a 1:1 propylene/ethylene glycol mix. Detection limits were 0.2 ng/ml (T), 0.13 ng/ml (5 α -dihydrotestosterone, DHT), 0.15 ng/ml (androstendione, AE), and 0.15 ng/ml (E₂). For statistical analysis, samples that were undetectable were set at the detection limit as a conservative estimate. Intraassay variations (using two standards per assay) were 6.7% (T), 9.1% (DHT), 0% (AE), and 8.7% (E₂). LH was determined in a single assay using the postprecipitation, double-antibody RIA devised by Follett *et al.* (1972) and Sharp *et al.* (1987) for avian LH. Detection limit for the LH assay was 0.08 ng/ml and intraassay variation was 12.5% (three high and low standards, respectively).

The preimplantation samples from the ATD + Flut study were analyzed in an indirect T assay without column separation (see Hau *et al.*, 1998) and the postimplantation samples in an assay with column separation after ether extraction. Detection limit for the preimplantation samples was 0.11 ng/ml and intraassay variation was below 10%. Detection limit for the postimplantation samples was 0.15 ng/ml and intraassay variation was 3.2%. Interassay variation between the two assays was 13.5%. The T antibody used in these assays (Wien Laboratories, Succasunna, NJ) cross-

reacts with 5α -DHT by about 60% and with 5β -DHT by about 6%. Therefore, since we measured total androgen in the preimplantation samples and only T in the postimplantation samples, we may have slightly underestimated the androgen content of postimplantation samples. Samples from the ATD + Flut study were analyzed in three LH assays (in which samples were randomly distributed), in which detection limits ranged between 0.08 and 0.26 ng/ml and intraassay variations between 2.45 and 12.5%. Interassay variation was 23.4%. In light of the clear changes in LH content in ATD + Flut-treated birds (see below), interassay variations appear not to have masked the outcome of the treatment on LH levels.

Statistical Analysis

Data were analyzed with SPSS Statistics (Version 7.5, SPSS Inc., Chicago). Only two-tailed, nonparametric tests were used. To analyze the effects of treatment on spontaneous behavior, hormone levels and body "condition" in each experiment, we first calculated the difference between pre- and posttreatment results for each individual. We then used Mann-Whitney U tests to find out whether these pre- and posttreatment differences were significantly different in the two groups. Behavioral data from the male-male encounters were also analyzed using Mann-Whitney U tests. Where appropriate, post hoc tests were performed using Wilcoxon sign ranks tests (abbreviated as WSR test).

RESULTS

T-Implant Experiment

Behavioral Effects

Spontaneous behavior. The data for group song were corrected for the total number of birds per group (see Fig. 1a), since females also sang occasionally. Group song strongly increased in T-treated birds, whereas song activity of control males remained on a low level (although control birds could hear T-implanted birds singing). Similar results were ob-

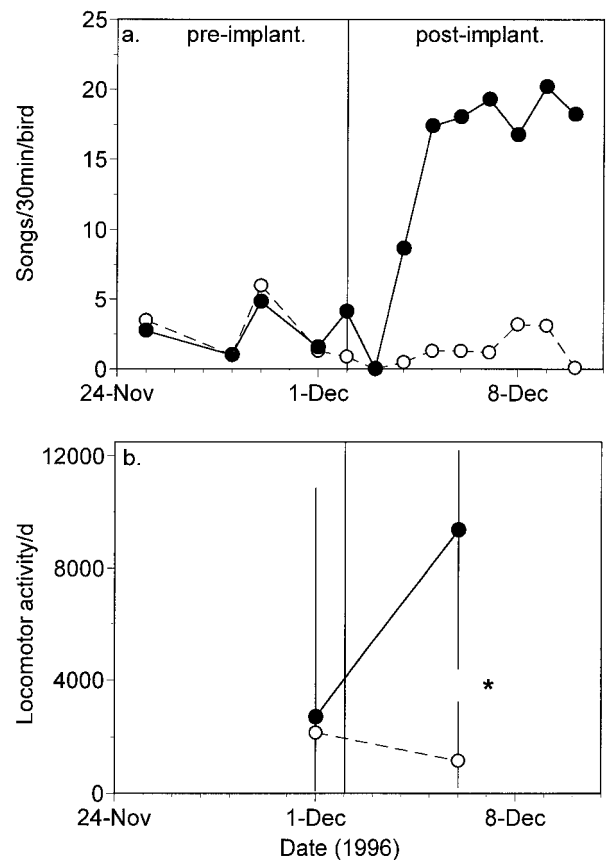


FIG. 1. Effects of T administration on spontaneous behavior of control (open circles, broken lines; $n = 8$) and T-implanted male Spotted antbirds (closed circles, solid line; $n = 7$). (a) Group song, corrected for number of birds present in each group; (b) locomotor activity of individuals (medians \pm quartiles). Vertical line indicates day of implantation. * Indicates a significant difference between groups, $P < 0.05$.

tained if song activity was corrected for the number of males present in each group. Locomotor activity was analyzed by comparing the mean daily activity level of each individual for 2 days before implantation (November 30–December 1) against its mean daily activity level for 3 days after implantation (December 5–7). T-treated birds significantly increased locomotor activity ($Z = -2.24$, $P = 0.025$; Fig. 1b).

Male-male encounter. T-implanted birds sang during the encounter, whereas control males did not sing at all ($Z = -3.16$, $P = 0.002$; Fig. 2a). Birds with T implants also displayed the white back patch faster than control birds (i.e., had a shorter latency time; $Z = -2.94$, $P = 0.003$; Fig. 2c). There was a trend in

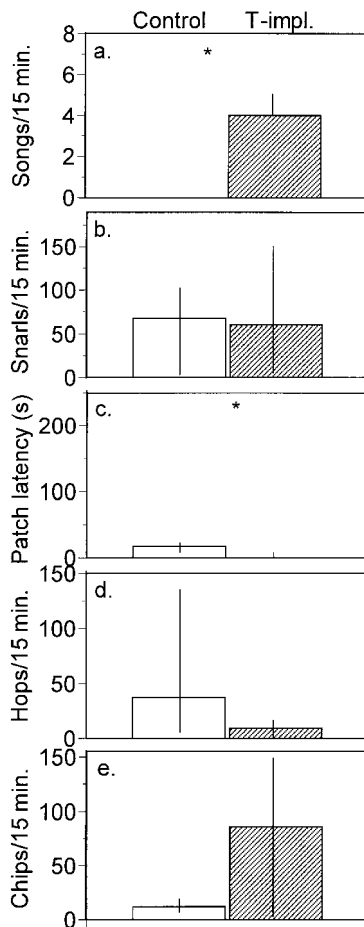


FIG. 2. Behavioral responses of control (open bars, $n = 8$) and T-implanted (hatched bars, $n = 7$) male Spotted antbirds during 15 min of a staged male-male encounter experiment (medians \pm quartiles). (a) Songs; (b) snarls; (c) latency to expose the white back patch; (d) hops away from the opponent; (e) chips. Scale of y-axis is matched to that of Fig. 4 to facilitate comparison. * Indicates a significant difference between groups, $P < 0.05$.

control males for increased hopping activity from the perch (toward the cage wall) compared to T-implanted males ($Z = -1.87$, $P = 0.061$; Fig. 2d). There were no differences between the two groups in number of snarls ($Z = -0.29$, $P > 0.7$; Fig. 2b) or chips ($Z = -1.57$, $P > 0.1$; Fig. 2e).

Physiological Effects

Implantation of a T-filled capsule significantly increased plasma T levels of male nonbreeding Spotted antbirds over those of control birds ($Z = -3.42$,

$P < 0.001$) (see Table 1). Plasma DHT levels also increased significantly in T-implanted birds compared to the low titers in control birds ($Z = -3.06$, $P = 0.002$). Plasma AE levels did not differ between groups ($Z = -1.74$, $P > 0.8$). Plasma levels of DHT and AE were significantly correlated with T levels (calculated only during implantation to avoid pseudoreplication; Spearman's ρ : 0.87, $P = 0.0001$ for DHT; 0.61, $P = 0.002$ for AE), with T levels generally being higher than the other androgens. Plasma levels of E_2 were hardly ever detectable and did not change ($Z = -0.93$, $P > 0.3$). Similarly, plasma LH levels remained low and unaffected by the manipulations in both groups ($Z = -0.84$, $P > 0.4$).

The size of the cloacal protuberance (CP) was not measured in the control group, but T-implanted birds increased CP length from 2.0 (2.0, 2.0) mm (median and quartiles, respectively) before implantation to 3.0 (2.0, 4.0) mm after implantation (WSR test, $Z = -2.06$, $P = 0.04$). The testes of all birds were regressed and testis volumes were not different between treatment groups ($Z = -0.39$, $P > 0.6$). There were no differences in hematocrit ($Z = -0.06$, $P > 0.9$), body mass ($Z = 0$, $P = 1.0$), or fat scores ($Z = -0.59$, $P > 0.5$) in the two groups. All birds had already molted wing and body feathers at the start of the experiment but our incomplete data and the short duration of the experiment did not allow us to analyze any effects of the T implants on molt in these birds.

ATD + Flut Experiment

Behavioral Effects

Spontaneous behavior. Group song (corrected for number of birds present in each group) tended to be lower in ATD + Flut-implanted than in control-implanted birds (Fig. 3a). Individual song activity differed between the two groups ($Z = -2.26$, $P = 0.024$; Fig. 3b): ATD + Flut-treated birds tended to decrease song activity (WSR test, $Z = -1.83$, $P = 0.068$), whereas control birds remained unchanged (WSR test, $Z = -1.29$, $P > 0.1$). Locomotor activity in both groups was analyzed by calculating the mean daily activity for 3 days before implantation (June 9–11) and for 3 days after implantation (June 14–16). Locomotor activity did not differ between the two groups ($Z = -1.21$, $P > 0.2$; Fig. 3c).

TABLE 1

Plasma Concentrations of Hormones, Percentage Detectability of Samples, and Several Physiological Measurements of Male Spotted Antbirds before and after Implantation of an Empty (Control) or a T-Filled Silastic Capsule

Median (quartiles)	Control-implanted ($n = 8$)		T-implanted ($n = 7$)	
	Before	After	Before	After
T (ng/ml)	0.2 (0.2, 0.02)	0.2 (0.2, 0.02)	0.2 (0.2, 0.02)	6.2 (3.4, 7.4)*
% det.	12.5	0	0	100
DHT (ng/ml)	0.13 (0.13, 0.13)	0.13 (0.13, 0.32)	0.13 (0.13, 0.53)	2.6 (1.97, 3.91)*
% det.	0	37.5	57	100
AE (ng/ml)	0.31 (0.27, 0.46)	0.23 (0.15, 0.7)	0.15 (0.15, 0.46)	0.43 (0.38, 1.02)
% det.	87.5	50	37.5	100
E ₂ (ng/ml)	0.15 (0.15, 0.15)	0.15 (0.15, 0.15)	0.15 (0.15, 0.15)	0.15 (0.15, 0.15)
% det.	12.5	0	0	0
LH (ng/ml)	0.2 (0.2, 0.3)	0.3 (0.2, 0.37)	0.3 (0.2, 0.6)	0.3 (0.2, 0.3)
% det.	100	100	100	100
Testis vol. (mm ³)	0.24 (0.14, 0.23)	0.4 (0.2, 1.03)	0.23 (0.2, 0.75)	0.68 (0.23, 0.79) ⁶
Hematocrit (%)	0.51 (0.47, 0.55)	0.5 (0.43, 0.54) ⁷	0.53 (0.51, 0.6)	0.5 (0.46, 0.54)
Body mass (g)	17.25 (16.5, 18.0)	17.5 (17.5, 18.1) ⁷	16.75 (15.5, 17.5) ⁶	17.0 (16.5, 17.5)
Fat score	0.5 (0.0, 1.0) ⁷	1.0 (0.5, 2.0) ⁷	0.5 (0.0, 1.0)	1.0 (0.5, 1.0)

Note. Numbers in superscript indicate sample size for this data point. For further explanations see text.

* Indicates a significant difference between treatment groups, $P < 0.05$.

Male-male encounter. During the staged male-male encounter, control-implanted males sang more than ATD + Flut-implanted males (which never sang, $Z = -2.03$, $P = 0.042$; Fig. 4a). Control birds also snarled about three times as much as ATD + Flut-implanted birds ($Z = -2.43$, $P = 0.01$; Fig. 4b). There were no significant differences between the two groups in the latency to expose the white back patch ($Z = -1.5$, $P > 0.1$; Fig. 4c) and in number of hops ($Z = -1.76$, $P = 0.078$; Fig. 4d) or chips ($Z = -0.65$, $P > 0.5$; Fig. 4e).

Physiological Effects

Total androgen levels did not differ between the two groups ($Z = -1.57$, $P > 0.1$) (see Table 2). However, LH levels increased in the ATD + Flut group after treatment (difference between treatment groups: $Z = -2.88$, $P = 0.004$, post hoc WSR test, $Z = -2.2$, $P = 0.028$) but not in the control group (post hoc WSR test, $Z = -0.94$, $P > 0.3$).

Testes of all birds were enlarged, but those of control birds did not differ from those of ATD + Flut implanted birds at the beginning of the study ($Z = -0.58$, $P > 0.5$). Testis sizes before implantation were (medians and quartiles): control group, 6.28 mm (1.05, 13.08); ATD + Flut group, 4.75 mm (1.57, 6.28). Testis sizes

were not measured after implantation. There was no change in hematocrit in either group after implantation ($Z = -0.24$, $P > 0.8$). Body mass did not differ between the two groups ($Z = -0.55$, $P > 0.5$), but control birds significantly increased (WSR test, $Z = -2.2$, $P = 0.28$) and ATD + Flut-treated birds tended to increase body mass during the experiment (WSR test, $Z = -1.83$, $P = 0.068$). Fat levels were not consistently measured during the experiments but were high after implantation in both groups. Birds showed some wing or body molt during the experiment but detailed analysis could not be carried out.

DISCUSSION

Spotted antbirds aggressively defend their territories in the rainforest year-round but can have low titers of plasma T throughout the year (Wikelski *et al.*, 1999a,b,c). The present experiments show that T implants increased song rate and aggressive behavior in nonbreeding male Spotted antbirds. Furthermore, the pharmacological blockade of T actions reduced song rate and aggressive behavior in individuals in breeding condition. Hence, these results suggest that despite naturally low plasma titers, T is involved in regulating

song and aggressive behavior in these tropical birds. However, it should be noted that to establish whether T indeed regulates aggression of male Spotted antbirds year-round, the effect of ATD + Flut treatment on behavior will also have to be examined during the nonbreeding season.

Effects of T Implants in Nonbreeding Birds

The effects of T implants on spontaneous song were rapid (within 2–3 days) and dramatic (Fig. 1a). During

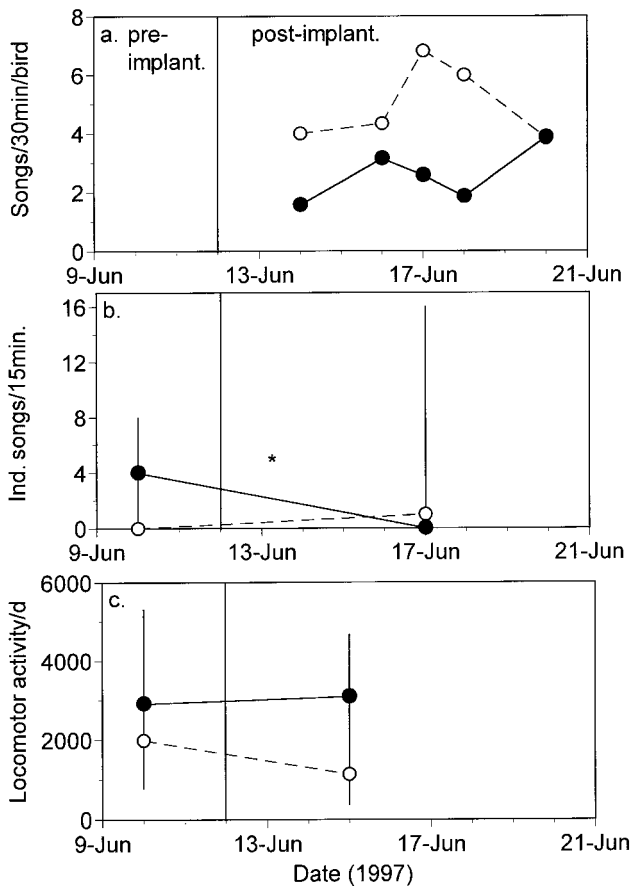


FIG. 3. Effects of T-blockade on spontaneous behavior of control (open circles, broken lines; $n = 7$) and ATD + Flut-implanted male Spotted antbirds (closed circles, solid line; $n = 7$). (a) Group song, corrected for number of birds present in each group; (b) individual song toward a female (medians \pm quartiles); (c) locomotor activity (medians \pm quartiles). Vertical line indicates day of implantation. No data could be obtained for group song prior to implantation. *Indicates a significant difference between groups, $P < 0.05$.

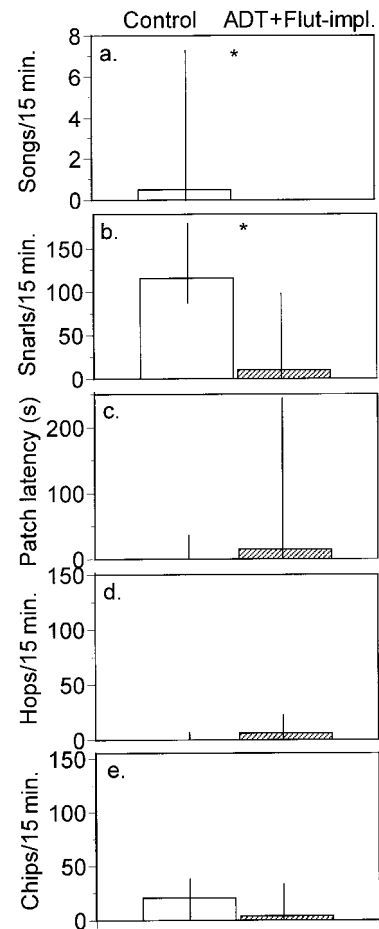


FIG. 4. Behavioral responses of control (open bars, $n = 6$) and ATD + Flut-implanted (filled bars, $n = 7$) male Spotted antbirds during 15 min of a staged male-male encounter experiment (medians \pm quartiles). (a) Songs; (b) snarls; (c) latency to expose the white back patch; (d) hops away from the opponent; (e) chips. Scale of y-axis is matched to that of Fig. 2 to facilitate comparison. *Indicates a significant difference between groups, $P < 0.05$.

male-male encounters, the increase in song activity and decrease in latency to show the white back patch of T-treated birds were also pronounced and highly significant (Fig. 2). In addition, there was a trend for T-treated birds to remain on their perch close to the opponent while aggressively displaying their white plumage markings, whereas control birds tended to hop more toward the back of the cage away from the opponent (Fig. 2d). All these lines of evidence indicate that exogenous T boosts aggressive behavior in these birds.

T implants increased circulating T and DHT levels

TABLE 2

Plasma Concentrations of Hormones, Percentage Detectability of Samples, and Several Physiological Measurements of Male Spotted Antbirds before and after Implantation of Four Empty (Control) or Four Silastic Capsules Filled with Either ATD or Flutamide

Median (quartiles)	Control-implanted (<i>n</i> = 6)		ATD + Flut-implanted (<i>n</i> = 7)	
	Before	After	Before	After
Androgens (ng/ml)	0.15 (0.15, 0.32) ⁷	0.14 (0.13, 0.15)	0.25 (0.15, 0.41)	0.42 (0.33, 0.78)
% det.	28.6	83.3	71.4	100
LH (ng/ml)	0.98 (0.5, 1.18) ⁷	1.79 (1.07, 2.2)	0.6 (0.4, 0.9)	4.07 (3.04, 5.4) ^{6*}
% det.	100	100	100	100
Hematocrit (%)	0.58 (0.52, 0.64)	0.57 (0.54, 0.61)	0.62 (0.56, 0.71)	0.6 (0.56, 0.67)
Body mass (g)	17.0 (14.7, 17.5) ⁷	17.8 (17.1, 19.8) [#]	16.7 (15.5, 17.7)	18.1 (17.2, 20.7) ⁵

Note. Numbers in superscript indicate sample size for this data point. For further explanations see text.

* Indicates a significant difference between treatment groups, $P < 0.05$.

Indicates a significant difference between treatments within a group, $P < 0.05$.

two to four times above maximal levels measured in free-living male Spotted antbirds (Wikelski *et al.*, 1999a,b,c) and resulted in titers that are comparable to those of many temperate male birds during the breeding season (Table 1). However, AE, E_2 , and LH levels of T-implanted Spotted antbirds stayed within the normal range of free-living birds (Wikelski *et al.*, 1999b). Furthermore, administration of T implants did not impair the well being of male Spotted antbirds as indicated by the lack of differences between groups in body mass, hematocrit levels, and fat scores (Table 1). Further experiments will examine the effectiveness of lower doses of exogenous T. Circulating plasma levels of the androgens AE and DHT were correlated with T levels and always remained at a lower level, supporting our previous interpretation that these androgens have not taken over the functions of T in these tropical birds (Wikelski *et al.*, 1999a,b,c). The increase in locomotor activity caused by T administration parallels results of studies on temperate birds (Wada, 1981, 1982, 1986; Wikelski *et al.*, 1999e).

Effects of Blockade of T in Breeding Birds

The pharmacological blockade of T actions through the combined administration of the aromatase inhibitor ATD and the androgen receptor antagonist flutamide decreased aggression in male Spotted antbirds, as suggested by a number of results. ATD + Flut-treated birds sang less spontaneously and less toward a female (Figs. 3a and 3b). Most importantly, during

the male–male encounter ATD + Flut-treated birds did not sing at all and snarled less than control birds (Figs. 4a and 4b). Currently, we cannot decide whether the reduction in aggression was due to the blockade of androgen receptors or the inhibition of aromatase, since we administered ATD and flutamide at the same time. In the future, these two pathways of T action will be blocked separately to distinguish between the two pathways.

Continuous administration of ATD + Flut-implants raised endogenous T and LH levels to naturally occurring levels (Table 2), presumably due to the lack of feedback mechanisms, as in many temperate birds (Archawaranon and Wiley, 1988; Johnson and Bottjer, 1993; Soma *et al.*, 1999). Similar treatment also decreased aggression in experiments on temperate birds (Beletsky *et al.*, 1990; Soma *et al.*, 1999). In a previous experiment on free-living Song sparrows aggressive territorial behaviors were reduced after 30 days but not after 7 days of treatment with similar doses of ATD and flutamide (Soma *et al.*, 1999). In our captive birds variability in behavior was likely reduced, perhaps allowing ATD + Flut effects to be detected earlier. Hence, a stronger difference in aggressive behavior of male Spotted antbirds may be obtained if the ATD + Flut treatment is continued for a longer period of time. Note that LH did not take over the role of T in regulating aggression in these birds (e.g., Crook and Butterfield, 1968; Levin and Wingfield, 1992), since ATD + Flut-treated birds had elevated LH titers but were less aggressive. ATD + Flut treatment did not affect locomotor activity, hematocrit, or body mass.

Instead, birds of both experimental groups increased body mass during the experiment (Table 2) and behaved normally (see also Searcy and Wingfield, 1980; Archawaranon and Wiley, 1988; Walters and Harding, 1988; Beletsky *et al.*, 1990; Schwabl and Kriner, 1991; Soma *et al.*, 1999).

T and Aggression in Spotted Antbirds

Our experiments suggest that T is involved in the regulation of aggressive behaviors of male Spotted antbirds. This interpretation is supported by evidence from free-living male Spotted antbirds, in which an increase in T during times of social instability at any time of year also suggested a role of T in territorial aggression year-round (Wikelski *et al.*, 1999a,c).

Three nonexclusive hypotheses are conceivable. (1) The birds increase plasma T only for short periods during "challenging situations," as suggested from our field data (Wikelski *et al.*, 1999a). (2) They may be highly sensitive to low levels of plasma T. This possibility is supported by the finding that control birds with low plasma T titers showed aggressive behavior in both experiments. One potential mechanism could be an increased density of androgen receptors in the brain of these birds. (3) Spotted antbirds secrete T precursors into the circulation and then convert them into T and E₂ in the brain during challenging situations. AE and dehydroepiandrosterone (DHEA) are T precursors that can be converted into T (e.g., Nakamura and Tanabe, 1974; Harding, 1986; Schlinger, 1997). However, plasma AE in Spotted antbirds (current data and Wikelski *et al.*, 1999b) and some temperate birds (e.g., Ramenofsky, 1984; Schlinger and Arnold, 1992) is generally lower than T. Plasma DHEA has been found in breeding quail (*Coturnix japonica*; Tsutsui and Yamazaki, 1995), and the enzyme that converts it into AE (which is a direct precursor of T) is present in songbird brains (Vanson *et al.*, 1996). Although direct evidence linking DHEA to aggressive behavior in birds is still missing, an interesting feature of DHEA is that it can be produced by peripheral glands, possibly by the avian adrenal (Nakamura *et al.*, 1978; Schlinger *et al.*, 1999). Hence, it could be available throughout the year, independent of breeding seasons and gonad sizes of the birds.

We hypothesize that year-round territorial birds

keep plasma levels of T low to avoid potential detrimental effects of continuously elevated plasma T levels (e.g., Zuk, 1994; Hillgarth and Wingfield, 1997). However, short-term increases in plasma T may be used to boost aggressive behavior and persistence in situations in which territory owners are confronted with a territorial challenge. In particular, Spotted antbirds often forage in association with raiding army ants and, in the course of this, temporally invade neighboring territories (Willis, 1972; Wikelski *et al.*, 1999a).

The current findings emphasize the need for more experimental studies to understand the hormonal control of territorial behavior and song in tropical birds in general. In particular, two intriguing questions emerge. How does T affect song in suboscine passerines such as Spotted antbirds, which are thought to lack a distinct song control center in the brain (e.g., Brenowitz, 1997)? Since female Spotted antbirds also sing and aggressively defend territories (Willis, 1972; Wikelski *et al.*, 1999b), what is the role of T in female territorial behavior in tropical birds?

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