

## Androgen and the elaborate courtship behavior of a tropical lekking bird

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### Abstract

In most bird species, male courtship behavior is controlled by testosterone (T) and its metabolites. In species breeding in temperate and arctic regions T circulates at high levels during a relatively short courtship period because high levels of T can be costly in terms of immunocompetence and parental care. Few studies have investigated androgen modulation of courtship behavior in tropical birds. Male golden-collared manakins (*Manacus vitellinus*) aggregate in leks for several months and perform spectacular, acrobatic courtship displays. Here we examined whether T is elevated in golden-collared manakins during the displaying period and if courtship behavior is modulated by androgen action on androgen receptors. We measured T levels in displaying males at the beginning of the breeding season and again, one month later. In addition, both wild and captive males were treated with the anti-androgen, flutamide, and their courtship behavior was recorded for several weeks. T levels were relatively high shortly after leks were established but decreased substantially a month later, even though the amount of courtship did not change. Flutamide reduced male courtship activity for one week, but display behavior then increased after two weeks of flutamide treatment. Our studies show that androgens modulate male manakin courtship, but the amount of courtship is not directly correlated with the concentration of circulating T. These results suggest that the relationships between androgen and courtship might differ between tropical and temperate birds.

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### Introduction

In species breeding in temperate and arctic regions males often have high circulating concentrations of testosterone (T) during a relatively short breeding season (Wingfield and Farner, 1993). The highest concentrations of T are recorded in the initial phases of the breeding season, during the establishment of territories, courtship, and pair formation (Wingfield et al., 1990). T decreases afterwards, probably because elevated T concentrations are costly in terms of parental care (reviewed in Wingfield et al., 2001) and immunocompetence (Folstad and Karter, 1992). By contrast, males of many tropical species with

prolonged breeding seasons typically have low levels of plasma T (reviewed by Goymann et al., 2004; Levin and Wingfield, 1992; Wikelski et al., 2003), unless they live at high elevations (Moore et al., 2002). However, high levels of T have been found in some tropical species during courtship (Chastel et al., 2005; Levin and Wingfield, 1992; Wiley and Goldizen, 2003).

The golden-collared manakin (*Manacus vitellinus*) is a subsong passerine bird found predominantly in lowland or foothill rainforests of Panama. Males of this species perform some of the most elaborate and spectacular courtship displays among birds (Chapman, 1935; Schlinger et al., 2001). During the long courtship season (January to July), males aggregate in leks within forests, clear a display arena on the ground between small vertical saplings and spend most of the day in and around the arena performing a variety of courtship and territorial displays that include production of mechanical sounds with their wings (“wingsnaps”) (Chapman, 1935).

We have substantial evidence that androgens activate courtship in males of this species. During the non-breeding

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season, when T levels are basal, males abandon their leks but display occasionally. Implantation with T, however, increases displaying activity up to breeding levels (Day et al., 2006). Sex steroids accumulate to a greater degree in the spinal cords of males than females (Schultz and Schlinger, 1999) and the motoneurons that innervate the wing muscles used for producing the wingsnaps contain androgen receptors (AR) (Schultz et al., submitted for publication). In addition, golden-collared manakins have a unique pattern of expression of AR in the brain, which may be related to the control of their elaborate displays (Fusani et al., 2003). Despite this evidence for androgen effects in males, we do not know whether courtship activity is correlated with high T levels during the lekking period. In the few lekking species of temperate regions that have been studied, males have high levels of androgen as long as they are actively displaying on their leks (Alatalo et al., 1996; Lisano and Kenamer, 1977). A recent study reported that in manakins T is elevated in males during parts, but not all, of the long breeding season (Wikelski et al., 2003), although the courtship activity of the individuals was not recorded.

In this report, we studied the relationships between androgens and the courtship activity of male golden-collared manakins. We measured T levels of male manakins that were actively displaying in their courts at the beginning of the breeding season and one month afterwards. According to the typical pattern of lekking birds in the temperate zones (Wingfield et al., 1990), we predicted that plasma levels of T would remain high throughout the courtship season. We then implanted some of these males with the anti-androgen flutamide (Miranda et al., 2002) and compared their courtship behavior with that of control males. We predicted that the treatment with flutamide would reduce courtship activity.

We found that T was elevated at the beginning of the displaying season but decreased significantly one month afterward although courtship activity did not change. In addition, we found that flutamide reduced courtship activity in the first week of implantation but the difference disappeared in the second week and was inverted in the third week, with flutamide implanted birds displaying more than untreated ones. To verify these unexpected effects of flutamide, we performed an additional experiment in which we implanted flutamide in captive manakins in which courtship behavior had been induced by T implantation. This study confirmed that courtship behavior may increase after two weeks of implantation with flutamide.

## Materials and methods

### Experimental design

The experiments were conducted near Gamboa, Republic of Panama, where we have studied manakins since 1996. In January, at the beginning of the dry season, golden-collared manakins establish leks in which each male clears a small arena on the forest floor between thin vertical saplings. In 2002, males began occupying leks in mid January. Over the next 4 weeks, we located 4 separate leks and we trapped 2 or 4 males from each of these leks using mist-nets. Courtship activity was initiated at slightly different times across leks but males were caught within one week of the beginning of evident courtship activity. Males were weighed and color banded. A blood sample (~100  $\mu$ l) was taken from the wing vein and the bird was then randomly assigned to the

flutamide ( $n=6$ ) or Control group ( $n=6$ ) (see below). The birds were released immediately after implantation and were seen displaying in their arenas as early as the day following capture. Behavioral observations were then conducted for three weeks as described below. Within a week after the conclusion of behavioral observations, all birds were recaptured. They were found to be in excellent conditions, a second blood sample was collected and the birds were euthanized by exposure to isoflurane and tissues collected for use in a separate study. We measured the length of tarsus, the weight of the syrinx, the length and diameter of the cloacal protuberance from which we calculated the cloacal volume using the formula for cylinders ( $\pi ab^2$ ;  $a$ =length,  $b$ =diameter/2) and the length and width of testes from which we calculated testis volume using the formula for ellipsoid cylinders ( $4/3\pi a^2 b$ ;  $a$ =length/2,  $b$ =width/2). We minimized the impact on natural populations by using the same animals for behavioral, endocrinological and neurobiological studies. Golden-collared manakins are common in central Panama and the collection of individuals was evaluated by the Smithsonian Tropical Research Institute and authorized by the Autoridad Nacional del Ambiente (ANAM) of the Republic of Panama and the University of California Chancellor's Animal Care and Use Committee.

### Flutamide treatment

Flutamide birds were implanted with time release pellets (1.6 mm  $\times$  5.0 mm; Innovative Research of America, Sarasota, FL) designed to release 167  $\mu$ g/day of flutamide for 60 days. The manakins were implanted subcutaneously at the base of the neck. The entire operation required less than 30 s and is comparable to a subdermal injection. Control birds were sham implanted. Each lek had an equal number of birds (1 or 2) assigned to each group. We released the birds immediately after the implantation and we did not observe any adverse effect of the implantation and/or flutamide. The efficacy of the pellets was tested in the laboratory by implanting one flutamide pellet in 3 male zebra finches. After 4 weeks the pellets were removed and sent to the laboratory of Dr. Isidoro Caraballo, University of Sevilla, Spain, for measurement of flutamide by means of HPLC (details in Miranda et al., 2002). Samples were analyzed in 3 replicates. The implanted pellets contained (mean  $\pm$  SEM) 3.25  $\pm$  0.17 mg of flutamide, i.e., 47.8% of the new pellet, which is very close to the 46.7% expected after 28 days of implantation. There was no sign of degradation of the flutamide as that the peak corresponding to its main catabolic product, hydroxyflutamide, was almost absent.

### Behavioral observations

The courtship behavior of the golden-collared manakin has been described in detail previously (Chapman, 1935; Schlinger et al., 2001). We focused our observations on 3 independent behaviors that could be identified unambiguously. In the "jump-snap" display, the male jumps between the vertical saplings that delimit the arena while producing loud "wingsnaps" in mid-air. The "rollsnap" is produced by perched manakins by a rapid fluttering of the wings. The "cheepoos" are vocalizations usually emitted following rollsnaps, but also independently of other courtship behaviors.

We did focal 30-min observations from the 2nd to the 21st day after implantation, performing at least 2 observations/bird for each 3-day period. Observations were done between 7:00 and 9:00 and between 12:30 and 15:30, when activity is known to peak within the lek (Chapman, 1935; Stein and Uy, 2006; personal observations). The observer sat about 10 m from the arena, from where he/she could always see the focal male in his arena. After 15 min, the observer recorded the behavior of the focal male on a notebook and on a tape recorder for 30 min. We recorded the number of (1) jump-snap displays; (2) wingsnaps during a jump-snap display; (3) rollsnaps; and (4) cheepoos. In addition, for each male we selected up to 5 displays on the tape recordings made during the first and third week of implantation. The interval between wingsnaps was measured on the oscillogram of the digitized recordings with an accuracy of 5 ms using Canary 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY).

### Flutamide implantation in captive birds

Because of an unexpected increase in displaying activity in flutamide-implanted birds in the third week (see Results), we conducted an additional experiment under controlled laboratory conditions in which six green-plumaged

manakins (2 females and 4 juvenile males, sexing procedure as described in Day et al., 2006) were implanted with T to activate courtship behavior and then with flutamide. Adult males do not display in captivity at this time of year (Day et al., 2007) however wingsnaps and rollsnaps can be induced in female and juvenile males by treatment with T (Day et al., 2006). Immediately upon capture, a blood sample was taken from the wing vein and birds were implanted with T pellet (1.5 mg, 60-day release, Innovative Research of America, FL) and housed in individual cages (details in Day et al., 2006). We recorded the behavior for 7 days after the T implantation, we took a blood sample, and we continued observations. At 13 to 23 days after T implantation (average: 18.5) all birds were bled again and implanted with flutamide pellets from the same lot of those used in the field experiment without removal of T implants. The behavior was observed for another 3 weeks, and after taking a final blood sample and removing the implants the birds were released at the original site of capture. Behavior was recorded on a Sony CCD-TRV68 camcorder for 2 h daily between 06:30 and 08:30. For each bird, we calculated the number of wingsnaps and roll snaps per minute during the recording sessions: T1= testosterone period 1 (days 1–7); T2= testosterone period 2 (days 8–17); Flu1= flutamide week 1; Flu2= flutamide week 2; and Flu3= flutamide week 3. Some birds had been implanted with T up to 23 days before flutamide implantation; however, we analyzed the behavior only up to 17 days post T implant so that all birds would have similar numbers of observations for the late T period.

### Hormone measurement

After centrifugation of the blood, plasma was collected, stored on dry ice and transported to the Research Centre for Ornithology of the Max-Planck Society, Andechs, Germany. T and 17 $\beta$ -estradiol (E<sub>2</sub>) were measured by radioimmunoassay (RIA) as described previously (Fusani et al., 2000). We measured E<sub>2</sub> to check whether the implantation of flutamide would affect the rate of conversion androgen into estrogen. The detection limits of the RIAs were 30 pg/ml for T and 26 pg/ml for E<sub>2</sub>. Water blanks were always below the lower detection limit. The average recoveries were between 87.3 $\pm$ 0.3% for T and 71.6 $\pm$ 0.3% for E<sub>2</sub>. The intra-assay variation was <8% for all assays. Inter-assay variation is not applicable because all the samples were run in a single assay.

In samples from the laboratory implantation experiments T levels were analyzed with a Coat-A-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA) previously validated for manakins (Day et al., 2006). Where possible, we used 50  $\mu$ l duplicate samples for each individual. We could not calculate the samples intra-assay variation as only one sample measured in duplicate was above the lower detection limit. The coefficient of variation for this sample was 13%. The intra-assay variation calculated for the standard curve was 1% and for 3 additional T plasma standards was 7%. The upper and lower detection limits were 10 pg/ml and 1600 pg/ml, respectively.

### Statistical analysis

Analyses were performed using SPSS 11.0 (SPSS, Inc.). The behavioral variables were analyzed with a two-way repeated measures ANOVA comparing between treatment groups (between-subjects) and between weeks of observation (weeks 1–3, within-subjects) using the individual week means. For the hormonal analysis the time of sampling (before implantation and at recapture) was the within-subjects factor. When there was a significant interaction effect, we performed post-hoc paired comparisons between groups and between weeks with one-way (repeated measures) ANOVA followed by Bonferroni-corrected pairwise comparisons. Significance was set at  $\alpha=0.05$ . The interval between wingsnaps was not normally distributed, and was compared between groups with a Mann–Withney *U* test.

For the laboratory implantation experiment, analyses were done with Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). We used the Friedman Test to examine changes in behavior and in T levels across the treatment period. Post-hoc analysis was performed with a Dunn's multiple comparison test (Dunn, 1964). In particular, we compared behavior between the first and last week of flutamide treatment, and T levels between the first week of T treatment and the end of the flutamide treatment. We limited the paired comparison to two time points because the power of the Dunn's test depends on the number of paired comparisons (Dunn, 1964; Siegel and Castellan, 1988).

## Results

### Plasma hormone levels

Plasma T levels were significantly higher in all birds at the beginning of the courtship season than four weeks later ( $F_{1,7}=8.38$ ,  $p<0.03$ ) (Fig. 1). There was no significant effect of the implantation ( $F_{1,7}=0.14$ , NS) and no significant interaction ( $F_{1,7}=0.00$ , NS) (Fig. 1). E<sub>2</sub> was below the lower detection limit of the assay (26 pg/ml) in all birds except 3 males before implantation (2 Control, 33.7 and 26.1 pg/ml; 1 flutamide, 55.0 pg/ml) and in one flutamide male at the end of the experiments (28.2 pg/ml).

### Courtship behavior

The courtship activity of untreated birds did not change significantly during the three weeks of observations (Fig. 2). Courtship activity was intense and we recorded about 2–3 displays with 20–30 wingsnaps during each 30-min observation (Fig. 2). Flutamide significantly reduced courtship activity during the first week of the treatment (Fig. 2). Overall, the ANOVA showed that courtship activity was not affected by the treatment (jump-snap displays,  $F_{1,10}=0.01$ , NS; wingsnaps,  $F_{1,10}=0.65$ , NS; rollsnaps,  $F_{1,10}=0.00$ , NS; cheepoos,  $F_{1,10}=1.82$ , NS). There was a significant effect of the week on the number of rollsnaps and of cheepoos ( $F_{2,20}=6.22$ ,  $p<0.01$ , and  $F_{2,20}=3.87$ ,  $p<0.05$ ) but not on the number of jump-snap displays and/or wingsnaps ( $F_{2,20}=1.19$ , NS, and  $F_{2,20}=3.01$ , NS). However, for all variables except the number of cheepoos there was a significant effect of the interaction between week and treatment (jump-snap displays,  $F_{2,20}=4.69$ ,  $p<0.03$ ; wingsnaps,  $F_{2,20}=9.64$ ,  $p<0.001$ ; rollsnaps,  $F_{2,20}=4.93$ ,  $p<0.02$ ; cheepoos,  $F_{2,20}=3.26$ , NS). The post-hoc tests showed that in the untreated birds there was no significant change for any behavioral variable during the whole duration of the experiment. The number of jump-snap displays, wingsnaps and rollsnaps of flutamide birds was lower during the first week of implantation compared to untreated birds (Fig. 2). This difference disappeared in the second week and was inverted in the third week (Fig. 2).

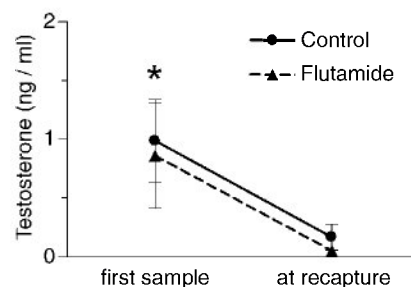


Fig. 1. Plasma levels of testosterone before implantation with flutamide and at the end of the experiments. In both flutamide-treated and control birds plasma testosterone levels were lower at the end of the experiments compared to pre-implantation levels. \* $p<0.03$ , before implantation vs. at recapture, two-way repeated measures ANOVA.

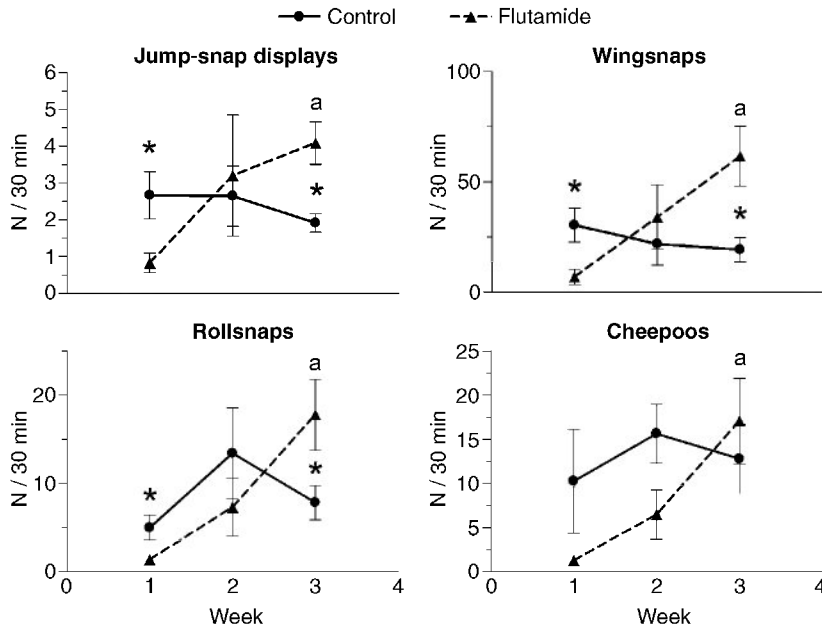


Fig. 2. Courtship displays of flutamide and control birds during the three weeks of treatment. The number of jump-snaps, wingsnaps, and rollsnaps was lower in flutamide birds during the first week of treatment. There was no difference between groups at week 2. During the third week, there was a reversal of the effects seen during the first week and flutamide birds displayed more than control birds. The courtship behavior of Control birds did not change during the experiments, with the exceptions of the number of cheepoos. \* $p < 0.05$ , flutamide vs. control males; <sup>a</sup> $p < 0.05$ , week 3 vs. week 1, flutamide birds only. Two-way repeated measures ANOVA followed by post-hoc pairwise comparisons.

During both the first and third weeks, we found no significant difference between untreated and flutamide implanted birds for the interval between wingsnaps (week 1: untreated,  $0.884 \pm 0.082$  s, flutamide,  $0.708 \pm 0.075$  s, Mann–Whitney  $U$  test,  $z = 1.43$ ,  $p = 0.15$ ; week 3: untreated,  $0.844 \pm 0.092$  s, flutamide  $0.753 \pm 0.034$  s,  $z = 0.64$ ,  $p = 0.52$ ). Paired comparisons also showed no significant differences in the interval between wingsnaps between weeks 1 and 3 (Wilcoxon signed ranks test  $z = 1.35$ ,  $p = 0.18$ ).

*Morphological variables*

We found no difference between groups for any of the morphological parameters. Flutamide implanted birds did not differ from untreated birds for body mass (mean  $\pm$  SEM, respectively:  $18.5 \pm 0.3$  vs.  $18.9 \pm 0.3$  g,  $t = 0.97$ ,  $p = 0.35$ ), tarsus length ( $21.53 \pm 0.14$  vs.  $21.54 \pm 0.20$  mm,  $t = 0.06$ ,  $p = 0.95$ ), average testis volume ( $52.83 \pm 7.20$  vs.  $48.40 \pm 5.44$  mm<sup>2</sup>,  $t = 0.49$ ,  $p = 0.63$ ), cloacal protuberance volume ( $30.11 \pm 3.30$  vs.  $37.47 \pm 3.07$ ,  $t = 1.51$ ,  $p = 0.16$ ) or syrinx weight ( $84.17 \pm 5.59$  vs.  $86.40 \pm 4.09$ ,  $t = 0.32$ ,  $p = 0.75$ ).

*Laboratory implantation experiment*

T treatment elevated plasma T concentrations in all implanted birds (Fig. 3). In one sample taken at the time of capture T was outside of the assay standard curve and was excluded from the analysis. The Friedman test showed a significant change in T levels over the treatment period ( $F_T = 10.33$ ,  $n = 6$ ,  $p < 0.002$ ; Fig. 3). Plasma T concentrations decreased significantly from the first week of T implantation

(T1) to the end of the flutamide implantation (Flu3) (Dunn’s Test,  $p < 0.01$ ). There were significant changes in the number of wingsnaps ( $F_T = 13.73$ ,  $n = 6$ ,  $p < 0.01$ ) and roll snaps ( $F_T = 12.43$ ,  $n = 6$ ,  $p < 0.02$ ) during the experimental period (Fig. 3). Paired comparisons showed that there was a significant increase in wingsnaps between Flu1 and Flu3 (Dunn’s test,

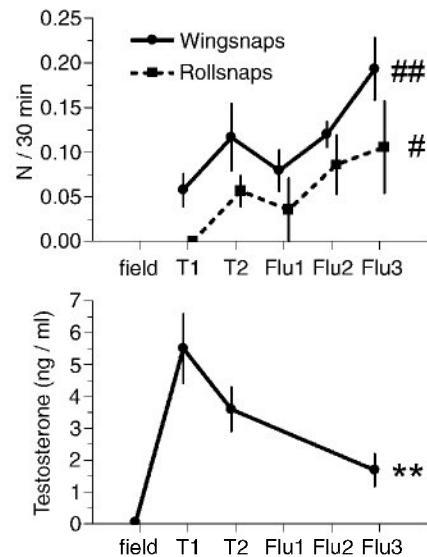


Fig. 3. Wingsnap and rollsnap frequency (top) and plasma levels of T (bottom) in captive female and juvenile male manakins implanted with T and flutamide. Time points are as follows: at capture = field; after implantation with T, days 1–7 = T1; days 8–17 = T2; and T+flutamide, days 1–7 = Flu1; 8–14 = Flu2; 15–21 = Flu3. <sup>##</sup> $p < 0.01$ , Flu3 vs. Flu1, Friedman Test followed by Dunn’s post-hoc comparison; <sup>#</sup> $p < 0.02$ , Friedman test. <sup>\*\*</sup> $p < 0.01$ , Flu3 vs. T1, Friedman’s test followed by Dunn’s post-hoc comparison.

$p < 0.01$ ) (Fig. 3). For roll snaps there was a numerical increase between Flu1 and Flu3 but the difference did not reach significance (Fig. 3).

## Discussion

Our data show that in the golden-collared manakin there is no direct relationship between plasma hormone levels and the intensity of the courtship displays. Whereas levels of display changed little over the first month of the breeding season, levels of T in plasma were initially relatively high and then decreased dramatically. Thus, the intense courtship behavior of adult male manakins is maintained by relatively low levels of circulating T. On the other hand, our work also shows that AR-mediated action influences the expression of courtship in male golden-collared manakins. Treatment with the AR antagonist flutamide caused an expected reduction in courtship behaviors, including vocal and mechanically produced acoustic features. Unexpectedly, most of the behaviors increased in frequency after 2 weeks treatment with flutamide. To test whether the paradoxical effects of flutamide were repeatable under more controlled conditions, we implanted flutamide into captive birds that had previously received a T implant to activate display behavior as shown in previous experiments (Day et al., 2006). The effects of flutamide were very similar to those found in adult manakins in the field. Courtship behaviors such as wingsnaps and rollsnaps showed a numerical decrease in the first week of flutamide implantation, followed by an increase in the third week that was significant for the wingsnaps.

### *Testosterone and display*

Birds breeding in temperate zones tend to have high levels of T during relatively short periods of reproductive activity (Wingfield et al., 1997). In the few lekking species that have been studied, males have high levels of androgen as long as they are actively displaying on their leks (Alatalo et al., 1996; Lisano and Kennamer, 1977). In general, tropical birds differ from their temperate-breeding counterparts by having relatively low levels of circulating T throughout the reproductive season (Goymann et al., 2004; Gwinner and Scheuerlein, 1999; Hau et al., 2000; Levin and Wingfield, 1992; Wikelski et al., 2003). This is particularly the case of species that, like the golden-collared manakin, live at low altitudes and have a long breeding season (Goymann et al., 2004). Golden-collared manakins seem to differ also from lekking species in the temperate-zone in that plasma levels of T declined significantly although the males continued to court females and interact with neighboring males. There is evidence that some tropical birds secrete T in high amounts for a brief period at the onset of reproduction (Chastel et al., 2005; Moore et al., 2002; Wiley and Goldizen, 2003). Results from our lab are consistent with this view. In this study, T levels at the time of lek establishment were around 0.75 ng/ml, with levels in 2 additional males above 3.5 ng/ml (Fusani and Schlinger, unpublished). T levels are consistently low during the non-breeding season (Day et al., 2007). Presumably,

elevated T levels at the beginning of the courtship period are important for initiating breeding or result from social instability during lek formation (see also Day et al., 2007; see also Wikelski et al., 1999). According to this hypothesis, in lekking species such as the golden-collared manakin males might require an increase in circulating androgen for a few weeks at the time of lek formation, when each male establishes his courting arena. After this initial period, T levels would decrease because of costs associated with long-term elevated T levels (Wingfield et al., 2001).

### *Flutamide treatment*

Previous studies have shown that flutamide reduces territorial and sexual behavior of male birds (Bottjer and Hewer, 1992; Hegner and Wingfield, 1987; Schwabl and Kriner, 1991). Our results during the first week after treatment of wild males are consistent with these other studies. Despite its effects on courtship activity, flutamide had no effect on the interval between wingsnaps suggesting that this feature of the courtship behavior is not directly modulated by androgen. This is consistent with previous studies that indicate that androgens influence the overall amount of courtship but have little effect on the nature of the displays themselves (Fusani and Hutchison, 2003; Hews and Moore, 1997).

We do not know why flutamide treatment stimulated an increase in courtship behavior after 3 weeks. The simplest explanation is that flutamide was no longer released by the pellets after the first week of implantation. The following increase in courtship behavior above control levels might have resulted from an increased sensitivity to androgen because of the up-regulation of AR expression described previously after anti-androgen treatment (Chen et al., 2004).

Several observations, however, indicate that indeed the pellets released flutamide throughout the experiments. The pellets were designed for a 60-day release, though they were only used for the 21-day experimental period. Furthermore, all pellets were in place at the end of the experiments and they were not encapsulated by connective tissue that might have impeded drug release. Finally, flutamide pellets implanted into zebra finches for 4 weeks showed no degradation of the drug and the expected amount of flutamide, as measured by HPLC, remained in the pellet.

Similarly, there are no reasons to suspect that the increase in courtship during the 3rd implantation week is due to an increased conversion of androgen into estrogen. In the field experiment we showed that plasma levels of both T and  $17\beta$ -estradiol, the main estrogen metabolite of T, were low in flutamide birds and did not differ from those of controls. We did not combine the flutamide treatment with aromatase inhibitors such as Fadrozole or androstratenedione (ATD) that block the conversion of T into  $E_2$  because such a treatment would not allow distinguishing between androgen and estrogen modulatory effects on courtship. In addition, treatment of intact male birds with aromatase inhibitors invariably results in an increase in circulating levels of T because neural aromatization is likely essential to create the negative feedback from circulating T (see

Fusani et al., 2001). Such an increase in peripheral T would mask the effects of flutamide due to its action as a competitive androgen receptor antagonist. For the same reasons, the increase in courtship behavior seen two weeks after the beginning of flutamide treatment cannot be accounted by an increase in circulating T. This was particularly evident in the laboratory experiment in which T levels decreased during the flutamide treatment, ruling out that the behavioral effects were due to a reduction in the flutamide/T ratio. In any case, it should be noted that the dose of flutamide used in the present study was relatively low, and was calibrated to obtain a modulation of androgen-dependent action rather than a complete inhibition. It is possible that flutamide became an androgen agonist after 2 weeks in vivo. Flutamide and other anti-androgens have been reported to have such time-dependent, paradoxical effects (reviewed in Shet et al., 1997; reviewed in Wirth and Froschermaier, 1997). Recent studies showed that anti-androgen can lead to a substantial upregulation of androgen receptor mRNA and protein expression, which results in a hypersensitization of tissues to very low concentration of androgen (Chen et al., 2004). Clearly, further experiments will be necessary to understand the mechanisms responsible for these paradoxical effects.

### General conclusions

Male manakins have some of the most complex and spectacular displays among birds making them excellent models to study the link between male courtship and T in tropical birds. Our work shows that the courtship behavior of male golden-collared manakins is influenced by androgen receptor-mediated action, but its maintenance does not require elevated T concentrations. These results are in agreement with those of another series of studies from our laboratory where we tested T-activation of courtships (Day et al., 2007). T treatment of adult males during the courtship season did not increase the amount of courtship behavior either in the field or in captivity (Day et al., 2007). Altogether, our studies show that courtship of male manakins is activated by T (Day et al., 2007) but high levels of T are not required to maintain courtship (this study) and treatment with T of males already engaged in courtship does not affect the amount of courtship behavior (Day et al., 2007). We are currently examining structural aspects of the courtship display (the specific elements of the display choreography) to determine if individuals differ and whether males are selected preferentially by females based on display characteristics. Finally, because of the potential for agonist effects like the ones we saw in this study, we suggest examination of long-term flutamide treatments with other bird species.

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### References

- Alatalo, R.V., Hoglund, J., Lundberg, A., Rintamaki, P.T., Silverin, B., 1996. Testosterone and male mating success on the black grouse leks. *Proc. R. Soc. Lond., Ser. B Biol. Sci.* 263 (1377), 1697–1702.
- Bottjer, S.W., Hewer, S.J., 1992. Castration and antisteroid treatment impair vocal learning in male zebra finches. *J. Neurobiol.* 23, 337–353.
- Chapman, F.M., 1935. The courtship of Gould's manakin (*Manacus vitellinus vitellinus*) on Barro Colorado Island, Canal Zone. *Am. Mus. Nat. Hist. Bull.* 68, 472–521.
- Chastel, O., Barbraud, C., Weimerskirch, H., Lormee, H., Lacroix, A., Tostain, O., 2005. High levels of LH and testosterone in a tropical seabird with an elaborate courtship display. *Gen. Comp. Endocrinol.* 140 (1), 33–40.
- Chen, C.D., Welsbie, D.S., Tran, C., Baek, S.H., Chen, R., Vessella, R., Rosenfeld, M.G., Sawyers, C.L., 2004. Molecular determinants of resistance to antiandrogen therapy. *Nat. Med.* 10 (1), 33–39.
- Day, L.B., McBroom, J.T., Schlinger, B.A., 2006. Testosterone activates courtship display but does not alter plumage in the tropical golden-collared manakin (*Manacus vitellinus*). *Horm. Behav.* 49 (2), 223–232.
- Day, L.B., Fusani, L., Hernandez, E., Billo, T.J., Sheldon, K.S., Wise, P.M., Schlinger, B.A., 2007. Testosterone and its effects on courtship in golden-collared manakins (*Manacus vitellinus*): seasonal, sex, and age differences. *Horm. Behav.* 51, 69–76.
- Dunn, O.J., 1964. Multiple comparisons using rank sums. *Technometrics* 5, 241–252.
- Folstad, I., Karter, A.J., 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* 139, 603–622.
- Fusani, L., Hutchison, J.B., 2003. Lack of changes in the courtship behaviour of male ring doves after testosterone treatment. *Ethol. Ecol. Evol.* 15 (2), 143–157.
- Fusani, L., Van't Hof, T., Hutchison, J.B., Gahr, M., 2000. Seasonal expression of androgen receptors, estrogen receptors and aromatase in the canary brain in relation to circulating androgens and estrogens. *J. Neurobiol.* 43, 254–268.
- Fusani, L., Gahr, M., Hutchison, J.B., 2001. Aromatase inhibition reduces specifically one display of the ring dove courtship behavior. *Gen. Comp. Endocrinol.* 122, 23–30.
- Fusani, L., Donaldson, Z., Schlinger, B., 2003. The distribution of steroid receptors and aromatase in the brain and spinal cord of a suboscine bird with elaborate courtship display, the Golden-collared manakin. *Horm. Behav.* 44, 50 (Society for Behavioral Neuroendocrinology 2003 Annual Meeting).
- Goymann, W., Moore, I.T., Scheuerlein, A., Hirschenhauser, K., Grafen, A., Wingfield, J.C., 2004. Testosterone in tropical birds: effects of environmental and social factors. *Am. Nat.* 164 (3), 327–334.
- Gwinner, E., Scheuerlein, A., 1999. Photoperiodic responsiveness of equatorial and temperate-zone stonechats. *Condor* 101, 347–359.
- Hau, M., Wikelski, M., Soma, K.K., Wingfield, J.C., 2000. Testosterone and year-round territorial aggression in a tropical bird. *Gen. Comp. Endocrinol.* 117 (1), 20–33.
- Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. *Auk* 104 (3), 462–469.
- Hews, D.K., Moore, M.C., 1997. Hormones and sex-specific traits: critical questions. In: Beckage, N.E. (Ed.), *Parasites and Pathogens. Effects on Host Hormones and Behavior*. Chapman and Hall, New York, pp. 277–292.
- Levin, R.N., Wingfield, J.C., 1992. The hormonal control of territorial aggression in tropical birds. *Ornis Scand.* 23, 284–291.
- Lisano, M.E., Kennamer, J.E., 1977. Seasonal variations in plasma testosterone level in male eastern wild turkeys. *J. Wildl. Manage.* 41, 184–188.
- Miranda, A., Caraballo, I., Millan, M., 2002. Stability study of flutamide in solid state and in aqueous solution. *Drug Dev. Ind. Pharm.* 28 (4), 413–422.

- Moore, I.T., Perfito, N., Wada, H., Sperry, T.S., Wingfield, J.C., 2002. Latitudinal variation in plasma testosterone levels in birds of the genus *Zonotrichia*. *Gen. Comp. Endocrinol.* 129 (1), 13–19.
- Schlinger, B.A., Schultz, J.D., Hertel, F., 2001. Neuromuscular and endocrine control of an avian courtship behavior. *Horm. Behav.* 40 (2), 276–280.
- Schultz, J.D., Schlinger, B.A., 1999. Widespread accumulation of [H-3] testosterone in the spinal cord of a wild bird with an elaborate courtship display. *Proc. Natl. Acad. Sci. U. S. A.* 96 (18), 10428–10432.
- Schultz, J.D., Fusani, L., Hau, M., and Schlinger, B., submitted. Androgen receptor expression in motor and sensory neurons innervating limb muscles of the golden-collared manakin, a bird with an acrobatic courtship display.
- Schwabl, H., Kriner, E., 1991. Territorial aggression and song of male European robins (*Erithacus rubecola*) in autumn and spring: effects of antiandrogen treatment. *Horm. Behav.* 25, 180–194.
- Shet, M.S., McPhaul, M., Fisher, C.W., Stallings, N.R., Estabrook, R.W., 1997. Metabolism of the antiandrogenic drug (flutamide) by human CYP1A2. *Drug Metab. Dispos.* 25 (11), 1298–1303.
- Siegel, S., Castellan, N.J.J., 1988. *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill, New York.
- Stein, A.C., Uy, J.A.C., 2006. Plumage brightness predicts male mating success in the lekking golden-collared manakin, *Manacus vitellinus*. *Behav. Ecol.* 17, 41–47.
- Wikelski, M., Hau, M., Wingfield, J.C., 1999. Social instability increases plasma testosterone in a year-round territorial neotropical bird. *Proc. R. Soc. Lond., B Biol. Sci.* 266 (1419), 551–556.
- Wikelski, M., Hau, M., Robinson, D.W., Wingfield, J.C., 2003. Reproductive seasonality of seven Neotropical passerine species. *Condor* 105, 683–695.
- Wiley, C.J., Goldizen, A.W., 2003. Testosterone is correlated with courtship but not aggression in the tropical buff-banded rail, *Gallirallus philippensis*. *Horm. Behav.* 43 (5), 554–560.
- Wingfield, J.C., Farner, D.S., 1993. Endocrinology of reproduction in wild species. In: Farner, D.S., King, J.R., Parkes, K.C. (Eds.), *Avian Biology*, vol. 9. Academic Press, San Diego, pp. 163–327.
- Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The challenge hypothesis-theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Am. Nat.* 136 (6), 829–846.
- Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997. Ecological constraints and the evolution of hormone-behavior interrelationships. *Integrative Neurobiology of Affiliation. Ann. N. Y. Acad. Sci.* 807, 22–41.
- Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the ‘costs’ of testosterone: ecological bases of hormone-behavior interactions. *Brain Behav. Evol.* 57 (5), 239–251.
- Wirth, M.P., Froschermaier, S.E., 1997. The antiandrogen withdrawal syndrome. *Urol. Res.* 25 (Suppl. 2), S67–S71.