SEX DIFFERENCES IN ANDROGEN ACTIVATION OF COMPLEX COURTSHIP BEHAVIOR

ABSTRACT

Sexual dimorphism is common but evolutionarily labile in vertebrates. While it is well established that gonadal hormones exert considerable influence on the development and expression of sexual traits, studies of animals with exceptional sexually dimorphic neural or neuromuscular phenotypes are limited. We studied the extent to which androgen treatment of female golden-collared manakins (*Manacus vitellinus*) activates specific elements of the elaborate, acrobatic courtship behavior characteristic of males. After one week, non-breeding females and juvenile males given implants containing testosterone (T) were observed for 3 weeks in an outdoor aviary situated in the middle of Panamanian rainforest. T-treated males performed the full suite of documented masculine courtship behaviors, whereas T-treated females performed only a few of these behaviors and then at much lower rates than males. T- treatment did increase aggressive behavior to a similar degree in both males and females. These results suggest that neuromuscular systems encoding elements of male courtship as well as aggressive behavior experience unique patterns of development from complete to limited to non-existent sexual differentiation. The basis of these patterns represents a unique opportunity for study.

Keywords: courtship; mating behavior; testosterone; hormonal activation; sexual differentiation; manakin.

24 INTRODUCTION

25

26 Males and females share most of their genome and this is expected to constrain the
27 evolution of sexually dimorphic traits (Ellegren and Parsch 2007; Cox and Calbeek
28 2009). In spite of this, sexual dimorphism is common and evolutionarily labile (Wiens
29 1999; Price et al. 2007; Odom et al. 2014). In vertebrates, two mechanisms appear to
30 mediate the development of sex-specific phenotypic traits with one linking the expression
31 of shared loci to gonadal hormones (Phoenix et al. 1959; Adkins-Regan 2012) and the
32 other stemming from expression of sex-specific loci arising from their position on sex
33 chromosomes (Arnold 2009; McCarthy 2016). Gonadal-hormone dependent behavioral
34 sexual differentiation is well established. In mammals, testosterone (T) circulates at
35 greater levels in males during early development to organize the masculine neural
36 circuitry, which is then activated in adulthood to enable male-specific behaviors to occur
37 in the presence of T. By contrast, in birds ovarian estrogens demasculinize female circuits
38 rendering them fully or partially insensitive to T such that experimentally increasing T
39 levels in adult females activates few or little if any masculine behavior (Lank et al. 1999;
40 Lahaye et al. 2012; Madison et al. 2015).

41

42 Organizational and activational effects of T are species, behavior, and tissue specific
43 (Adkins-Regan 2012; McCarthy 2016). In the case of behavior, traits may be part of
44 different ‘modules’, allowing them to evolve independently in the face of sexual selection
45 pressures and thus to differ in the extent to which they acquire steroid-sensitivity or fall
46 under sex-chromosome dependent control (West-Eberhard 2003). These levels of control

likely depend on whether these traits are expressed exclusively in a reproductive context, like copulatory behaviors, or can be expressed in non-reproductive contexts, like aggressive or singing behaviors (Kendrick and Schlinger 1996; Schlinger 1998; Adkins-Regan 2012).

Courtship behaviors can be striking composites of reproductive and non-reproductive behavioral elements, or modules (Huxley 1914; Tinbergen 1951), and thus, courtship behaviors might be organized developmentally in diverse ways involving different modes of hormone-dependence or sex-chromosomal control (Arnold 2009; Schlinger 1998; Adkins-Regan 2012). Moreover, whereas courtship behavior is usually expressed only by males and serves as a basis for mate-choice by females, in some species, both males and females participate together in such displays, especially as a mechanism for establishment of pair-bonds (Langmore 1998). Thus, the neural and muscular machinery underlying the motivation and capacity to perform courtship displays are subject to unique selection pressures that make them available for capture by hormone-independent or hormone-dependent organizational/activational mechanisms.

To understand the degree to which aggressive and courtship behaviors are expressed in adults in a sex-specific pattern, we have examined the extent to which T can activate in females several male-typical behaviors of golden-collared manakins (*Manacus vitellinus*; hereafter GCmanakin). Many species of manakins (family Pipridae) are characterized by polygynous mating systems in which males aggregate at leks where they defend individual courts and perform complex courtship displays (Prum 1990). Male

GCmanakins perform an exquisitely acrobatic courtship routine with multiple display elements (Chapman 1935). Conspicuously, males produce wingsnaps created by forcefully beating their wings together above their heads to produce loud clapping sounds. Wingsnaps are produced singly, in mid-flight, or as ‘rollsnaps’ which consist of a series of 17-25 wing-snaps produced rapidly (50-60Hz) in sequence. Acrobatic features of the courtship dance, or jump-snap display, includes males swiftly leaping between saplings when the single wing-snap is produced midflight. Males also perform a jumpsnap with a half-twist landing on the ground followed by a grunt-jump back onto a sapling. The last move involves flying vertically to a perch with the ‘grunt’ sound produced by flapping of the wings (Bostwick and Prum, 2003). Thus, manakin courtship displays utilize a variety of neuromuscular systems for use in a mating context (Fuxjager and Schlinger 2015). To date, little is known about the extent that these elements may be developmentally organized differently between the sexes or whether differences in adult androgen levels maintain sexual dimorphism in courtship behavior.

Previous studies of wild male GCmanakins show that circulating T is elevated during the breeding season (Day et al. 2006; Schlinger et al. 2013) and that exogenous T, acting through androgen-receptors (AR), activates courtship behaviors in non-breeding and juvenile males with low circulating T levels (Fuxjager et al. 2013). Interestingly, female GCmanakins have little circulating T but they express AR at levels comparable to those of males in skeletal muscle and spinal cord (Feng et al. 2010; Fuxjager et al. 2012). Thus, it is plausible that androgens could activate these neuromuscular systems to produce ‘male-like’ behavior. Females do join males in their jump-snap display before allowing

some males to copulate, but they are not known to perform wingsnaps, rollsnaps or to jump (they only fly) between saplings with or without males. In small cages, T-treated females do occasionally produce wing- and roll-snaps (Day et al. 2007). Thus, females and males may overlap in the development of at least some neuromuscular systems controlling courtship display.

In this study, we observed females and juvenile males treated with or without T, in a large tropical forest aviary that permits the full range of behaviors under highly natural conditions. We asked whether T activates the entire repertoire of courtship elements in juvenile males and whether females perform some or all of the male's behavioral repertoire.

METHODS

The work was conducted at the Smithsonian Tropical Research Institute (STRI) in Gamboa, Panama. Experiments were conducted during the non-breeding season (July to mid-Dec) in both 2014 and 2015. During this season, lek activity is considerably reduced compared to the breeding season (Schlinger et al. 2013) and circulating T levels are basal or near so in all birds (Day et al. 2006). Moreover, T-treatment of juvenile males at this time effectively activates several elements of adult courtship, such as wing-snaps and rollsnaps (Day et al. 2006, 2007). GCmanakins were captured using passive mist netting, individually marked with plastic colored leg-bands, weighed, and transferred to STRI

facilities in Gamboa, where they were housed individually in cages ~36 x 29 x 32 cm. Birds were in visual and auditory contact in cages set on a table in a room with open screened windows (allowing ventilation and natural lighting). Supplemental lighting was added and timed to coincide with the ambient sunrise and sunset. Fresh papaya with vitamin supplements and water were provided twice daily (*ad libitum*).

Ethical Note

The work was conducted under permits from local Panamanian authorities (Autoridad Nacional del Ambiente and MiAmbiente permits SE/A-4-14 and SE/A-55-15) and in accordance with animal care policies at the Smithsonian Tropical Research Institute (Animal Care and Use Committee Protocol number 2013-0315-2016) and at UCLA (Office of Animal Research Oversight, ARC Protocol number 2009-123-21), which adhere to the standards set by ASAB/ABS (2012). All birds were released at the point of capture following the end of experiments.

Sex determination

Females and juvenile males are superficially indistinguishable from one another bearing an overall olive-green coloration. Thus, we determined each bird's sex using a molecular approach that involves amplifying homologous sections of the CHD gene, which is present on both Z and W chromosomes, but which differs in length. Products appear as one or two bands respectively for males and females (Griffiths et al. 1998). After capture, ~5-20µL of blood was collected by puncture of the brachial vein and stored in 100µL Queen's lysis buffer at 4°C until analysis. DNA was extracted using the Sigma Aldrich

DNeasy Blood and Tissue kit and the concentration was adjusted to 20ng/μL. The CHD-gene was PCR-amplified (2.5μL DNA solution, 3.0 μL of 20 mM MgSO₄, 1.0 μL of 10X buffer solution, 2.9 μL water, 0.2 μL dNTPs solution, 0.2 μL of each P2 and P8 primers, 0.05 μL Taq Master Mix (Qiagen)) using the following protocol: 2 min denaturing step at 94°C followed by 35 cycles of 20 s at 72°C, 20 s at the annealing temperature, 62°C, and 30 s at 72°C. PCR products were separated by electrophoresis for 45-60 min at 60V in a 3% gel stained with GelRed (Biotium Inc.). Samples were run in duplicate together with DNA from 1-2 known sex males or 1 known male and 1 known female.

T-implant administration

Once 3-4 same sex birds were available, they were implanted at the base of the neck with silastic tubes (Dow Corning Inc., 10mm long, 0.76 mm inner diameter, and 1.65 mm outer diameter) filled with crystalline T (Steraloids Inc.) or empty as control. Details of the implantation procedure were described previously (Fuxjager et al. 2012) and show that levels of T were elevated in females on average to 2.24 ng/ml as compared levels in unimplanted females of 0.51 ng/ml. In separate studies using different implants, male-like behaviors were detected in T implanted birds 7 days post-treatment and peaked at day 13 (Day et al. 2007), so here, implanted birds were relocated to the outdoor aviary 7 days following implantation where they were observed for 21 days (17 days for one group, see below).

161

162 *Aviary setup*

163 The outdoor aviary (Appendix fig. A1) is a 10 m x 5 m x 4.5 m tall enclosure located ~2
164 km inside Soberania National Park near the town of Gamboa. It has a 0.5 m cement base
165 with chain link fence walls and ceiling covered on the inside with soft plastic mesh
166 (1cm). A sheet of corrugated steel covers ~ one-third of the enclosure, including over a
167 1m² double-door entrance area. The base was filled with soil in which we planted 45
168 small saplings (< 2 m) with straight, unbranching stems preferred by males for
169 performing courtship displays, as well as 7 trees (2-4 m tall) that the birds use to rest.
170 Fresh papaya and banana slices with liquid vitamin supplement were provided at four
171 ant-proof and rain-protected feeders. To ensure feeding on the first day of introduction,
172 additional papaya was presented on skewers throughout the aviary as well as some
173 berries (*Miconia* or *Psychotria* spp.), typical food of manakins in the wild. Fresh food
174 was provided twice a day, in the morning and at midday. A water bath was provided,
175 cleaned and refilled daily.

176

177 *Behavioral observations*

178 We observed a total of 3 groups of T-treated individuals, 2 groups of females (4 birds,
179 from Oct 9-25 2014; and 3 birds, from Nov 19 to Dec 9, 2015) and one group of juvenile
180 males (4 birds, from Nov 25 to Dec 14, 2014). Observations of the first T-treated group of
181 females was terminated after 17 days (all others 20-21 days) because the aggressiveness
182 of one female prevented the other females from accessing the feeders. During the second
183 experiment with T-treated females, one female was also more aggressive than the others,

which restricted their use of feeders, so we added 8-10 feeding locations (skewer feeders) throughout the aviary, which allowed the other females access to food in spite of increased agonistic interactions. We also observed two groups with blank implants, one group of females (4 birds, Sept 1-21, 2015) and one group of males (3 birds, Oct 8-28, 2015).

Birds were introduced to the aviary between 8:30AM and 12:00PM and observed for 1-2 hrs to ensure they fed. Afterwards, 4 hrs of behavioral observations were performed daily after morning food provision at ~6:30AM. The observer sat in the double door entrance. A tripod-mounted Sony Handycam CX220, set near the entrance, recorded each observation period, capturing activity in ~ 70% of the aviary. We documented the occurrence of previously described behaviors (Chapman 1935; Day et al. 2006, 2007; Schlinger et al. 2013): 1) rollsnaps, which consist of a series of quick wing-snaps; 2) jumpsnap displays, which consist of hopping from one sapling to another while wing-snapping midflight; we included here jumps with no wing-snapping, which may be rarely performed by males just prior to launching into a jump-snap display; 3) half-flip jumps to the ground and grunt-jump displays; and 4) arena cleaning, which involves removing leaves or other dead plant materials from the court or biting and pulling at shoots or leaves of live plants. To ensure that plant material of different types was available for removal, and because leaves can become covered in mud during the rainy season, we augmented dead leaves throughout the aviary, twice during each experiment (~ every 7 days from the experiment's onset).

Typically in lekking animals, males compete for access to females. In GCManakins this involves gaining dominance for possession of a court so courtship displays can be performed without interruption during female visits (see Foster 1981). We examined the frequency of displacements and chases (agonistic behaviors), both on and away from the court (including at feeders) as all likely contributed towards establishment of dominance. A displacement involved one bird being replaced by another on a perch (or less frequently leaving the perch in anticipation of another landing nearby) without leaving the immediate area. Chases were defined as one bird pursuing another in flight, sometimes protracted and culminating in a fight. Male GCManakins also produce snips infrequently, which are quieter wing-snaps that can be performed while perched or while individuals hop from branch to branch while chasing each other at the lek (Chapman 1935). Thus we recorded their occurrence for both sexes and treatments.

Statistical analysis

Some behaviors were performed only by males and statistics were not used for comparison with females. Females performed some recorded behaviors (rollsnaps, jump displays, snips) infrequently and due to many zero values, we were unable to transform the data to attain normality. We therefore used mixed-effects logistic regression analyses to determine whether T-treatment increased the probability of females performing male-like behaviors. Secondly, to explore the effects of T-treatment on the rate of behavioral performance (the number of occurrences observed per day), we modeled the subset of observations that were greater than zero using a generalized linear mixed-effects model. Since data were counts of observations, we used a Poisson or negative binomial error

distribution (if the data were overdispersed). For both types of analyses, we modeled each behavior separately and included sex, treatment, day in the aviary, and day x treatment interaction as factors with bird id as a random variable. For each behavior, we initially included all factors in the model and assessed the contribution of each of the variable by comparing models with and without each variable using a chi-square test. If sex or treatment were significant predictors, we then performed simplified models that included only 2 of the 4 groups (control-females, T-females, control-males, T-males) at a time and this allowed us to determine which pairs showed significant differences in daily probability of behaviors. Blank-treated females did not perform some of the behaviors of interest (rollsnaps, jump-displays, snips, or chases), therefore, models examining differences in daily rates only included 3 groups (T-females, control-males, and T-males) in these cases. All statistical analyses were conducted in R, version 3.3.0.

RESULTS

During a pilot study (March 1 – May 15, 2014), we assessed the utility of the aviary for studies of GCmanakin behavior by releasing three T-implanted juvenile males into the aviary. Within 24 hrs of introduction, two birds performed jumpsnap displays. Daily observations (15-30 min) indicated that all had established arenas and performed courtship displays most days we visited. The aviary is located within forest near (~10-20m) an established lek of wild GCmanakins. Occasionally, adult males and green-plumaged birds were attracted to the aviary stimulating captive males to call in response.

These observations gave us confidence to proceed with further investigations of courtship display and social behavior in this semi-natural setting.

Courtship display behavior

Juvenile males with blank implants initiated courtship behaviors starting on day 3 of observations, whereas males treated with T initiated display on day 1. Juvenile males in both treatments performed the full extent of the courtship display seemingly indistinguishable from that of adult males (Supplementary material video). The typical courtship routine starts with 1-2 jumps between saplings with no wing-snap midflight, followed by jump-snaps, and occasionally a half-flip jump and a grunt-jump display element. For juvenile males, jumps not accompanied by wing-snaps were infrequent (< 4% of total jumps based on 1243 jump/jump-snap displays by 3 males observed over 5 days), so we used the total number of jumps and jump-snaps in our analysis of display behavior.

When treated with T, females also showed some male-like behaviors starting on day 2 of observations whereas no courtship behaviors were observed in blank-treated females. Five of the 7 females performed rollsnaps, one performed jumps between saplings close to the ground (<1m) and another 4 performed jumps between branches >1m in trees. None of the females performed jump-snaps, or jumps/jump-snaps landing on the ground, or half-flip jumps or grunt-jump displays. Also notably, when females performed jumps, they flapped their wings midflight, thus this behavior differs from that of adult males jumping between saplings, as adult males do not flap their wings (Fusani et al. 2007).

Nevertheless, our observations (including examination of video recordings at 30 frames per second) indicate that juvenile males also flap their wings during some jumps that are not accompanied by a wing-snap. Female rollsnaps were quieter than those of males and shorter; we examined the number of snaps in a rollsnap by inspecting sonograms of audio collected during observations in the aviary (using the software Audacity 2.0.0), and for females, rollsnaps contained 3.92 ± 2.17 (S.D.) snaps (range of 2 to 10 snaps, $n = 26$ rollsnaps) compared to 7.61 ± 3.12 (S.D.) snaps (3 – 18 snaps, $n = 71$ rollsnaps) for males (Wilcoxon rank sum test $W = 1611$, $P < 0.001$).

The probability of performing courtship behaviors in both sexes was significantly impacted by T-treatment (fig. 1A, rollsnaps $\chi^2 = 9.95$, $P = 0.002$; jumps/jump-snaps: $\chi^2 = 7.91$, $P = 0.004$). Males were more likely to perform rollsnaps and jump-snap displays compared to females (rollsnaps: $\chi^2 = 17.26$, $P < 0.001$; jumps/jump-snaps: $\chi^2 = 29.86$, $P < 0.001$). Comparisons of simplified models including only 2 groups at a time (of the 4 groups: blank-females, T-females, blank-males, and T-males), indicate that all pair-wise comparisons are significant for jump-displays and rollsnaps (all $P < 0.001$). In addition, our results indicate that the probability of individuals performing rollsnaps and jump/jump-snap displays increased significantly with time in the aviary (rollsnaps: $\chi^2 = 15.81$, $P < 0.001$, $P < 0.001$; jumps/jump-snaps $\chi^2 = 26.58$, $P < 0.001$).

For the subset of days when individuals were observed to perform the behavior of interest, the number of rollsnaps and jump-displays in a day by an individual increased

297 significantly with T treatment (fig. 1B, rollsnap rate: $\chi = 3.9$, $P = 0.048$; jump-snap rate:
 298 $\chi = 5.04$, $P = 0.025$), however only the jump-display rate was significantly different
 299 between the sexes ($\chi = 20.37$, $P < 0.001$). Follow-up comparisons of simplified models
 300 including only 2 groups at a time (of the 3 groups: T-females, control-males and T-males)
 301 indicate that for jump-snap displays all groups are significantly different from each other
 302 (all $P < 0.001$), whereas for rollsnaps, control males showed no significant difference in
 303 daily rate of roll-snaps from T-treated females ($\chi = 2.35$, $P = 0.50$). T-treated males
 304 showed significant differences from T-treated females ($\chi = 21.19$, $P < 0.001$) and control
 305 males ($\chi = 25.26$, $P < 0.001$).

306 The probability of individuals performing rollsnaps and jump/jump-snap displays
 307 increased significantly with time in the aviary (rollsnaps: $\chi = 15.81$, $P < 0.001$, $P < 0.001$;
 308 jumps/jump-snaps $\chi = 26.58$, $P < 0.001$). The number of jump-displays performed in a
 309 day by an individual also showed a significant increase with time in the aviary and
 310 similarly, the number of rollsnaps also increased over time in the aviary (fig. 3 A and B,
 311 jump/jump-snaps: $\chi = 34.87$, $P < 0.001$, rollsnaps: $\chi = 15.44$, $P < 0.001$), however for
 312 rollsnaps, there was an interaction with treatment, such that blank-treated individuals
 313 showed a decrease in daily rollsnap rate over time in the aviary ($\chi = 4.53$, $P = 0.033$).

314

315

316 *Aggressive behaviors and vocalizations*

317 Males in both treatment groups established arenas where they performed most of the
 318 recorded display behavior (97% of 1344 jump-snap displays over 4 days). For females,
 319 and particularly for the one that performed jumps between saplings, no court
 320 establishment was observed and jumps were performed at different locations throughout
 321 the aviary. Nevertheless, the probability of displacements was not different between the
 322 sexes or between treatments (fig. 2A, sex: $\chi = 1.33$, $P = 0.25$, treatment: $\chi = 0.40$, $P =$
 323 0.53). However, the probability of chases increased significantly with T-treatment ($\chi =$
 324 16.17 , $P < 0.001$) and was higher for males ($\chi = 9.48$, $P = 0.0021$). Follow-up models
 325 including only 2 groups at a time indicate that all groups were significantly different from
 326 each other ($P < 0.001$).

327 The number of displacements by individuals did not differ with treatment or
 328 between the sexes (fig 2B, treatment: $\chi = 0.01$, $P = 0.94$; sex: $\chi = 0.10$, $P = 0.76$). The
 329 number of chases observed in a day was significantly higher for T-treated females than
 330 for males ($\chi = 7.49$, $P = 0.006$). Follow-up models including only 2 groups at a time (of
 331 the 3 groups: T-females, blank-males, and T-males) showed that T-females differed
 332 significantly from T-males ($\chi = 4.13$, $P = 0.042$) but not from blank-males ($\chi = 5.87$, $P =$
 333 0.053).

334 Over the course of observation in the aviary, the probability of displacements did
 335 not change ($\chi = 2.97$, $P = 0.085$), however the probability of chases increased

significantly ($\chi = 17.07$, $P < 0.001$). The number of displacements also did not change over the observation period but the number of chases showed an increase for T-females relative to both groups of males (fig. 3C and D, $\chi = 6.49$, $P = 0.011$).

The probability of snips increased with T-treatment, however it was not different between the sexes (fig 2A, treatment: $\chi^2 = 4.83$, $P = 0.028$, sex: $\chi^2 = 0.12$, $P = 0.73$). The number of snips was also not different with treatment or sex (fig. 2B) and the daily rate of snips was not significantly predicted by time in the aviary (fig. 3E).

343

344

345 **DISCUSSION**

Long-term behavioral studies of wild female golden-collared manakins are challenging because they disperse after release following capture and their dull-green plumage makes them cryptic in the forest they inhabit. Thus, a major objective of this study was to provide suitable captive conditions that would promote our ability to study female behavior and the possible expression of the male behavioral phenotype after treatment with testosterone.

In the absence of T, female GCmanakins performed no male-typical courtship displays. Following treatment with T, females still did not claim a court, clear an arena (the subject of a follow-up study), perform half-flip jumps or grunt-jump displays. Females treated with T did, however, perform several male-typical courtship elements, usually at lower rates than juvenile males. Females performed single wing-snaps while perched (a

behavior rarely observed in males), as well as rollsnaps and jumps without wingsnaps. In particular, in the absence of a male, jumps have not previously been reported for females with or without T-treatment. Thus, whereas females displayed some motivation to engage in male courtship, either they were insufficiently motivated or physically incapable of performing the full suite of male behaviors, even in the presence of elevated plasma T. Juvenile males, with and without T-treatment, performed the complete courtship display, which includes complex moves such as jump-snaps, grunt jumps, and half-flip jumps. Thus, our observations indicate that female treatment with T results in partial expression of male courtship display elements. This indicates that some neuromuscular systems supporting courtship behavior are present in females and are partially coupled to T. Nevertheless, some male-like neuromuscular tissues are, in females, either insensitive to T or are absent altogether. These results indicate that there is partial developmental sexual differentiation of the neuromuscular systems involved in display behavior. Females treated with T were as aggressive as males, suggesting that the development of neuromuscular components supporting aggression may differ from those supporting courtship and copulatory behaviors.

Courtship

An initial point of interest is that juvenile males perform male courtship during the non-breeding season when T levels are basal. While we have seen evidence for this before (Day et al. 2006), these observations document quite clearly that both central motivational and peripheral neuromuscular systems are relatively intact and functional in

379 these males. Males probably develop their adult plumage after their 2nd year, so these
380 males could have been a few months to over a year in age. Thus, it is possible that some
381 males had experienced elevated levels of T at earlier months and/or had some time to
382 practice courtship routines. Nevertheless, T-treatment significantly amplified the overall
383 production of these behaviors. In skeletal muscles involved in courtship, androgens
384 significantly upregulate genes encoding proteins involved in energy utilization (Fuxjager
385 et al. 2016), thus one main function of androgenic activation of male courtship may lie in
386 the regulation of optimal muscle metabolism required for sustained behavioral
387 performance.

388 Conspicuously, female GCmanakins naturally perform little that resembles male-typical
389 courtship, even here in the presence of T that effectively activated courtship behavior in
390 males and that activated aggressive behavior in females. In female GCmanakins, the
391 activation of male courtship behaviors seem to fall into two categories, those never
392 performed by females and those performed by females, but significantly less frequently
393 than by males. With or without T, females seem to have no motivation to establish a court
394 and keep it cleared of debris. Presumably, this failure to claim a court represents a fully
395 sexually-dimorphic motivational system, one present in males, but not in females.
396 Females also never perform the half-flip to the ground and grunt-jump back to a perch.
397 When a female has joined a male in his courtship dance, this latter behavior is often
398 performed by the male just prior to copulation, so its performance by males may be a
399 component of copulatory behavior per se, and thus subject to gonadal hormone-
400 dependent organizational mechanisms as are sex-specific copulatory behavior in other

401 species (Balthazart et al. 1996; Adkins-Regan 2012). In GCManakins it has become one
402 element in the male's courtship display, performed with or without a female partner.

403 We cannot dismiss the idea that T-treated females might have displayed male copulatory
404 behaviors had they been housed together with non T-treated, and thus possibly receptive,
405 females. Some females did, however, clearly dominate others, and they might have
406 attempted to court and copulate with these subordinate females. Over many hours of
407 observations, our data suggest that copulatory behavior, as in adults of other species, is
408 fully sexually dimorphic (Adkins-Regan 2012) unlike perched snaps and rollsnaps that
409 can be activated by T in females.

410 Although performed less frequently, T-treated females did perform both rollsnaps (5 of 7
411 females) and one performed jumps between saplings, though in no single location as is
412 done by males. It appears that females are unable to perform the midflight single
413 wingsnap, unless perhaps considerable practice is involved requiring longer-term T-
414 treatment. Some females did produce rollsnaps, a complex behavior. Presumably,
415 females possess the full androgen-dependent neuromuscular system (and forelimb
416 anatomy, Bodony et al. 2016) to achieve this high degree of forelimb neuromuscular
417 speed and accuracy required to produce these behaviors, even when they seldom, if ever,
418 naturally perform this behavior.

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421

422 *Aggressive behaviors*

423 Females were aggressive, displacing other females throughout the aviary as often as did
424 males. With T-treatment, females showed an increase in the level of aggressive behavior,
425 specifically chases, such that the number per day was higher than that displayed by
426 males. T-treated females also produced snips, which in males are observed in male-male
427 interactions near the court (Chapman 1935). However, no females showed behavior that
428 resembled establishment and defense of a specific court suggesting female aggression
429 was not driven by the motivation to establish a court. In females, snips might function
430 under circumstances unrelated to mate competition, such as part of a distraction display
431 when defending a nest (see Bodony et al. 2016). Observations of female behavior in the
432 wild are limited, however they do not appear normally aggressive, so our results were a
433 surprise. The data suggest that female manakin aggressiveness requires additional
434 investigation. The data do point to a specific T-dependent activation of aggression in
435 females, suggesting that the underlying neural circuits involved in aggressive motivation
436 and/or motor output are sensitive to this hormone in female GCmanakins, as they are in
437 males. Thus, developmental mechanisms organizing neural circuits controlling
438 aggression differ from those underlying most courtship display and copulatory-related
439 behavioral elements.

440

441 *General conclusions and discussion*

442 The many notable elements of male GCmanakin social behavior, their creation,
443 maintenance and territoriality around a courtship arena, the performance of their
444 elaborate, acrobatic courtship display and their production of acoustic signals using both
445 syringeal-based (vocal) and forelimb-based (wingsnap) mechanisms, appear to express
446 varying degrees of sexual dimorphism in response to T-activation (Table 1). Previous
447 studies show that both males and females possess a radius morphology unique among
448 birds, and that appears to be the source of the wingsnap sound (Bodony et al. 2016). In
449 addition, male and females both possess forelimb muscles that exhibit elevated AR
450 expression (Feng et al. 2010) upon which T acts to facilitate the production of wingsnaps
451 (Fuxjager et al. 2013). Thus, these birds have evolved the peripheral neuromuscular
452 capacity for hormone-dependent control of wingsnap production. The fact that the
453 probability of wingsnap and rollsnap production differs across the sexes, likely stems
454 from differences in central motivation centers and the degree to which androgens activate
455 those circuits. It is possible that wingsnaps evolved originally for non-reproductive
456 purposes (such as for contact maintenance between individuals or for predator
457 deterrence) and were later incorporated into the male's courtship display, an evolutionary
458 perspective on courtship applied previously (Tinbergen 1951).

459 In contrast to these behaviors, females appear incapable of expressing some male-typical
460 behaviors either because central motivational circuits are lacking or are T insensitive.
461 These latter behaviors show substantial overlap with those behavioral elements that
462 appear to provide unique information to females about male quality (Fusani et al. 2007;

463 Barske et al. 2011) and thus developed exclusively in males by independent mate choice
464 pressures.

465 Neuroanatomical studies reveal characteristics that may underlie some of these
466 behavioral phenotypes. Males exhibit greater aromatase activity in the hypothalamus
467 pre-optic area than do females (Saldanha et al. 2000), a pattern consistent with other
468 species where estrogens promote masculine copulatory behaviors (Balthazart et al. 1996).
469 Whereas we have found a prominent role for androgens, and not estrogens, in the
470 activation of GCmanakin male courtship (Schlinger et al. 2013), we cannot dismiss the
471 possibility that estrogens promote some male behavioral elements. The arcopallium is
472 larger in males than females and this is a likely site for pre-motor control of some display
473 elements (Day et al. 2011). Additional work is required to establish just how different
474 brain regions participate in controlling the discrete elements of courtship. Moreover,
475 hormonal manipulations of GCmanakin embryos are needed to elucidate how sex
476 differences, where they exist, are produced and lead to the complex sexually dimorphic
477 and hormone-dependent behavioral phenotype we observe.

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Figure 1

Figure 1. A) The percentage of days (mean $\pm$ S.E.) for which courtship behavior was observed (presence or absence) by sex and treatment. T-treatment included 7 females (total of 130 observation days) and 4 juvenile males (80 observation days). Control-treatment included 4 females (84 observation days) and 3 juvenile males (63 observation days). B) Box plots of daily rate of courtship behavior by sex and treatment. Only days when a behavior was performed are included (non-zero observations), and sample sizes are shown at the bottom (number of females/total days when behavior was observed). Data points representing individual rate are superimposed on the plot.

Figure 2

Figure 2

Figure 3