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Sex differences in androgen activation of complex courtship behaviour

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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 behaviour characteristic of males. After 1 week, nonbreeding 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 behaviours, whereas T-treated females performed only a few of these behaviours and then at much lower rates than males. T treatment did increase aggressive behaviour to a similar degree in both males and females. These results suggest that neuromuscular systems encoding elements of male courtship as well as aggressive behaviour experience unique patterns of development from complete to limited to nonexistent sexual differentiation. The basis of these patterns represents a unique opportunity for study.

Males and females share most of their genome and this is expected to constrain the evolution of sexually dimorphic traits (Cox & Calsbeek, 2009; Ellegren & Parsch, 2007). In spite of this, sexual dimorphism is common and evolutionarily labile (Odom, Hall, Riebel, Omland, & Langmore, 2014; Price, Friedman, & Omland, 2007; Wiens, 1999). In vertebrates, two mechanisms appear to mediate the development of sex-specific phenotypic traits with one linking the expression of shared loci to gonadal hormones (Adkins-Regan, 2012; Phoenix, Goy, Gerall, & Young, 1959) and the other stemming from expression of sex-specific loci arising from their position on sex chromosomes (Arnold, 2009; McCarthy, 2016). Gonadal hormone-dependent behavioural sexual differentiation is well established. In mammals, testosterone (T) circulates at greater levels in males during early development to organize the masculine neural circuitry, which is then activated in adulthood to enable male-specific behaviours to occur in the presence of T. By contrast, in birds, ovarian oestrogens demasculinize female circuits, rendering them fully or partially insensitive to T such that experimentally increasing T levels in adult females activates few or little, if any, masculine behaviour (Lahaye, Eens, Darras, & Pinxten, 2012; Lank, Coupe, & Wynne-Edwards, 1999; Madison, Rouse, Balthazart, & Ball, 2015).

Organizational and activational effects of T are species, behaviour and tissue specific (Adkins-Regan, 2012; McCarthy, 2016). In the case of behaviour, traits may be part of different 'modules', allowing them to evolve independently in the face of sexual selection pressures and thus to differ in the extent to which they acquire steroid sensitivity or fall 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 behaviours, or can be expressed in nonreproductive contexts, like aggressive or singing behaviours (Adkins-Regan, 2012; Kendrick & Schlinger, 1996; Schlinger, 1998).

Courtship behaviours can be striking composites of reproductive and nonreproductive behavioural elements, or modules (Huxley, 1914; Tinbergen, 1951), and thus, courtship behaviours might be organized developmentally in diverse ways involving different modes of hormone dependence or sex chromosomal control (Adkins-Regan, 2012; Arnold, 2009; Schlinger, 1998). Moreover, whereas courtship behaviour is usually expressed only by males and serves as a basis for mate choice by females, in some

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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 behaviours are expressed in adults in a sex-specific pattern, we have examined the extent to which T can activate in females several male-typical behaviours of golden-collared manakins, *Manacus vitellinus*. 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 golden-collared manakins perform an exquisitely acrobatic courtship routine with multiple display elements (Chapman, 1935). Conspicuously, males produce 'wing snaps' by forcefully beating their wings together above their heads to produce loud clapping sounds. Wing snaps are produced singly, in midflight, or as 'roll snaps', which consist of a series of 17–25 wing snaps produced rapidly (50–60 Hz) 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 jump snap 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 & Prum, 2003). Thus, manakin courtship displays utilize a variety of neuromuscular systems for use in a mating context (Fuxjager & Schlinger, 2015). To date, little is known about the extent to which these elements may be developmentally organized differently between the sexes or whether differences in adult androgen levels maintain sexual dimorphism in courtship behaviour.

Previous studies of wild male golden-collared manakins show that circulating T is elevated during the breeding season (Day, McBroom, & Schlinger, 2006; Schlinger, Barske, Day, Fusani, & Fuxjager, 2013) and that exogenous T, acting through androgen receptors, activates courtship behaviours in nonbreeding and juvenile males with low circulating T levels (Fuxjager, Longpre, Chew, Fusani, & Schlinger, 2013). Interestingly, female golden-collared manakins have little circulating T, but they express AR at levels comparable to those of males in skeletal muscle and spinal cord (Feng, Katz, Day, Barske, & Schlinger, 2010; Fuxjager et al., 2012). Thus, it is plausible that androgens could activate these neuromuscular systems to produce 'male-like' behaviour. Females do join males in their jump-snap display before allowing some males to copulate, but they are not known to perform wing snaps, roll snaps or to jump (they only fly) between saplings with or without males. In small cages, T-treated females do occasionally produce wing snaps 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 behaviours 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 behavioural repertoire.

METHODS

The work was conducted at the Smithsonian Tropical Research Institute (STRI) in Gamboa, Panama. Experiments were conducted during the nonbreeding season (July to mid-December) 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 roll snaps (Day et al., 2006, 2007). Golden-collared manakins were captured using passive mist netting, individually marked with plastic coloured leg bands, weighed and transferred to STRI facilities in Gamboa, where they were housed individually in cages ~36 × 29 × 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 the University of California Los Angeles (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, Double, Orr, & Dawson, 1998). After capture, ~5–20 μl of blood was collected by puncture of the brachial vein and stored in 100 μl of Queen's lysis buffer at 4 °C until analysis. DNA was extracted using the Sigma Aldrich DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, U.S.A.), and the concentration was adjusted to 20 ng/μl. The *CHD* gene was PCR amplified (2.5 μl of DNA solution, 3.0 μl of 20 mM $MgSO_4$, 1.0 μl of 10× buffer solution, 2.9 μl of water, 0.2 μl of dNTPs solution, 0.2 μl each of P2 and P8 primers, 0.05 μl of 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 60 V in a 3% gel stained with GelRed (Biotium, Inc., Hayward, CA, U.S.A.). Samples were run in duplicate together with DNA from one to two known males, or one known male and one known female.

T-implant Administration

Once three to four same-sex birds were available, they were implanted at the base of the neck with Silastic tubes (Dow Corning, Midland, MI, U.S.A., 10 mm long, 0.76 mm inner diameter and 1.65 mm outer diameter) filled with crystalline T (Steraloids Inc., Newport, RI, U.S.A.) or left empty as controls. Details of the implantation procedure have been described previously (Fuxjager et al., 2012) and showed that levels of T were elevated in females, on average, about 2.24 ng/ml as compared to levels in non-implanted females of 0.51 ng/ml. In separate studies using different implants, male-like behaviours were detected in T-implanted birds 7 days post-treatment and peaked at day 13 (Day et al., 2007), so