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RESEARCH ARTICLE

Peripheral androgen action helps modulate vocal production in a suboscine passerine

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ABSTRACT
Androgenic activation of intracellular androgen receptors (AR) influences avian vocal production, though this has largely been investigated at the level of the brain. We investigated the influence of predominantly peripheral AR on vocal output in wild Golden-collared Manakins (*Manacus vitellinus*). In this suboscine species, males court females by performing acrobatic displays and by producing relatively simple *chee-poo* vocalizations. To assess whether peripheral AR influences the acoustic structure of these vocal signals, we treated reproductively active adult males with the peripherally selective antiandrogen bicalutamide and then measured phonation performance. Inhibiting AR outside of the central nervous system increased the duration of the *chee* note and decreased the fundamental frequency of the *poo* note. This treatment caused no discernable change to *chee-poo* frequency modulation or entropy. Our results show that activation of peripheral AR mediates note-specific changes to temporal and pitch characteristics of the Golden-collared Manakin's main sexual call. Thus, our study provides one of the first demonstrations that androgenic action originating outside of the brain and likely on musculoskeletal targets can modulate avian vocal production.

Keywords: androgen receptors, birdsong, Golden-collared Manakin, musculoskeletal system, peripheral nervous system, skeletal muscle, social behavior, song control system, syrinx, testosterone

La acción de andrógenos periféricos ayuda a modular la producción vocal en un ave paserina suboscina

RESUMEN
La activación androgénica de los receptores de andrógenos (RA) intracelulares influye en la producción de vocalizaciones en las aves, aunque muchos de los trabajos que han investigado este asunto lo han hecho a nivel cerebral. Investigamos la influencia de los RA, predominantemente en la periferia, sobre la producción vocal en *Manacus vitellinus*. En esta especie de ave suboscina, los machos cortejan a las hembras haciendo despliegues acrobáticos y produciendo vocalizaciones relativamente simples (*chii-pu*). Para determinar si los RA periféricos influyen en la estructura acústica de estas señales vocales, tratamos machos adultos reproductivamente activos con bicalutamida, un antiandrógeno periféricamente selectivo, y luego medimos el desempeño de fonación. La inhibición de los RA por fuera del sistema nervioso central incrementó la duración de la nota *chii* y disminuyó la frecuencia fundamental de la nota *pu*. Este tratamiento no causó cambios discernibles en la modulación de frecuencias del llamado ni en su entropía. Estos datos muestran que la activación de los RA periféricos produce cambios específicos de cada nota en las características temporales y tonales del principal llamado sexual de *M. vitellinus*. Por lo tanto, nuestro estudio provee una de las primeras demostraciones de que la acción androgénica que se origina por fuera del cerebro, probablemente en blancos músculo-esqueléticos, puede modular la producción vocal en las aves.

Palabras clave: canto de aves, comportamiento social, *Manacus vitellinus*, músculo esquelético, receptores de andrógenos, siringe, sistema músculo-esquelético, sistema nervioso periférico, sistemas de control del canto, testosterona

INTRODUCTION

Androgenic hormones act via intracellular androgen receptors (AR) to influence vertebrate social behavior (Adkins-Regan 2005), and avian vocal production is a prime example of this trait. In some species, vocalizations are acoustically complex and require exquisite coordination between (1) central systems that govern sensorimotor

and motor programming and (2) peripheral systems that govern the generation of sound (Schlinger 1997). In passerine birds, for example, males sing and/or call to attract mates (Catchpole and Slater 2008), and research has shown that androgens influence such behavior by changing the number of times that individuals sing and/or call (Silverin 1980, Nowicki and Ball 1989, Ketterson et al. 1992, P. G. McDonald et al. 2001, Kurvers et al. 2008), as well as the acoustic structure or makeup of these songs and/or calls (Deviche and Schumacher 1982, Groothuis and Meeuwissen 1992, Fusani et al. 1994, Galeotti et al. 1997, Cynx et al. 2005, Apfelbeck et al. 2012). However, in general, we know relatively little about where and how androgens act within the body to mediate acoustic parameters of bird songs and calls.

Most studies that have examined how androgens influence avian vocal production have focused at the level of the brain. The midbrain nucleus intercollicularis (nICO), for example, is an androgen-sensitive premotor region that regulates the calls of many species (Brown 1965, Cohen 1981, Cohen and Cheng 1982, Panzica et al. 1991). Additionally, in oscine songbirds, a higher-level song-control system underlies the learning and production of complex songs (see Jarvis et al. 2005), and androgenic hormones modulate this system in a way that presumably influences when and how songs are produced (Nottebohm 1980, Tramontin et al. 2003, Sartor et al. 2005). Despite this focus on the brain, it is also possible that androgens signal via AR in peripheral parts of the body. Musculo-skeletal systems, for instance, can express abundant AR (Michel and Baulieu 1980, Brantley et al. 1993, Regnier and Herrera 1993, Bland 2000, Kawano et al. 2003, Monks et al. 2004, Feng et al. 2010, Wyce et al. 2010), and some of the tissues that compose these systems are essential for avian vocal production. The avian vocal organ, the syrinx, is a prime example: It not only expresses AR (Wade and Buhlman 2000, Veney and Wade 2004) but also influences acoustic parameters of vocal output, such as fundamental frequency (F_0), frequency modulation (FM) and entropy (Goller and Suthers 1996, Riede et al. 2006, Elemans et al. 2008, Secora et al. 2012). Thus, in principle, this means that androgens have the capacity to act not only on the brain, but also on the musculature and cartilaginous structures that influence vocal filtering and production (Deviche and Schumacher 1982, Fusani et al. 1994). To date, such effects have been studied infrequently.

Here, we examine how activation of AR mainly outside of the central nervous system (CNS) influences acoustic production in the Golden-collared Manakin (*Manacus vitellinus*). This suboscine passerine species inhabits Panamanian rainforests. Males regularly perform elaborate courtship displays that involve mechanical "wing-snaps" and rapid dancing routines over the forest floor (Schlinger et al. 2013). As part of this sexual repertoire, males also
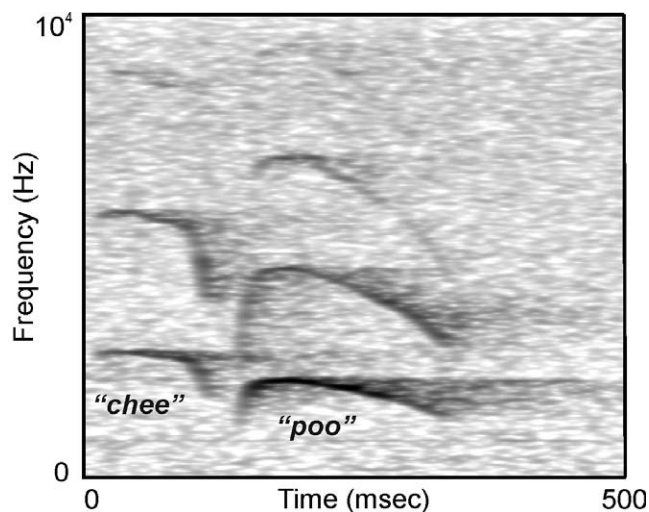
FIGURE 1. Narrow-band spectrograph of a *chee-poo* call from a reproductively active adult male Golden-collared Manakin.

broadcast simple *chee-poo* calls (Figure 1), although such vocalizations are produced independently of physical maneuvering. Females use the *chee-poo* in choosing mates, which suggests that these calls are adaptive and that their underlying mechanisms are influenced by sexual selection (Barske et al. 2011). Thus, like many other manakin species (Durães et al. 2011), Golden-collared Manakins utilize vocalizations as an important component of their reproductive and territorial behavior.

To inhibit AR primarily in the periphery, we treated reproductively active adult male Golden-collared Mana-kins with the antiandrogen bicalutamide (BICAL). This pharmacological agent blocks AR peripherally without affecting AR centrally (Freeman et al. 1989, Furr 1989). We have verified that BICAL acts in a peripherally selective manner in the study species, in that it significantly disrupts the expression profiles of known androgen-dependent genes peripherally but does not significantly affect expression of androgen-dependent genes in the brain (Fuxjager et al. 2013). Additionally, we have shown that, within days of treatment, BICAL decreases the rates at which males perform wing-snaps and courtship dances; BICAL administration does not, however, significantly influence the rate at which males produce *chee-poos* (Fuxjager et al. 2013). Those results suggest that inhibition of peripheral AR changes the physicality of male courtship behavior. Given that the syrinx (the avian vocal organ) of the Golden-collared Manakin expresses large amounts of AR compared with other passerine species (Feng et al. 2010), we asked, in the present study, whether blocking AR in peripheral tissues that contribute to phonation, such as the syrinx, over the same period similarly disrupts acoustic production.

To address this question, we assessed the acoustic features of the *chee-poos* recorded from those wild males treated with BICAL or control implants (from Fuxjager et al. 2013). We specifically focused on measurements of *chee-poo* pitch (F_0), degree of sound change over time (FM), and tonal purity (entropy), because these characteristics can be affected by peripheral sound-generating structures (Goller and Suthers 1996, Riede et al. 2006, Elemans et al. 2008, Secora et al. 2012). We also measured the duration of the notes within the *chee-poo*.

METHODS

Experimental Design

We studied reproductively active adult male Golden-collared Manakins during the height of the breeding season (February–April) at the Smithsonian Tropical Research Institute in Gamboa, Panama. Birds were captured via passive mist netting and then weighed, uniquely leg-banded for future identification, and randomly assigned to 1 of 2 treatment groups. In the first group, males ($n = 6$) received a time-release implant that emitted 0.25 mg day^{-1} of the peripherally selective antiandrogen BICAL for 21 days (Innovative Research of America, Florida, USA; dose = 12.5 mg kg^{-1} day^{-1}). In the second group, males ($n = 6$) received a control implant that was identical in every way but emitted no BICAL. Implants measured 1.6 × 5 mm (height × diameter) and were placed subcutaneously on the bird's back at the base of its neck. Implantation procedures are described in detail elsewhere (Fusani et al. 2007, Fuxjager et al. 2013). Notably, implantation is quick (~2 min) and does not complicate the birds' health or activity levels (Fuxjager et al. 2013).

Birds came from a total of 7 leks, with at least 2–10 birds lek^{-1}. In 5 of these leks that contained ≥4 displaying males, we used 2 birds lek^{-1} (each of these birds was assigned to a different treatment group). In 2 of these 7 leks that contained ≤3 displaying males, we used only 1 bird lek^{-1}. In one instance, this bird was assigned to the BICAL group, and in the other instance this bird was assigned to the control group. Ultimately, we obtained data from 4 males group^{-1}, given that some males ($n = 2$ group^{-1}) did not *chee-poo* during the tape-recorded observational session (see below).

Bicalutamide

In vertebrates, BICAL acts as a potent antiandrogen that blocks AR exclusively outside of the CNS (Freeman et al. 1989, Furr 1989, Furr and Tucker 1996). For example, Freeman et al. (1989) injected animals with radio-labeled BICAL and found significant accumulation of radioactivity in all of the peripheral organs examined, but not within the brain. Moreover, treatment with modest amounts of BICAL (sufficient to block peripheral AR) had no effect on the androgen-dependent mammalian hypothalamic–pituitary–gonadal axis (Freeman et al. 1989, Furr 1989). As noted above, we had previously validated the efficacy of BICAL in the study species by examining central and peripheral androgen-dependent gene expression: The BICAL-treated birds appeared to be healthy and displayed the same overall activity and locomotor abilities as nontreated birds (Fuxjager et al. 2013).

After implantation, males were immediately released onto the lek from which they were captured. Each bird returned to its respective display arena, and some individuals were witnessed displaying within minutes of implantation and release.

Chee-poo Recordings

Each bird was observed for a 10-day period after implantation. We selected this time frame because past work had shown that BICAL inhibited display behavior beginning on the first day after treatment and through the following 10 days thereafter (these data, including the frequency of *chee-poo* production, are provided in Fuxjager et al. 2013). Each observation session lasted 30 min, occurring between 0700 and 0900 hours and between 1200 and 1630 hours, when the birds' activity levels were highest (Stein and Uy 2006, Fusani et al. 2007). Observers sat ~10 m from the display arena and provided birds with a 15-min habituation period before collecting data. In a randomly selected subset of observation sessions over the 10-day period, *chee-poos* were tape-recorded using a Sennheiser microphone (K6 series, model ME66) and a Sony TC-D5M Professional tape recorder (sample rate = 48 KHz; 16-bit dynamic range). Sound files were digitized from the recorder using Audacity Audio Editor. Multiple *chee-poos* from each individual were recorded (range: 3–6), and only *chee-poos* that were definitively determined to be from the focal animal were used for analysis.

We used Praat Phonetics software to generate spectrograms of each *chee-poo* (window length = 5 msec; dynamic range = 70 dB), and from these we measured both the duration and the F_0 of each note. Duration was determined by selecting the beginning and end of each note with the cursor; we carefully avoided inclusion of the echo at each note's end. The F_0 for each note was computed by averaging F_0 measurements determined (via Praat) at 10-msec intervals along each note's base (fundamental) frequency band. Finally, we used the free, open-code software Sound Analysis Pro to calculate the FM and Weiner entropy of each note.

Statistical Analysis

For each acoustic variable, we used a separate general linear mixed model to examine the effects of both BICAL treatment and note (*chee* vs. *poo*). As such, BICAL

TABLE 1. Sound characteristics of the Golden-collared Manakin's *chee-poo* vocalization and linear mixed-model results for the effects of bicalutamide (BICAL) treatment and note (*chee* vs. *poo*). Statistical results in bold denote significant effects ($P < 0.05$) of each model.

Metric	*Chee* note (mean ± SE)		*Poo* note (mean ± SE)		Statistics		
	Control	BICAL	Control	BICAL	Treatment	Note	Interaction
Duration (msec)	91.1 ± 6.6	110.5 ± 5.8	176.5 ± 5.4	175.6 ± 6.0	$F_{1,\,5.87} = 0.24$	$\mathbf{F_{1,\,55.91} = 206.95}$	$\mathbf{F_{1,\,55.91} = 4.04}$
F_0 (Hz)	2,913.1 ± 8.6	2,909.9 ± 7.7	2,207.1 ± 4.6	2,092.4 ± 4.7	$F_{1,\,6.50} = 1.85$	$\mathbf{F_{1,\,241.55} = 896.65}$	$\mathbf{F_{1,\,241.55} = 6.07}$
FM (arbitrary units)	25.3 ± 1.7	22.9 ± 1.6	27.3 ± 1.2	24.81 ± 1.38	$F_{1,\,4.32} = 2.50$	$F_{1,\,48.72} = 2.19$	$F_{1,\,48.72} = 0.001$
Entropy (arbitrary units)	−1.95 ± 0.18	−1.90 ± 0.12	−2.6 ± 0.3	−3.00 ± 0.13	$F_{1,\,5.81} = 0.49$	$\mathbf{F_{1,\,48.83} = 102.85}$	$F_{1,\,48.83} = 3.78$

treatment and note were included in each model as a fixed factor, whereas bird identity was also included as a random factor. Significant interactions were followed by a calculation of the percent change between control and BICAL treatment for each note. We were unable to collect large numbers of *chee-poos* from each focal individual across the entire 10-day observation period, so we were unable to include treatment time in our model as a fixed factor.

RESULTS

The *chee-poo* is a 2-note call in which a *chee* note always precedes a *poo* note (Figure 1). On average, the *chee* note by itself is shorter in duration than the *poo* note. The *chee* note also has a higher F_0 and greater entropy than the *poo* note. There is, however, no discernable difference in FM between these 2 separate notes (Table 1).

Although BICAL treatment does not induce a singular main effect on any of the measured acoustic parameters (Table 1), this antiandrogen does exert note-specific effects on specific acoustic characteristics. Namely, both the duration and F_0 of the *chee-poo* are influenced by a significant BICAL × note interaction (Table 1). With respect to call duration, this effect is driven by a BICAL-induced increase in the duration of the *chee* by ∼21% (∼20 msec), with virtually no effect on the duration of the *poo* (Figure 2A). The magnitude of this note-specific change in duration is greater than the likely error in measuring the onset and offset of sound in relatively low signal-to-noise ratios, which in our experience is ∼3 msec. Next, for call F_0, BICAL has a negligible effect on the chee but decreases the F_0 of the *poo* by >5% (∼115 Hz; Figure 2B). Neither FM nor entropy is affected by a BICAL × note interaction (Table 1).

DISCUSSION

We examined how AR influences the structure and sound features of the Golden-collared Manakin's adaptive *chee-poo* call. We found that by using BICAL to block AR in a peripherally selective manner, we changed the call's temporal and pitch characteristics. These results suggest that activation of AR populations outside of the brain and spinal cord has the ability to mediate acoustic properties of a vocal signal in this species. Our results also show that BICAL treatment does not affect other sound elements of the *chee-poo*, such as the degree of frequency change over time (FM) and/or tonal purity (entropy). Effects on entropy may, in theory, be difficult to discern, because our recordings were obtained from animals in nature, a setting which is relatively more "noisy" than a controlled laboratory environment. However, metrics such as FM should be easily detected from wild birds in field settings; the absence of any effect of BICAL on this vocal parameter therefore points to the generally selective influence of BICAL on vocal output. In other words, the blockade of AR outside of the CNS does not induce a dramatic change in the *chee-poo* itself, but instead subtly changes the way in which certain components of vocal signals are produced. Our results provide compelling evidence that peripheral activation of AR plays a role in guiding avian phonation more-or-less independently of central activation of AR.

BICAL does not affect acoustic output by suppressing an individual's health or by altering its social motivation, the latter of which is likely driven by central actions of androgen (Fusani et al. 2007). Previous studies of these birds show that BICAL treatment does not change the rates at which males broadcast *chee-poos* around their lek (Fuxjager et al. 2013) and has no effect on an individual's activity, feeding behavior, and general social arousal (Fuxjager et al. 2013). Thus, BICAL treatment appears to drive the observed effects on sound characteristics of the *chee-poo* by inhibiting androgenic activity via AR on peripheral substrates that are either directly or indirectly related to sound generation.

Peripheral Androgens and Vocal Control
Birds given BICAL not only increase the duration of *chee* notes by ∼20 msec (21%), but also reduce the F_0 of *poo* notes by ∼115 Hz. These findings are consistent with other work that similarly shows androgen-dependent changes in the acoustic "content" of vocal production. In male Black Redstarts (*Phoenicurus ochruros*), for instance, inhibition
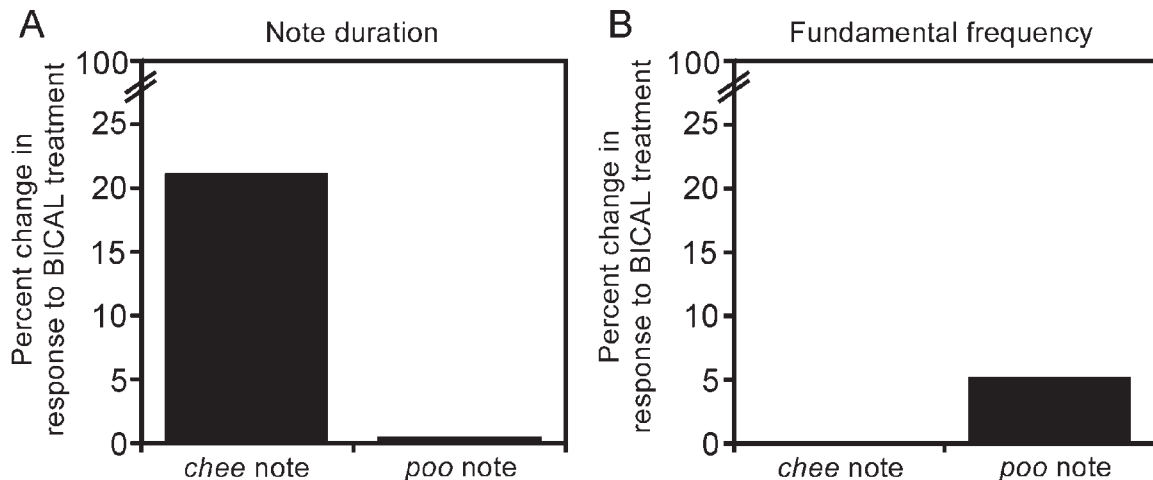
FIGURE 2. Note-specific changes in acoustic parameters of the *chee-poo* after bicalutamide (BICAL) treatment. (**A**) Percent change in the length, or duration, of each note. (**B**) Percent change in the fundamental frequency (F_0) of each note.

of androgenic and estrogenic action induced shifts of ∼300 Hz in frequency parameters of aggressive song (Apfelbeck et al. 2012). Likewise, in male Zebra Finches (*Taeniopygia guttata*), long-term testosterone implantation caused a ∼100 Hz decrease in the F_0 of directed sexual song (Cynx et al. 2005). Given that the effects reported in these past studies generally mirror, in magnitude, those reported here, it is tempting to conclude that the peripheral AR influences song production in numerous avian species.

Equally interesting is that the effects of BICAL on acoustic output occur within days of treatment, which is consistent with BICAL's impact on physical display behavior (Fuxjager et al. 2013). Nonetheless, this result stands in contrast to work by Cynx et al. (2005), which showed that some effects of testosterone on acoustic output require a month to emerge. This difference may result from interspecific variation in AR expression in peripheral sound-producing tissues such as the syrinx, given that Golden-collared Manakins express more AR mRNA in this organ than Zebra Finches (Feng et al. 2010). Of course, we cannot rule out the possibility that effects of BICAL on manakin vocal production differ in other ways in response to longer-term AR blockade.

These data raise two important questions: (1) Where do androgens act in the periphery to influence vocal duration and pitch? and (2) What do androgens do to these tissues to effectuate such changes? Musculoskeletal systems that modify sound production include the syrinx, expiratory and intercostal muscles, and upper vocal tract (Wild et al. 1998, Suthers et al. 2002, Goller and Riede 2013); thus, these tissues may be substrates on which peripheral AR influences vocal production. We suspect that the syrinx is the prime organ through which this occurs, because it sits at the tracheo–bronchial junction and controls intertra-

cheal sound-generating labia (Goller and Riede 2013). The muscles and labia of the syrinx contain AR, which makes these tissues susceptible to functional and/or morphological changes in response to androgenic action (Veney and Wade 2004, Feng et al. 2010). Blocking syringeal AR may therefore alter (1) the ability of the organ's musculature to appropriately control labial movement during expiration and/or (2) the structural constitution of the extracellular matrix and epithelium that make up and determine the labia's oscillatory (i.e. sound-generating) properties. Both of these tissues respond to steroid hormones, including androgens (Luine et al. 1983, Abitbol et al. 1999, Wade and Buhlman 2000, Chan et al. 2007).

These results do not exclude the possibility that activation of peripheral AR modulates central systems that regulate manakin vocal production. Stimulation of AR in skeletal muscle can influence the morphology of innervating motor neurons (Rand and Breedlove 1995), such that inhibition of peripheral AR may change the properties of the retrograde signaling that underlies muscle–CNS feedback. These effects may influence how the *chee-poo* is produced and may even explain the change in call duration, given evidence that the brain controls this acoustic variable (Long and Fee 2008).

Functional Significance

In Golden-collared Manakins, circulating testosterone is elevated at the onset of the breeding season and activates display behavior (Schlinger et al. 2013). We suspect that elevated androgen levels act to fine-tune acoustic performance. As a consequence, BICAL treatment likely induces a peripherally specific "nonreproductive" state by blocking AR exclusively outside of the brain (Day et al. 2007, Schlinger et al. 2013). The shifts in acoustic parameters

that we document here appear to deviate significantly from the apparent natural variation that otherwise exists in control birds, even though the magnitude of these changes in and of themselves is relatively small. Birds listening to the calls of treated males are therefore likely to perceive such differences in acoustic content that we document (Nelson 1988), and this may explain why information from the *chee-poo* is supposedly used both by females in choosing mates (Barske et al. 2011) and by males in competing with one another (D. B. McDonald et al. 2001).

It is more difficult to assess how acoustic content affects *chee-poo* function, because we know so little about how note duration, F_0, FM, and entropy are related to female choice and/or male–male interactions. Most studies that have attempted to manipulate these acoustic parameters did so in a way that simultaneously altered other factors intrinsic to male quality (McDonald 1989, Alatalo et al. 1990). Work on Zebra Finches has avoided this limitation and shown that gross manipulations of vocal production dramatically shape courtship success (Tomaszycki and Adkins-Regan 2005). In the case of the *chee-poo*, we expect that call duration and F_0 similarly contain salient information that is relevant to social interactions, including the solicitation of female copulations. In particular, these features of the call may be honest indicators of male quality, given that they are guided by androgenic action, which is considered "costly" (Ketterson et al. 1992).

Phylogenetic Considerations

Our results highlight that androgens are capable of modulating the song of a suboscine passerine. Most work investigating the effects of sex steroids on vocal performance have utilized oscine passerine birds (Barker et al. 2004). One of the main functional characteristics that distinguish these suborders is the inability of suboscine birds, including Golden-collared Manakins (Saldanha et al. 2000), to learn songs during development and the lack of any defined song-control system in the brain (Kroodsma and Konishi 1991). In suboscines, it is likely that androgens act primarily on the midbrain nICO to drive the motor programming of call production (Cohen 1981, Cohen and Cheng 1982). It is also possible that suboscine birds rely on androgenic mediation of peripheral substrates as a means of sound control. Future work should more closely consider the contributions of peripheral and central androgenic action on avian vocal production, particularly in the suboscine avian suborder.

ACKNOWLEDGMENTS

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