Androgens modulate song effort and aggression in Neotropical singing mice

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ABSTRACT

Androgens are an important class of steroid hormones involved in modulating the expression and evolution of male secondary sex characters. Vocalizations used in the context of aggression and mate attraction are among the most elaborate and diverse androgen-dependent animal displays as reflected in a rich tradition of studies on bird song and anuran calls. Male Alston's singing mice (Scotinomys teguina) commonly emit trilled songs that appear to function in male–male aggression. In this study, we experimentally manipulated androgens in singing mice to assess their role in modulating aggression and song effort. Testosterone- and DHT-treated animals retained aggressive and song attributes similarly. However, castrated mice administered empty implants showed more subordinate behavior and sang fewer songs that were shorter, lower in power, higher in frequency, and less stereotyped. The extensive effects of androgens on a suite of phenotypes highlight their role in linking gonadal status with decisions about investment in reproductive behaviors.

Introduction

Androgens are critically involved in the expression and evolution of secondary sex characters (Adkins-Regan, 2005), including vocalizations produced in the context of aggression and mate attraction (Ketterson et al., 1996; Ball et al., 2003; Moore et al., 2005; Wilczynski et al., 2005). In birds, frogs, and fish, androgens act on neural pathways and peripheral musculature to influence vocalization rate, signal duration (Bass and Remage-Healey, 2008), amplitude (Connaughton et al., 1997), and dominant frequency (Fusani et al., 1994; Beani et al., 1995; Cynx et al., 2005). In turn, males that vocalize often and produce longer, louder signals with lower frequencies are often perceived as greater threats by rival males and preferred by females (Klump and Gerhardt, 1987; Clayton and Prove, 1989; Ryan and Keddy-Hector, 1992; Reby et al., 2005; Apicella et al., 2007; Mager et al., 2007; Wyman et al., 2008). Androgens thus play a key role in modulating information content of vocal signals, and their manipulation enables exploration into how hormonal mechanisms influence behavior.

In rodents, androgens modulate the rate of ultrasonic vocalizations (USV) produced in reproductive contexts (Sales, 1972; Floody, 1981; Nyby et al., 1992; Yamaguchi and Kelley, 2002; James et al., 2006; Hammerschmidt et al., 2009). However, examples of androgenic effects on other attributes of mammalian vocalizations are limited to frequency changes observed in human "castratis" of the 17–18th centuries (Jenkins, 1998; Nelson, 2005) and adult men administered testosterone therapy (King et al., 2001; Akcam et al., 2004; Hartgens and Kuipers, 2004). While dominant frequency and amplitude features are correlated with fighting ability and female mate choice in some mammals (Reby et al., 2005; Wyman et al., 2008), the role of androgens in controlling such vocal attributes is poorly known.

Herein, we investigate androgenic effects on vocalizations of Neotropical singing mice (genus Scotinomys), diurnal insectivorous rodents distributed throughout Central American cloud forests (Wilson and Reeder, 2005). Singing mice provide a tractable mammalian model because adult males commonly emit loud, frequency-modulated trills that span wide bandwidths (10–43 kHz; Hooper and Carleton, 1976; Miller and Engstrom, 2007; Campbell et al., 2010; Fig. 1A; Appendix A), features characteristic of advertisement signals used to maximize transmission distance and facilitate localization (Bradbury and Vehrencamp, 1998). Presentation and subsequent removal of females increase male singing rate (Fernandez-Vargas et al., 2008), and males counter-sing in response to broadcast songs of intruding males in the field and laboratory (Pasch, in preparation). Thus, singing mouse trills appear to function similarly to frog and bird trills used in male–male aggression and mate attraction (Emerson, 2001; Collins, 2004).

In this study, we tested the hypothesis that androgens modulate vocal behavior, song characteristics, and aggression in Alston's singing mouse (Scotinomys teguina) by experimentally manipulating circulating androgen concentrations. We predicted that males receiving no androgen replacement following castration would sing fewer songs and be less aggressive, whereas androgen replacement would maintain or

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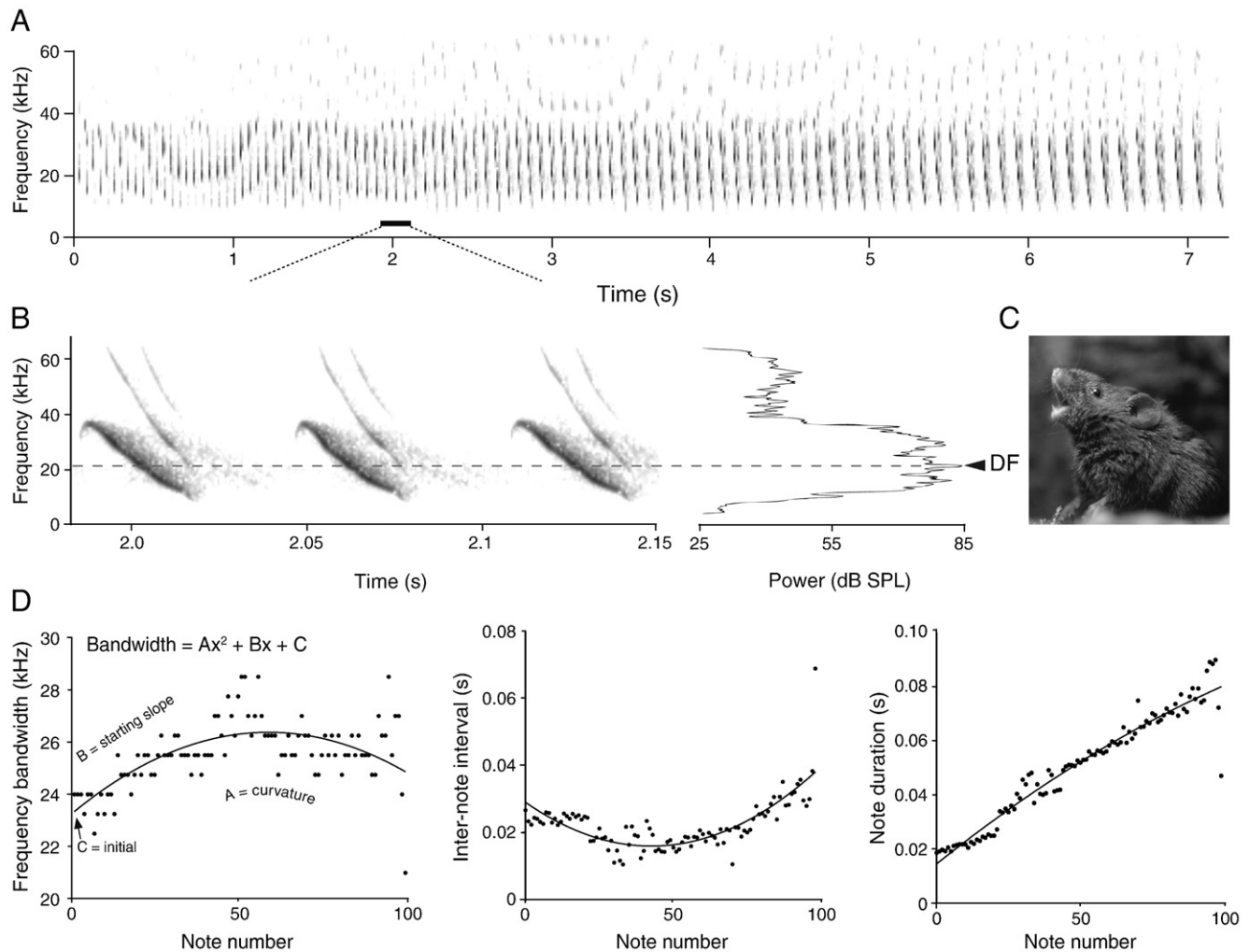
Fig. 1. The song of Alston's singing mouse (*S. teguina*). (A) Spectrogram of Alston's singing mouse (*S. teguina*) song. (B) Spectrogram and power spectrum of 3 notes underlined in panel A. (C) Adult male *S. teguina* singing. (D) Note measurements on change in frequency bandwidth, inter-note interval, and note duration over the course of song. DF = dominant frequency.

increase song effort and aggression. Lastly, we examined whether individual differences in androgen titers predicted individual differences in behavior.

Materials and methods

Study animals

Experimental animals used in the study were laboratory-reared offspring (F1) of wild-captured mice from Cerro La Carpintera, Costa Rica. Sexually experienced adult males were assigned randomly to treatment groups and housed individually in cages for 1 month prior to experimentation. Animals were given cat chow and water *ad libitum* and maintained on a 12 L:12D cycle at 20 ± 3 °C. The Institutional Animal Care and Use Committee at the University of Florida (UF #200801939) approved all procedures.

Surgical procedure

Mice were anesthetized with isoflurane (SurgiVet Isotec T^3 Classic Isoflurane Vaporizer) and castrated (Cast) bilaterally through a 1-cm scrotal incision. We placed a 10-mm silastic implant (1.47 mm i. d. $\times$ 1.96 mm o.d., Dow Corning Corporation, Midland, Michigan) subcutaneously along the dorsal midline. Implants were filled with either 1 mm (15 mg) of testosterone (T; Sigma T1500), 2 mm (30 mg)

of testosterone, 1 mm of dihydrotestosterone (DHT; Sigma A8380), or left empty ($n = 9$ per treatment). The DHT treatment group was included to identify whether androgens were sufficient to cause behavioral and acoustic changes in the absence of aromatization (Nyby et al., 1992). All implants were sealed with silicon adhesive, sterilized with ethylene oxide, and soaked in 0.1% saline solution at 37 °C for 12 h prior to implantation. Researchers were blind to animal treatment and implant concentrations approximated physiological doses of plasma T from adult field-captured animals (see Results).

Song recording and measurement

Fourteen days pre- and post-surgery, we placed mouse cages inside an acoustic chamber ($42 \times 42 \times 39$ cm) made from expanded PVC and lined with anechoic foam. Between 0700 and 1000 h, we recorded spontaneous song rate for 30 min, broadcast a single conspecific song, and measured the latency to counter-sing for 5 min. The broadcast song was randomly chosen from among 51 laboratory recordings of 12 wild-caught males. The stimulus was within 2 standard deviations of the population mean for all song parameters (Campbell et al., 2010). We played stimuli at 50-dB SPL at 1 m from a Pioneer TS-250 speaker (flat frequency response 6– 40 kHz) via an external soundcard (Edirol FA-66). We used an ACO Pacific microphone and preamplifier (Model 7016 and 4116) connected to a laptop running Raven Pro 1.3 via an external

soundcard (Edirol FA-66) to record mouse songs at 30 cm. Microphones were calibrated prior to recording sessions with a Brüel and Kjær sound level meter (Type 2219) and calibrator (Type 4230). All songs at were sampled at 96 kHz and 24 bits.

To analyze recordings, we used automated code written in MATLAB (Phelps, available upon request) to quantify a variety of song parameters including note number, trill rate (note/s), power (dB SPL), frequency bandwidth (kHz), and dominant frequency (kHz) from three spontaneous songs per individual pre- and post-treatment. We then measured a subset of parameters for every note in the trill and used a quadratic function, $f(x) = Ax^2 + Bx + C$, to summarize how parameters changed over the course of a song. For example, to describe frequency bandwidth over the course of the song, $f(x)$ is defined as the bandwidth at note number x, C represents the bandwidth of the first note, B corresponds to change in bandwidth between the first and second notes, and A describes how rapidly bandwidth changes over the course of a song (Fig. 1D). We refer to these parameters as note bandwidth A, B, and C in Table 1. Finally, we averaged the coefficients of variation ($CV = [SD/mean] \times 100$) from each song parameter to assess the degree of song variability pre- and post-treatment.

Resident–intruder trials

We performed resident–intruder trials to test whether androgens mediate aggressive behavior in *S. teguina*. The day following pre-surgery song recording, we introduced a random novel male (intruder) to the home cage of the treatment animal (resident) and videotaped their interactions for 5 min. Cages of residents were transferred from isolation to the behavior room 30 min prior to initiation of trials. We recorded number of attacks, latency to attack, and number of submissive supine displays of treatment animals. We repeated the same procedure 15 days post-treatment but presented treatment animals with novel intruders so that each intruder was used only once.

Table 1
Results of paired t tests of acoustic variables for male singing mice post−pretreatment. Bolded values are significant after Benjamini-Hochberg procedure ($Q = 0.10$). INI = inter-note interval, RMS amp = root–mean–square amplitude, DF = dominant frequency.

Acoustic variable	Empty		DHT (1 mm)		T (1 mm)		T (2 mm)	
	t_8	p	t_8	p	t_8	p	t_8	p
Amplitude measures								
No. of notes	−6.42	0.0002	0.13	0.89	−0.37	0.72	−0.58	0.57
Trill rate (notes/s)	−1.16	0.27	−2.03	0.07	−2.01	0.07	−0.23	0.82
Power	−5.34	0.0007	1.76	0.11	0.78	0.45	0.23	0.82
Note duration A	−1.51	0.16	0.88	0.41	2.06	0.07	−0.04	0.96
Note duration B	2.51	0.03	0.19	0.85	−0.77	0.46	0.67	0.51
Note duration C	1.37	0.02	−0.24	0.81	2.10	0.06	−0.15	0.88
INI A	2.02	0.07	1.38	0.20	2.82	0.02	1.13	0.28
INI B	0.66	0.52	−1.12	0.29	−2.92	0.01	−1.21	0.25
INI C	2.59	0.03	1.16	0.27	1.50	0.17	2.03	0.07
Note RMS Amp A[a]	–	–	–	–	–	–	–	–
Note RMS Amp B	−3.32	0.01	1.28	0.23	0.15	0.88	0.70	0.49
Note RMS Amp C	0.63	0.54	−0.55	0.59	−0.47	0.65	−1.74	0.11
Frequency measures								
Bandwidth	−1.19	0.08	1.05	0.32	3.48	0.008	−0.27	0.79
Dominant frequency	1.46	0.18	0.74	0.47	−1.29	0.23	−3.72	0.005
Note bandwidth A	0.15	0.88	0.46	0.65	−0.28	0.78	−0.71	0.49
Note bandwidth B	0.28	0.78	−0.32	0.75	0.07	0.94	0.40	0.69
Note bandwidth C	0.07	0.93	0.66	0.52	1.15	0.28	−0.21	0.83
Note DF A	0.61	0.55	−0.63	0.54	−0.06	0.94	0.37	0.71
Note DF B	0.13	0.89	1.30	0.22	−0.25	0.80	−1.12	0.29
Note DF C	0.38	0.71	−1.40	0.19	0.58	0.57	2.51	0.03
Stereotypy measure								
Song variability	2.77	0.02	−0.50	0.62	−1.45	0.18	−0.49	0.64

[a] Change is RMS Amp is linear (values = 0).

Testosterone radioimmunoassay

All animals were euthanized 16 days post-surgery, and trunk blood was collected with heparinized capillary tubes. We centrifuged blood at 2500 rcf for 15 min. We used a solid-phase, 96-well plate (Perkin Elmer, Boston, MA, Protein A Flash Plate Plus) radioimmunoassay (RIA) to determine plasma T concentrations as previously described (Hamlin et al., 2010). Briefly, an antibody specific to T (Fitzgerald Industries, Concord, MA, Cat # 20-TR05T) was diluted in phosphate-buffered saline gelatin to a concentration of 1:15,000. We incubated plates with antibody for 2 h at room temperature. ^{3}H-labeled steroid was then added at 12,000 counts/min per 100 μl and plates were incubated for 3 h at room temperature. Prior to sample analyses, we serially diluted a plasma pool to ensure parallelism with the standard curve. Interassay variance wells were similarly prepared from a plasma pool of 5 animals. Intraassay variance averaged 3.7%, whereas interassay variance averaged 6.4%. T concentrations of DHT-treated males reflected in part the cross-reactivity of the assay and are reported below but not included in our analyses. Although the T antibody used in our RIA is reported to have 1% cross-reactivity with DHT (Fitzgerald Industries), the "T" levels detected in our DHT-treated castrates suggest androgen cross-reactivity is more substantial. It is worth noting, however, that other studies show DHT-treated castrates to have substantial levels of T (e.g., Crews et al., 1978). We cannot rule out the possibility that DHT promotes T release from non-testicular sources. We compared T concentrations of our experimental animals to those of adult males ($n = 12$) sampled in the field and processed in an identical manner.

Data analysis

We first used analysis of variance (ANOVA) and Kruskal–Wallis tests to assess variation of all pre-treatment measures among groups. Paired t tests and Wilcoxon signed-rank tests were then used to assess differences within groups pre- and post-treatment. We corrected for multiple comparisons of song parameters using the Benjamini and Hochberg (1995) procedure. ANOVAs were used to compare post-treatment T concentrations and post−pre differences within groups among treatments. We conducted post hoc comparisons using Tukey's test. To assess the association between circulating concentrations of T and post-measures on song characteristics, we fit a logarithmic curve to individuals across treatments and used linear regressions to highlight patterns within treatment groups. All statistics were performed in JMP, Version 7 (SAS Institute Inc., Cary, NC, 1989–2007). We report means ± standard error (SE) throughout the text unless otherwise noted.

Results

Plasma androgen concentrations

T concentrations (± SD) varied significantly among groups post-treatment (cast + empty: 0.39 ± 0.1 ng/ml, cast + DHT: 2.03 ± 3.3 ng/ml, cast + T (1 mm): 5.33 ± 2.8 ng/ml, Cast + T (2 mm): 13.28 ± 3.1 ng/ml; $p < 0.0001$) and approximated the physiological range of adult males sampled in the field (0.76–6.35 ng/ml).

Resident–intruder trials

The number of attacks or latency to attack intruding males did not differ pre- and post-treatment in any group ($p > 0.05$). However, empty-implanted males engaged in more submissive supine displays post-treatment (pre: 0.1 ± 0.1, Post: 1.1 ± 0.3; $W = 14.0$, $p = 0.01$; Fig. 2).
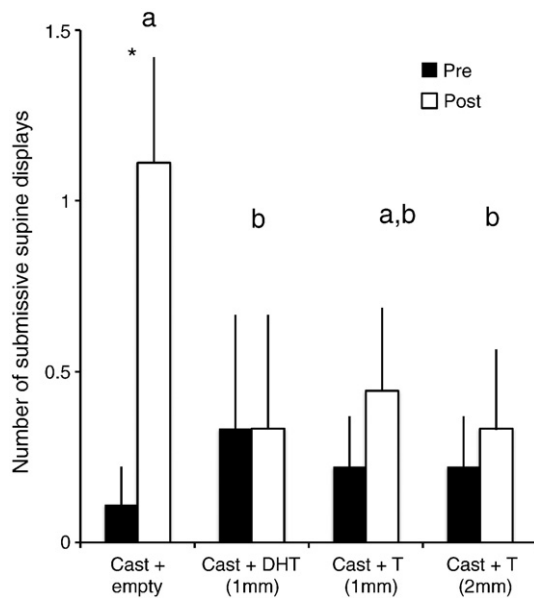
Fig. 2. Androgenic effects on aggressive behavior. Number of submissive supine displays by resident male singing mice to intruding male conspecifics pre- and post-treatment. Error bars are +1 SE *$p<0.05$ for post−pre differences within groups. Groups not sharing the same letter have significantly different post−pre values (Tukey HSD, $p<0.05$). Cast = castrate, DHT = dihydrotestosterone, T = testosterone.

Singing behavior

Pre-treatment measures for all behavioral variables did not differ among groups ($P>0.05$). Empty-implanted males decreased spontaneous song rate (pre, 1. 6±0.3 songs/10 min; post, 0.48±0.2 songs/10 min; $W=−22.5$, $p=0.004$; Fig. 3A) and increased latency to sing in response to a conspecific song (pre, 217.6±35 s; post, 275.3±23 s; $W=7.5$, $p=0.03$; Fig. 3B), whereas DHT, T (1 mm), and T (2 mm)-implanted males increased spontaneous song rate ($W=9.5, 14.0, 17.5$ respectively, $p<0.05$) but exhibited no change in latency to respond ($W=−1.5, 1.5, −7.5$ respectively, $p>0.05$; Fig. 3).

Song parameters

Experimental manipulation had significant effects on a variety of song parameters in empty-implanted males, as exemplified in spectrograms of one male's song pre- and post-treatment (Fig. 4; Appendix B and C). Empty-implanted males sang songs with fewer notes (pre, 101.4±6 notes; post, 54.7±6 notes; $p>0.001$) that were lower in power (pre, 71.6±1 dB SPL; post, 62.6±1.9 dB SPL; $p>0.001$) with slightly higher dominant frequencies (pre, 19.9±0.8; post, 22.5±2 kHz; $p=0.18$; Table 1; Figs. 5A–C). Such changes were manifest in animals starting songs with longer note durations and internote intervals that quickly decreased in power (Note Duration C, INI C, and RMS amplitude B; Table 1). Empty-implanted males also sang less stereotyped songs after treatment (pre, 6.8±0.3%; post, 11.1±1.7%; $p>0.05$; Table 1; Fig. 5D). Conversely, implants maintained the majority of song parameters in DHT- and T-treated groups, with the exception of T (1 mm)-treated males exhibiting increased frequency bandwidth (pre, 25.2±0.6 kHz; post, 26.2±0.5 kHz; $p>0.01$) and T (2 mm)- treated males showing decreased dominant frequency post-treatment (pre, 21.2±0.7 kHz; post, 18.8±0.5 kHz; $p>0.01$; Table 1; Fig. 5c).

The relationship between circulating T concentrations and number of notes post-treatment showed a highly non-linear effect across treatments ($y=10.5(\ln)x+68$, $r^2=0.52$, $F_{1,23}=24.73$, $p<0.0001$; Fig. 6A), reflected in varying slopes of the relationship within treatment groups. Among empty-implanted individuals, for example, low circulating concentrations (below physiological range of adult males
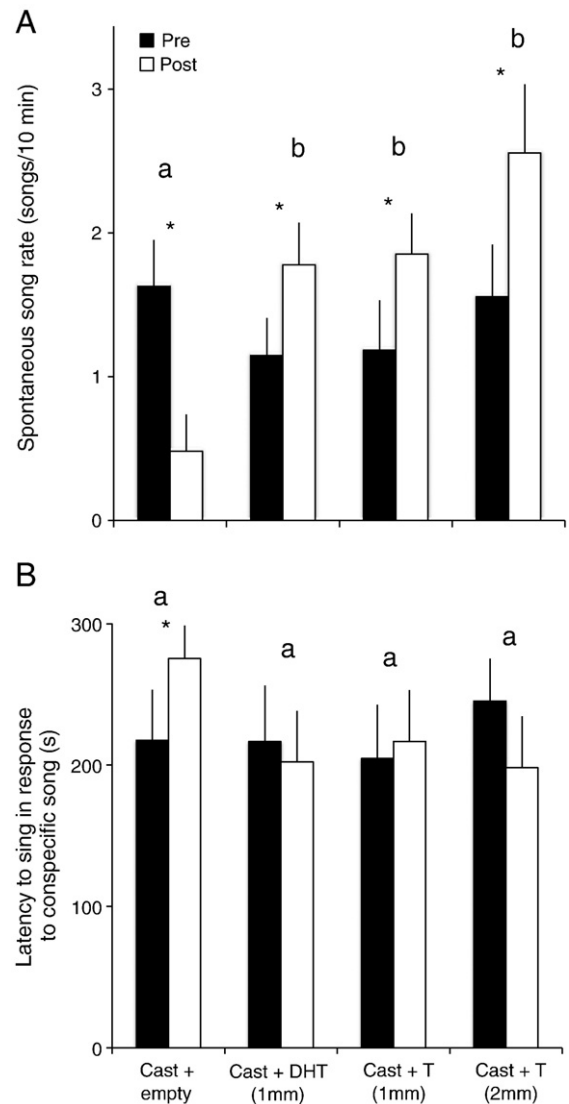

Fig. 3. Androgenic effects on singing behavior. (A) Spontaneous song rate (songs/10 min) and (B) latency to sing (s) in response to conspecific song of male singing mice pre- and post-treatment. Error bars are +1 SE * $p<0.05$ for post−pre differences within groups. Groups not sharing the same letter have significantly different post−pre values (Tukey HSD, $p<0.05$). Cast = castrate, DHT = dihydrotestosterone, T = testosterone.

captured in the field) of androgens showed a steep positive correlation with number of notes ($m=137.6$, $r^2=0.63$, $p=0.02$). T (1 mm)-treated males showed a less steep association ($m=1.5$, $r^2=0.11$, $p=0.4$), whereas T (2 mm)-treated males that were above physiological levels of T showed no relationship ($m=−0.71$, $r^2=0.05$, $p=0.56$). We found similar non-linear effects between circulating T concentrations and power ($y=2.6(\ln)x+65.8$, $r^2=0.57$, $F_{1,23}=7.53$, $p<0.0001$), dominant frequency $y=21.6(\ln)x−1.1$, $r^2=0.26$, $F_{1,23}=7.55$, $p=0.01$, and song variability ($y=−0.017(\ln)x+0.08$, $r^2=0.37$, $F_{1,23}=11.21$, $p=0.003$; Fig. 6B).

Discussion

We explored the role of androgens in modulating singing behavior, song characteristics, and associated aggressive behavior in Alston's singing mouse. We found that androgens influenced a suite of measures, highlighting their role in linking gonadal status to decisions about investment in reproductive behaviors. Some effects of castration were profound and conspicuous (e.g., reductions in song rate,
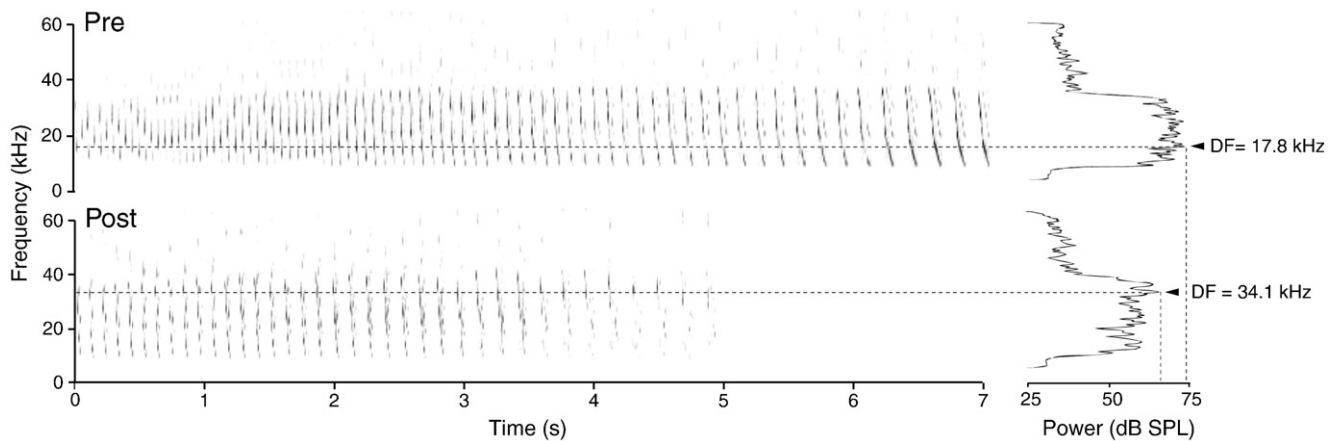
Fig. 4. Mouse song pre- and post-castration. Representative songs of an individual male singing mouse castrated + empty-implant pre- and post-treatment. df = Dominant frequency.

length, and power), whereas their influence on aggression was subtler.

Administration of T and DHT influenced measures of song rate and response latency similarly. Conversely, empty-implanted males sang less and showed longer latencies to respond to conspecific songs. Because DHT cannot be aromatized to estrogen, androgens appear to act directly on androgen receptors to mediate vocal behavior in singing mice. USVs are androgen-dependent in other rodents,

although estrogens facilitate the production of vocalizations in rats and gerbils (Floody, 1981; Nyby et al., 1992). Androgenic effects on song rate and latency reflect motivational state in many taxa including rodents (Nunez et al., 1978) and likely act on neural pathways that govern vocal production.

Androgens had a profound influence on the structure of vocalizations. When empty-implanted males sang, their trills were shorter, lower in power, higher in frequency, and more variable. Song length is
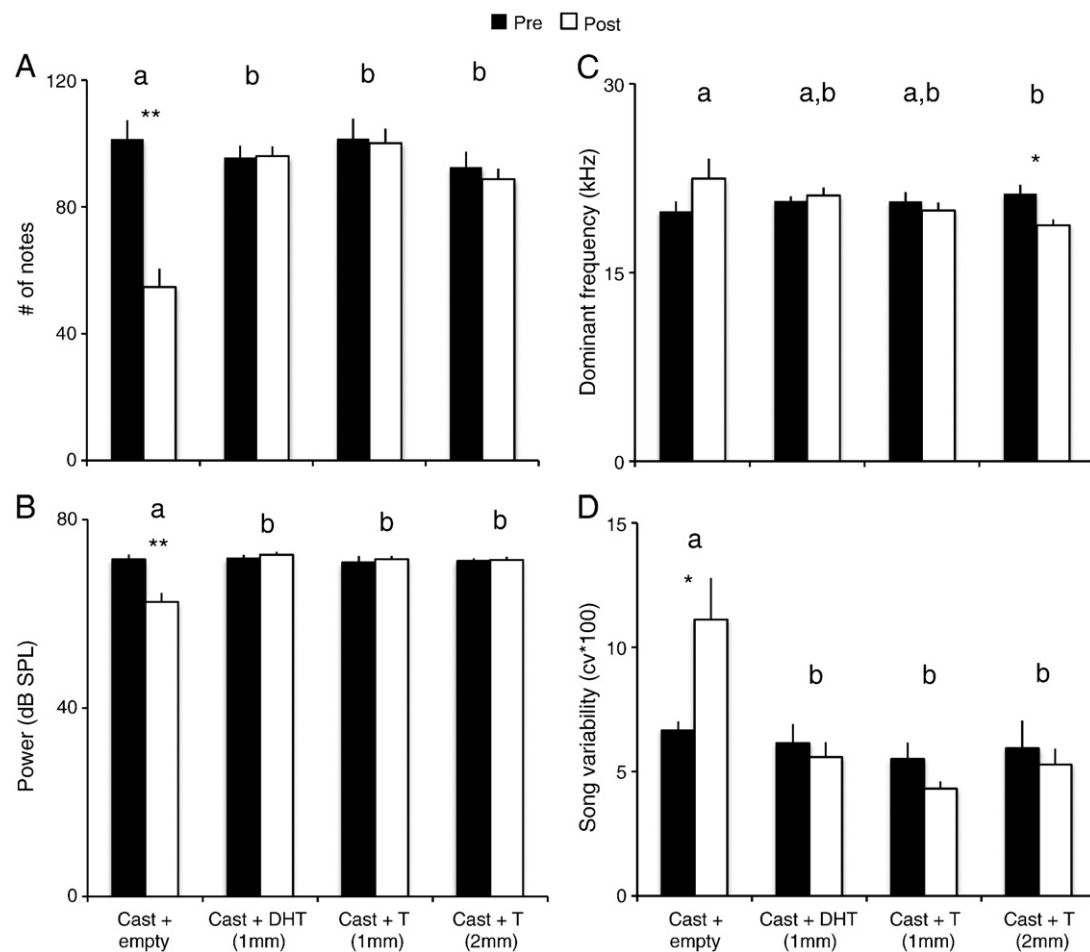

Fig. 5. Androgenic effects on song parameters. (A) Number of notes, (B) power (dB SPL at 30 cm), (C) dominant frequency (kHz), and (D) song variability (CV *100) of male singing mice pre- and post-treatment. Error bars are +1 SE **$p<0.001$, *$p<0.05$ for post − pre differences within groups. Groups not connected by same letter have significantly different post − pre values (Tukey HSD, $p<0.05$). Cast = castrate, DHT = dihydrotestosterone, T = testosterone.
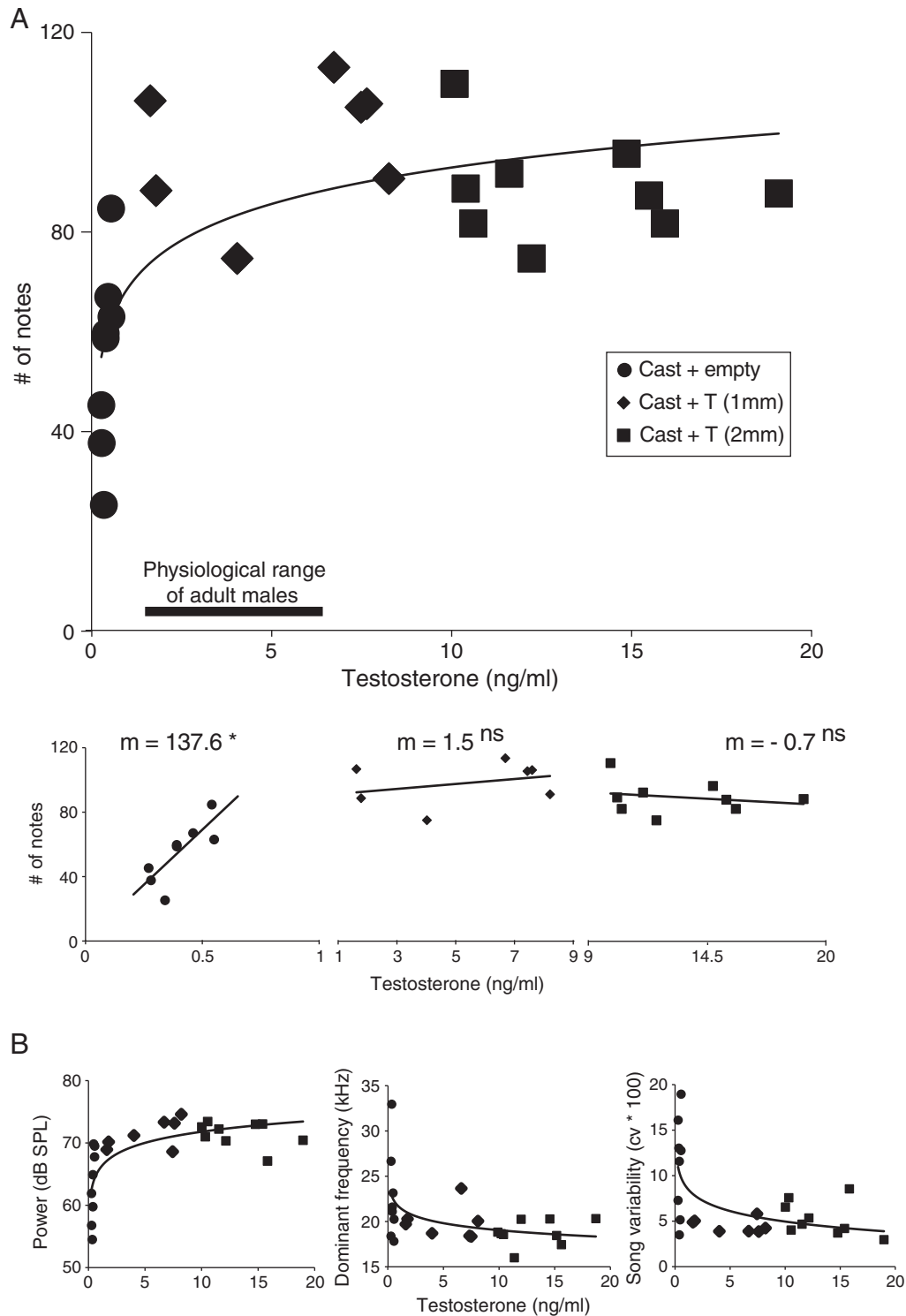
Fig. 6. Relationship between androgen concentrations and song parameters in singing mice. (A) Testosterone concentrations (ng/ml) of hormone-treated male singing mice by number of notes. Black line along *x*-axis indicates the range of physiological concentrations of testosterone from field-captured mice in Costa Rica and Panamá. *m* = slope, *p* < 0.10. (B) Testosterone concentrations (ng/ml) of hormone-treated male *S. teguina* by power (dB SPL at 30 cm), dominant frequency (kHz), and song variability (CV *100).

a classic indicator of motivation in anurans and birds (Catchpole et al., 1986; Bensch and Hasselquist, 1991; Welch et al., 1998), and power can convey information on receiver intent and condition (Gerhardt and Huber, 2002; Forstmeier et al., 2002; Brumm and Todt, 2004; Wyman et al., 2008). The fact that empty-implanted males sang at higher frequencies ('castrati effect') while T (2 mm) males sang at lower frequencies after treatment indicates that the larynx remains androgen responsive after puberty. Androgens may influence larynx

size or modulate expression of myosin heavy chain isoforms that govern the velocity and force of muscular contractions (Fischer et al., 1993; Dabbs and Mallinger, 1999), thus providing a potential mechanism for changes in song attributes. The high song variability of empty-implanted males suggests that androgens are involved in coordinating both the fine neuromotor control and highly motivated state associated with song stereotypy (Kao and Brainard, 2006; Woolley and Doupe, 2008). Thus, the extensive effects of androgens

on vocalizations suggest that they act on a suite of targets, including brain regions that relay impulses to the larynx and peripheral muscles that influence dominant frequency and amplitude during exhalation (Beani et al., 1995).

While androgens had a profound influence on song, their effects on aggression were significant but subtler. Androgens significantly reduced the probability of submissive supine behavior, but we found relatively little offensive aggression in any treatment group. Moreover, animals exhibited low levels of offensive aggression prior to castration, indicating that the surgery itself was not causing low aggression levels. We suspect that our testing procedures contributed to low levels of aggression. Blondel (2004) placed male residents of *Scotinomys xerampelinus*, a sister species, in the testing room the day before testing, performed trials under red light, and found that animals were very aggressive. The fact that we staged encounters 30 min after placing males in the novel testing room may have led subjects to behave as intruders rather than residents. Nevertheless, the ability of castration to promote supine displays indicates androgens modulate aggression as well as vocalization in singing mice.

Interestingly, the influence of androgens on vocal attributes followed a strongly non-linear relationship (Fig. 6). The relationship between number of notes and plasma testosterone titers, for example, is steepest in the range of hormone concentrations exhibited by castrates, shallow in the range of intact males, and flat at supra-physiological levels. We interpret such a curve as a type of "reaction norm", which describes how animals translate variation in circumstance (in this case reproductive status signaled by androgen concentrations) into changes in phenotype (vocal effort). If this relationship is adaptive, the steep increase in vocal effort elicited by T concentrations just below those of healthy adult males may be a means of ensuring that males engage in high vocal effort when testes are producing gametes, or none at all when conditions are unfavorable for reproduction. The more shallow relationship within the range of adult T titers suggests that natural variation in androgens coordinates subtle differences in vocal effort among males. Lastly, the flat relationship at supra-physiological doses suggests that increasing vocal effort beyond that plateau will provide no further increase in fitness regardless of reproductive condition or social status. Such non-linear curves were consistent across measures of vocalization and have long been observed in behavioral endocrinology (Beach, 1948; Adkins-Regan, 2005; Ball and Balthazart, 2008). Reaction norms could provide a useful and general framework for investigating the relationship between hormone concentrations and behavior.

The hormonal coordination of multiple dimensions of vocal effort is similar to proposed endocrine control over vocalization rates in anurans (Marler and Ryan, 1996; Emerson, 2001; Leary et al., 2004). The model suggests that androgens have graded effects on vocal characteristics and are suppressed by negative feedback from corticosterone associated with energetic constraints of calling. Future studies that incorporate androgenic interactions with other hormones and neuromodulators will provide important insight into the full suite of mechanisms involved in signal production. By introducing a novel mammalian species, we hope to broaden the taxonomic scope of work in acoustic signaling to facilitate integration of hormones, behavior, and evolution.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yhbeh.2010.10.011.

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