Endocrinology. First published ahead of print January 15, 2010 as doi:10.1210/en.2009-0901

Limb Muscles Are Androgen Targets in an Acrobatic Tropical Bird

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Spectacular athleticism is a conspicuous feature of many animal courtship displays yet surprisingly little is known about androgen dependence of skeletal muscles underlying these displays. Testosterone (T) acts through androgen receptors (ARs) to stimulate muscular male Golden-collared manakins of Panama to perform a remarkably athletic courtship display that includes loud wing-snaps generated by the rapid and forceful lifting of the wings. We tested the hypothesis that androgen sensitivity, reflected in the expression levels of AR mRNA, is a muscular adaptation supporting these courtship displays. Quantitative PCR showed substantially greater AR mRNA expression in all limb muscles of wild male and female manakins compared with two other avian species that do not perform athletic displays, zebra finches and ochre-bellied flycatchers. AR expression levels in the massive skeletal muscles were comparable with the minute oscine syringeal muscle but greater than levels in nonmuscular androgen targets that did not differ across species. Compared with zebra finches, male manakins also had greater activity of the T-activating enzyme 5α-reductase in a wing-lifting muscle. In addition, low levels of estrogen receptor α (ER) mRNA were detected in all muscles of control, T-treated, and estradiol-treated manakins. Treatment of manakins with T, but not estradiol, significantly increased skeletal muscle ER expression, suggesting that ER expression is AR-dependent. These results confirm manakin limb muscles as important androgen targets where T may act to promote the speed, force, and/or endurance required for the manakin display. Androgen-sensitive muscular phenotypes may adapt males of many species to perform impressive athletic displays. **(*Endocrinology* 151: 0000–0000, 2010)**

Androgens act on intracellular androgen receptors (ARs) to masculinize and activate numerous vertebrate tissues, including some skeletal muscles. The majority of studies showing muscle androgen dependence have examined muscles that exclusively or temporarily support masculine reproductive functions, such as copulatory perineal muscles of rodents, sexual clasping muscles of frogs, or, in several species, vocal muscles used for mate-attraction (1–7). Typically, androgens have trophic effects on these muscles, where androgen-deprivation induces their atrophy and testosterone (T) replacement increases muscle mass and contractility, fiber size and number, and changes in fiber type (1–6). In humans, androgens can increase lean muscle mass and improve strength of limb and trunk muscles to promote athletic performance (8–10). Few animal models exist, however, to examine natural androgen actions in such a wide diversity of skeletal muscles and on the enhancement of muscle performance (11). Furthermore, the role of androgens acting on AR in promoting sex, individual, and age related differences in body composition and athletic abilities have not been well studied (9).

Males of many animal species perform physical courtship displays that, in some cases, resemble human athletes in their complex neuromuscular coordination, strength, and stamina. We study such a species, the Golden-collared

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0901 Received July 30, 2009. Accepted December 8, 2009.

Abbreviations: AR, Androgen receptor; 5α-A, 5α-androstanedione; Ct, threshold cycle number; E2, estradiol; ER, estrogen receptor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, gluteal; PEC, pectoralis; SC, supracoracoideus; SH, scapulohumeralis caudalis; SYR, syringeal muscle; T, testosterone.

manakin (*Manacus vitellinus*), a suboscine bird of lowland rainforests of Panama. Males of this species are unusually muscular (12, 13). When courting, the males jump acrobatically, rapidly, and precisely from branch to branch around an arena that they clear in the forest floor (described in detail in Refs. 14 and 15). As part of the display, males forcefully and rapidly flip their wings above their backs to produce loud firecracker-like "wingsnaps" or "wingrolls," a series of 12 or more wingsnaps at 50–70 Hz (wingsnaps per second). This courtship display is highly energetic and noisy (including many vocalizations) and can be performed for several hours each day over a prolonged 6–8 month reproductive season. We have considerable evidence that T naturally activates male manakin courtship and does so primarily through AR. First, plasma T levels are low in adult males during the nonbreeding season and elevated during the breeding season when the males are courting (16–18). Second, noncourting females and juvenile males have low levels of T (16, 17). Third, T-treatment of nonbreeding males and females activates courtship behavior (16). Finally, courtship behavior is disrupted by treatment of adult breeding males with flutamide, an AR-antagonist (18).

Because limb muscles are the principal effectors that produce manakin courtship, we tested the hypothesis that androgen sensitivity, reflected in the expression levels of AR mRNA, is a muscular adaptation in several manakin wing and leg muscles involved in courtship. We predicted that 1) sexually-dimorphic limb muscles used in generating wingsnaps express AR and do so at higher levels than sexually-monomorphic muscles not used for wingsnaps, 2) male wing muscles express higher levels of AR than female wing muscles, and 3) manakin muscles express higher levels of AR than the same muscles of birds that do not perform athletic courtship displays. We examined three muscles that raise [the supracoracoideus (SC) and scapulohumeralis caudalis (SH)] and retract [the pectoralis (PEC)] the wings during wingsnapping. These muscles exhibit conspicuous and sexually dimorphic morphological adaptations for rapid and forceful contraction in males, such as greater mass, larger fiber diameter, and greater expression of a fast-twitch myosin isoform (12, 13). We also examined the gluteal (GL), a leg muscle used for jumping during the courtship dance that is hypertrophied in manakins when compared with zebra finches. The GL is sexually-monomorphic and lacks morphological specializations for rapid and forceful contraction so we predicted AR-expression in this muscle would be low (12). As a positive control, we examined syringeal muscles (SYRs) used in avian vocalizations and that express AR in oscine songbirds (7). Finally, we examined testes, brain, and spinal cord as nonmuscular androgen-dependent tissues.

Quantitative PCR was used to measure AR mRNA levels in male and female manakin muscles, which were compared to expression levels in the same muscles in two other passeriform species, the oscine zebra finch (*Taenopygia guttata*) and the subocine ochre-bellied flycatcher (*Mionectes oleagieus*). Male zebra finches perform a simple dance during courtship that is not highly athletic and do not use their wings for mechanical sound production (16). Zebra finches are otherwise similar in size to manakins and are readily available from our breeding colony. Like the manakin, the flycatcher is a small tropical breeding subocine species. They lift their wings while feeding, possibly adding demands on their SC and SH, but these wing-lifts do not produce sound and lack the power and intensity of the manakin wing snap. Additionally, we measured AR and estrogen receptor α (ER) mRNA levels in male manakins treated with T or estradiol (E2) to look for steroid regulation of receptor expression. Finally, because T potency can be increased by local conversion into 5α-dihydrotestosterone by the enzyme 5α-reductase (17), we used a radioenzymatic assay to measure 5α-reductase activity in the SC and GL of female and male manakins and zebra finches (13).

Materials and Methods

Tissues

Reproductively active male (n = 9) and female (n = 6) manakins were collected from the field in March, 2000 and 2008. Reproductively inactive male manakins were collected in the nonbreeding season, August, 2006, and June, 2008, serving as untreated controls (n = 3), or were implanted with T (n = 3) or E2 (n = 3) for 3 wk before tissues were collected (steroids were purchased from Steraloids Inc., Wilton, NH). Steroids were delivered in 10-mm SILASTIC brand (Dow Corning, Midland, MI) silicon implants assembled in our laboratory following procedures described previously (18). In addition, we were able to collect four reproductively-active male flycatchers. After capture, all birds were returned to laboratory facilities of the Smithsonian Tropical Research Institute in Gamboa, Panama, where they were killed by decapitation and tissues collected as described previously (13, 18). Specifically, for sex and species comparisons of AR expression as well as enzyme activity, SC and GL were collected from breeding male and female manakins and zebra finches (n = 6/group). For comparisons of AR expression across species, SC, GL, PEC, and SH were collected from an additional three breeding and three nonbreeding male manakins, three breeding male zebra finches, and four breeding male flycatchers. SYR were collected from two breeding manakins, three nonbreeding manakins, eight breeding zebra finches, and two breeding flycatchers. Testes was collected from two breeding manakins and three breeding zebra finches; brain regions (telencephalon, optic tecta, hypothalamus, and cerebellum) and spinal cord regions (cervical, thoracic, and lumbar) were collected from three breeding manakins.

Tissues were dissected bilaterally and frozen immediately on dry-ice where they remained until placed in a −80 C freezer at Smithsonian Tropical Research Institute facilities in Panama City. Tissues were shipped to University of California, Los Angeles (UCLA), on dry-ice and held again at −80 C until use. We replicated these procedures as closely as possible when collecting tissues from zebra finches taken from our breeding colony at UCLA. Dissected muscles were frozen first on dry-ice and then stored at −80 C until use. All procedures were approved by the UCLA Chancellor's Animal Research committee.

RT-PCR

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Tissues were homogenized for 45 sec at medium speed by a rotor/stator homogenizer. Total RNA concentration was determined spectophotometrically, and RNA integrity was verified by gel electrophoresis.

After DNase treatment (Promega, Madison, WI), 1 μg of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). The reaction was run for 50 min at 42 C followed by 15 min at 70 C. To confirm tissue expression, the resulting cDNA was used for PCR amplification of AR using AR primers designed from the Canary (*Serinus canaria*) (GenBank accession no. L25901): forward, 5′-TGACGTGTGGGAGCTGCAAA; AR reverse, 5′-GGCCATCCACTGGAATAATACTGA, which amplified a 741-bp product. ER amplification used primers designed from zebra finch ER (GenBank accession no. NM_001076701) and amplified a 370-bp product; ER forward, 5′-TGTCCCTGACAGCAGAACAG; ER reverse, 5′-GTAGCCAGCAGCATGTCAAA. Each 25 μl of PCR reaction contained 0.375 mM each of deoxynucleotide triphosphate, 0.4 μM each of primers, 50 ng of cDNA as a template, 0.06 u/ml of the enzyme DNA taq polymerase (Bioline, Randolph, MA), and buffer. Reactions were carried out at 95 C for 5 min, then 38 cycles of 95 C for 30 sec/63 C for 30 sec/72 C for 1 min, followed by 72 C for 10 min. AR and ER products were extracted from 2% agarose gel using QIAquick Gel Extraction kit (QIAGEN Inc., Valencia, CA) and sequenced at the UCLA Sequencing and Genotyping Core. Product sequences were then aligned against annotated zebra finch AR (GenBank accession no. NM 001076688) and ER using NCBI BLAST 2 Sequences to determine sequence identity.

Quantitative PCR

Quantitative PCR analysis was performed on an ABI 7300 sequence detection system by using the SYBR Green PCR Master Mix kit (Applied Biosystems Inc., Foster City, CA) with 5 ng of template and a varying concentration of primers determined by primer optimization. All primers were designed from the annotated zebra finch sequences: AR forward (0.3 μM), 5′-ATGAGTACCGCATGCACAAA and AR reverse (0.3 μM), 5′-AACTCCTGGGGTGTGATCTG (amplifying a 100-bp product); ER forward (0.9 μM), 5′-TGAAAGGTGGAATCCGAAAAGA and ER reverse (0.9 μM), 5′-TTGGCGTTTTTGTTTCATCACT (amplifying a 59-bp product), in a total volume of 25 μl. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. AF255390) amplification served as the internal control using 0.3 μM of each primer: GAPDH forward, 5′-TGACCTGCCGTCTGGAAAA; GAPDH reverse, 5′-CCATCAGCAGCAGCCTTCA (70-bp product). Reactions were carried out at 50 C for 2 min, 95 C for 10 min, and 40 cycles at 95 C for 15 sec/60 C for 1 min, followed by 95 C for 15 sec, 60 C for 30 sec, and 95 C for 15 sec. Dissociation curves of PCR products were assessed to ensure the absence of DNA contamination. Samples were run in duplicate.

Standard curves with correlation coefficients of >0.99 were generated with known concentrations of cDNA generating slopes that were used to calculate amplification efficiency ($E = 10^{-1/\text{slope}} - 1$) for AR, ER, and GAPDH reactions (19). Because all primers were near 100% efficient in all species, the ΔCt method was used to quantify the relative abundance of AR, where the ΔCt value was taken as the threshold cycle number (Ct) for AR relative to the Ct for GAPDH: $2^{-(\text{Ct,AR} - \text{Ct,GAPDH})} \times 1000$.

Biochemistry

SC and GL muscles from male and female manakins and zebra finches were homogenized for 45 sec at medium speed in sucrose phosphate buffer (pH 7.4). 5α-Reductase activity was analyzed in tissue homogenates by measuring the metabolism of [^{3}H]androstenedione ([^{3}H]A; specific activity 91 Ci/mmol; PerkinElmer, Boston, MA) to [^{3}H]5α-androstanedione (5α-A) using procedures used routinely in our laboratory and described in detail previously (20). Reactions were carried out at 41 C for 90 min and were started by the addition of [^{3}H]A in an reduced nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide phosphate generating system. Preliminary studies established the optimal incubation duration and substrate concentration for muscle assays to be 90 min and 100 nM [^{3}H]A, respectively. Control tubes contained substrate and cofactors but no tissue. For determination of procedural losses, parallel incubates without substrate received approximately 200,000 cpm [^{3}H]A. Reactions were terminated by snap-freezing in a dry-ice/methanol bath and samples were stored at −20 C until analysis.

Radioinert 5α-A was added to each tube as carriers, the samples were extracted with diethyl ether, ether residues were chromatographed on thin-layer silica gel plates using ether/hexane (3:1), and carriers were visualized under UV light after spraying with primulin. Silica gel from each product region was scraped into test tubes and eluted in methanol. The eluate was added to scintillation cocktail (PerkinElmer) for estimation of radioactivity in the scintillation counter (Beckman LS-3100). Total protein content of homogenates was determined by the Bradford protein assay. Specific activities of enzymes were presented as fmol/mg protein. To verify the authenticity of our products, we recrystallized [^{3}H]5α-A products to constant specific activity from manakin muscles as described previously (21). 5α-A products were recrystallized with an 82.3% recovery with 0.2% error. In one assay, we measured enzyme activity in SC and GL muscles from male and female manakins. In a second assay, we measured enzyme activity in SC and GL muscles from male manakins and zebra finches.

Statistical analyses

Data are presented as means ± SEM. Data were analyzed using Statview 4.37. Two-way ANOVA was used to evaluate quantitative PCR ΔCT values and enzyme activities across muscle and species or across sex and muscle. Three-way ANOVA was used to compare quantitative PCR ΔCT values across muscle, sex, and species. *Post hoc* comparisons used Fisher's projected least significant difference with the exception of second biochemistry assay with significant interactions where *post hoc t* tests was used with Bonferonni corrections.

Results

AR

PCR-amplification of AR showed a single band of the expected size (741 bp) in each lane for SC, GL, PEC, SH, SYR, and testes of all three species (data not shown). Products sequenced from tissues of all three species were confirmed as AR. Alignment of the 741-bp product from each species to the annotated zebra finch AR sequence showed 98% identity with our zebra finch AR product, 94% identity with our manakin AR product, and 92% identity with our flycatcher AR product. Alignment of the quantitative PCR products (100 bp) from zebra finch and manakin showed 98% identity with each other and 98% identity to the flycatcher product; 100% identity was found between primer sequences and AR sequences from all species. In addition to calculating the primer efficiencies from slopes of standard curves (see *Materials and Methods*), our alignment results indicate that, although the primers were designed from the zebra finch sequence, they worked with equivalent efficiency across all species.

We used quantitative PCR to measure AR mRNA expression in muscles of male manakins, zebra finches, and flycatchers to look for species and muscle differences. For AR expression (Fig. 1), two-way ANOVA identified significant species ($F_{2,52} = 30.41$, $P < 0.0001$) and muscle effects ($F_{4,52} = 13.229$, $P < 0.0001$), with no species* muscle interaction ($F_{8,52} = 1.62$, $P = 0.14$). Manakin tissues had significantly higher AR expression than both flycatchers and zebra finches (manakin>zebra finch: $P < 0.0001$; manakin>flycatcher: $P < 0.0001$). Overall, SYR had higher AR expression than other muscles ($P < 0.0001$ for all comparisons). Mean AR expression levels in manakin limb muscles were strikingly similar to levels seen in the oscine zebra finch syrinx. Zebra finch syrinx AR expression was similar to that seen in the syrinx of flycatchers but lower than that of manakins. Elevated levels of AR in the manakin syrinx are not unexpected given the importance of vocalizations to the rich acoustic output of the male's display.

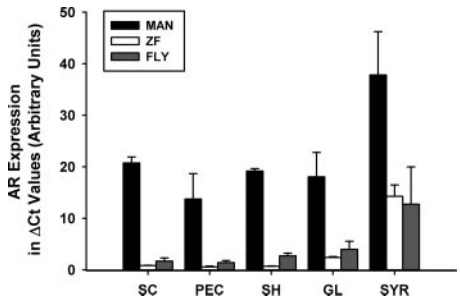

FIG. 1. AR mRNA expression levels relative to GAPDH (ΔCt values) in SC, PEC, SH, GL, and SYR of male manakins (MAN), zebra finches (ZF), and ochre-bellied flycatchers (FLY). AR expression in manakin muscles was significantly higher than in zebra finches and flycatchers, and syrinx had significantly higher expression than all other muscles. For manakin tissues, n = 3 for all limb muscles, n = 5 for SYR. For zebra finch tissues, n = 6 for SC and GL, n = 3 for PEC and SH, and n = 8 for SYR. For flycatcher tissues, n = 4 for all limb muscles, and n = 2 for SYR.

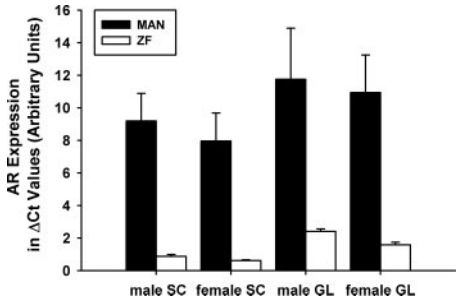

FIG. 2. AR mRNA expression levels in SC and GL of male and female manakins and zebra finches (n = 6 for all groups). We found no sex difference in AR expression of SC and GL. AR expression was significantly higher in manakin muscles compared with the same muscles of zebra finches.

We compared AR expression in male and female manakin and zebra finch SC and GL to look for sex, species, and muscle differences. Three-way ANOVA identified a significant effect of species ($F_{1,40} = 56.16$, $P < 0.0001$) but not muscle ($F_{1,40} = 3.07$, $P = 0.09$) or sex ($F_{1,40} = 0.46$, $P = 0.50$), with manakin muscles having higher AR expression than zebra finch muscles (Fig. 2). We found no significant interactions (species*muscle: $F_{1,40} = 0.44$, $P = 0.51$; species*sex: $F_{1,40} = 0.05$, $P = 0.83$; muscle*sex: $F_{1,40} = 0.001$, $P = 0.98$; species*muscle*sex: $F_{1,40} = 0.05$, $P = 0.83$).

To compare AR expression in skeletal muscles with other known androgen target tissues, we measured AR expression in four brain regions (telencephalon, optic tecta, hypothalamus, and cerebellum; n = 3 each), three spinal cord regions (cervical, thoracic, and lumbar; n = 3 each), and compared the combined averages of all brain, all spinal cord, with all skeletal muscles, as well as testis (n = 2) of breeding male manakins (Fig. 3). One-way ANOVA identified a significant difference between these tissue regions ($F_{3,48} = 5.687$, $P = 0.002$) with skeletal muscles having the highest AR. Fisher's projected least significant difference showed that skeletal muscles were significantly higher than brain ($P = 0.0007$) and spinal cord ($P = 0.009$). The hypothalamus, a well-established neural androgen target, had the highest AR expression out of the four manakin brain regions examined (data not shown). Skeletal muscles had a combined mean ΔCt value of 18.26 ± 2.43, more than 3-fold greater than the hypothalamus with a mean ΔCt value of 5.13 ± 1.34. There was no difference in AR expression levels in testes of manakins (ΔCt = 8.07 ± 0.39) and zebra finches (ΔCt = 4.39 ± 2.04; $t_4 = 1.77$, $P = 0.15$; data not shown); in manakins, AR expression levels in testes were decidedly lower than in limb

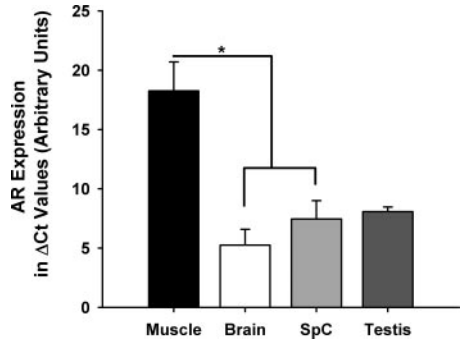

FIG. 3. Combined average AR mRNA expression levels of male manakin muscles (combined average from nine SC, nine GL, three PEC, three SH, and five SYR), brain regions (combined average of cerebellum, hypothalamus, telencephalon, and optic tectum from three breeding manakins), spinal cord regions (SpC; combined average from cervical, thoracic, and lumbar regions from three breeding manakins), and testes (n = 2). Manakin muscles express the highest AR with significantly higher expression than brain and spinal cord (*, $P < 0.009$).

muscles, whereas in zebra finches, AR expression levels in testes were slightly higher than that seen in limb muscles.

We found no significant differences in manakin limb muscle AR expression across season ($F_{1,16} = 0.18$, $P = 0.68$), or muscle ($F_{3,16} = 1.56$, $P = 0.24$), and there was no interaction between factors ($F_{3,16} = 1.04$, $P = 0.40$; Fig. 4A).

ER

PCR-amplification of ER showed a single band of the expected size (370 bp) in each lane for all skeletal muscles from male manakins (data not shown). Products sequenced from muscle tissues were confirmed as ER. Our manakin ER product showed 95% identity to the annotated zebra finch ER sequence, confirming the presence of ER in manakin muscles.

Levels of ER mRNA were low compared with AR but similar to levels expressed in some other estrogen targets. For example, ΔCt values for ER expression in the hypothalamus (data not shown) was 0.12 ± 0.04 with that of skeletal muscles on average 0.17 ± 0.06. We detected no significant difference in seasonal expression of ER in manakin muscles ($F_{1,20} = 3.06$, $P = 0.096$), no difference between muscles ($F_{3,20} = 0.19$, $P = 0.90$), and no interaction ($F_{3,20} = 1.03$, $P = 0.40$; Fig. 4B).

Upon detecting ER, we used RT-PCR to examine skeletal muscle expression of the estrogen synthetic enzyme aromatase. Aromatase was undetected in all muscles (data not shown) so the role of E2 in avian male muscle function is unknown.

Regulation

We quantified both AR and ER mRNA from nonbreeding manakin limb muscles with or without treatment of T or E2. We found a significant treatment effect on AR expression ($F_{2,24} = 19.41$, $P < 0.0001$) with no effect of tissue ($F_{3,24} = 1.41$, $P = 0.27$) or interaction ($F_{6,24} = 1.857$, $P = 0.13$; Fig. 5A). T and E2 significantly down-regulated AR from control levels (T, $P < 0.0001$; E2, $P = 0.0002$). This suggests that AR expression can be regulated by an estro-

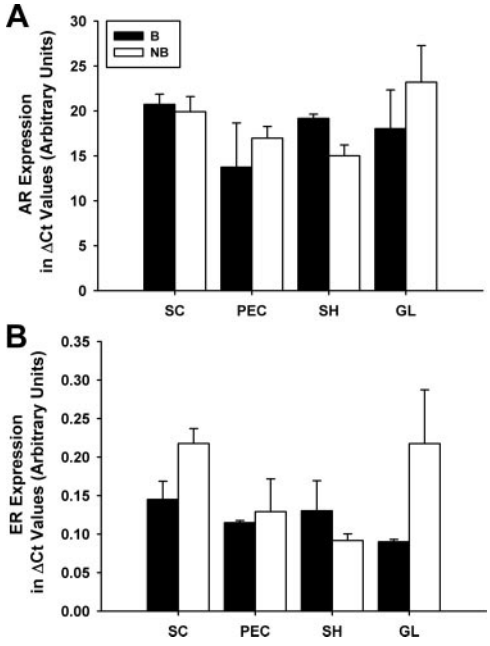

FIG. 4. A, AR mRNA expression in limb muscles of breeding (B) and nonbreeding (NB) male manakins (n = 3 for all groups). No significant seasonal differences were detected. B, ER mRNA expression in limb muscles of B and NB male manakins (n = 3 for all groups). No significant seasonal differences were detected.

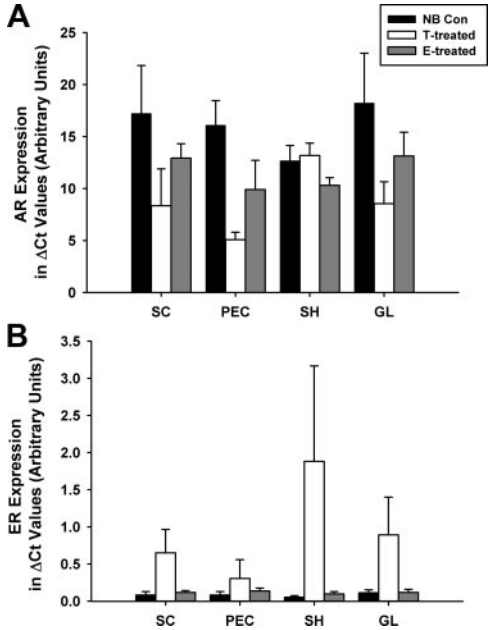

FIG. 5. A, AR mRNA expression in T-treated (n = 3 each), E2-treated (n = 3 each), and nontreated nonbreeding control (NB Con; n = 4 each) male manakin limb muscles. AR expression was significantly reduced in T and E2 treated birds. B, ER mRNA expression in T-treated (n = 3 each), E2-treated (n = 3 each), and NB Con (n = 4 each) male manakin limb muscles. ER expression was significantly increased in T-treated, but not E2-treated birds.

gen-dependent mechanism, confirming the presence of functional ER protein in skeletal muscle.

In addition, we found a significant treatment effect for ER expression in T and E2 treated birds ($F_{2,24} = 4.57$, $P = 0.02$; Fig. 5B): T significantly up-regulated ER in manakin muscles ($P = 0.02$), whereas E2 had no effect ($P = 0.87$). This suggests ER expression can be regulated by an androgen-dependent mechanism, confirming the presence of functional AR protein in skeletal muscle.

5α-Reductase

In one assay, we measured 5α-reductase activity in SC and GL of male and female manakins (Fig. 6A). Two-way ANOVA revealed a significant effect of muscle ($F_{1,20} = 11.91$, $P = 0.003$) with activity in the SC higher than the GL. There was no significant effect of sex ($F_{1,20} = 0.004$, $P = 0.95$) and no interaction between factors ($F_{1,20} = 0.01$, $P = 0.93$).

In a second assay, we measured 5α-reductase activity in SC and GL of male manakins and zebra finches (Fig. 6B). Two-way ANOVA revealed significant effects of both muscle ($F_{1,20} = 27.10$, $P < 0.0001$) and species ($F_{1,20} = 4.71$, $P = 0.04$) with a significant muscle*species interaction ($F_{1,20} = 78.45$, $P < 0.0001$). The manakin SC had significantly higher enzyme activity than the GL ($t_{10} = 2.82$, $P = 0.02$), confirming our results from the first experiment (Fig. 6A), and activity in the male manakin SC was significantly higher than that found in the male zebra finch SC ($t_{10} = 7.31$, $P < 0.0001$). There was significantly greater activity in zebra finch GL than in manakin GL ($t_{10} = 5.10$, $P = 0.0005$) and in zebra finch SC ($t_{10} = 9.22$, $P < 0.0001$).

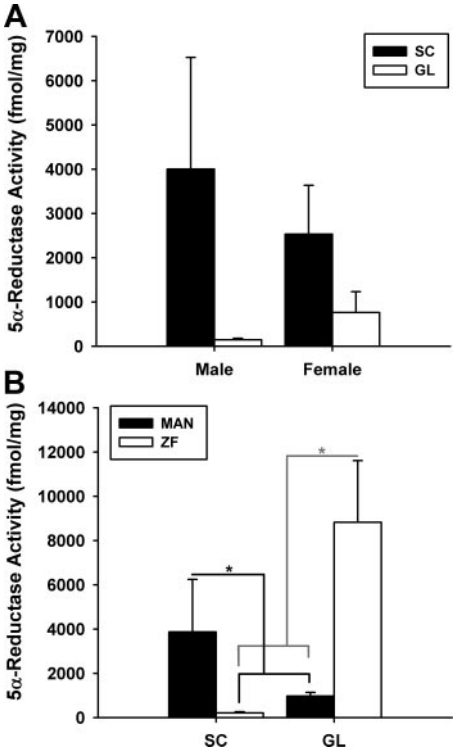

FIG. 6. A, 5α-Reductase activity in SC and GL of male and female manakins (n = 6 for all groups). Activity was significantly higher in SC than in GL but was not different between males and females. B, 5α-Reductase activity in SC and GL of male manakins and zebra finches (n = 6 for all groups). Male manakin SC had significantly higher 5α-reductase activity than GL and male zebra finch SC (*black lines*). Zebra finch GL had higher enzyme activity than SC and the GL of manakins (*gray lines*). *, $P < 0.025$ for all.

Discussion

Results presented here confirm our prediction that several limb muscles in male manakins express AR at substantially higher levels than in the same muscles of species that do not perform athletic courtship displays. AR levels found in manakin limb muscles are comparable with that of the zebra finch syrinx, a known androgen target muscle (of relatively small mass), but conspicuously greater than present in nonmuscular androgen targets such as the testis, brain, and spinal cord. This manakin AR expression profile differs strikingly from that found in other species, such as humans and mice, whose skeletal muscles generally express low levels of AR (22). Thus, elevated AR expression, and perhaps also elevated 5α-reductase activity, may be part of an underlying androgen-dependent muscle phenotype that evolved in manakins to enable their physically-intensive courtship display. Elevated skeletal muscle AR expression may be a characteristic of other species with elaborate courtship, such as members of the Pipridae family to which the manakins belong, and the charismatic birds of paradise in Australasia (23, 24). Thus, manakins offer us valuable insights into differential muscle hormone dependence as demanded by animals with different life histories and reproductive strategies, making them useful models for studying individual, sex, species, and age-related differences in hormonal influence on athleticism and muscle physiology.

We predicted that males would have higher AR expression than females and that the wing muscles would have higher AR expression than the GL. Incongruent with these predictions, comparable levels of AR expression were found in all manakin limb muscles examined from both sexes, including the GL, a leg muscle that, unlike manakin wing muscles, exhibits no sexually dimorphic or species differences in mass, fiber size, or myosin expression (13). Thus, elevated levels of 5α-reductase in the SC compared with the GL may be required to masculinize the sexually dimporphic characteristics found in the SC by providing the potent androgen 5α-dihydrotestosterone to activate AR. There is little evidence for a role for 5α-reductase in skeletal muscle in any species as opposed to its crucial

function in most other androgen targets (9, 17, 25). This enzyme may have a more widespread role in muscle function in species that employ numerous neuromuscular systems in complex physical displays.

Female manakins showed similarly elevated AR expression and 5α-reductase activity as in males and greater than those seen in both sexes of zebra finches and in male flycatchers. Females naturally have low levels of T year round, levels apparently insufficient to activate AR and wingsnapping behavior. Notably, wingsnapping can be induced in captive females treated with T (26). Presumably, high AR expression and 5α-reductase activity in the SC contribute to the female's capacity to perform behavioral elements of male courtship in the presence of exogenous T.

The exact mechanisms promoting elevated AR in manakin skeletal muscles are unknown. T treatment, electrical stimulation, or exercise can up- or down-regulate AR in skeletal muscles of other species (9, 11, 27–31). T might influence muscle hypertrophy in male manakins in part due to its actions on central targets that motivate performance of display behavior and the exercise of limb muscles. However, our results suggest that muscles are important peripheral targets of direct T action, and that the anabolic effects of T acting on AR may be responsible for inducing hypertrophy and sexually dimorphic characteristics seen in male manakin muscles. Furthermore, the elevated AR levels that we see in muscles of manakins with low T (females and nonbreeding males) who do not normally wingsnap, as well as in the GL, argues that the increased androgen responsiveness in manakin muscles is a constitutive property of this species, and not a by-product of exercise. Androgens acting on AR have been shown to promote muscle hypertrophy through multiple pathways, including increasing contractile protein synthesis, stimulating satellite cell differentiation to increase fiber size or number, and recruiting pluripotent cells to the myogenic lineage (9, 10, 32, 33). These androgen-mediated mechanisms may operate in manakin skeletal muscles to induce their hypertrophy, predisposing these birds to engage in the physically complex behaviors for which they are so well known. Future studies that directly investigate the effects of exogenous T on expression of genes involved in myotrophy in manakin muscles are now warranted.

Birds evolved extensive anatomical and physiological adaptations to enable flight, including many that minimize body mass (34). Accordingly, for most flighted birds, skeletal muscles became the most massive organs in the body (up to 40% of total body mass) with those muscles that lift and retract the wings having proportionately the greatest mass (35). With this is mind, and given the elevated AR expression we have found, manakins can now be seen as possessing an extraordinary body-wide degree of androgen-sensitivity. As a consequence, when T is elevated during the prolonged courtship season, there is likely an inordinately large impact on AR-dependent genes and muscular functions. Such actions are probably energetically demanding and could be viewed as important "costs" of T, a well-recognized by-product of androgen-dependent masculine stimulation (36). Females may actually select males based on their ability to withstand these added costs of T (36). We have evidence that females prefer males that perform their courtship display more rapidly (37), so circulating T levels and the degree of muscle AR activation may impact the speed of courtship displays to make males more attractive to females.

Few studies have examined ER expression in skeletal muscle. ER and aromatase were undetected in zebra finch syrinx (7), suggesting limited estrogen-dependent muscular support of songbird vocalizations. By contrast, we detected ER expression in all limb muscles examined, but these levels were low and were not coupled with expression of aromatase. Thus, it is unclear whether male muscles are normally exposed to significant levels of circulating estrogen to impact this minimal ER expression. The fact that T up-regulated ER and E2 down-regulated AR in manakin skeletal muscles is evidence that estrogens act on these limb muscles. Muscle responsiveness to estrogens is poorly understood (38, 39) so manakins may be a useful model for exploring estrogen action on muscle.

Many animals engage in physically complex courtship displays that, like manakins, often involve mechanical sound production (sonation) using their limbs, tails, or other structures (23, 40). Because adaptations in the size and physiology of muscles must be closely fitted to the lifestyle of any given species (35), we believe that the degree of androgen sensitivity in muscles directly influence these adaptive differences. More than has been previously appreciated, androgens may have important control over a diverse array of neuromuscular systems in many vertebrate species.

Acknowledgments

We thank Dr. Xuqi Chen for assistance with the ABI 7300 sequence detection system.

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This work was supported by the National Science Foundation Grants IBN-0213194 and IBN-0646459. This work was also supported by the Undergraduate Research Center/Center for Academic and Research Excellence Office, the Rose Hills Foundation, and the Amgen Foundation.

Disclosure Summary: The authors have nothing to disclose.

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