Research Resource: Hormones, Genes, and Athleticism: Effect of Androgens on the Avian Muscular Transcriptome

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Male vertebrate social displays vary from physically simple to complex, with the latter involving exquisite motor command of the body and appendages. Studies of these displays have, in turn, provided substantial insight into neuromotor mechanisms. The neotropical golden-collared manakin (*Manacus vitellinus*) has been used previously as a model to investigate intricate motor skills because adult males of this species perform an acrobatic and androgen-dependent courtship display. To support this behavior, these birds express elevated levels of androgen receptors (AR) in their skeletal muscles. Here we use RNA sequencing to explore how testosterone (T) modulates the muscular transcriptome to support male manakin courtship displays. In addition, we explore how androgens influence gene expression in the muscles of the zebra finch (*Taenopygia guttata*), a model passerine bird with a limited courtship display and minimal muscle AR. We identify androgen-dependent, muscle-specific gene regulation in both species. In addition, we identify manakin-specific effects that are linked to muscle use during the manakin display, including androgenic regulation of genes associated with muscle fiber contractility, cellular homeostasis, and energetic efficiency. Overall, our results point to numerous genes and gene networks impacted by androgens in male birds, including some that underlie optimal muscle function necessary for performing acrobatic display routines. Manakins are excellent models to explore gene regulation promoting athletic ability. (***Molecular Endocrinology*** **30: 254–271, 2016)**

Complex social behavior in vertebrates, including humans, relies on the exquisite integration of higher-level processes that govern decision making and downstream motor processes that control movement and behavioral output (1–3). Exploration of such mechanisms focuses predominantly on the former (4–7), with little work examining the way in which peripheral motor systems contribute to the production of physically intensive and complex behavior. This leaves a gap in our understanding of the physiological basis of animal athleticism and how organisms produce extraordinary feats of physical balance, postural control, and limb movement (8).

Gene expression profiles in musculoskeletal tissues are key determinants of physical ability and prowess. Engineered variation in the transcription of only one or two genes, for example, significantly enhances organismal performance and endurance (9–12). This suggests that such altered gene expression in select muscles underlies an

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Abbreviations: AR, androgen receptor; ARE, androgen response element; ER, estrogen receptor; FDR, false discovery rate; GO, Gene Ontology; NTC, novel transcript cluster; PC, principal component; PCA, principal component analysis; PEC, pectoralis; RNA-Seq, RNA sequencing; RPKM, reads per kilobase of exon per million mapped reads; SH, scapulohumeralis caudalis; SLC, solute carrier membrane transport protein.

ability to produce behavioral displays, particularly those that are athletic, by incorporating unusual movement patterns not otherwise performed in everyday life. Little is known about either the way in which muscle-wide gene expression relates to extreme physical movements or how such gene expression patterns are modulated to fine-tune these behaviors.

Courtship behaviors are performed in a reproductive context, and in males, the steroid hormone testosterone (T) often activates such behavior by acting via either androgen receptors (AR) or estrogen receptors (ER). Once bound to T, the AR functions as a potent transcription factor in target cells throughout the body (13). This means that circulating T adjusts gene expression in select tissues that express high levels of AR, and thereby alters tissue functionality in a way that supports the masculine reproductive phenotype (14–17). However, the degree to which androgens transcriptionally impact distinct skeletal muscle systems with mechanically unique properties to promote animal courtship and territoriality is unknown.

In the current study, we examine how androgens affect the muscular transcriptome to accommodate the production of an adaptive acrobatic sexual display. We investigate the golden-collared manakin (*Manacus vitellinus*) because males of this passerine avian species perform an especially acrobatic dance to court females and compete with rivals (18, 19). A main feature of this display is the wing snap, whereby males rapidly and forcefully hit their wing together above their heads either once or up to 23 times (at approximately 55 Hz) to produce loud, firecracker-like sonations (20, 21). This maneuvering requires contractions of muscles that lift (supracoracoideus; scapulohumeralis caudalis [SH]) and depress (pectoralis [PEC]) the wings, and these tissues possess anatomical and biochemical specializations to support this behavior (22–24). Foremost among these traits is that all three of these muscles express significantly more AR, compared to similar bird species with less robust motoric wing displays (8, 25). Our studies indicate that the androgen activation of AR in the manakin forelimb musculature promotes both gross- and fine-motor control during wing-snap production (21, 26). This work supports the conclusion that elevated AR expression in the SC, SH, and PEC is an adaptive trait that facilitates the production of elaborate wing movements by promoting a robust transcriptional response to elevated T during the breeding season (8, 25, 27).

Here we use next-generation RNA-Sequencing (RNA-Seq) to perform two studies that address the issue described above. In the first study, we treat nonbreeding male golden-collared manakins with exogenous T. We then measure the effects of this treatment (+T group vs −T control group) on the transcriptome of the SH and PEC because these muscles guide wing extension and contraction during the wing snap (19). In the second study, we specifically examine the AR-dependent transcriptomic profiles of the SH and PEC in the zebra finch (*Taenopygia guttata*), another passerine bird species that is a well-established model in avian research and the first songbird with a sequenced genome (28). Moreover, the zebra finch expresses far less AR in its skeletal muscles than the manakin, giving us the opportunity to examine androgen effects on the same muscles that vary with regard to the amount of AR they contain. To this end, it is well established that the zebra finch performs no significant appendage movement in courtship (29), which also allows us to make some general comparisons with the acrobatic manakin.

To perform this latter experiment, we assess the transcriptome of the SH and PEC in male zebra finches treated with both T and the AR antagonist flutamide (+Flut group) or with T by itself (−Flut group). Although the treatments of these two experiments are different, their ultimate endocrine effects on muscle are in theory highly similar. In other words, the effect of the +T manipulation in manakins is fully comparable with that of the −Flut manipulation in zebra finches, whereas the effect of the −T (control) manipulation in manakins is indirectly comparable with the +Flut manipulation in zebra finches. Furthermore, avian wing muscles contain little aromatase, the enzyme that converts T into estradiol, which activates ER (25). Thus, although our manipulations are unlikely to activate estrogenic pathways, inhibition of AR allows us to directly assess this possibility.

One obstacle to our transcriptome analyses is the incomplete annotation of their genomes. To overcome this problem, we conducted in-depth transcriptome reconstruction to improve gene annotation using RNA-Seq data. In addition to routine differential gene expression analysis, we analyzed gene coexpression networks to infer potential functional pathways. We also elucidate genes specifically related to display behavior in manakins. Altogether, these studies identify specific genes and gene networks that are androgen dependent both across muscle and species. The experiments similarly confirm, using the zebra finch, that most effects observed in golden-collared manakins are mediated by AR itself, rather than by alternate pathways (eg, ER), if T is aromatized to estradiol (25). Finally, our work points to a suite of candidate genes that are likely integral to muscle performance in a manner that supports the production of the manakin acrobatic display routine.

Materials and Methods

Animals

All experiments and animal work were conducted according to the relevant national and international authorities (University of California, Los Angeles, and Smithsonian Tropical Research Institute Animal Research Committee protocol number 2009–123-13). Bird euthanasia was performed via rapid decapitation (see below) according to this protocol.

Male golden-collared manakins were captured via passive mist netting in the lowland rainforests of Panama, near the town of Gamboa. All captures occurred in the nonbreeding season (August through September), when adult males cease producing their elaborate courtship displays for females and when their circulating T levels are basal (30). Male zebra finches were collected from a laboratory colony at University of California, Los Angeles (Los Angeles, California), at which they were housed in open-flight aviaries (1.8 × 1.8 × 1.2 m).

Experiment 1: androgenic manipulation in golden-collared manakins

Upon capture, male manakins were immediately brought to the Smithsonian Tropical Research Institute facilities in Gamboa where they were housed individually in cages 32 cm × 29 cm. All cages were held in the same room in visual and acoustic contact. Birds were given water and fed papaya ad libitum. Additional housing conditions are described in detail elsewhere (27).

Upon arriving at the laboratory, males were assigned at random to receive either a T implant (n = 3) or a blank (control) implant (n = 3). Implants were made from 12-mm SILASTIC brand tubing (Dow Corning Corp; 0.76 mm inner diameter, 1.65 mm outer diameter) filled with 10 mm of crystalline T (or nothing). The ends of each implant were sealed with 1 mm of SILASTIC brand adhesive. Both T-filled and control implants were inserted sc at the base of the neck according to standard procedures described elsewhere (21, 26, 27, 31). Past work shows that identical T implants replicate male breeding levels of plasma T (31, 32), activate male manakin courtship (32), and influence gene expression in manakin skeletal muscle (27); given these previous validations of these implants in golden-collared manakins, we did not measure plasma T from these individuals. All birds were euthanized by rapid decapitation 9–16 days after initiation of T treatment, shortly after birds were observed routinely producing wing snaps and thus confirming sufficient muscle androgen exposure to promote courtship (21, 25). Whole bodies were immediately flash frozen on dry ice and were stored at −80°C until tissues were dissected and RNA extracted (see below).

A muscle that provides lift (the SH) and depression (the PEC) were later dissected from these individuals for transcriptomic analysis. Therefore, for this experiment, we obtained T-treated SH and PEC muscles (hereafter referred to as $+T_{SH}$ and $+T_{PEC}$, respectively) and non-T-treated SH and PEC (hereafter referred to as $-T_{SH}$ and $-T_{PEC}$, respectively).

Experiment 2: hormone manipulation in zebra finches

Male zebra finches were taken from the open-flight aviaries and placed in smaller cages so that all individuals were in constant visual and acoustic contact with each other. Birds were provided water and seed ad libitum.

Each bird was assigned at random to receive either a flutamide implant (n = 3) or a blank (control) implant (n = 3). On the subsequent day, every male received an implant filled with T. All implants (including control implants) were identical with those described in experiment 1. In the zebra finches, however, implants were placed sc in the fat pads of the left and right flanks, immediately above the hip and below the wing. Further details regarding the implantation of males and the dose of the implants is described in detail elsewhere (27). Importantly, prior studies have validated that the amount of flutamide we administer is sufficient to block all AR in the body of a zebra finch and induce no obvious adverse side effects (27, 33). This treatment was designed to assess whether there is a population of genes in zebra finch skeletal muscles that are subject to AR control, despite the somewhat lower levels of AR present in skeletal muscles of this species, and whether genes regulated by T in manakins are similarly AR dependent in zebra finches.

The SH and PEC were dissected from males 7 days after the implantation of flutamide, and they were flash frozen on dry ice to preserve RNA. In this experiment, we therefore obtained AR-inhibited SH and PEC (hereafter $+Flut_{SH}$ and $+Flut_{PEC}$) and AR-activated SH and PEC muscles (hereafter $-Flut_{SH}$ and $-Flut_{PEC}$), respectively.

RNA extraction and sequencing

Total RNA was extracted from zebra finch and golden-collared manakin muscle tissues using Trizol (Life Technologies). Additional column purification and deoxyribonuclease I treatment were applied using a Direct-zol RNA kit (Zymo Research). The integrity of the extracted total RNA was analyzed by BioAnalyzer, and the standard Illumina protocol was used to prepare strand-specific libraries for RNA-Seq (TruSeq mRNA library preparation kit, with ribosomal RNA removal and polyA selection). Using gel electrophoresis, the DNA fragments in the libraries with an insert size of approximately 250 bp were isolated, amplified, and sequenced on the Illumina HiSeq 2000 sequencer in the paired-end sequencing mode (2 × 100 bp reads).

To reduce potential batch effects, RNA extraction and library generation were carried out for all samples using the same batch of reagents on consecutive days. The samples were handled in a randomized order in these procedures. Once the libraries were finished, sequencing was carried out by which four libraries were pooled in one lane. A total of six lanes were sequenced for the 24 samples. Importantly, each lane contained one replicate from each group of samples (eg, 1 of each from $-T_{SH}$, $+T_{SH}$, $-Flut_{SH}$, $+Flut_{SH}$ in one lane). Thus, biological replicates were sequenced in separate lanes. This design minimizes the potential batch effects that may confound analyses comparing between species or treatment. Note that the two muscle types were sequenced in different lanes.

RNA-Seq mapping

Raw sequencing reads were first processed by cutadapt (version 1.2.1, https://code.google.com/p/cutadapt/) to remove any adapter sequences. The parameters for cutadapt are as follows; -e 0.2 -a AGATCGGAAGAGCACACGTCTGAACTC CAGTCAC -m 36 -n 7 -O 5, -e 0.2 -a GATCGTCGGACTG-

TAGAACTCTGAACCTGTCG -m 36 -n 7 -O 5. Zebra finch RNA-Seq reads were aligned to the zebra finch genome (WUGSC 3.2.4/taeGut1) and transcriptome (Ensembl release 68, http://www.ensembl.org/) using a method described previously (34, 35); Blat and Bowtie were used for read alignment and the mapping parameters are as follows: BLAT (version 3.4): -minIdentity = 75 -stepSize = 8; Bowtie (version 0.12.9): -samnosq -S -k 80 -e 140 -n 3 -l 20 -q -phred33-quals -p 1 -y. Uniquely mapped pairs of reads were retained for further analysis. For golden-collared manakin RNA-Seq read mapping, the newly assembled golden-collared manakin genome was used as reference sequences (36). The mapping method was otherwise similar as for the zebra finch data.

Expression of genes and analysis of androgen-responsive differential gene expression

Gene annotations were obtained from Ensembl for zebra finch (release 68) and Beijing Genome Institute for golden-collared manakin (36). To assess the expression levels of genes, their reads per kilobase of exon per million mapped reads (RPKMs) values were calculated (37) and normalized by the trimmed mean method implemented in the edgeR package in R (38). To test the efficacy of this normalization procedure, we examined the expression levels of a set of known housekeeping genes (39). The correlation of RPKM values of these genes across samples prior to and after normalization was then examined (Supplemental Figure 1). We reasoned that the intercept of the linear regression line should be closer to zero after normalization if potential batch effects were alleviated by this procedure (see *Results*).

Using the gene RPKM values, a principal component analysis (PCA) was performed using the prcomp module in R. To investigate the impact of androgen on gene expression in each species, we tested differential gene expression between (−Flut vs +Flut) or (+T vs −T) groups for each species. To this end, we used the edgeR method (implemented in R) that assumes a negative binomial distribution for read counts of genes or transcripts. EdgeR estimates the genewise dispersions by conditional maximum likelihood. Differential expression was then assessed for each gene using an exact test analogous to the Fisher's exact test but adapted for the overdispersed data (38). *P* values resulted from the exact test were further corrected via the Benjamini and Hochberg method (40) that estimates the false discovery rate (FDR) based on the *P* values. To define significantly differentially expressed genes, we required an FDR cutoff of 5% and a minimum expression fold change of 2. This fold change was calculated as the ratio between the average RPKM values of each gene in the two groups under comparison. The minimum fold change cutoff was determined by examining levels of expression changes of a few known androgen-responsive genes (*MMP9*, *CKS2*, *SDC1*, *BIRC5*) (41). All these genes had expression changes of at least 2-fold between the control and the treated samples in zebra finch.

Analysis of species-specific genes and their androgenic response

To define a set of species-specific genes, we examined the control samples of each species and analyzed differential gene expression between the $-T_{SH}$ and $+Flut_{SH}$ and between the $-T_{PEC}$ and $+Flut_{PEC}$ groups. Similarly as described above, trimmed mean normalization and the edgeR method in R (38) were used and differentially expressed genes were defined as those that passed the cutoffs: FDR of 5% or less and RPKM fold change of 2 or greater. The resultant genes are termed as species-specific genes. It should be noted that the comparisons between species may be confounded by batch effects due to differences in the sample acquisition time and location for the golden-collared manakin and zebra finch. We evaluated this potential issue and the normalization procedure using a set of housekeeping genes (Supplemental Figure 1) (see *Results*).

After defining the species-specific genes using the control samples, we analyzed whether these genes responded to androgen manipulation in each species. Specifically, we compared their expression levels between the −T and +T or the +Flut and −Flut groups for each muscle type of each species. This analysis was again carried out using edgeR in R (38) and the same significance cutoffs as described above (FDR $\leq$ 5 and fold change $\geq$ 2). The final identified genes are those that are species specific and androgenic responsive in each species.

We also predicted whether the species-specific, androgen-responsive genes are potentially direct targets of AR. Promoter sequences of these genes were analyzed to identify androgen response elements (AREs) using the CisGenome program (42). AREs are specific DNA response elements recognized by AR receptors (43). Three distinct ARE sequences (MA0007.1, MA0007.2, and MA0007.3) are available in the JASPAR database (44), which were used in this analysis. Putative direct AR target genes were predicted as those that have at least one ARE with the motif score significantly higher than expected by chance (likelihood ratio $\geq$ 1000). A similar analysis was also carried out for all orthologous genes of manakin and zebra finch.

Gene Ontology (GO) analysis

GO refers to a set of structured and controlled vocabularies used in describing the attributes of genes and gene products for all species (45). GO descriptions fall into three domains: cellular component, molecular function, and biological processes. Thus, each known gene is annotated for its likely involvement in one or more of the three domains. We downloaded GO term annotations of each gene from the Ensembl database and carried out GO enrichment analysis similarly as described previously (46). Specifically, to identify GO terms that are enriched in a specific set of genes (eg, those that are differentially expressed between two groups), the number of genes in the set with a particular GO term was compared with that in the control gene sets. A control gene set was constructed so that the randomly picked controls and the test genes have one-to-one matched gene length. Control of gene length in this analysis is appropriate because it is known that GO enrichment analysis of differentially expressed genes resulted from RNA-Seq experiments can be confounded by gene length (47). Based on 10 000 randomly selected control sets, a *P* value for enrichment of each GO term in the test gene set was calculated as the fraction of times that F_{test} was lower than or equal to $F_{control}$, where F_{test} and $F_{control}$ denote, respectively, the fraction of genes in the test set or a random control set associated with the current GO category. A *P* value cutoff (1/total number of GO terms considered) was applied to choose significantly enriched GO terms.

Transcriptome reconstruction

Because transcript annotation for zebra finch or golden-collared manakin may not be complete, we conducted RNA-Seq-based transcript reconstruction using methods described by Lee et al (46) to achieve a comprehensive identification of all exons. Briefly, the expressed regions (based on the uniquely mapped RNA-Seq reads) in each known annotated gene were analyzed to identify possible novel exons in known genes (a method called guided transcriptome reconstruction [46]). This type of isoform reconstruction was informed by spliced junction reads between known exons or novel exons. In addition, we carried out a de novo isoform reconstruction that is independent of transcript annotation in intergenic/unannotated regions of the zebra finch or golden-collared manakin genome (46). This method identified novel transcript clusters (NTCs) that corresponded to novel genes. For NTCs, we compared their sequence similarity with known genes in other organisms via a basic local alignment search tool search against human, mouse, rat, and chicken Ensembl cDNA sequence databases and Ref-Seq mRNA databases. Default basic local alignment search tool parameters were used.

Gene coexpression network analysis

To understand the relationships between genes at the genome-wide level, we constructed weighted gene coexpression network using the WGCNA method (48, 49). All known genes (Ensembl r68) and newly identified NTCs (≥3 RPKM) in this study were included in this analysis. A total of 28 and 41 network modules were identified for golden-collared manakin and zebra finch, respectively. For network modules associated with the different categories ($+T_{SH}$, $-T_{SH}$, $+T_{PEC}$, and $-T_{PEC}$ in golden collared manakin and $-Flut_{SH}$, $+Flut_{SH}$, $-Flut_{PEC}$, and $+Flut_{PEC}$ in zebra finch), we calculated their eigengenes and correlations between eigengenes and sample categories. We defined a significant association as those that have r (correlation) greater than 0.5 and a value of $P < .1$. To analyze the module preservation between the two species, we defined orthologous genes according to gene annotations from a previous study (36). In the previous study, the whole genome was annotated by the Genewise method with Ensembl genes from three species; zebra finch, human, and chicken. We used the zebra finch Ensembl genes as orthologous genes for both species and the orthologous genes that were expressed in our samples were used for the module preservation analysis. In the WGCNA package, we used the module preservation function to calculate Zsummary. If the Zsummary of the module is 10 or greater, the module is strongly preserved between two species. The module is moderately preserved if the Zsummary is between 2 and 10. Otherwise, the module is considered as not preserved (48, 49). To identify hub genes in each significantly associated network module, we calculated gene significance, which indicates the biological relevance of a gene with respect to the trait of interest based on the correlation between gene expression profiles and the trait, and intramodular gene connectivity, which is a measurement of module membership by correlating its gene expression profile with the module eigengene of a given module. We defined the gene as a hub gene if the gene significance is greater than 0.6 and the intramodular gene connectivity is greater than 0.9.

Real-time quantitative PCR validations

RNA extracted from the muscle tissues (see above) was used to perform real-time quantitative PCR for the validation of differential expression results. Methods used to carry such procedures out are described in detail elsewhere (21, 25, 27, 31). A total of eight genes (four from each species) related to muscle functioning and/or basic cellular processing was selected for validation. Species-specific primers were used for both the manakins and zebra finches, both of which were designed from each species respective genome (Supplemental Table 1).

Results

RNA-Seq reads mapping

In all treatment groups of golden-collared manakins and zebra finches, we obtained total RNA samples from the SH and PEC, muscles involved in lift and depression, respectively. Three biological replicates were collected for each sample type. Sample handling was randomized and sequencing libraries were pooled to minimize potential batch effects across groups (see *Materials and Methods*). A total of approximately 343 (an average per sample: 29 million pairs of reads) and approximately 356 (an average per sample: 30 million pairs of reads) million pairs of reads (2 × 100 bp) were obtained for golden-collared manakin and zebra finch respectively. Approximately 154 (manakin, an average per sample: approximately 13 million pairs of reads) and 209 million pairs (finch, an average per sample: approximately 17 million pairs of reads) of reads were uniquely mapped and properly paired corresponding to the paired-end mode (Table 1). For the manakin, 79% of the uniquely mapped reads overlapped the putative gene annotations based on the assembled genome draft. For the zebra finch, 69% of the uniquely mapped read pairs overlap known genes and exons.

Principal components analysis (PCA)

Prior to the PCA, the expression levels (RPKM) of annotated genes in all samples were normalized by the trimmed mean method to minimize potential batch effects

Table 1. RNA-Seq Read Mapping Summary

	Total Sequencing Pairs	Unique Pairs	Multiple Pairs	Low-Quality Pairs or no Pairs	Unmapped Reads
Golden-collared manakin	343, 979, 761	154, 900, 601	21, 376, 372	20, 709, 007	293, 987, 562
Zebra finch	356, 429, 620	209, 266, 962	60, 263, 547	11, 287, 572	151, 223, 078

(see *Materials and Methods*). Batch effects may exist between samples derived from the two species because they were inevitably acquired at different sites and different times. To evaluate the effectiveness of the normalization procedure, we examined a set of housekeeping genes (39), assuming their expression levels are similar across species. After normalization, the correlation of their expression values across species was less biased compared with that prior to normalization, as reflected by the intercept values being closer to zero (Supplemental Figure 1). The improvement in the intercept values was especially evident for the control samples (−T and +Flut), and the across-species comparisons were highly correlated. Thus, the normalization procedure was relatively effective in reducing potential batch effects.

PCA was first carried out for all samples in this study. This analysis reveals that 83% of the variation in gene expression is explained by the first three principal components (PCs) (Supplemental Figure 2). Interestingly, the first PC separates the data according to species (manakin vs zebra finch) (Figure 1A). To better resolve the variation across samples within a species, we conducted a PCA again, using samples from each species separately (Figure 1B). We observed that the second PC resulted from these analyses separates the data according to muscle (PEC vs SH) in both species, similarly as in Figure 1A. Importantly, the third PC separates the data according to androgen treatment; however, this effect is evident only in the golden-collared manakin and not in the zebra finch (Figure 1B). Together these findings identify the dominant factors in our study that influence gene expression profiles of avian wing muscles. Although the two main factors are species and muscle, androgenic action is the third most influential factor that impacts transcriptomic variation. Most importantly, the third PC (androgen treatment) clusters the data only in the golden-collared manakin, likely highlighting the strong species specificity in which androgenic steroids modify the transcriptional machinery of target tissues. As might be expected, modulation occurs to a greater degree in the species and tissues that not only express higher amounts of AR but also that produce the complex courtship display (25, 31).

Differential gene expression

The transcriptomic profile of the golden-collared manakin wing musculature is highly responsive to T treatment (Figure 2 and Table 2). In the SH muscle involved in wing lift, 534 (~4.21%) genes in the annotated transcriptome are differentially regulated in response to T implantation. Most genes (496, ~3.91%) are up-regulated by T, whereas a lower number of genes (38, ~0.3%) are down-regulated (Figure 2A). In the PEC muscle involved in wing

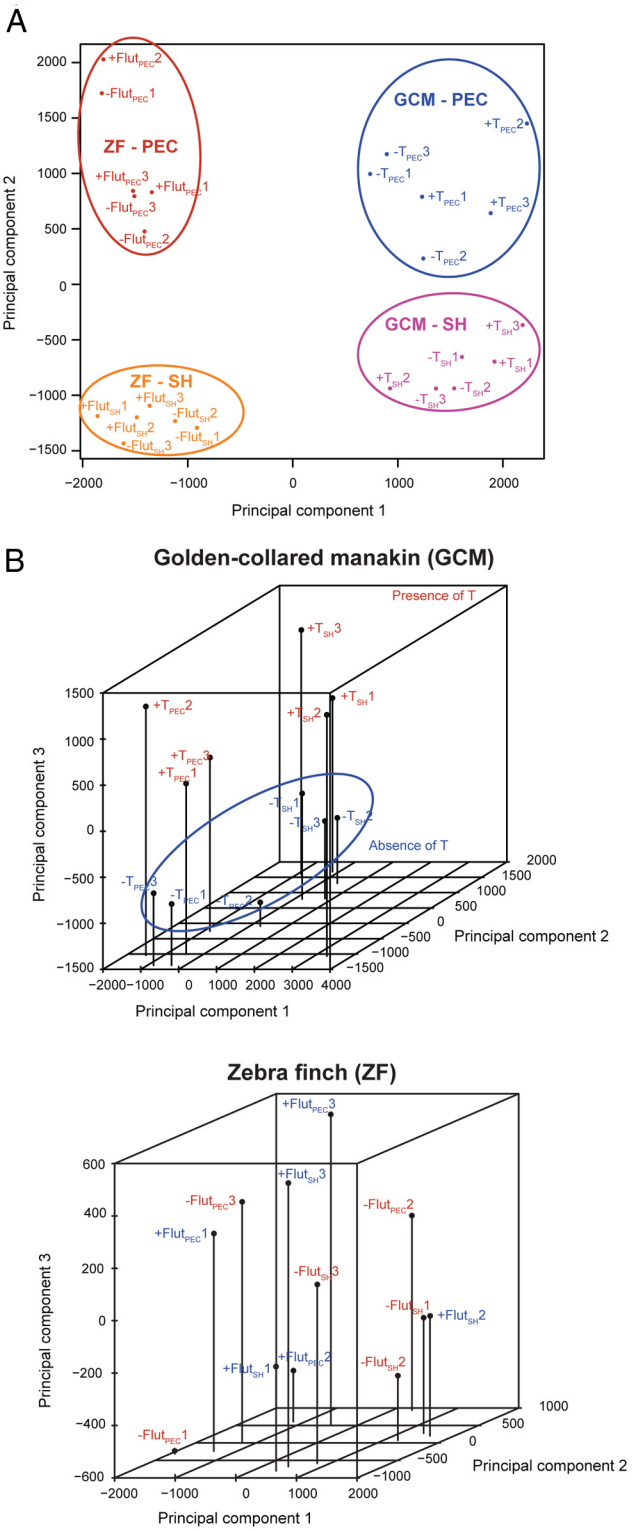

Figure 1. PCA of zebra finch (ZF) and golden-collared manakin (GCM) gene expression profiles for different samples (three biological replicates in each group). A, Two-dimensional scatter plots for (x-axis) and PC2 (y-axis). PC1 classifies the species between ZF and GCM, and PC2 differentiates two types of the muscle, pectoralis (PEC) and scapulohumeralis caudalis (SH). B, Three-dimensional scatter plots for three principal components. For GCM but not ZF, the third principal component (PC3) classifies the two groups of samples treated with T or otherwise.

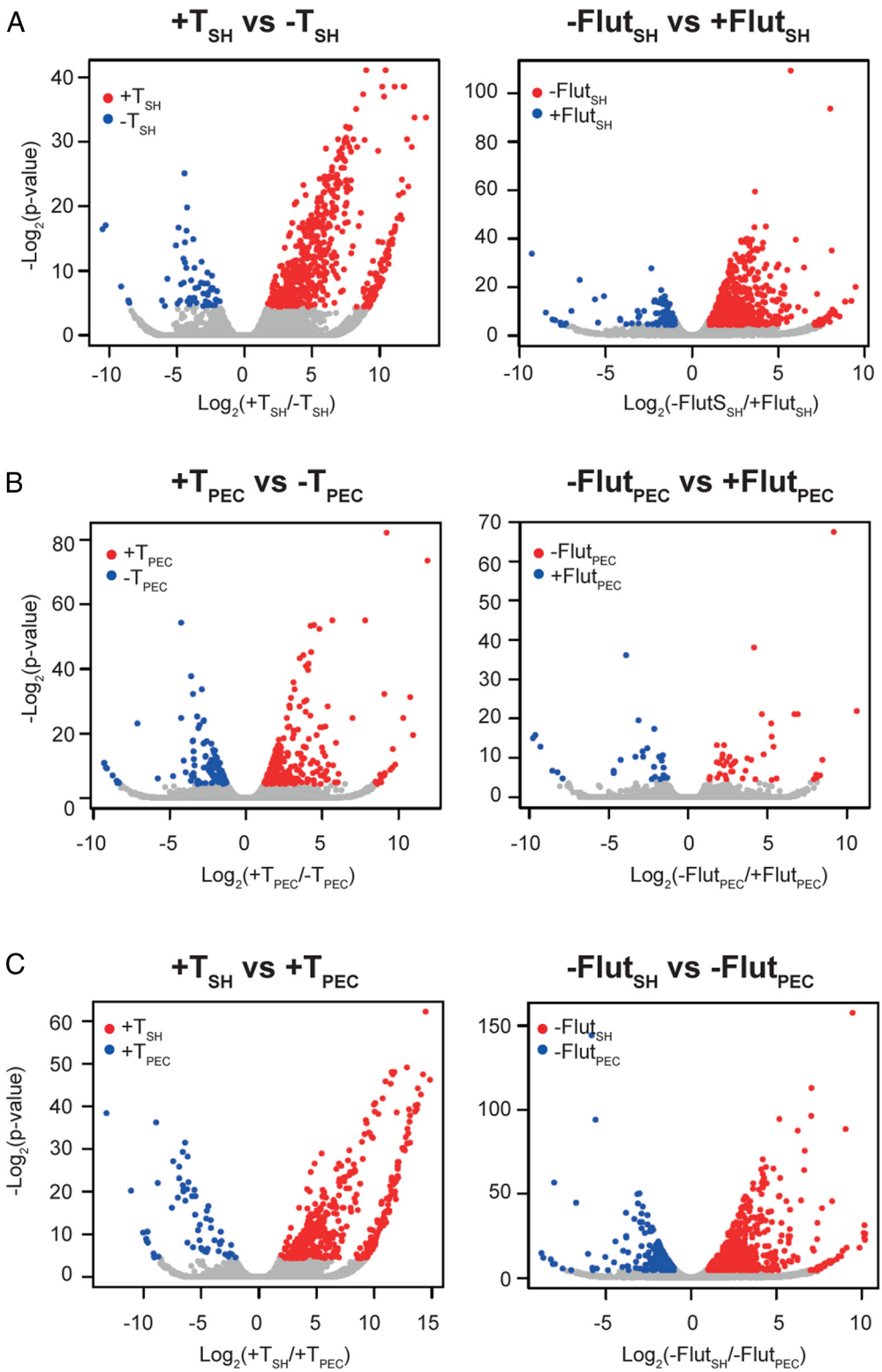

Figure 2. Differential gene expression between pairs of sample groups in golden-collared manakin (GCM) or zebra finch (ZF). The volcano plots show the differentially expressed genes as red and blue colors. The x- and y-axes represent the magnitude of fold changes (log_2 transformed) and the adjusted *P* value (−log2) by Benjamini-Hochberg correction, respectively. A, Differential gene expression in SH between the samples treated with T and otherwise. Left panel, Golden-collared manakin ($+T_{SH}$ vs $-T_{SH}$); right panel, zebra finch ($-Flut_{SH}$ vs $+Flut_{SH}$). B, Differential gene expression in PEC between the samples treated with T and otherwise. Left panel, Golden-collared manakin ($+T_{PEC}$ vs $-T_{PEC}$); right panel, zebra finch ($-Flut_{PEC}$ vs $+Flut_{PEC}$). C, Differential gene expression between the muscle samples, SH and PEC, in the presence of T. Left panel, Golden-collared manakin ($+T_{SH}$ vs $+T_{PEC}$); right panel, zebra finch ($-Flut_{SH}$ vs $-Flut_{PEC}$).

Table 2. Number of Differential Expressed Genes

Comparison (A vs B)	Overexpressed in A Group	Overexpressed in B Group	Total
Golden-collared manakin			
$+T_{SH}$[a] vs $-T_{SH}$[b]	496[c] + 77[d]	38 + 20	534 + 97
$+T_{PEC}$[e] vs $-T_{PEC}$[f]	222 + 51	93 + 28	315 + 79
$+T_{SH}$ vs $+T_{PEC}$	418 + 61	50 + 11	468 + 72
Zebra finch			
$-Flut_{SH}$[g] vs $+Flut_{SH}$[h]	582 + 55	95 + 16	677 + 71
$-Flut_{PEC}$[i] vs $+Flut_{PEC}$[j]	28 + 14	22 + 6	50 + 20
$-Flut_{SH}$ vs $-Flut_{PEC}$	685 + 63	431 + 52	1117 + 115

[a] $+T_{SH}$: SH muscle in the presence of T treatment in golden-collared manakin.
[b] $-T_{SH}$: SH muscle in the absence of T treatment in golden-collared manakin.
[c] Number of differentially expressed annotated genes.
[d] Number of differentially expressed NTCs.
[e] $+T_{PEC}$: PEC muscle in the presence of T treatment in golden-collared manakin.
[f] $-T_{PEC}$: PEC muscle in the absence of T treatment in golden-collared manakin.
[g] $-Flut_{SH}$: SH muscle in the presence of T treatment in zebra finch.
[h] $+Flut_{SH}$: SH muscle in the presence of T treatment with flutamide in zebra finch.
[i] $-Flut_{PEC}$: PEC muscle in the presence of T treatment in zebra finch.
[j] $+Flut_{PEC}$: PEC muscle in the presence of T treatment with flutamide in zebra finch.

depression, 315 genes (~2.61%) are differentially regulated in response to T, with the majority (222, ~1.84%) being up-regulated and fewer (93, ~0.77%) being down-regulated (Figure 2B). Thus, T induces mostly positive effects on gene expression, consistent with the known function of transcriptional activation by androgens (50, 51). Importantly, T has a relatively greater impact on the SH than the PEC (P = 4.8E-12, Fisher's exact test). Androgens therefore seem to modify gene expression patterns differently in wing muscles that serve distinct biomechanical purposes. Our results also reveal significant differences in the way that androgens affect gene expression in the manakin SH and PEC, namely, only 1% of genes are differentially expressed when these two muscles are directly compared with each other without T treatment (ie, −T groups); however, approximately 4% of genes are differentially expressed when the two muscles are compared after T treatment (ie, +T groups) (Figure 2C). This means that T increases the number of genes that are differentially expressed between the SH and PEC and that T has muscle-specific effects on gene expression ($P <$ 2.2E-16, Fisher's exact test). If T were to have the same transcriptional effect on each of these muscles, then we would expect the percentage of differentially expressed genes between the muscles to be the same with or without T (−T vs +T groups).

Although patterns of differential gene expression in zebra finch wing muscles are similar to those in the manakins, their magnitude is noticeably different between SH and PEC (Figure 2A). In the zebra finch SH, for example, 677 genes in the annotated transcriptome (~5.02%) are differentially regulated when ARs are activated (−Flut) vs when AR are inhibited (+Flut). Most of these genes (582, ~4.32%) are up-regulated, whereas relatively few (95, ~3.21%) are down-regulated. By contrast, only 50 (~0.38%) genes are differentially regulated by AR activation (−Flut) in the PEC, with 28 of these genes (~0.22%) being up-regulated and 22 (~0.17%) being down-regulated (Figure 2B). Furthermore, when we compare the SH and PEC in AR inhibited (+Flut) groups, we find that 394 genes (~3%) are differentially regulated. When we compare these two muscles in AR-activated (−Flut) groups, we find that 1116 genes (~8.32%) are differentially regulated (Figure 2C). These data bear a close resemblance to those collected in manakins; that is, the activation of AR appears to elicit different transcriptional responses in the SH and PEC (Figure 2C).

Notably, our results also highlight that androgenic treatment differentially affects the SH and PEC between species. These finding is shown through species comparisons of the relative number of genes within the genome affected by androgens. Consequently, androgen action (ie, +T or −Flut) influences the expression of a significantly greater proportion of the genome in the golden-collared manakin PEC, compared with the zebra finch PEC ($P <$ 2.2E-16, Fisher's exact test). These species differences in the impact of androgenic action within the SH are much smaller compared with PEC (P = .002, Fisher's exact test). Although direct species comparisons in our analysis should be considered cautiously because AR manipulations differed across species (see *Discussion*), these results reveal substantial variation in androgenic responsiveness of muscles across birds. Accordingly, these findings are consistent with the observations that PC3 in our

PCA analysis above cannot separate samples in the zebra finch but can separate samples in the manakin.

To assess the validity of these differential expression analyses, we also examined androgen-dependent changes in the expression of eight genes using quantitative PCR (four genes each from the manakin and zebra finch SH; Supplemental Figure 3 and Supplemental Table 1). The results from both methods were significantly correlated ($R = 0.73$, $P = .033$; slope = 1.923), which corroborates the RNA-Seq analysis.

Gene ontology analysis

To assess the functional effects of T on muscle, we performed a GO enrichment analysis on the genes that were differentially expressed between treatment and tissues (Supplemental Table 2). In manakins, genes up-regulated in the SH of the +T group were enriched in a total of 44 GO terms, whereas genes that were down-regulated in this muscle and group were enriched with four GO terms. By contrast, genes up-regulated in the PEC of the +T group were enriched in 11 GO terms, whereas genes down-regulated in this muscle and group enriched five GO terms. GO terms identified in the SH included several linked to metabolic processing as well as ion and protein transport and binding. GO terms identified in the PEC included starvation responsiveness as well as cholesterol storage and processing. Furthermore, we assessed GO terms enriched from genes that were differentially expressed between the SH and PEC specifically within the +T group. In this case, genes expressed more abundantly in the SH were enriched in 31 GO terms, whereas genes expressed more abundantly in the PEC were enriched in only 11 GO terms. Many of the SH GO terms were related to metabolic processing and ion and protein transport, whereas the PEC GO terms were related to protein metabolism. Thus, it appears that T mediates the metabolic capability of both forelimb muscles but through different pathways.

In the zebra finch, we performed a similar GO enrichment analysis of differentially expressed genes. In the SH, we identified 53 GO terms enriched by genes that were up-regulated in −Flut group and 11 GO terms enriched by genes up-regulated in the +Flut group (Supplemental Table 3). Many of these GO terms were linked to the maintenance of cell structure and general cellular processes. In the PEC, we identified seven GO terms that were enriched in the −Flut group and another seven GO terms enriched by genes up-regulated in the +Flut group (Supplemental Table 3). Several of these GO terms were related to synaptic transmission. In addition, we examined GO terms enriched by genes differentially regulated between the zebra finch SH and PEC. In the −Flut groups, we identified 37 GO terms enriched by genes more abundantly expressed in the SH, relative to the PEC. We also identified 59 GO terms enriched by genes more abundantly expressed in the PEC, relative to the SH (Supplemental Table 3). The GO terms associated with genes expressed more in the SH were linked with muscle filament structure and sliding mechanics as well as components of glucose metabolism. The GO terms associated with genes abundantly expressed in the PEC were largely involved in mitochondrial physiology and aerobic respiration. Importantly, these results resemble those obtained in the manakin, wherein androgens have robust effects on skeletal muscle gene expression that differ between the biomechanically different SH and PEC.

RNA-Seq-based annotation of transcriptomes

Because the golden-collared manakin and zebra finch genomes are relatively new and not yet fully annotated, we sought to improve the annotations of their transcriptomes using the RNA-Seq data to obtain a more complete view of the AR-related transcriptome variations. Applying our previously developed transcriptome reconstruction methods (43), we identified a large number of novel isoforms for known genes of the two genomes. Transcriptome reconstruction for golden-collared manakin was carried out using the partially assembled genome scaffold sequences as reference. In the regions with gene annotations (36), we reconstructed 7449 multiexon genes, and among them, 5440 (73%) genes were expressed at three or more RPKMs. Among the reconstructed genes, 4946 (66%) have at least one alternatively spliced exon. In genomic regions without any known genes (ie, intergenic regions), we discovered 6398 NTCs, potentially representing novel genes (Supplemental Table 4). A total of 1524 NTCs were expressed at three or more RPKMs in our RNA-Seq data.

For the 6594 multiexon genes in zebra finch (defined by Ensembl) with an expression level of three or more RPKMs, 4468 (68%) were detected with novel isoforms as a result of alternative splicing, 5682 (86%) as a result of alternative initiation or termination, and 3944 (60%) with both types of novel isoforms. Similarly as golden-collared manakin transcript reconstruction in intergenic regions, 7316 NTCs were discovered based on RNA-Seq (Supplemental Table 5). Importantly, many NTCs had detectable expression in other zebra finch tissues. In public RNA-Seq data derived from testes, spleen, muscle, liver, skin, and embryo (52), 1906 NTCs were detected with RNA-Seq reads in at least one tissue, among which more than 60% were expressed at three or more RPKMs with two or more reads per exon (Supplemental Figure 4).

Among all NTCs discovered above, a total of 2916 in golden-collared manakin (of 6398, 46%) and 3485 in zebra finch (of 7316, 48%) were identified with sequence similarity to known genes in other species (see *Materials and Methods*) (Supplemental Tables 4 and 5). These putative annotations of NTCs may help to predict their function (see below).

Differential expression of NTCs

For all NTCs identified in each species, we compared their expression levels across different sample groups using the same method as for known genes. A total of 197 and 353 NTCs were differentially expressed between at least one comparison in the manakin and zebra finch, respectively (Supplemental Tables 4 and 5). Although many of these NTCs were similar to genes associated with cellular signaling and basic cellular processing, a number were also similar to genes that play a role in muscle contraction. For example, in manakins, +T individuals showed a greater than 6000-fold increase in the expression of an NTC with a sequence homology to the human myosin 18A (*MYO18A*) gene. Likewise, when comparing SH and PEC muscles in +T manakins, the SH showed a greater than 1000-fold increase in the expression of an NTC similar to the human myosin 5B (*MYO5B*) gene. These data indicate that androgens increase the expression of novel myosin-like transcripts that may be involved in motor control. Unfortunately, given their novelty, it is not yet possible to determine whether these NTCs help generate movement like classical myosin filaments in striated skeletal muscle or whether they are unconventional myosins that guide cellular trafficking (53, 54).

The results in the zebra finch paralleled those in the manakin. We found that −Flut individuals increased the expression of NTCs that were similar to human myosin genes. These include myosin heavy chain 2 (*MYH2*) and myosin 1F (*MYO1F*), which show 7- and 34-fold greater expression in the SH compared with +Flut individuals, respectively. Additionally, we found that NTCs with similarity to human myosin light chain kinase 2 (*MLCK2*) and myosin heavy chain 6 (*MYH6*) were expressed approximately 133- and 4-fold more in the PEC of −Flut individuals, respectively. One of these myosins, *MYH2*, is a component of the skeletal muscle contractile apparatus (55), suggesting that generally, as in manakins, activation of the AR modulates the contractility of zebra finch skeletal muscle. Notably, however, the novel AR-dependent myosin genes differed between the zebra finch and manakin. Together these data suggest that the AR-dependent myosin isoforms in manakins and zebra finches likely enhance muscle contraction ability, and such effects are more pronounced in manakins that require rapid muscle contraction-relaxation cycling to produce their displays.

Gene coexpression network

Coexpression patterns of genes may help reveal gene networks or pathways that are related to sample phenotypes. Using the combination of known genes and NTCs, we identified 28 and 41 significant coexpression network modules for golden-collared manakin and zebra finch, respectively. The hierarchical clustering dendrogram and corresponding network modules are shown in Supplemental Figure 5. We then calculated the module eigengenes and the correlations between eigengenes and sample types. This analysis resulted in 17 network modules in the manakin and 20 in the zebra finch that are strongly associated with one of the four sample categories in each species (manakin: $+T_{SH}$, $-T_{SH}$, $+T_{PEC}$, $-T_{PEC}$; zebra finch: $+Flut_{SH}$, $-Flut_{SH}$, $+Flut_{PEC}$, $-Flut_{PEC}$,) (r; correlation > 0.5 and $P < .1$; Supplemental Tables 6 and 7 and Supplemental Figure 6).

For the significant network modules associated with $+T_{SH}$, $+T_{PEC}$, $-Flut_{SH}$, and $-Flut_{PEC}$, we performed a GO enrichment analysis (Supplemental Tables 8 and 9). For both species, enriched GO terms involved mitochondrial function and cellular metabolic processes, including both carbohydrate and lipid metabolism, as well as general cellular function. These results suggest that hormonal control of muscle fiber metabolic efficiency may be an especially important target of regulation in wing muscles used for flight in these birds and for courtship in manakins.

We next extracted and examined hub genes from these network modules (Supplemental Tables 10 and 11). Such genes are centrally located in their respective modules and might be essential nodes in the network. Interestingly, many identified hub genes were up-regulated genes in the differential expression tests. For example, in the manakin SH in +T group, the turquoise cluster was enriched with several myosin transcripts, including myosin 7B (*MYO7B*), myosin 7A (*MYO7A*), myosin heavy chain 9 (*MYH9*), and myosin 5B (*MYO5B*) (Supplemental Table 10). These are mainly unconventional myosins that serve a variety of motor and regulatory functions (56, 57). The turquoise cluster was also enriched with a variety of solute carrier membrane transport proteins (SLCs). Of particular interest in this latter group are the SLCs that belong to family 25 because these genes play an integral role in oxidative phosphorylation and thus support aerobic respiration (58). This suggests that a principal action of androgens on manakin muscle fibers is the transcriptional regulation of the muscular motor complex and energy-producing capacity.

In the SH of −Flut zebra finches, hub genes similar to those in the golden-collared manakin enrich the turquoise cluster, including *MYH9*, myosin 5A (*MYO5A*), and myosin 9B (*MYO9B*) (Supplemental Table 11). Because these myosins are considered unconventional (56, 57), their role in skeletal muscle performance is unclear. Another hub gene that enriched the turquoise module was tropomyosin 4 (*TPM4*), a marker of muscle growth and repair in response to muscular damage and/or stress (59). Finally, various modules (turquoise and blue) expressed SLCs from the family 25. These results suggest the transcriptional regulation of hub genes related to the muscle motor complex and aerobic capacity is indeed dependent on ARs because they are seen in both manakins and zebra finches.

Potential function of NTC hub genes

Previously we proposed a method to infer the potential function of NTCs based on WGCNA network module analysis (43). This method is built upon the assumption that functionally related genes involved in the same biological pathways or protein interaction networks often demonstrate correlated expression patterns (60). Thus, one approach to infer the potential function of novel genes is by determining whether their expression patterns correlate with those of known genes of certain function, based on coexpression analysis. We applied this method to predict the potential function of NTCs, focusing on those that are hub genes in the network. In the golden-collared manakin, 100 NTCs were identified as hub genes, 21 of which had at least 10 neighboring genes as known genes. The GO analysis results of these 21 NTCs are included in Supplemental Table 12, with the network of one example NTC (scaffold 427:1335928–1338736−) shown in Figure 3A. Notably, many of these NTC hub genes regulated downstream gene products, with enriched GO terms again related to the myosin complex and the sarcolemma. All of these NTC hubs were clustered into the turquoise module, suggesting that T induced broad modulation of genes linked to the fiber complexity and structure of muscle fibers.

In the zebra finch networks, a total of 67 NTCs were identified as hub genes. To predict the function of the NTCs, we further required that at least 10 neighboring genes of an NTC were known genes. After this filter, 37 NTCs were retained and GO enrichment analysis of the neighboring genes of each NTC was performed. The enriched GO terms associated with each of the 37 NTCs are listed in Supplemental Table 13, and the network module of an example NTC (chrZ: 39643962–39656303+) is shown in Figure 3B. Many of these NTC hubs affected the downstream products with enriched GO terms linked to muscle function, such as the sarcomere structure and ac-

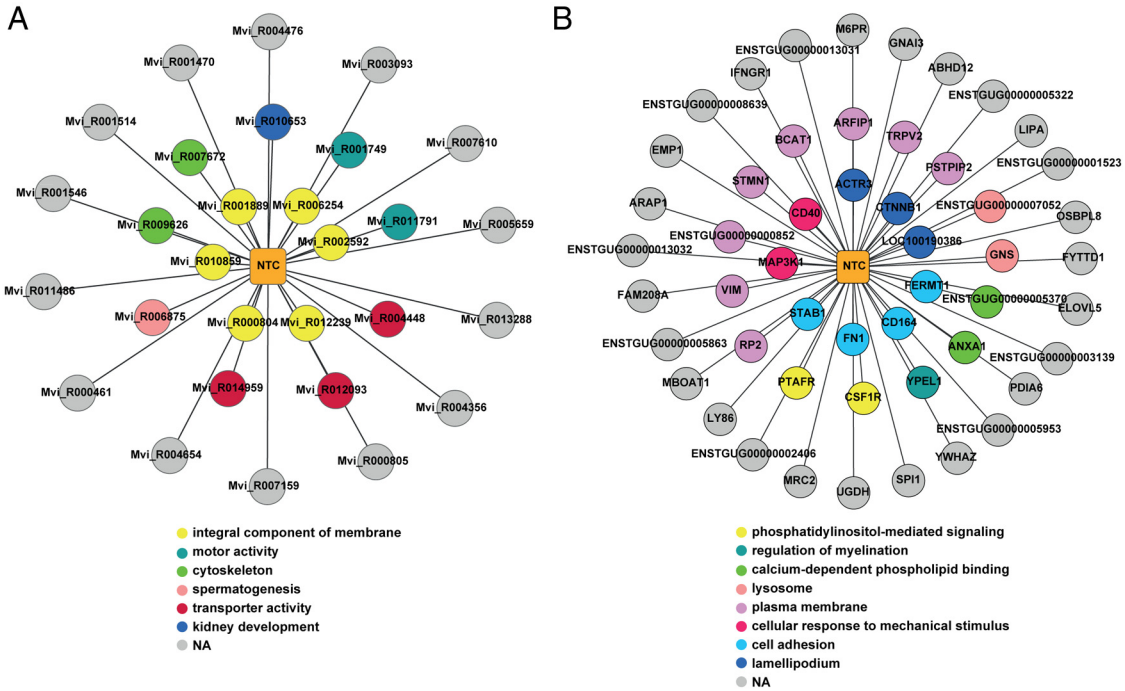

Figure 3. NTCs identified via RNA-Seq. Two examples of the NTCs and their neighboring genes in the gene coexpression networks are shown. The NTC is placed at the center of the network. Colored nodes represent genes that are involved in significant GO terms listed below the networks. A, Golden-collared manakin (NTC identification: scaffold 427: 1335928–1338736−). B, Zebra finch (NTC identification: chrZ: 39643962–39656303+).

tin binding. Moreover, one NTC affected a number of myosin and myosin-like genes integrally related to skeletal muscle structure, function, and performance.

Importantly, in both the manakin and zebra finch, not all of the enriched GO terms were related to muscle function per se. Many enriched GO terms were related to processes involved in traditional cell maintenance, signaling, and ion trafficking, which suggests that androgenic action may have a larger impact on avian skeletal muscle than previously recognized. Alternatively, the mechanisms by which androgens mediate the physiological changes in muscle tissue may be highly varied and not specifically linked to reorganization of muscle structure and contractile kinetics.

More broadly, the results collectively indicate that RNA-Seq-based gene annotation expanded the repertoire of genes that are functionally important to the transcriptomic signatures of the androgenic response. This deepens the framework in which we can consider the physiological effects of androgens on skeletal muscles and their saliency to each animal's phenotype.

Module preservation between zebra finch and golden-collared manakin coexpression networks

Next, to better assess the degree to which androgen-dependent gene expression in the muscle of manakins and zebra finches is a conserved phenomenon, we quantified the level of similarity in the coexpression network properties of both species. We approached this analysis in two ways. First, we used the golden-collared manakin coexpression network and the ortholog gene expression profile from the zebra finch and calculated a Zsummary preservation score (Figure 4A) via the WGCNA package. Then we performed the same analysis using the zebra finch coexpression network and the ortholog gene expression profile from the golden-collared manakin (Figure 4B). In the first case, a total of four network modules (white, brown, magenta, and turquoise) were strongly preserved (Zsummary score $\geq$ 10) with three modules associated with the +TS category and one module associated with −TS. Seven network modules (cyan, grey60, saddlebrown, dark gray, dark magenta, dark olive green, and red) were weakly preserved (2 $\leq$ Zsummary score $<$ 10). Of these, one was associated with $+T_{SH}$ and two were associated with $-T_{SH}$. The other 17 modules were not preserved. In the second analysis, we identified a total of three network modules (blue, turquoise, and dark orange) that were strongly preserved, and all three modules were significantly associated with the $-Fut_{SH}$ category. Seven network modules (green, dark green, salmon, light cyan 1, plum 2, dark turquoise and violet) were weakly preserved (Zsummary score was between 2 and 10). Two of seven weakly preserved modules were associated with $-Flut_{SH}$ (green) or $-Flut_{PEC}$ (violet). The other 31 network modules were not preserved.

Notably, most (three of four) significantly preserved network modules with a significant sample association were associated with the $+T_{SH}$ group in manakin. Similarly, all of the preserved modules with a significant sam-

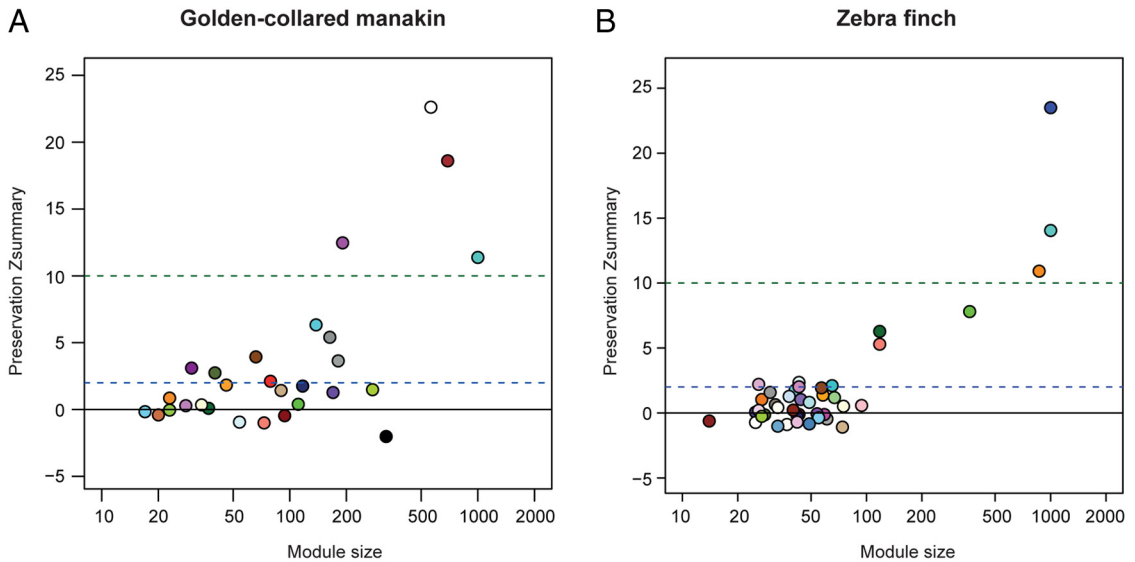

Figure 4. Module preservation between golden-collared manakin and zebra finch gene coexpression networks. The module preservation score, Zsummary (y-axis) for each network module was plotted relative to network module size (x-axis). If the Zsummary score is greater than 10, the module is considered as strongly preserved. A score between 2 and 10 indicates weak to moderate preservation, and a score below 2 suggests no preservation. A, Preservation analysis of the golden-collared manakin coexpression network. B, Preservation analysis of the zebra finch coexpression network.

ple association were associated with the $-Flut_{SH}$ group in zebra finch. Likewise, the network modules that were significantly associated with the zebra finch $-Flut_{SH}$ group were preserved in golden-collared manakin. Interestingly, no modules were preserved that were associated with $+T_{PEC}$ group, except the moderately preserved violet module in the zebra finch $-Flut_{PEC}$ group. These data provide support for the conclusion that T influences common mechanisms in the SH muscle of both species but is functionally different in the PEC.

Identification of transcripts related to manakin courtship

We identified transcripts linked specifically to acrobatic courtship displays in golden-collared manakins. Accordingly, we first examined the number of transcripts in each muscle that are constitutively expressed at different levels between the two species in the −T and +Flut conditions (see *Materials and Methods* for details about the expression profile normalization between taxa). In the SH, we found a total of 2527 manakin-specific genes and 1635 zebra finch-specific genes. In the PEC, we found a total of 2485 manakin-specific genes and 1661 zebra finch-specific genes. Next, within each species, we examined which of these differentially expressed (ie, species specific) transcripts were responsive to androgen manipulation. Because androgens act on the SH and PEC to modulate performance of manakin displays (8, 21), we reasoned that genes expressed in both a manakin-specific and androgen-responsive manner likely represent candidate genes that contribute to the physiological framework that underlies acrobatic display production. Thus, in the golden-collared manakin, we found 111 of these candidate genes in the SH, and 73 of these candidate genes in the PEC. Hypergeometric tests revealed that manakin-specific genes are significantly enriched with androgen-responsive genes compared with nonspecies-specific genes (SH: $P = .0059$; PEC: $P = .00077$). For zebra finch, 80 of the candidates genes in SH and seven of the candidates genes in PEC were identified as zebra finch specific and androgen responsive. Hypergeometric tests show that, for either muscle, zebra finch-specific genes were either not significantly enriched or were enriched with a much smaller degree of significance as were androgen-responsive genes in the manakin (SH: $P = .95$, compared with $P = .0059$ in manakin; PEC: $P = .016$, compared with $P = .00077$ in manakin). Altogether these findings demonstrate that, in either muscle, genes specifically expressed in golden-collared manakins are more androgen responsive than genes specifically expressed in zebra finches. Furthermore, genes expressed specifically in the golden-collared manakin are generally more androgen responsive, compared with genes that are not expressed specifically in this species. This is not the case in the zebra finch because relatively fewer genes specifically expressed in this species are also androgen responsive.

We next sought to examine the functionality of the candidate genes for acrobatic courtship that were derived in the above analysis (Supplemental Table 14). In the manakin SH, the 111 candidate genes were enriched in 26 GO terms linked to steroid metabolism, sodium channel activity, and membrane transport. Especially notable is the number of GO terms related to lipid metabolism, particularly through the expression of apolipoprotein B (*APOB* gene) and microsomal triglyceride transfer protein (*MTTP* gene). These GO terms (and the genes with which they are associated) facilitate fatty acid oxidation in skeletal muscle (61), which produces energy to fuel myocyte function and thus help sustain rigorous physical activity (62–64). Accordingly, our data suggest that modulation of the machinery that governs fatty acid metabolism underlies the athletic-like nature of the manakin display. In the manakin PEC, the 73 candidate genes are enriched in seven GO terms. Although the GO terms in the PEC differ from those in the SH, many of them are also linked to muscle fuel metabolism. Namely, we found GO terms centered on glycogen metabolic processes as well as ion transmembrane transport and activity. Of particular interest within these terms is the presence of solute carrier 22A5, which helps mediate carnitine-based mechanisms (65) of lipid oxidation (65, 66). These findings again support the idea that fatty acid oxidation helps form the basis of acrobatic display performance.

In this same vein, we also identify a number of GO terms that identify androgen-responsive genes that are not expressed in a species-specific way (Supplemental Table 15). We assume that these terms represent relatively conserved functional effects of androgens on avian skeletal muscle, and they include a variety of processes (eg, membrane structure and composition, citrate transport, transporter activity, etc). Notably, some of these GO terms are also related to lipid metabolism (triglyceride homeostasis, very long chain fatty acid-CoA activity). This suggests that the regulatory processes to enhance lipid-based fuel acquisition in manakins may be evolutionarily enhanced and/or modified to support energetic demands required for display performance.

Putative genes directly affected by AR

Overall, we found more orthologous genes having promoter ARE motifs in the golden-collared manakins (5168) compared with the zebra finch (3473), a finding consistent with the notion that the manakin is generally

more responsive to androgenic action than the zebra finch.

Using a similar analysis, we next focused on the species-specific and androgen-responsive genes highlighted above. We found that, in the manakin SH, 61 of 111 genes (55%) contained AREs in their promoter regions. This represents a significant proportion of genes enriched with promoter binding motifs that potentially respond directly to AR compared with the whole gene set (hypergeometric test, $P = .011$). In the manakin PEC, however, we found that only 31 of 73 genes (42.5%) contained AREs in their promoter, a nonsignificant effect (hypergeometric test, $P = .64$). In the zebra finch, the SH had 29 of 80 genes (36.3%) with at least one putative promoter ARE, whereas the PEC had two of seven of these genes (28.6%); compared with whole-gene sets, neither of these results was significant (hypergeometric tests; SH: $P = .11$, PEC: $P = .66$). These results suggest that AR has the ability to directly regulate a variety of genes in wing muscles, but in the manakin SH, AR can directly modulate transcription of relatively more genes compared with the PEC and the zebra finch. These results are consistent with other results indicating that manakins have evolved mechanisms to enable especially potent androgen-dependent gene regulation within a key muscle used in their athletic display performance.

Discussion

The genomic and physiological mechanisms that facilitate or constrain motor systems underlying complex social behavior are poorly studied. To investigate this issue, we integrate RNA-Seq experiments with powerful bioinformatic approaches and study how androgenic hormones regulate the muscle transcriptome of a passerine bird with an acrobatic and physically intensive courtship behavior as well as a related passerine species with no such display. In our studies, we identify numerous genes that are transcriptionally regulated by androgen action within the main forelimb musculature. Androgen effects are noticeably varied, modulating a range of gene pathways that regulate diverse cellular processes, including muscle fiber structure and general muscle fiber metabolism. When we focus specifically on those androgen-dependent genes that are specific to the golden-collared manakin, that is, those genes most closely associated with the capacity to perform physically intensive courtship, we identify a suite of genes that are functionally related to lipid and glycogen metabolism. Thus, androgens seem to generally exert effects on tissue size and strength (67) but also impact processes related to energy mobilization and cellular respiration to facilitate courtship. These findings support the hypothesis that T promotes adaptive motor skills (17) by both modifying muscle contractility and enhancing muscle energetic efficiency.

Androgens and skeletal muscle gene expression

The effects of T on the manakin muscular transcriptome are mediated through AR, a conclusion based both of past work and our current results. First, we know that manakins and zebra finches express no aromatase in their skeletal muscles and thus are unable to locally convert T into an active estrogenic ligand (25). Male golden-collared manakins also express significantly more AR in forelimb muscles compared to other avian species that have similar levels of circulating androgen but that use their wings less for courtship and territorial behavior (8, 25). Experimental studies also demonstrate that AR activation is required for male manakins to perform both gross- and fine-motor skills during displays (21, 26). Second, results presented here indicate that the elevated levels of AR in manakin wing muscles are quantitatively associated with their transcriptional response to circulating androgens (21); that is, compared with zebra finches with lower muscle AR expression, androgens regulate a larger proportion of the muscular transcriptome in manakins. This relationship encompasses all differences we find with respect to previously annotated transcripts, novel transcripts, and module hub genes. Furthermore, we also find evidence that manakins, particularly in the SH muscle, are more susceptible to direct modulation of gene expression by AR itself. Nevertheless, our preservation analysis between coexpression networks of manakins and zebra finches reveals significant similarity in gene modules that were differentially expressed in response to each species' androgenic treatment (ie, +T vs −T in the manakins and −Flut vs +Flut in the zebra finch). Importantly, and as discussed below, we also identify a suite of manakin-specific/androgen-dependent genes that likely underlie the athletic muscle use of males of this species.

Despite this large body of compelling evidence that the endocrine manipulations used in both studies are functionally the same (ie, they both activate exclusively AR dependent pathways), we cannot definitely rule out that flutamide did not block all AR in zebra finches. Thus, our treatment in zebra finches is not perfectly matched with those in manakin muscle. While our results should be viewed through this lens, there is considerable evidence for the efficacy of flutamide antagonism of AR (68, 69). Moreover, effects of flutamide given at the dose we selected are functionally comparable with elimination of T in a variety of experimental paradigms (26, 33, 70, 71).

Notwithstanding this caveat, our findings highlight intriguing species commonalities and differences that likely contribute to the evolution of species-specific motor needs.

Another notable finding is that, in both manakins and zebra finches, androgens exert different effects on the transcriptomes of a muscle involved in elevating the wing (the SH) as compared with a muscle involved in depression of the wing (the PEC). Not only do androgens influence the expression of a greater number of genes in the SH than in the PEC, but they also affect different functional endpoints. In manakins, for example, T influenced genes related to ion transport and trafficking in the SH but influenced protein metabolism in the PEC. Moreover, these effects were usually mutually exclusive, in that T did not impact protein metabolism in the SH, nor did T extensively impact ion transport in the SH. The functional significance of these effects may be rooted in differences in each muscle's physiological make-up, given that they serve unique biomechanical roles. Limited past work in both species supports this view by showing some muscle-specific variation in morphological and histological traits, including relative muscle mass and fiber type (22). Further study of the biomechanical properties of these muscles is now warranted.

Equally interesting is that, although absolute AR expression levels differ markedly across species, within a species, SH and PEC AR levels are indistinguishable (8, 25, 31). This suggests that other mechanisms, such as transcription factor profiles or androgen metabolic machinery, adjust the nature of androgen action on a given muscle to drive tissues-specific gene expression. Nonetheless, these findings highlight the dynamic way through which the level of circulating androgen can influence unique functional properties of distinct skeletal muscles crucial for optimal behavioral performances. This represents a fascinating potential line of future research.

Androgens, muscular gene expression, and athletic courtship

Although we document many commonalities across species with regard to androgen action on muscle, we also identify a suite of manakin-specific androgen-dependent genes that we hypothesize underlie the intensive muscle use associated with the manakin's athletic-like courtship. We show that many of these genes are especially susceptible to direct modulation by AR because there is a significantly greater proportion of genes in the manakin SH that contain putative AREs in their promoter regions. From a functional perspective, these genes are largely related to lipid metabolism. Our previous work showing that the muscle-intensive manakin display poses little energetic demand, even though it is performed repeatedly and at great speed (72), is consistent with the idea that enhanced energy use represents a significant androgen-dependent function in the muscles of these animals. Thus, on top of their species-wide effects on size and contractility, androgens seems to promote courtship, and perhaps other muscle-dependent reproductive functions, by specifically enhancing energy use.

It is interesting to note that, compared with the PEC, the zebra finch SH also shows a relatively high transcriptional response to T. However, these birds do not use their wings in courtship (29) suggesting that, across birds, androgens modify the SH to permit seasonal or contextual fine-tuning of wing kinematic ability, perhaps contributing to wing movements used in the social signaling behaviors of other bird species that birds incorporate wing movements (73–77). By contrast, selection may have maintained more limited androgen responsiveness of the PEC to preserve its crucial flight-related functions. With these ideas in mind, we suspect that specialized wing movements and/or flight routines are integrated into sociosexual displays in many species as a result of AR expression and AR-dependent transcriptional regulation in the SH and other wing muscles (8). These ideas merit further investigation, given that they may help unlock the way in which animal motor systems are able to evolve and accommodate specialized reproductive movement.

Obviously we cannot rule out the impact of factors other than courtship behavior that might also account for some of the species variation in gene expression. Genetic drift or other phylogenetic factors, for example, may explain some species specificity of certain differentially expressed genes. Yet, because the two species differ so dramatically in the physicality of courtship routines and because that many of the genes we identified influence muscle performance and endurance, we view variation in courtship behavior as the primary factor linked to species-specific profiles of gene expression.

Androgenic effects by way of the spinal cord

It is important to note that the spinal cord may have influenced some of our results. For example, species variation in gene expression might be driven by noncell autonomous mechanisms, the source of which could be motoneurons that innervate the wing muscles and whose cell bodies reside in the spinal cord. Golden-collared manakins and many of its close relatives express AR in their spinal cords (8), with somewhat greater AR expression in golden-collared manakins than in zebra finches (8, 31). Thus, species differences in muscle gene expression may be attributed, in part, to androgen action on the

spinal cord, with downstream, transsynaptic regulation of the SH or PEC.

Our results also suggest that there may be species differences in the way that androgens influence spinal motor and sensory neurons via actions at the level of the muscle. Studies show not only that AR activation of some target muscles triggers axonal retrograde transport of signals from muscle to the spinal cord (14, 78) but also that these effects are vital for appropriate motor function (79, 80). With this in mind, because our results indicate that the functional impact of androgen on skeletal muscle is in some ways fundamentally different across species, we expect that the mechanisms that underlie such muscle-to-spinal signaling may similarly vary. If so, then altering the level of androgen sensitivity in a muscle might affect the degree to which androgens maintain motor unit capacity. Such bottom-up maintenance of motor control may be especially important to the golden-collared manakins (21), considering its need for rapid, agile movements during display performances (20, 81, 82).

Conclusions

In summary, we use RNA-Seq to identify androgen-dependent genes and gene networks in the skeletal muscles of two passerine species. We find striking differences by which androgens impact genes and gene networks in a muscle that lifts vs a muscle that depresses the wing. Because only one of these species, the golden-collared manakin, engages these muscles in a physically complex courtship display, our results demonstrate genes likely involved in the muscular control of this behavior. To our knowledge, this study is the first to investigate how steroid hormones regulate the transcriptome of skeletal muscle to support the production of physically elaborate social behavior. In addition to our transcriptome reconstruction improving gene annotation of the golden-collared manakin and zebra finch genomes, providing a valuable resource for the research community, this work reveals hormonal and molecular skeletal muscle pathways underlying an extraordinary vertebrate behavior.

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Data accessibility included the following: RNA-Seq data (record number GSE73660) are stored on Gene Expression Omnibus at the National Center for Biotechnology Information (Bethesda, MD).

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References

1. **O'Connell LA, Hofmann HA.** The vertebrate mesolimbic reward systems and social behavior network: a comparative synthesis. *J Comp Neurol*. 2011;519:3599–3639.
2. **O'Connell LA, Hofmann HA.** Evolution of vertebrate social decision-making network. *Science*. 2012;336:1154–1157.
3. **Goodson JL, Evans AK, Lindberg L, Allen CD.** Neuro-evolutionary patterning of sociality. *Proc R Soc B*. 2005;272:227–235.
4. **Bass AH, Chagnaud B.** Shared developmental and evolutionary origins of neural basis of vocal-acoustic and pectoral-gestural signaling. *Proc Natl Acad Sci USA*. 2012;109:10677–10684.
5. **Remage-Healey L, Coleman ME, Oyama RK, Schlinger BA.** Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. *Proc Natl Acad Sci USA*. 2010;107:3852–3857.
6. **Fuxjager MJ, Forbes-Lorman RM, Coss DJ, Auger CJ, Auger AP, Marler CA.** Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. *Proc Natl Acad Sci USA*. 2010;107:12393–12398.
7. **Lim MM, Wang ZX, Olazabal DE, Ren XH, Terwilliger EF, Young LJ.** Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature*. 2004;429:754–757.
8. **Fuxjager MJ, Eaton J, Lindsay WR, et al.** Evolutionary patterns of adaptive acrobatics and physical performance predict expression profiles of androgen receptor—but not oestrogen receptor—in the forlimb musculature. *Funct Ecol*. 2015;29:1197–1208.
9. **Wang YX, Zhang CL, Yu RT, et al.** Regulation of muscle fiber type and running endurance by PPAR δ. *PLoS Biol*. 2004;2:1532–1539.
10. **Pearen MA, Goode JM, Fitzsimmons RL, et al.** Transgenic muscle-specific Nor-1 expression regulates multiple pathways that effect adiposity, metabolism, and endurance. *Mol Endocrinol*. 2013;27:1897–1917.
11. **Musaro A, McCullagh K, Paul A, et al.** Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. *Nat Genet*. 2001;27:195–200.
12. **Whittemore LA, Song KN, Li XP, et al.** Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem Biophys Res Commun*. 2003;300:965–971.

13. **Chang C.** *Androgens and Androgen Receptor: Mechanisms, Functions, and Clinical Applications*. New York, NY: Springer; 2002.
14. **Rand MN, Breedlove SM.** Androgen alters the dentritic arbors of SNB motoneurons by acting upon their target muscles. *J Neurosci*. 1995;15:4408–4416.
15. **Tobias ML, Marin ML, Kelley DB.** The role of sex, innervation, and androgen in laryngeal muscle of *Xenopus laevis*. *J Neurosci*. 1993;13:324–333.
16. **Brantley RK, Marchaterre MA, Bass AH.** Androgen effects on vocal muscle structure in a teleost fish with intersexual and intrasexual dimorphism. *J Morphol*. 1993;216:305–318.
17. **Fuxjager MJ, Heston JB, Schlinger BA.** Peripheral androgen action helps modulate vocal production in a suboscine passerine. *Auk*. 2014;131:327–334.
18. **Fusani L, Barske J, Day LD, Fuxjager MJ, Schlinger BA.** Physiological control of elaborate male courtship: female choice for neuromuscular systems. *Neurosci Biobehav Rev*. 2014;46:534–546.
19. **Schlinger BA, Barske J, Day L, Fusani L, Fuxjager MJ.** Hormones and the neuromuscular control of courtship in the golden-collared manakin (*Manacus vitellinus*). *Front Neuroendocrinol*. 2013;34:143–156.
20. **Fusani L, Giordano M, Day LB, Schlinger BA.** High-speed video analysis reveals individual variability in the courtship displays of male golden-collared manakins. *Ethology*. 2007;113:964–972.
21. **Fuxjager MJ, Longpre KM, Chew JG, Fusani L, Schlinger BA.** Peripheral androgen receptors sustain the acrobatics and fine motor skill of elaborate male courtship. *Endocrinology*. 2013;154:3168–3177.
22. **Schultz JD, Hertel F, Bauch M, Schlinger BA.** Adaptations for rapid and forceful contraction in wing muscles of the male golden-collared manakin: sex and species comparisons. *J Comp Physiol A*. 2001;187:677–684.
23. **Chapman FM.** The courtship of Gould's manakin (*Manacus vitellinus vitellinus*) on Barro Colorado Island, Canal Zone. *Bull Am Museum Nat Hist*. 1935;68:472–521.
24. **Lowe PR.** The anatomy of Gould's manakin (*Manacus vitellinus*) in relation to its display. *Ibis*. 1942;6:50–83.
25. **Feng NY, Katz A, Day LB, Barske J, Schlinger BA.** Limb muscles are androgen targets in an acrobatic tropical bird. *Endocrinology*. 2010;151:1042–1049.
26. **Fusani L, Day LB, Canoine V, Reinemann D, Hernandez E, Schlinger BA.** Androgen and the elaborate courtship behavior of a tropical lekking bird. *Horm Behav*. 2007;51:62–68.
27. **Fuxjager MJ, Barske J, Du S, Day LB, Schlinger BA.** Androgens regulate gene expression in avian skeletal muscles. *PLoS One*. 2012;7:e51482.
28. **Warren WC, Clayton DF, Ellegren H, et al.** The genome of a songbird. *Nature*. 2010;464:757–762.
29. **Williams H.** Choreography of song, dance and beak movements in the zebra finch (*Taeniopygia guttata*). *J Exp Biol*. 2001;204:3497–3506.
30. **Day LB, Fusani L, Hernandez E, et al.** Testosterone and its effects on courtship in golden-collared manakins (*Manacus vitellinus*): seasonal, sex, and age differences. *Horm Behav*. 2007;51:69–76.
31. **Fuxjager MJ, Schultz JD, Barske J, et al.** Spinal motor and sensory neurons are androgen targets in an acrobatic bird. *Endocrinology*. 2012;153:3780–3791.
32. **Day LB, McBroom JT, Schlinger BA.** Testosterone increases display behaviors but does not stimulate growth of adult plumage in male golden-collared manakins (*Manacus vitellinus*). *Horm Behav*. 2006;49:223–232.
33. **Tomaszycki ML, Banerjee SB, Adkins-Regan E.** The role of steroids in courtship, pairing and pairing behaviors in the socially monogamous zebra finch. *Horm Behav*. 2006;50:141–147.
34. **Bahn JH, Lee JH, Li G, Greer C, Peng G, Xiao X.** Accurate identification of A-to-I RNA editing in human by transcriptome sequencing. *Genome Res*. 2012;22:142–150.
35. **Li G, Bahn JH, Lee JH, et al.** Identification of allele-specific alternative mRNA processing via transcriptome sequencing. *Nucleic Acids Res*. 2012;2012:1–13.
36. **Jarvis ED, Mirarab S, Aberer AJ, et al.** Whole-genome analyses resolve early branches in the tree of life of modern birds. *Science*. 2014;346:1320–1331.
37. **Mortazavi A, Williams BA, McCue K, Schaeffer L, Worl B.** Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. 2008;5:621–628.
38. **Robinson MD, McCarthy DJ, Smyth GK.** edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26:139–140.
39. **Zinzow-Kramer WM, Horton BM, Maney DL.** Evaluation of reference genes for quantitative real-time PCR in the brain, pituitary, and gonads of songbirds. *Horm Behav*. 2014;66:267–275.
40. **Benjamini Y, Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc Ser B Stat Method*. 1995;57:289–300.
41. **Stanbrough M, Bubley GJ, Ross K, et al.** Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res*. 2006;66:2815–2825.
42. **Ji H, Jiang H, Ma W, Johnson DS, Myers RM, Wong WH.** An integrated software system for analyzing ChIP-chip and ChIP-seq data. *Nat Biotechnol*. 2008;26:1293–1300.
43. **Matsumoto T, Sakari M, Okada M, et al.** The androgen receptor in health and disease. *Annu Rev Physiol*. 2013;75:201–224.
44. **Portales-Casamar E, Thongjuea S, Kwon AT, et al.** JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. *Nucleic Acids Res*. 2010;38:D105–D110.
45. **Harris MA, Deegan JI, Lomax J, et al.** The Gene Ontology project in 2008. *Nucleic Acids Res*. 2008;36:D440–D444.
46. **Lee J-H, Gao C, Peng G, Greer C, Ren S, Wang Y, Xiao X.** Analysis of transcriptome complexity through RNA sequencing in normal and failing murine hearts. *Circ Res*. 2011;109:1332–1341.
47. **Young MD, Wakefield MJ, Smyth GK, Oshlack A.** Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol*. 2010;11:R14.
48. **Langfelder P, Horvath S.** WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 2008;9:959.
49. **Langfelder P, Luo R, Oldham MC, Horvath S.** Is my network module preserved and reproducible? *PLoS Comput Biol*. 2011;7(1):e1001057.
50. **Yoshioka M, Boivin A, Ye P, Labrie F, St Amand J.** Effects of dihydrotestosterone on skeletal muscle transcriptome in mice measured by serial analysis of gene expression. *J Mol Endocrinol*. 2006;36:247–259.
51. **Wyce A, Bai YC, Nagpal S, Thompson CC.** Research resource: the androgen receptor modulates expression of genes with critical roles in muscle development and function. *Mol Endocrinol*. 2010;24:1665–1674.
52. **Ekblom R, Balakrishnan CN, Burke T, Slate J.** Digital gene expression analysis of the zebra finch genome. *BMC Genomics*. 2010;11:219.
53. **Watanabe S, Mabuchi K, Ikebe R, Ikebe M.** Mechanoenzymatic characterization of human myosin Vb. *Biochemistry*. 2006;45:2729–2738.
54. **Mori K, Furusawa T, Okubo T, et al.** Genome structure and differential expression of two isoforms of a novel PDZ-containing myosin (MysPDZ) (Myo18A). *J Biochem*. 2003;133:405–413.
55. **Tajsharghi H, Hilton-Jones D, Raheem O, Saukkonen AM, Oldfors A, Udd B.** Human disease caused by loss of fast IIa myosin heavy chain due to recessive MYH2 mutations. *Brain*. 2010;133:1451–1459.
56. **Bement WM, Hasson T, Wirth JA, Cheney RE, Mooseker MS.** Identification and overlapping expression of multiple unconventional myosin genes in vertebrate cell types. *Proc Natl Acad Sci USA*. 1994;91:6549–6553.

57. **Bloemink MJ, Geeves MA.** Shaking the myosin family tree: biochemical kinetics defines four types of myosin motor. *Semin Cell Dev Biol*. 2011;22:961–967.
58. **Palmieri F.** The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pfluegers Arch*. 2004;447: 689–709.
59. **Vlahovich N, Schevzov G, Nair-Shaliker V, et al.** Tropomyosin 4 defines novel filaments in skeletal muscle associated with muscle remodelling/regeneration in normal and diseased muscle. *Cell Motil Cytoskeleton* 2008;65:73–85.
60. **Stuart JM, Segal E, Koller D, Kim SK.** A gene-coexpression network for global discovery of conserved genetic modules. *Science*. 2003; 302:249–255.
61. **Bartels ED, Ploug T, Størling J, Mandrup-Poulsen T, Nielsen LB.** Skeletal muscle apolipoprotein B expression reduces muscular triglyceride accumulation. *Scand J Clin Lab Invest*. 2014;74:351–357.
62. **Horowitz JF, Klein S.** Lipid metabolism during endurance exercise. *Am J Clin Nutr*. 2000;72:558S–563S.
63. **Tunstall RJ, Mehan KA, Wadley GD, et al.** Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2002;283:E66–E72.
64. **Watt MJ, Hoy AJ.** Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *Am J Physiol Endocrinol Metab*. 2012;302:E1315–E1328.
65. **Nezu JI, Tamai I, Oku A, et al.** Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. *Nat Genet*. 1999;21:91–94.
66. **Bremer J.** Carnitine: metabolism and functions. *Physiol Rev*. 1983; 63:1420–1480.
67. **Herbst KL, Bhasin S.** Testosterone action on skeletal muscle. *Curr Opin Clin Nutr Metab Care*. 2004;7:271–277.
68. **Peets EA, Henson MF, Neri R.** On the mechanism of the antiandrogenic action of flutamide (α-α-α-trifluoro-2-methyl-4′-nitrom-propionotoluidide) in the rat. *Endocrinology*. 1974;94:532–540.
69. **Sufrin G, Coffey DS.** Flutamide. Mechanism of action of a new nonsteroidal antiandrogen. *Invest Urol*. 1976;13:429–434.
70. **Trainor BC, Bird IM, Marler CA.** Opposing hormonal mechanisms of aggression revealed through short-lived testosterone manipulations and multiple winning experiences. *Horm Behav*. 2004;45: 115–121.
71. **Sperry TS, Wacker DW, Wingfield JC.** The role of androgen receptors in regulating territorial aggression in male song sparrows. *Horm Behav*. 2010;57:86–95.
72. **Barske J, Fusani L, Wikelski M, Feng NY, Santos M, Schlinger BA.** Energetics of the acrobatic courtship in male golden-collared manakins (*Manacus vitellinus*). *Proc R Soc B* 2014;281:20132482.
73. **Clark CJ.** Courtship dives of Anna's hummingbird offer insights into flight performance limits. *Proc R Soc B*. 2009;276:3047–3052.
74. **Nuechterlein GL, Storer RW.** The pair-formation displays of the western grebe. *Condor*. 1982;84:350–369.
75. **Beehler B, Pruett-Jones SG.** Display dispersion and diet of birds of paradise: a comparison of nine species. *Behav Ecol Sociobiol*. 1983; 13:229–238.
76. **Bostwick KS, Prum RO.** High-speed video analysis of wing-snapping in two manakin clades (*Pipridae: Aves*). *J Exp Biol*. 2003;206: 3693–3706.
77. **Bostwick KS, Prum RO.** Courting bird sings with stridulating wing feathers. *Science*. 2005;309:736–736.
78. **Verhovshek T, Sengelaub DR.** Androgen action at the target musculature regulates brain-derived neurotrophic factor protein in the spinal nucleus of the bulbocavernosus. *Dev Neurobiol*. 2013;73: 587–598.
79. **Dobrowolny G, Giacinti C, Pelosi L, et al.** Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model. *J Cell Biol*. 2005;168:193–199.
80. **Kaspar BK, Llado J, Sherkat N, Rothstein JD, Gage FH.** Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science*. 2003;301:839–842.
81. **Barske J, Schlinger BA, Fusani L.** The presence of a female influences courtship performance of male manakins. *Auk*. 2015;132: 594–603.
82. **Barske J, Schlinger BA, Wikelski M, Fusani L.** Female choice for male motor skills. *Proc R Soc B*. 2011;278:3523–3528.