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Evolutionary patterns of adaptive acrobatics and physical performance predict expression profiles of androgen receptor - but not oestrogen receptor - in the forelimb musculature

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Abstract

1. Superior physical competence is vital to the adaptive behavioral routines of many animals, particularly those that engage in elaborate socio-sexual displays. How such traits evolve across species remains unclear.

2. Recent work suggests that activation of sex steroid receptors in neuromuscular systems is necessary for the fine motor skills needed to execute physically elaborate displays. Thus, using passerine birds as models, we test whether interspecific variation in display complexity predicts species differences in the abundance of androgen and estrogen receptors (*AR* and *ER*α) expressed in the forelimb musculature and spinal cord.

3. We find that small-scale evolutionary patterns in physical display complexity positively predict expression of the *AR* in the main muscles that lift and retract the wings. No such relationship is detected in the spinal cord, and we do not find a correlation between display behavior and neuromuscular expression of *ER*α. Also, we find that *AR* expression levels in different androgen

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Data Accessibility

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targets throughout the body – namely the wing muscles, spinal cord, and testes – are not necessarily correlated, providing evidence that evolutionary forces may drive *AR* expression in a tissue-specific manner.

4. These results suggest co-evolution between the physical prowess necessary for display performance and levels of *AR* expression in avian forelimb muscles. Moreover, this relationship appears to be specific to muscle and *AR*-mediated, but not *ER*α-mediated, signaling.

5. Given that prior work suggests that activation of muscular *AR* is a necessary component of physical display performance, our current data support the hypothesis that sexual selection shapes levels of *AR* expressed in the forelimb skeletal muscles to help drive the evolution of adaptive motor abilities.

Graphical abstract

Keywords

social display; neuromuscular physiology; sexual selection; testosterone; tropical birds; manakins

Introduction

Sexual selection shapes performance phenotypes in a manner that guides or constrains the evolution of elaborate socio-sexual tactics and strategies (Lailvaux & Irschick 2006; Irschick *et al.* 2007). Although research in this area has deepened our understanding of why and how physical competence contributes to animal design, far less is understood about the mechanisms by which sexual selection modifies endogenous aspects of adaptive prowess and motor ability. Hints about these mechanisms come from studies in physiological genetics that demonstrate how engineered variation in the transcription of select genes establishes significant individual differences in endurance capacity (Wang *et al.* 2004; Pearen *et al.* 2013) and strength performance (Musaro *et al.* 2001; Whittemore *et al.* 2003). These observations suggest that interspecific variation in performance phenotypes result in part from selection for specific gene expression profiles in tissues related to movement, balance, and proprioception. However, we know little about the link between athletic-like behavioral traits in wild vertebrates and an organism's transcriptional milieu in neuromuscular systems.

Males of many species produce physically elaborate displays to court females and compete with rivals (Nuechterlein & Storer 1982; Beehler & Pruettjones 1983; Hoglund & Lundberg 1987; Vliet 1989; Walls & Semlitsch 1991; Voigt *et al.* 2001; How *et al.* 2008; Clark, Feo & Bryan 2012). This behavior often incorporates complex and/or unusual body and limb movements that require exceptional strength, agility, and dexterity. Modification of anatomical and physiological systems that control or contribute to an organism's kinematic and proprioceptive features are likely required to guide the emergence of such behavioral elements (Losos 1990; James, Navas & Herrel 2007). Candidate substrates through which these effects likely occur include skeletal muscles and spinal motor and sensory neurons, since these tissue systems directly control nearly all movement associated with the physical output of behavior. Yet, the way in which evolutionary forces refine these neuromuscular systems to drive species diversification of elaborate and complex display repertoires is poorly understood.

The androgen receptor (*AR*) and estrogen receptor α (*ER*α) are two genes known for regulating motivational and neuro-motor systems that underlie vertebrate social behavior. They encode the *AR* and *ER*α transcriptional regulatory proteins, which are expressed throughout the body and in most of the vertebrate muscular and spinal systems. Activation of *AR* in these tissues, in particular, mediates simple movement patterns and reflexes necessary for successful copulation (Rand & Breedlove 1992; Brantley, Marchaterre & Bass 1993; Regnier & Herrera 1993; Tobias, Marin & Kelley 1993; Oki *et al.* 2013). In wild animals, the relationship between neuromuscular *AR* and socio-sexual motor control has been well studied in the golden-collared manakin (*Manacus vitellinus*), given that males of this tropical bird perform elaborate displays to court females and compete with rivals. This behavior is guided in large part by androgenic signaling via *AR* expressed in both the animal's skeletal musculature and its spinal motor neurons that innervate these tissues (Schlinger *et al.* 2013; Fusani *et al.* 2014a). For example, inhibiting *AR* exclusively within the periphery not only alters gene expression profiles in the main muscles that raise [*supracoricoidieus* (SC), *scapulohumeralis caudalis* (SH)] and retract the wings [*pectoralis* (PEC)], but also disrupts fine motor movements of the wings that are essential to the bird's most complex display maneuvers (Fuxjager *et al.* 2013). Considering that female golden-collared manakins preferentially mate with males that produce faster and more agile displays (Barske *et al.* 2011), *AR* expression within the muscles and spinal motor neurons that control body and limb movement is likely a sexually selected trait (Schlinger *et al.* 2013; Fusani *et al.* 2014a). This concept is not only bolstered by evidence showing that golden-collared manakins maintain relatively high levels of *AR* transcripts in their main wing musculature and regions of the spinal cord that innervate these tissues (Schultz & Schlinger 1999; Feng *et al.* 2010; Fuxjager *et al.* 2012b), but it is also reinforced by work suggesting that this elevated *AR* expression is a constitutive trait that is unaffected by endogenous androgen levels or aspects of muscle use (Feng *et al.* 2010; Fuxjager *et al.* 2012b). At the same time, the role of estrogens in guiding the motor control of display behavior is more elusive, with past work suggesting that these hormones prime the motor circuits that underlie the production of display routines (Schlinger *et al.* 2013; Fusani *et al.* 2014a). Such effects may be mediated through *ERα* which is readily expressed in the male-golden-collared manakin's wing musculature and spinal cord (Feng *et al.* 2010; Fuxjager *et al.* 2012b). Based on this

collective body of work, we hypothesize that the evolution of elaborate display repertoires is specifically linked to transcriptional profiles of *AR* – but not ERα − within the neuromuscular system. Accordingly, selection may influence diversification in display capability by interacting with *AR* expression patterns in these tissues that govern body and limb movement.

We investigate this idea by comparing profiles of *AR* and *ERα* expression in skeletal muscles and spinal cords of seven separate avian species that each incorporates a different level of physicality into their socio-sexual displays (Fig. 1). We focus on passerine birds, paying particular attention to species within the manakin (Pipridae) family (including the golden-collared manakin). In neotropical manakins, adult males of nearly all species produce dramatic dance and flight routines for courtship and territorial competition (Prum 1990; Prum 1994; Prum 1998). The nature and physical complexity of the movement patterns that underlie these displays vary dramatically across species. To increase our study's taxonomic and phylogenetic reach, we included another sub-oscine species, the ochre-bellied flycatcher, which produces a relatively moderate physical display to attract mates and defend territories (Westcott & Smith 1994). We also included two closely related oscine species that differ in terms of the courtship display they perform: the pin-tailed whydah and zebra finch (Fig. 1). The former performs a modest behavioral display (Shaw 1984), while the latter performs a minimal physical display (Williams 2001). This array of passerines collectively represents species at both ends of the continuum of display complexity, as well as species in-between these ends.

In the current study, we measure (*i*) whether wing muscle and spinal cord *AR* and *ERα* expression varies among the species described in Figure 1, and (*ii*) how interspecific differences in the motor complexity of display behavior predict such variation in steroid receptor levels. Our focus primarily centers on the SC, SH, and PEC wing muscles, given that past work in golden-collared manakins implicate these tissues as sites of androgen-mediated display output and that the majority of maneuvering in avian behavioral displays relies on wing kinematics (~68%, see Table 1). We focus on the spinal cord, because *AR* in spinal motor and sensory neurons is thought to contribute to display acrobatics (Schultz & Schlinger 1999; Fuxjager *et al.* 2012b). If there is a relationship between the evolution of elaborate display behavior and neuro-motor *AR* and/or *ERα* expression profiles, then we expect that display complexity itself will positively predict the level at which these genes are expressed in either the wing muscles or the spinal cord (or both).

Materials and Methods

Animals

This work was conducted with approval of appropriate governmental agencies and Animal Care and Use Committees at the University of California, Los Angeles (UCLA), the University of Mississippi (UM), and the Smithsonian Tropical Research Institute (STRI). We used males that were actively courting and sexually capable, the latter of which we confirmed by visually inspecting the gonads during dissections (see below) to ensure that they were enlarged in a manner consistent with a breeding bird. Wild manakins [golden-collared (*Manacus vitellinus*), *n*=4; red-capped (*Ceratopipra mentalis*), *n*=3-4; lance-tailed

(*Chiroxiphia lanceolata*) *n*=4; blue-crowned (*Lepidothrix coronata*) *n*=6-7)] and ochre-bellied flycatchers (*Mionectes oleaginous; n*=3-4) were captured passively with mist-nets on breeding grounds (leks) near Gamboa, Panama (February-July). Wild pin-tailed whydahs (*Vidua macroura, n*=2-6) were passively captured with mist-nets in Puerto Rico (July). These birds were singly housed in cages (60 × 40 × 40 cm); males were maintained for one week prior to prior to euthanasia, so that their song could be recorded for another study. Zebra finches (*Taeniopygia guttata, n*=4-5) were collected from a breeding colony at UCLA, in which males were group-housed in large, open-flight aviaries adjacent to an aviary that contained only females. Sample sizes vary as a result of certain tissues not being collected from all individuals.

Tissue collection

Birds were euthanized via rapid decapitation. Tissues were quickly dissected from the carcasses and either flash frozen on dry ice or immersed in *RNALater*. Those samples placed in *RNAlater* were treated according to the manufacturer's instructions for long-term storage. We first dissected the PEC, SC, and SH. We then dissected the cervical/thoracic spinal cord (tissue rostral of segment 18) and the lumbar/sacral spinal cord (tissue caudal of 18). We finally dissected the (enlarged) testes. Whole pin-tailed whydahs bodies were flash frozen after decapitation, and tissues were removed (still frozen) from these animals in the lab using a Dremel™ tool. Samples were stored at −80°C until RNA extraction.

To validate that the two methods of RNA preservation described above did not differentially affect the RNA quality or gene expression readings, we dissected the left PEC from 5 adult male zebra finches and immersed half of this sample in *RNALater* and placed the other half on dry ice (flash freeze). In both cases, samples were treated identically to those collected in the field. We found no effect of preservation technique on RNA $A_{260/280}$ values (paired *t*-test;t_4=0.76, p=0.49), or in *AR* (paired *t*-test; t_4=−0.50, p=0.65) and ERα (paired *t*-test; t_4= −1.29, p=0.27) expression levels. As such, we conclude that the methods of tissue (RNA) preservation did not affect RNA integrity or abundance of *AR* and ERα transcripts.

RNA extraction and real-time PCR (RT-PCR) amplification

Total RNA was extracted from tissue using TRIzol Reagent™ (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Accordingly, tissues were homogenized for 40 sec at medium speed using a rotor/stator homogenizer, and the final RNA concentration of each sample was measured using a Nanodrop System (Thermo Scientific, Wilmington, DE, USA). RNA integrity was verified through gel electrophoresis and evaluation of $A_{260/280}$ values. Samples were treated with DNAse (Promega, Madison, WI) and reverse transcribed using Superscript Reverse Transcriptase II (Invitrogen).

RT-PCR was performed using methods established for passerine species, including those within the *Taeniopygia*, *Manacus*, and *Mionectes* genera (Feng *et al.* 2010; Fuxjager *et al.* 2012a; Fuxjager *et al.* 2012b; Fuxjager *et al.* 2013). Reactions occurred at 42°C for 50 min and then 70°C for 15 min. We used primers for *AR* (forward: TGACGTGTGGGAGCTGCAAA, reverse: GGCCATCCACTGGAATAATACTGA) and *ERα* (forward: TGTCCCTGACAGCAGAACAG, reverse:

GTAGCCAGCAGCATGTCAAA) that are designed from the zebra finch genome and are used regularly on passerine muscle and spinal cord tissue (Feng *et al.* 2010; Fuxjager *et al.* 2012b). Each reaction contained 0.38 mM of deoxynucleotide triphosphate, 0.4 μM of forward and 0.4 μM of reverse primer, 50 ng of sample cDNA, 0.06 ng of DNA taq polymerase (Bioline, Randolph, MA), and buffer. Reactions were first run at 95°C for 5 min and then subjected to 38 cycles of 95°C for 30 sec, ~64°C for 30 sec, and 72°C for 1 min. Completion of reactions occurred at 72°C for 10 min. Gel electrophoresis was used to verify that the length of amplified transcripts matched the predicted lengths. RT-PCR products were sequenced (Genewiz Inc., La Jolla, CA, USA) and blasted against the zebra finch to assess homology.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed according to methods outlined previously (Feng *et al.* 2010; Fuxjager *et al.* 2012a; Fuxjager *et al.* 2012b; Fuxjager *et al.* 2013). Reactions were performed on an ABI 7300 sequence detection system using SYBR Green PCR Master Mix kits (Applied Biosystems Inc., Foster City, CA). Each reaction was run at a total of 25 μl, with 5 ng of template primer concentrations that depended on optimizations. Primers for qPCR were designed from zebra finch genome, using regions of the *AR* (forward: ATGAGTACCGCATGCACAAA; reverse: AACTCCTGGGGTGTGATCTG) and *ERα* (forward: TGAAAGGTGGAATCCGAAAAGA; reverse: TTGGCGTTTTTGTTTCATCACT) genes that are highly conserved and nearly identical among species. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated from the zebra finch genome (forward: TGACCTGCCGTCTGGAAAA; reverse: CCATCAGCAGCAGCCTTCA), we found no differences in levels of muscular or spinal GAPDH gene expression among species. Past work has used these primers successfully on other passerines and showed that they work across avian species with similar efficiency (Feng *et al.* 2010; Fuxjager *et al.* 2012a; Fuxjager *et al.* 2012b; Fuxjager *et al.* 2013). Each reaction was run at 50°C for 2 min and 95°C for 10 min. Reactions were next subjected to 40 cycles of 95°C for 15 sec and 60°C for 1 min. A final dissociation stage was added to the end of the reaction, whereby samples were run at 95°C for 15 sec, 60°C for 30sec, and 95°C for 15 sec. Reaction efficiencies were between 90%-110%, and dissociation curves were inspected to verify that samples were not contaminated. Samples were run in duplicate, and the average delta CT for each sample $[1000\times(2^{-(\text{CT Gene of Interest} - \text{CT GAPDH})}]$ was used to quantify relative transcript abundance.

Calculations of display complexity

The level of complexity with which species perform social displays varies across taxa, and thus is likely an evolutionarily labile trait (Prum 1990; Kusmierski *et al.* 1997). We quantified such variation using as framework developed by Lindsay *et al.* (in press), in which the level of display complexity is assigned a numerical score. Higher scores in this system correspond to more complex displays, whereas lower scores correspond to less complex displays. The framework itself is based on indices published previously that measure various forms of physical behavior that are incorporated into avian display routines (Shaw 1984; Prum 1990; Prum 1994; Westcott & Smith 1994; Prum 1998; Williams 2001;

Bostwick & Prum 2003; Day, McBroom & Schlinger 2006; DuVal 2007; Fusani *et al.* 2007; Durães 2009). This scoring system is conceptually similar to other indices that have also been developed to examine taxonomic variation in multifaceted behavioral traits (Madden 2001; Day, Westcott & Olster 2005).

Thus, for each species included in our analysis, we compute a score that described (*i*) the complexity of wing movement within a display routine (i.e., *wing movement complexity score*) and (*ii*) the overall motor complexity of a given display routine (i.e., *total motor complexity score*). To generate these scores, we assigned a value (*1 point = presence, 0 point = absence*) to discrete behavioral characters that make up a species' physical signaling repertoire (Table 1). The majority of display characters are originally described and operationally defined by Prum (1990), while the remaining characters are defined in more recent accounts of social behavior relevant to our study species (Shaw 1984; Westcott & Smith 1994; Williams 2001; Bostwick & Prum 2003; Day, McBroom & Schlinger 2006; DuVal 2007; Fusani *et al.* 2007; Durães 2009). As we combined behavioral characters from these independent sources, we carefully avoided adding redundant maneuvers that represented the same behavior, but that were given different names by separate researchers or research groups. We also assigned one point for each unique mechanical sonation that a species can produce during its display. Double-counting of these display/behavioral elements with discrete display characters described above was avoided; for instance, wing-snaps produced by golden-collared manakins were counted as a sonation within the "sonation repertoire size," but not as their own behavioral character *per se* (Table 1; see Lindsay *et al.* in press).

To produce a wing movement complexity score for each species, we summed the points accumulated in each category that either directly incorporated wing movement or overtly relied on wing movement (as indicated by the movement's operational definition). Such characters made up roughly ~68% of all behavioral elements outlined in the display complexity framework (Table 1). To produce a *total motor complexity score*, we summed the points accumulated in all categories. The use of duel scoring systems provides a comprehensive picture of variability in motoric complexity associated with behavioral display performance across the taxa of interest. To this end, given that the display repertoire of each species in our study has been well described (Shaw 1984; Westcott & Smith 1994; Williams 2001; Bostwick & Prum 2003; Day, McBroom & Schlinger 2006; DuVal 2007; Fusani *et al.* 2007; Durães 2009), these metrics account completely for each species display routine.

Two separate individuals blindly and independently quantified complexity scores for each species, and the numerical values obtained by these individuals were highly correlated with each other (R^2=0.93, p=0.002) and showed little variation (CV=7.3%). Furthermore, it is important to note that the scoring system and the scores themselves were generated without anticipation or knowledge of our current hypotheses and results, as the framework developed by Lindsay *et al.* (in press) was intended to examine other aspects of manakin behavior, morphology, and physiology.

Statistical analyses

We performed linear mixed-model ANOVAs to compare *AR* and *ERα* transcript levels across species and tissue/region (fixed factors). Bird identity was included in these models to account for within-individual variation across tissues. Significant main effects and interactions were followed by post-hoc pairwise comparisons, in which we used Shaffer-Holm procedures to adjust α for multiple comparisons (Shaffer 1986; Holland & Copenhaver 1987; Shaffer 1995). Receptor expression measurements were log transformed [LOG (X+1)], as *Q-Q* plots revealed that these transformations rendered data that better conformed to the parameters of normality (Zar 1999). Estimated marginal means (EMM) derived from these models were used to represent species' averages for *AR* and *ERα* expression in subsequent analyses.

We used a phylogenetic generalized least-squares (PGLS) approach to test predictive relationships between display complexity and steroid receptor expression (Pagel 1999; Freckleton, Harvey & Pagel 2002), given that analyses of comparative data can be confounded by non-independence of data points due to common ancestry (Felsenstein 1985). PGLS analyses were performed using CAPER version 0.5.2 (Orme 2013) and APE version 0.6 (Paradis, Claude & Strimmer 2004) packages in R Studio version 0.98. Each PGLS model produces a maximum likelihood estimate of the phylogenetic scaling parameter λ, in which values of λ close to 0 indicate complete phylogenetic independence (star phylogeny) and values of λ close to 1 indicate complete phylogenetic dependence. For all significant models, we used maximum likelihood ratio tests to determine whether the model in which λ is estimated differs from models with λ values set at either 0 or 1. Our analyses were based on a 50% majority rules consensus phylogeny, which was generated in Geneious Pro v5.6 (Biomatters, Ltd.) using 10,000 trees of the seven taxa downloaded from birdtree.org (Jetz *et al.* 2012). The branch lengths of our consensus tree are depicted in Figure 1, and the phylogenetic relationships among taxa within our tree are consistent with such relationships depicted in current avian systematical analyses (Barker *et al.* 2004; Jetz *et al.* 2012; Ohlson, Fjeldsa & Ericson 2013). Display complexity scores were log transformed [LOG (X+1)], as *Q-Q* plots revealed that these transformations rendered data that better conformed to the parameters of normality (Zar 1999).

Results

Presence of androgen receptor (AR) and estrogen receptor α (ERα) mRNA in the passerine neuromuscular system

We confirmed that all seven passerines in our study express both *AR* and *ERα* in their respective neuromuscular systems. Using real-time PCR (RT-PCR), we amplified fragments of each gene's mRNA in the SH wing muscle and lumbar/sacral spinal cord of all seven species. We detected a single band for both *AR* and *ERα* RT-PCR reactions in all tested samples, and sequencing of these products confirmed that these bands were indeed their predicted receptor mRNA sequence. Furthermore, alignment analyses revealed that *AR* and *ERα* transcript are highly homologous among taxa (>90%). These results demonstrate not only that a wide variety of passerine species express *AR* and *ERα* within their

neuromuscular tissues, but also that each of these two genes are highly conserved among the sampled birds.

Variation in neuromuscular androgen receptor (AR) and estrogen receptor α (ERα) among species and between tissues

We tested whether patterns of *AR* expression in the neuromuscular system differed among the sampled taxa. In the wing muscles (Fig. 2A), we detected significant variation in *AR* expression across species ($F_{6,27.5}$=8.72, p<0.001), with golden-collared and red-capped manakins having the most *AR* mRNA in these tissues compared to the majority of the other taxa (see Fig. 2A for post-hoc comparisons). Other species, such as the lance-tailed manakins and ochre-bellied flycatchers, maintained relatively intermediate levels of *AR* in these tissues (p>0.15). We detected no effect of muscle on *AR* expression ($F_{2,50.22}$=2.34, p=0.11), nor did we uncover a species × muscle interaction ($F_{612,49.96}$=1.42, p=0.187). These data are consistent with past work showing that *AR* levels do not vary among the separate wing muscles (Feng *et al.* 2010). In the spinal cord (Fig. 2B), we also found that *AR* expression varied among the sampled species ($F_{6,21.26}$=14.67, p<0.001). In this case, however, golden-collared manakins, red-capped manakins, and ochre-bellied flycatchers maintain the highest levels of *AR* mRNA, relative to many of the other species (see Fig. 2B for post-hoc comparisons). In addition, *AR* expression was generally higher in the cervical/thoracic region of the cord, compared to the lumbar/sacral region of the cord ($F_{1,22.04}$=4.80, p=0.039). We also detected a significant species × spinal cord region interaction ($F_{6,22.17}$=3.27, p=0.019), as pin-tailed whydah and blue-crowned manakins showed strong regional differences in spinal *AR* mRNA levels (see Fig. 2B for post-hoc results).

We tested whether *ERα* expression in the neuromuscular system differed across species. In the muscles (Fig. 2C), we discovered significant variation among taxa ($F_{6,23}$=3.24, p=0.020), with ochre-bellied flycatchers maintaining the highest levels of this transcript (see Fig.2C for post-hoc comparisons). There was no overall effect of muscle ($F_{2,23}$=2.94, p=0.064), nor was there a significant species × muscle interaction ($F_{6,23}$=0.73, p=0.71). In the spinal cord (Fig. 2D), we found robust species differences in *ERα* mRNA levels ($F_{6,23}$=7.11, p<0.001), with both blue-crowned manakins and zebra finches expressed relatively high levels of *ERα* transcript (see Fig.2D for post-hoc comparisons). Regionally, the lumbar/sacral portion of cord expressed relatively more *ERα*, compared to the cervical/thoracic region ($F_{6,23}$=10.36, p=0.002). Finally, we detected a significant species × spinal cord region interaction ($F_{6,23}$=9.22, p<0.001), such that both blue-crowned manakins and pin-tailed whydahs expressed significantly more *ERα* in their lumbar/sacral cords than in their cervical/thoracic cords (see Fig. 2D for post-hoc comparisons).

Relationship between the physical complexity of socio-sexual displays and neuromuscular androgen receptor (AR) and estrogen receptor α (ERα)

We next tested whether species differences in the physicality of socio-sexual display behavior predicted such differences in neuromuscular *AR* and *ERα* expression. To begin this analysis, we quantified levels of wing movement complexity and total motor complexity for each species' display (Lindsay *et al.* in press; see Methods and Table 1). With respect to *AR*, we found that species that produce displays with greater wing complexity transcribed

collectively higher levels of this receptor in their SC, SH, and PEC (Fig. 3A; adjusted r^2=0.70, t=3.88, p=0.011, λ<0.01). This regression model (with a maximum likelihood estimate of λ) was a statistically better fit than a model in which we manually set λ to 1 (p=0.020), but not to a model in which we manually set λ to 0 (p=1.0). Furthermore, in a separate analysis, we found that the positive association between display behavior and muscular *AR* expression was evident in the context of overall motor complexity (Fig. 3B; adjusted r^2=0.81, t=5.16, p=0.0036, λ<0.01). This model with a maximum likelihood estimate of λ was a marginally better fit than a model in which we manually set λ to 1 (p=0.087), but not to a model in which we set λ to 0 (p=1.0). Meanwhile, in the spinal cord, we found that both wing movement complexity (Figs. 3C; adjusted r^2=−0.16, t=0.40, p=0.70, λ<0.01) and total motor complexity (Fig. 3D; adjusted r^2=−0.068, t=0.78, p=0.47, λ<0.01) failed to predict levels of *AR* expression.

In addition, we measured whether display complexity scores predicted measurements of muscular and spinal ERα expression. We found that neither wing movement complexity, nor total motor complexity predicted the collective amount of ERα mRNA expressed in the SC, SH, and PEC (Fig. 3E and 3F; wing movement complexity: r^2=−0.12, t=−0.60, p=0.58, λ<0.01; total motor complexity: adjusted r^2=−0.092, t=−0.70, p=0.51, λ<0.01) or the spinal cord (Fig. 3G and 3H; wing movement complexity: adjusted r^2=−0.12, t=−0.60, p=0.58, λ<0.01; total motor complexity: adjusted r^2=−0.092, t=−0.70, p=0.51, λ<0.01).

Evidence for tissue-specific regulation of androgen receptor (AR)

Given the apparent link between *AR* and display complexity, we examined whether expression of *AR* is subject to tissue-specific modification by evolutionary forces. Thus, we tested whether *AR* levels in the wing muscles and spinal cord are related not only to each other, but also to *AR* levels in another androgen target in the periphery: the testes (Table 2; Nastiuk & Clayton 1994; Leska *et al.* 2012). We found a positive predictive relationship between collective *AR* mRNA in the wing muscles and collective *AR* mRNA in the spinal cord (adjusted r^2=0.57, t=3.03, p=0.029; λ=1.0). At the same time, we did not detect a significant relationship between *AR* mRNA levels in either of these neuromuscular tissues and *AR* mRNA levels in the testes (muscle vs. testes: adjusted r^2=0.14, t=1.34, p=0.25; λ=0.85; spinal cord vs. testes: adjusted r^2=−0.24, t=0.19, p=0.86; λ<0.01).

Discussion

Our results provide the first evidence that androgenic sensitivity in select parts of the neuromotor system is an evolved mechanism to facilitate performance abilities and acrobatics in physically elaborate socio-sexual displays. Both *AR* and *ERα* genes are expressed in the neuromuscular tissues of all seven passerine birds that we sample; however, the level of this expression varies markedly across the taxa and in a way that is predicted by species variation in measures of display complexity. Namely, we found that birds that perform highly complex displays express relatively greater levels of *AR* in the three main skeletal muscles that control wing movement. This relationship exists independently of the species' phylogenetic history, and it persists whether we apply either a narrow definition of display complexity to account for only wing kinematics or a broad definition of display complexity

to account for overall motor output. At the same time, we found that species differences in display complexity did not predict levels of *AR* expression in the spinal cord, the immediate up-stream level of motor control for the muscles. Moreover, display complexity scores failed to predict *ERα* expression in both the wing muscles and spinal cord. Taken together, these data suggest that there is co-evolution between elaborate behavioral displays in passerine birds and the degree to which *AR* is expressed in specific parts of the neuromuscular architecture.

Muscular androgen receptor (AR) expression and the evolution of elaborate behavioral displays

Given the link we uncover between species variation in behavioral display complexity and muscular *AR* expression, our data support the hypothesis that sexual selection adjusts levels of *AR* expression in the wing muscles to influence the evolution of adaptive motor skills. In particular, we suspect that sexual selection increases the sensitivity of the wing muscles to androgenic hormones as a way of enhancing these tissues' performance limit to accommodate adaptive acrobatics and rapid wing kinematics. In support of this view, we find that the two species that express the most *AR* in the SC, SH, and PEC – the golden-collared and red-capped manakins – both rely on incredibly rapid wing movements to generate mechanical sonations that echo loudly throughout the rainforest (Bostwick & Prum 2003; Fusani *et al.* 2007). Recent physiological work in wild adult male golden-collared manakins also lends credence to this idea by showing that individuals treated with a drug to block peripheral *AR* experienced not only a dramatic change in the molecular composition of their muscles, but also a reduced capacity to perform the motor skills necessary for their most complex wing displays (Fuxjager *et al.* 2013). This, of course, does not mean that muscular *AR* is unimportant for many of the other species in our analysis that also produce elaborate displays, but that may not require extraordinarily rapid wing movements to do so. For example, male lance-tailed manakins must have remarkable flight agility to perform carefully-timed leap-frog displays with conspecifics (DuVal 2007), while male ochre-bellied flycatcher need wing mobility and endurance to produce both hover-flight and butterfly-flight displays (Westcott & Smith 1994). Selection may therefore favor relatively "intermediate" levels of *AR* expression in the wing musculature to support the kinematics of such behavior, as they likely require muscular performance that is relatively greater than that of a bird who does not display (i.e., zebra finch; Williams 2001), but that is relatively less than that of a bird that sonates using rapid wing movement (i.e., golden-collared and red-capped manakin; Bostwick & Prum 2003; Fusani *et al.* 2007).

Future work is needed to fully explore the ideas described above. In particular, a better appreciation for the kinematics of each species' behavioral display is likely necessary to understand when selection favors *AR* as a means to modify muscle performance. This does not detract from the importance of our current findings, which highlight the androgenic system as a main physiological trait that may help govern adaptive motor command. Thus, the nexus among selection, androgenic action, and muscle physiology might provide a common pathway through which motor skills are incorporated, refined, or removed from reproductive behavior in birds and other vertebrates. Additional phylogenetic analysis within other animal clades that show *AR*-dependent, sex-related motor skills will help confirm the

validity and generality of this hypotheses (Rand & Breedlove 1992; Brantley, Marchaterre & Bass 1993; Tobias, Marin & Kelley 1993).

Muscular androgen receptor (AR) as a target of selection

Important to the ideas outlined above, we present evidence that levels of *AR* expression are responsive to effects of selection. Amounts of *AR* transcribed in the forelimb muscles and spinal cord are decoupled from the amounts of *AR* transcribed in the testes, a known androgen target in birds (Nastiuk & Clayton 1994; Leska *et al.* 2012). Thus, this suggests that species with high levels of *AR* in their neuromuscular system do not necessarily maintain proportionately high levels of *AR* in all other parts of the body. To this end, such differential regulation of *AR* itself may not negatively impact the functional harmony of the androgenic system throughout the whole organism. Our results, however, do point to some degree of constraint in *AR* expression across tissues, as the abundance of muscular and spinal *AR* is related across species. In light of these findings, it is possible that selection acts on *AR* expression in a subset of spinal motor and sensory neurons that control muscles directly involved in display production (Fuxjager *et al.* 2012b), rather than at motor circuits within the spinal cord that have little involvement in reproductive systems. This topic is a focus of future research.

Although muscular *AR* levels appear to have co-evolved with display complexity, we did not discover any relationship between species variation in neuromuscular *ERα* expression and metrics of this behavior. This result is important because it highlights the relative selectivity with which sexual selection likely acted on androgenic systems – and not other sex steroid systems – to influence physical display ability. It is still possible, however, that *ERα* plays a role in regulating neuromuscular functionality (Evrard & Balthazart 2004; Svensson *et al.* 2010) in some of the species we examined. For example, we find that both blue-crowned manakins and pin-tailed whydahs show unique *ERα* expression profiles in their spinal cord, whereby males of both species express this receptor much more in their lumbar/sacral cords than their cervical/thoracic cords. Most of the other birds express relatively similar levels of *ERα* across these two regions. Lumbosacral motor and sensory neurons mainly relay information to and from the leg musculature, suggesting that *ERα* may mediate neuro-motor activity of the hind limbs. Neither blue-crowned manakins, nor pin-tailed whydahs use their hind limbs more than most of the other species to perform the complex elements of their displays, so it remains unclear whether estrogenic sensitivity in the lower spinal cord is linked to the evolution of the species' respective displays. At the same time, the lumbosacral spinal cord houses the motor and sensory neurons that innervate the cloacal musculature, and prior work in quail suggests that estrogenic action at these neurons may influence sexual abilities (Evrard & Balthazart 2002; Evrard & Balthazart 2003). It is therefore possible that similar mechanism has evolved in the blue-crowned manakin and pin-tailed whydah, though future work is needed to explore this intriguing idea.

Finally, although not investigated here, we cannot dismiss the possibility that ERβ is acted upon in avian skeletal muscle and/or spinal cord. Further work is needed to assess its functional expression in these tissues and whether it too is a target of sexual selection as *AR* appears to be.

Endogenous control of muscular androgen receptor (AR) and estrogen receptor α (ERα) expression levels

There is considerable evidence that expression levels of *AR* and *ERα* in wing muscles and spinal cord are independent of circulating androgens. First, despite significant differences in circulating androgen levels with sex and breeding season for golden-collared manakins and zebra finches, *AR* expression levels in skeletal muscle are similar between adult males and females and between males during the breeding and non-breeding season (Feng *et al.* 2010). Second, testosterone treatment of non-breeding male golden-collared manakins (with low circulating testosterone) produces no change in muscular or spinal *AR* expression (Feng *et al.* 2010; Fuxjager *et al.* 2012b). Finally, from comparative perspective, published studies in reproductively active adult male zebra finches, golden-collared manakins, and lance-tailed manakins indicate that testosterone circulates at similar levels in all species (Vleck & Priedkalns 1985; Schlinger, Day & Fusani 2008; DuVal & Goymann 2010), even though these birds differ in the amounts of steroid receptors they express in their wing muscles and spinal cords.

Furthermore, we believe that muscle use can also be ruled out as a contributing factor in neuromuscular steroid-receptor expression. As mentioned previously, *AR* levels in wing muscles of golden-collared manakins do not differ between sexes, even though females seldom, if at all, perform the masculine courtship displays (Schlinger *et al.* 2013; Fusani *et al.* 2014a). It may seem counterintuitive that neuromuscular steroid-receptor expression is sexually monomorphic if muscular *AR* expression is shaped by sexual selection. However, only adult breeding males have elevated levels of circulating testosterone (Day *et al.* 2007) that activates their courtship behavior; consequently, increased muscular *AR* expression would benefit male reproductive success with no 'cost' to females, a condition that is susceptible to shaping by forces of sexual selection. Regardless of these considerations, the muscles we examined are also used for non-reproductive functions (i.e. flight), so it stands to reason that steroid-receptor expression would be decoupled from this non-reproductive muscle use. Thus, we view skeletal muscle *AR* expression in these birds as a constitutive trait on which selection can readily act to adjust how androgens impact these tissues (Feng *et al.* 2010; Fuxjager *et al.* 2012a; Fuxjager *et al.* 2013).

Physiological significance of elevated androgen receptor (AR) in the muscles

How might activation of *AR* in the skeletal muscles enhance motor capability? There are likely two non-mutually exclusive ways in which this might occur. The first is through direct modulation of muscle itself, whereas the second is through indirect modulation of the spinal motor circuitry that innervates the muscles used to execute display maneuvering. Both of these events occur when androgens bind to *AR* in the myocyte and thereby up-regulate the expression of genes that enhance the strength and contractile properties of the muscle fiber (Wyce *et al.* 2010) and/or induce retrograde transport of signaling molecules that travel from the muscle to the spinal cord via motoneurons to maintain spinal motor circuitry (Rand & Breedlove 1995). Both of these mechanisms are potentially at play in golden-collared manakins, as muscular *AR* up-regulates parvalbumin and IGF-I (Fuxjager *et al.* 2012a). Parvalbumin is a calcium buffer that increases the speed of muscle contraction cycling (Muntener *et al.* 1995), whereas IGF-I not only increases muscle size (Adams & McCue

1998), but also maintains spinal motor circuits via retrograde transport to the spinal cord (Dobrowolny *et al.* 2005).

We cannot rule out the possibility that skeletal muscle systems in addition to the PEC, SC, and SH are androgen sensitive targets of sexual selection. The leg muscles are such an example, considering that both the golden-collared and red-capped manakins use their hind limbs to display (Fusani *et al.* 2007; Bostwick *et al.* 2010). In the golden-collared manakins, past work shows that the gluteal muscle expresses elevated *AR*, similar to the wing muscles. We focused on the latter for this analysis, because wing movements contribute to the majority of display moves (Table 1). Undoubtedly, selection also targets the brain to influence adaptive motor programming of display behavior in birds and other vertebrates (Fusani *et al.* 2014b; Lindsay *et al.* in press). Recent work highlights the stunning flexibility of central motor programs that control limb gestural movements in response to evolutionary forces (Bass & Chagnaud 2012), which of course applies to the displays of birds given their reliance on extensively on wing movements (Prum 1990; Prum 1994; Prum 1998). Future work is currently in progress to elucidate some of these higher-level adaptations.

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References

Adams GR, McCue SA. Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. Journal of Applied Physiology. 1998; 84:1716–1722. [PubMed: 9572822]

Barker FK, Cibois A, Schikler P, Feinstein J, Cracraft J. Phylogeny and diversification of the largest avian radiation. Proceedings of the National Academy of Sciences, USA. 2004; 101:11040–11045.

Barske J, Schlinger BA, Wikelski M, Fusani L. Female choice for male motor skills. Proceedings of the Royal Society of London, Series B: Biological Sciences. 2011; 278:3523–3528. [PubMed: 21508030]

Bass AH, Chagnaud B. Shared developmental and evolutionary origins of neural basis of vocal-acoustic and pectoral-gestural signaling. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:10677–10684. [PubMed: 22723366]

Beehler B, Pruettjones SG. Display dispersion and diet of birds of paradise: a comparison of 9 species. Behavioral Ecology and Sociobiology. 1983; 13:229–238.

Bostwick KS, Elias DO, Mason A, Montealegre F. Resonating feathers produce courtship song. Proceedings of the Royal Society of London, Series B: Biological Sciences. 2010; 277:835–841. [PubMed: 19906670]

Bostwick KS, Prum RO. High-speed video analysis of wing-snapping in two manakin clades (Pipridae: Aves). Journal of Experimental Biology. 2003; 206:3693–3706. [PubMed: 12966061]

Brantley RK, Marchaterre MA, Bass AH. Androgen effects on vocal muscle structure in a teleost fish with intersexual and intrasexual dimorphism. Journal of Morphology. 1993; 216:305–318. [PubMed: 8315650]

Clark CJ, Feo TJ, Bryan KB. Courtship displays and sonations of a hybrid male broad-tailed and black-chinned hummingbird. Condor. 2012; 114:329–340.

Day LB, Fusani L, Hernandez E, Billo TJ, Sheldon KS, Wise PM, Schlinger BA. Testosterone and its effects on courtship in golden-collared manakins (*Manacus vitellinus*): seasonal, sex, and age differences. Hormones and Behavior. 2007; 51:69–76. [PubMed: 17022984]

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*). Hormones and Behavior. 2006; 49:223–232. [PubMed: 16085049]

Day LB, Westcott DA, Olster DH. Evolution of bower complexity and cerebellum size in bowerbirds. Brain, Behavior and Evolution. 2005; 66:62–72.

Dobrowolny G, Giacinti C, Pelosi L, Nicoletti C, Winn N, Barberi L, Molinaro M, Rosenthal N, Musaro A. Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model. Journal of Cell Biology. 2005; 168:193–199. [PubMed: 15657392]

Durães R. Lek structure and male display repertoire of blue-crowned manakins in eastern Ecuador. Condor. 2009; 111:453–461.

DuVal EH. Cooperative display and lekking behavior of the Lance-tailed Manakin (Chiroxiphia lanceolata). Auk. 2007; 124:1168–1185.

DuVal EH, Goymann W. Hormonal correlates of social status and courtship display in the cooperatively lekking lance-tailed manakin. Hormones and Behavior. 2010; 59:44–50. [PubMed: 20950621]

Evrard HC, Balthazart J. Localization of oestrogen receptors in the sensory and motor areas of the spinal cord in Japanese quail (Coturnix japonica). Journal of Neuroendocrinology. 2002; 14:894–903. [PubMed: 12421343]

Evrard HC, Balthazart J. Aromatase (estrogen synthase) activity in the dorsal horn of the spinal cord: Functional implications. Steroids and the Nervous System. 2003:263–271.

Evrard HC, Balthazart J. Rapid regulation of pain by estrogens synthesized in spinal dorsal horn neurons. Journal of Neuroscience. 2004; 24:7225–7229. [PubMed: 15317848]

Felsenstein J. Phylogenies and the comparative methods. American Naturalist. 1985; 125:1–15.

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. [PubMed: 20080872]

Freckleton RP, Harvey PH, Pagel M. Phylogenetic analysis and comparative data: a test and review of evidence. American Naturalist. 2002; 160:712–726.

Fusani L, Barske J, Day LD, Fuxjager MJ, Schlinger BA. Physiological control of elaborate male courtship: Female choice for neuromuscular systems. Neuroscience & Biobehavioral Reviews. 2014a; 46:534–546. [PubMed: 25086380]

Fusani L, Donaldson Z, London SE, Fuxjager MJ, Schlinger BA. Expression of androgen receptor in the brain of a sub-oscine bird with an elaborate courtship display. Neuroscience Letters. 2014b; 578:61–65. [PubMed: 24954076]

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.

Fuxjager MJ, Barske J, Du S, Day LB, Schlinger BA. Androgens regulate gene expression in avian skeletal muscles. PloS One. 2012a; 7:e51482. [PubMed: 23284699]

Fuxjager MJ, Eaton J, Lindsay WR, Salwiczek LH, Rensel MA, Julia Barske J, Sorenson L, Day LB, Schlinger BA. Evolutionary patterns of adaptive acrobatics and physical performance predict expression profiles of androgen receptor - but not estrogen receptor - in the forelimb musculature. Dryad Digital Repository. 2015 (doi:10.5061/dryad.1bk06).

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. [PubMed: 23782945]

Fuxjager MJ, Schultz JD, Barske J, Feng NY, Fusani L, Mirzatoni A, Day LB, Hau M, Schlinger BA. Spinal motor and sensory neurons are androgen targets in an acrobatic brid. Endocrinology. 2012b; 153:3780–3791. [PubMed: 22635677]

Hoglund J, Lundberg A. Sexual selection in a monomorphic lek-breeding bird: correlates of male mating success in the great snipe, *Gallinago media*. Behavioral Ecology and Sociobiology. 1987; 21:211–216.

Holland BS, Copenhaver MD. An Improved Sequentially Rejective Bonferroni Test Procedure. Biometrics. 1987; 43:417–423.

How MJ, Hemmi JM, Zeil J, Peters R. Claw waving display changes with receiver distance in fiddler crabs, *Uca perplexa*. Animal Behaviour. 2008; 75:1015–1022.

Irschick DJ, Herrel A, Vanhooydonck B, Van Damme R. A functional approach to sexual selection. Functional Ecology. 2007; 21:621–626.

James RS, Navas CA, Herrel A. How important are skeletal muscle mechanics in setting limits on jumping performance? Journal of Experimental Biology. 2007; 210:923–933. [PubMed: 17337705]

Jetz W, Thomas GH, Joy JB, Hartmann K, Mooers AO. The global diversity of birds in space and time. Nature. 2012; 491:444–448. [PubMed: 23123857]

Kusmierski R, Borgia G, Uy A, Crozier RH. Labile evoltion of display traits in bowerbirds indicates reduced effects of phylogenetic constraint. Proceedings of the Royal Society of London, Series B: Biological Sciences. 1997; 264:307–313. [PubMed: 9107048]

Lailvaux SP, Irschick DJ. A functional perspective on sexual selection: insights and future prospects. Animal Behaviour. 2006; 72:263–273.

Leska A, Kiezun J, Kaminska B, Dusza L. Seasonal changes in the expression of the androgen receptor in the testes of the domestic goose (*Anser anser f. domestica*). General and Comparative Endocrinology. 2012; 179:63–70. [PubMed: 22885558]

Lindsay WR, Giuliano CE, Houck JT, Day LB. Acrobatic courtship display coevolves with brain size in manakins (Pipridae). Brain, Behavior and Evolution. in press.

Losos JB. The evolution of form and function: morphology and locomotor performance in West Indian Anolis lizards. Evolution. 1990; 44:1189–1203.

Madden J. Sex, bowers and brains. Proceedings of the Royal Society of London, Series B: Biological Sciences. 2001; 268:833–838. [PubMed: 11345329]

Muntener M, Kaser L, Weber J, Berchtold MW. Increase of skeletal muscle relaxation speed by direct injection of parvalbumin cDNA. Proceedings of the National Academy of Sciences, USA. 1995; 92:6504–6508.

Musaro A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, Barton ER, Sweeney HL, Rosenthal N. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nature Genetics. 2001; 27:195–200. [PubMed: 11175789]

Nastiuk KL, Clayton KL. Seasonal and tissue-specific regulation of canary androgen receptor messenger ribonucleic acid. Endocrinology. 1994; 134:640–649. [PubMed: 8299561]

Nuechterlein GL, Storer RW. The pair-formation displays of the western grebe. Condor. 1982; 84:350–369.

Ohlson JI, Fjeldsa J, Ericson PGP. Molecular phylogeny of the manakins (Ayes: Passeriformes: Pipridae), with a new classification and the description of a new genus. Molecular Phylogenetics and Evolution. 2013; 69:796–804. [PubMed: 23831559]

Oki K, Wiseman RW, Breedlove SM, Jordan CL. Androgen receptors in muscle fibers induce rapid loss of force but not mass: Implications for spinal bulbar muscular atrophy. Muscle & Nerve. 2013; 47:823–834. [PubMed: 23629944]

Orme, D. The CAPER package: comparative anlysis of phylogenetics and evoltuion in R. 2013. http://www.cran.r-project.org/web/packages/caper/vignettes/caper/pdf

Pagel M. Inferring the histroical patterns of biological evolution. Nature. 1999; 401:877–884. [PubMed: 10553904]

Paradis E, Claude J, Strimmer K. APE: analysis of phylogenetics and evoltuion in R language. Bioinformatics. 2004; 20:289–290. [PubMed: 14734327]

Pearen MA, Goode JM, Fitzsimmons RL, Eriksson NA, Thomas GP, Cowin GJ, Wang SCM, Tuong ZK, Muscat GEO. Transgenic Muscle-Specific Nor-1 Expression Regulates Multiple Pathways That Effect Adiposity, Metabolism, and Endurance. Molecular Endocrinology. 2013; 27:1897–1917. [PubMed: 24065705]

Prum RO. Phylogenetic analysis of the evolution of display behavior in the neotropical manakins (Aves, Pipridae). Ethology. 1990; 84:202–231.

Prum RO. Phylogenetic analysis of the evolution of alternative social behavior in the manakins (Aves: Pipridae). Evolution. 1994; 48:1657–1675.

Prum RO. Sexual selection and the evolution of mechanical sound production in manakins (Aves : Pipridae). Animal Behaviour. 1998; 55:977–994. [PubMed: 9632483]

Rand MN, Breedlove SM. Androgen locally regulates rat bulbocavernosus and levator ani size. Journal of Neurobiology. 1992; 23:17–30. [PubMed: 1564453]

Rand MN, Breedlove SM. Androgen alters the dentritic arbors of SNB motoneurons by acting upon their target muscles. Journal of Neuroscience. 1995; 15:4408–4416. [PubMed: 7540674]

Regnier M, Herrera AA. Changes in the contractile properties by androgen hormones in sexually dimorphic muscles of male frogs (*Xenopus laevis*). Journal of Physiology (Cambridge). 1993; 461:565–581.

Schlinger BA, Barske J, Day L, Fusani L, Fuxjager MJ. Hormones and the neuromuscular control of courtship in the golden-collared manakin (*Manacus vitellinus*). Frontiers in Neuroendocrinology. 2013; 34:143–156. [PubMed: 23624091]

Schlinger BA, Day LB, Fusani L. Behavior, natural history and neuroendocrinology of a tropical bird. General and Comparative Endocrinology. 2008; 157:254–258. [PubMed: 18579141]

Schultz JD, Schlinger BA. Widespread accumulation of [H-3]testosterone in the spinal cord of a wild bird with an elaborate courtship display. Proceedings of the National Academy of Sciences, USA. 1999; 96:10428–10432.

Shaffer JP. Modified Sequentially Rejective Multiple Test Procedures. Journal of the American Statistical Association. 1986; 81:826–831.

Shaffer JP. Multiple Hypothesis-Testing. Annual Review of Psychology. 1995; 46:561–584.

Shaw P. The social-behavior of the pin-tailed whydah, vidua macroura, in northern Ghana. Ibis. 1984; 126:463–473.

Svensson J, Movérare-Skrtic S, Windahl S, Swanson C, Sjögren K. Stimulation of both estrogen and androgen receptors maintains skeletal muscle mass in gonadectomized male mice but mainly via different pathways. Journal of Molecular Endocrinology. 2010; 45:45–57. [PubMed: 20435684]

Tobias ML, Marin ML, Kelley DB. The role of sex, innervation, and androgen in laryngeal muscle of *Xenopus laevis*. Journal of Neuroscience. 1993; 13:324–333. [PubMed: 8423478]

Vleck CM, Priedkalns. Reproduction in zebra finches: hormone levels and effect of dehydration. The Condor. 1985; 87:37–46.

Vliet KA. Social displays of the American alligator (*Alligator mississippiensis*). American Zoologist. 1989; 29:1019–1031.

Voigt CC, von Helversen O, Michener R, Kunz TH. The economics of harem maintenance in the sac-winged bat, Saccopteryx bilineata (Emballonuridae). Behavioral Ecology and Sociobiology. 2001; 50:31–36.

Walls SC, Semlitsch RD. Visual and movement displays function as agonistic behavior in larval salamanders. Copeia. 1991; 1991:936–942.

Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR, Ham J, Kang H, Evans RM. Regulation of muscle fiber type and running endurance by PPAR delta. PLoS Biology. 2004; 2:1532–1539.

Westcott DA, Smith JNM. Behavior and social organization during the breeding season in *Mionectes oleagineua*, a lekking flycatcher. Condor. 1994; 96:672–683.

Whittemore LA, Song KN, Li XP, Aghajanian J, Davies M, Girgenrath S, Hill JJ, Jalenak M, Kelley P, Knight A, Maylor R, O'Hara D, Pearson A, Quazi A, Ryerson S, Tan XY, Tomkinson KN, Veldman GM, Widom A, Wright JF, Wudyka S, Zhao L, Wolfman NM. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochemical and Biophysical Research Communications. 2003; 300:965–971. [PubMed: 12559968]

Williams H. Choreography of song, dance and beak movements in the zebra finch (Taeniopygia guttata). Journal of Experimental Biology. 2001; 204:3497–3506. [PubMed: 11707499]

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. Molecular Endocrinology. 2010; 24:1665–1674. [PubMed: 20610535]

Zar, JH. Biostatistical Analysis. Prentice Hall; Upper Saddle River: 1999.

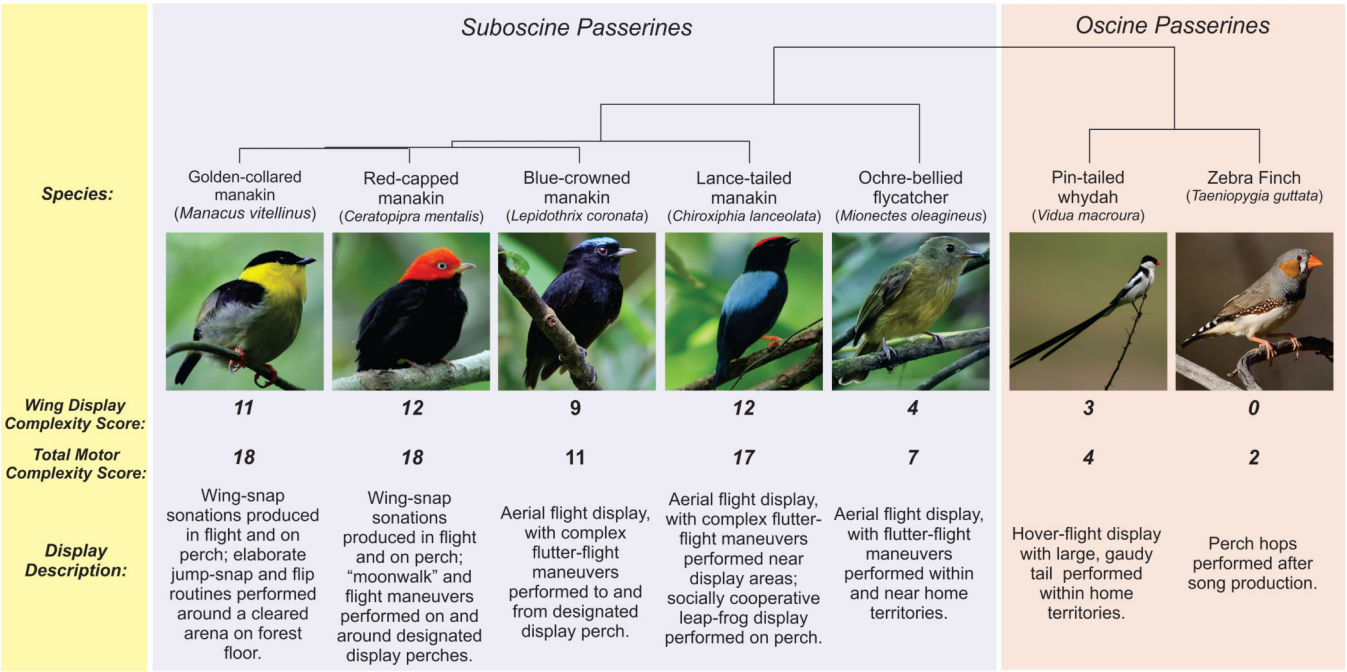

Figure 1.

Passerine species included in the current study. At top is our reconstructed phylogenetic tree that depicts the relatedness among species (Jetz *et al.* 2012). Each species' image is included under its common and scientific name. Under each image are the species' scores on the two display complexity indices: the *wing movement complexity score* and *overall motor complexity score* (see Table 1 and Methods). At the bottom is a brief description of the bird's display. Species included in the light blue box are suboscine passerines, whereas species in the pink box are oscine passerines. Manakin and flycatcher photographs from Nick Athanas; pin-tailed whydah photograph from Jody de Bruyn; zebra finch photograph from Mat Gilfedder.

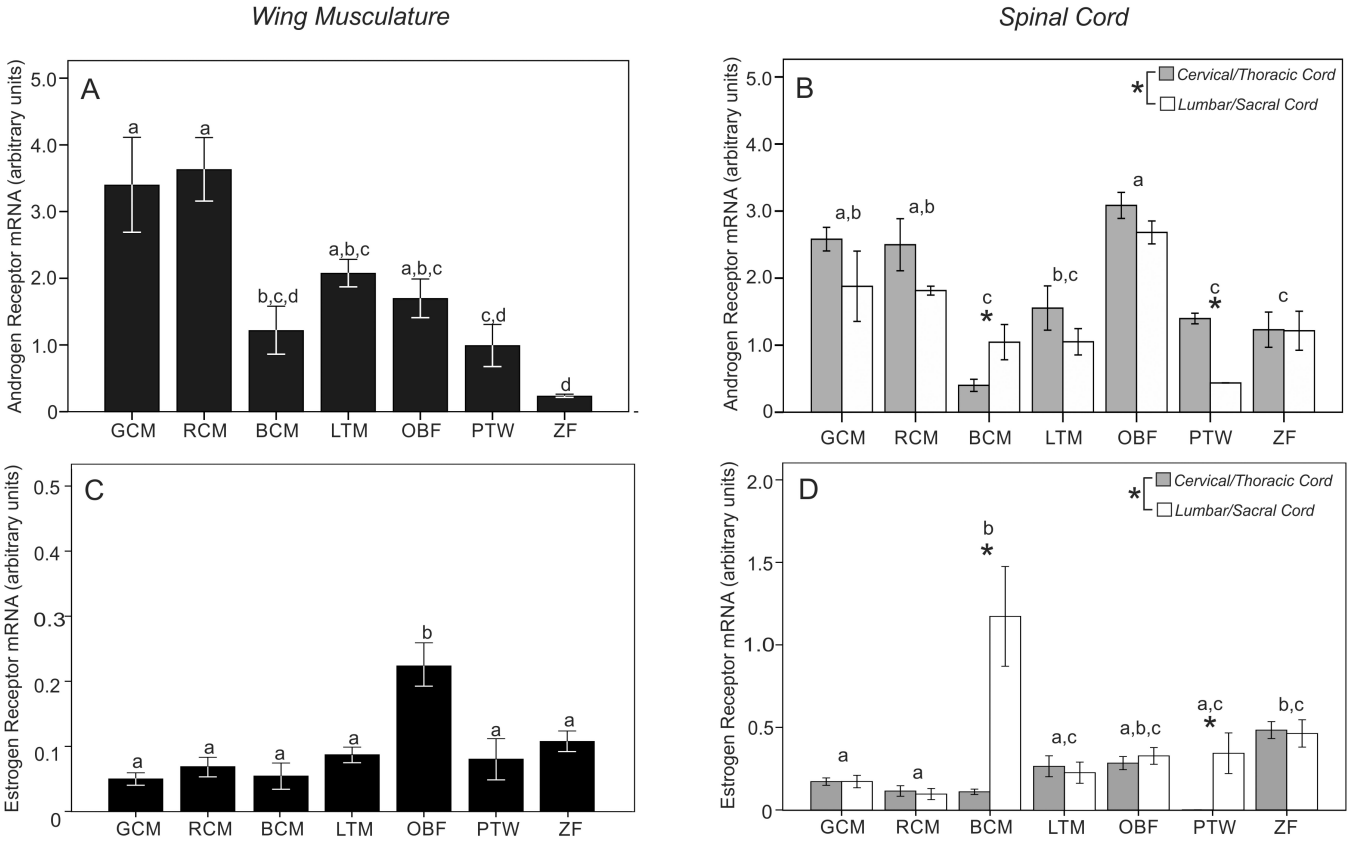

Figure 2.

Androgen receptor (*AR*) and estrogen receptor α (*ERα*) mRNA expression in the different species' (A and C) wing muscles and (B and D) spinal cords. Both *AR* (A) and *ERα* (C) levels in the *supracoricoidieus* (SC), *scapulohumeralis caudalis* (SH) and *pectoralis* (PEC) are collapsed into one group for each species, as we find no effect of muscle on either gene's expression. In the spinal cord, gray bars indicate *AR* (B) and *ERα* (D) levels in cervical/thoracic region, whereas white bars indicate these genes' expression in the lumbar/sacral region. Note that the axes between these two graphs are different, as *ERα* was abundantly expressed in the blue-crowned manakin lumbar/sacral cord. In all graphs, differences in letters atop error bars depict significant differences between species (Shaffer-Holm correction), whereas asterisks (*) under a species' respective letter depict species-specific regional differences. Species are indicated on the horizontal axis (GCM=golden-collared manakin; RCM = red-capped manakin; BCM = blue-crowned manakin; LTM = lance-tailed manakin; OBF = ochre-bellied flycatcher; PTW = pin-tailed whydah; and ZF = zebra finch). Data represent means ± 1SEM.

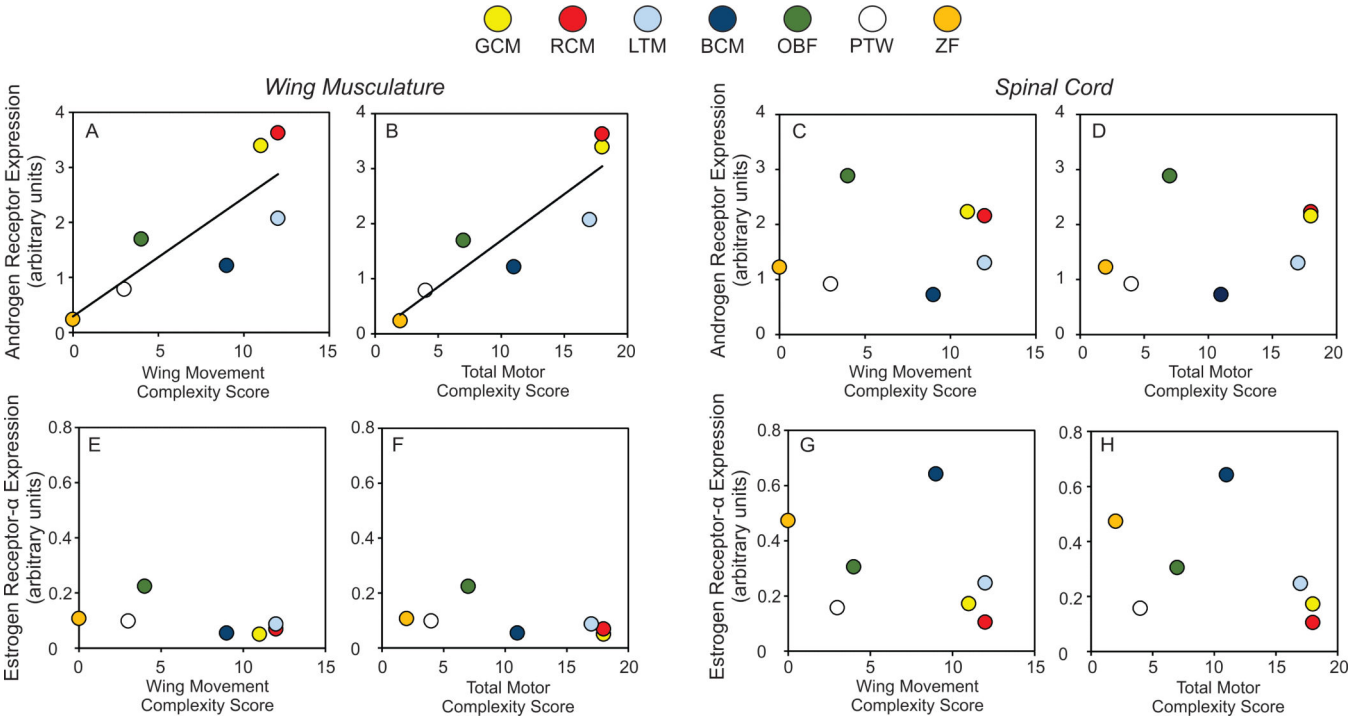

Figure 3.

Relationship between species variation in neuromuscular androgen receptor (*AR*) expression and display complexity. (A) Overall display complexity and (B) wing movement display complex and their relationships to levels of wing muscle *AR* expression. (C) Overall display complexity and (B) wing movement display complex and their relationships to levels of spinal cord *AR* expression. Graphic representations do not correct for phylogenetic relatedness, although these corrections are implemented statistically using phylogenetic generalized least-squares (PGLS) models. Best-fit lines represent significant models ($p<0.05$). GCM=golden-collared manakin; RCM = red-capped manakin; BCM = blue-crowned manakin; LTM = lance-tailed manakin; OBF = ochre-bellied flycatcher; PTW = pin-tailed whydah; and ZF = zebra finch. *AR* mRNA levels represent each species' estimated marginal means (EMM) derived from mixed-model analyses.

Table 1

Display complexity scoring system and its application to each species in our study.

DISPLAY CHARACTER*	SPECIES AND THEIR SCORES†							CITATION REFLECTING SCORE'S ORIGIN
	Golden-collared manakin	*Red-capped manakin*	*Blue-crowned manakin*	*Lance-tailed manakin*	*Ochre-bellied flycatcher*	*Pin-tailed whydah*	*Zebra finch*	
Up-right Body Posture	0	1	0	0	0	1	0	Described and scored by Prum (1990)
Horizontal Posture	0	1	0	0	0	0	0	
Hunched Posture	0	0	0	0	1	0	0	
Throat Feathers Erected/Extended	1	0	0	0	0	0	0	
About-Face Display	1	1	1	0	0	0	0	
Slide-Down Display	1	0	0	0	0	0	0	
Backward Slide Display	0	1	0	0	0	0	0	
Side-To-Side Jump Display	0	0	1	1	0	0	0	
Stationary Display	0	0	0	0	0	1	0	
Bow Display	0	0	1	1	0	0	0	
Grunt-Jump Display§	1	0	0	0	0	0	0	
Frenzied-Flutter/Hover Display	0	1	1	0	1	1	0	
Cart-Wheel Display	0	0	0	1	0	0	0	
Butterfly-Flight Display	0	0	1	1	1	0	0	
Whirring-To-And-Fro Flight Display	0	0	1	0	0	0	0	
To-And-Fro Flight Display	1	1	1	1	1	0	0	
Jump-Snap Display§	1	0	0	0	0	0	0	
'S'-Curve or Swoop-In Flight Display	0	1	1	0	0	0	0	
Exaggerated or Soft Wing-Flicks	0	1	1	1	1	1	0	Described by Westcott and Smith (1994)
Trailing	1	0	1	1	1	0	0	
High and Low Perch-To-Perch Flight Display	0	0	1	0	0	0	0	Described by Duraes (2009)
Up and Down	0	0	0	1	0	0	0	Described by Duval (2007)
Pip Flight	0	0	0	1	0	0	0	
Swoop Maneuver	0	0	0	1	0	0	0	
Eek Display	0	0	0	1	0	0	0	

DISPLAY CHARACTER*	SPECIES AND THEIR SCORES†							CITATION REFLECTING SCORE'S ORIGIN
	Golden-collared manakin	*Red-capped manakin*	*Blue-crowned manakin*	*Lance-tailed manakin*	*Ochre-bellied flycatcher*	*Pin-tailed whydah*	*Zebra finch*	
Bounce Move	0	0	0	1	0	0	0	
Somersault Maneuver	1	0	0	0	0	0	0	Described by (Fusani et al. 2007)
Jump-snip§	1	0	0	0	0	0	0	Described by Day et al. (2006) and Chapman (1935)
Hops	0	0	0	0	1	0	1	Described by Williams (2001)
Side-To-Side Head Movements	0	0	0	0	0	0	1	
Sonation Repertoire Size (*1 point for each unique mechanical sonation produced*)§	4	5	0	2	0	0	0	Described by Prum (1998)
Sonation Pulse Type (*7 = single pulse, 2 = single and multiple pulse*)	2	2	0	1	0	0	0	
Wing Movement Complexity Score (SUM of CHARACTERS in BOLDFACE)	**11**	**12**	**9**	**12**	**4**	**3**	**0**	
Total Motor Complexity Score (SUM of ALL CHARACTERS)	**18**	**18**	**11**	**17**	**7**	**4**	**2**	

*Characters and elements written in boldface typesetting indicate maneuvers that involve/require wing movement, and that are used to compute a wing-motor complexity score.

†Scores updated in manakins and generated in non-manakins using current descriptions of each species social display repertoire (see Shaw 1984; Westcott and Smith 1994; Williams 2001; Bostwick and Prum 2003; Day et al. 2006; Duval 2007; Fusani et al. 2007; Duraes 2009).

§Display characters that require rapid wing movements.

Table 2

Estimated marginal means (±1SEM) of gonadal androgen receptor (AR) expression levels across species*.

Species	Relative AR expression (arbitrary units)
Golden-collared manakin (GCM)	3.68±0.50
Red-capped manakin (RCM)	2.60±0.62
Blue-crowned manakin (BCM)	1.84±0.44
Lance-tailed manakin (LTM)	2.04±0.62
Pin-tailed whydah (PTW)	6.05±0.87
Zebra Finch (ZF)	1.47±0.55

*Note that ochre-bellied flycatchers are omitted because be did not have access to gonadal tissues from these birds.