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# Androgenic signaling systems and their role in behavioral evolution

Matthew J. Fuxjager*, Eric R. Schuppe

*Department of Biology, Wake Forest University, 455 Vine Street, Winston-Salem, NC 27101, United States*

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ABSTRACT

Sex steroids mediate the organization and activation of masculine reproductive phenotypes in diverse vertebrate taxa. However, the effects of sex steroid action in this context vary tremendously, in that steroid action influences reproductive physiology and behavior in markedly different ways (even among closely related species). This leads to the idea that the mechanisms underlying sex steroid action similarly differ across vertebrates in a manner that supports diversification of important sexual traits. Here, we highlight the *Evolutionary Potential Hypothesis* as a framework for understanding how androgen-dependent reproductive behavior evolves. This idea posits that the cellular mechanisms underlying androgenic action can independently evolve within a given target tissue to adjust the hormone's functional effects. The result is a seemingly endless number of permutations in androgenic signaling pathways that can be mapped onto the incredible diversity of reproductive phenotypes. One reason this hypothesis is important is because it shifts current thinking about the evolution of steroid-dependent traits away from an emphasis on circulating steroid levels and toward a focus on molecular mechanisms of hormone action. To this end, we also provide new empirical data suggesting that certain cellular modulators of androgen action—namely, the co-factors that dynamically adjust transcritpional effects of steroid action either up or down—are also substrates on which evolution can act. We then close the review with a detailed look at a case study in the golden-collared manakin (*Manacus vitellinus*). Work in this tropical bird shows how androgenic signaling systems are modified in specific parts of the skeletal muscle system to enhance motor performance necessary to produce acrobatic courtship displays. Altogether, this paper seeks to develop a platform to better understand how steroid action influences the evolution of complex animal behavior.

## 1. Introduction

Sex steroid signaling plays a fundamental role in regulating many aspects of reproductive phenotypes across vertebrates. This is because sex steroids guide the organization of sexual structures early in life, and then later activate key traits during specific moments in an animal's life, when reproductive opportunities are greatest [1–8]. In this sense, sex steroids can be viewed as molecular agents that mediate animal traits based on the external environment, and ultimately help generate behavior in a context-specific manner [9,10]. This framework has defined our thinking about steroidal regulation of adaptive animal behavior for decades. However, there are still many unanswered questions about the role played by sex steroids to influence the remarkable behavioral diversity in the natural world. How do sex steroids—which are highly conserved in their molecular structure—generate so many different behavioral traits across the vertebrate tree of life? Part of the answer likely lies in the sheer diversity of cellular mechanisms by which steroids operate, which inevitably varies among these taxa. As a result, the impact of steroidal signaling can generate an impressive range of both physiological and behavioral outcomes.

This review aims to probe this question, and then create a framework for understanding how mechanistic diversity in steroid action is borne out in animal physiology and behavior. Through this lens, we can begin to conceptualize the many routes by which steroid signaling systems change in functionally meaningful ways to influence phenotypic evolution. This is by no means the first attempt to discuss this topic; rather, the review revisits past ideas framed in our most current understanding of species variation in the properties of sex steroid action and its effect on adaptive behavioral output [11–16]. We hope that this work stimulates further investigations into the intricacies of steroid physiology and its relationship to the diversification of organismal form, function, and performance.

We focus most of the review specifically on genomic signaling mechanisms of androgenic hormones, discussing recent studies that advanced our understanding of how these processes can change over time to support the diversification of reproductive phenotypes. We then present original data that expand our knowledge of how evolution can shape expression of key molecular players in this signaling system.

* Corresponding author.
*E-mail address:* fuxjagmj@wfu.edu (M.J. Fuxjager).

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Finally, we close the review with a case study in a tropical bird called the golden-collared manakin (*Manacus vitellinus*) [17]. This species performs an acrobatic courtship dance that appears to have evolved in part due to androgen-dependent regulation of extraordinary physiological performance. Overall, our aim is to develop a framework grounded in empirical evidence that elucidates how sex steroid systems are exploited by evolution to drive behavioral adaptation and diversification, particularly in terms of reproductive traits.

## 2. Evolutionary potential of androgenic hormone systems

Androgens are the primary sex steroids that regulate the masculine reproductive phenotype [9,18]. They do this largely by altering local patterns of gene expression (genomic signaling), and thus reconfiguring the parameters that determine how a given target cell functions. However, they can also exert rapid non-genomic effects to alter cellular functioning. Regardless of the route of action, androgenic effects at the cell-level ultimately balloon up to alter how entire tissues work, which ultimately influences both physiology and behavior [19]. Accordingly, altering the mechanisms of androgenic action at a local target tissue is one way that evolution can shape the way by which androgens regulate a broad range of reproductive phenotypes.

### 2.1. Evolutionary potential hypothesis

For decades, studies of species variation in androgenic systems have largely focused on circulating levels of androgens, such as testosterone (T) [20–27]. Although this work has certainly contributed to our understanding of how androgenic profiles may evolve to accommodate taxonomic differences in behavior, it has also periodically fallen short of achieving these goals. For example, there are many instances in which species differences in important aspects of reproductive biology are not predicted by differences in circulating levels of T [28–30]. This has lead researchers to rightfully speculate that other factors likely modulate androgen signaling across species (beyond circulating levels alone) to influence the expression of complex traits essential to reproductive success. In turn, these alternative mechanisms should underlie behavioral differences, and even potentially support the processes by which behavioral traits, strategies, and syndromes are adapted or diversified. This idea is cogent from an evolutionary perspective, considering that the genes that encode proteins related to androgen biosynthesis, detection, and signal transduction should evolve faster than the evolution of steroid hormone structure.

One of the most important models of steroid system evolution is captured through the "*Evolutionary Potential Hypothesis*" [12]. The overarching idea of this hypothesis is compellingly straightforward: *i*) evolutionary forces can modify the molecular elements that comprise the androgenic signaling cascade independently of each other, and *ii*) such modifications can occur in a tissue-specific manner. This means that a given species will maintain its own androgenic phenotype that presumably evolves in response to selection for certain suites of reproductive traits. This model is exciting is because it showcases a seemingly endless array of possible androgenic phenotypes, which in theory can liberate the processes of reproductive adaptation and explain the diversity of complex traits used for courtship, sex and competition that we see in the nature. Indeed, the *Evolutionary Potential Hypothesis* was first posed in the context of life history theory, which predicts that selection favors efficient physiological mechanisms to govern the strategies animals use for allocation. This frequently occurs through the coupling of various life history traits into trade-offs [31,32], with androgens acting as a prime mediator between many of these competing traits [33–36]. Nonetheless, the model also applies more broadly to the evolution of androgen-dependent behavior, or suites of behavior, even if they are not classically studied life history traits *per se*.

Of course, it is also important to mention that the *Evolutionary Potential Hypothesis* was first discussed considering a contrasting view, referred to as the *Evolutionary Constraint Hypothesis* [12,see also 13,16]. Under this framework, androgenic signaling systems evolve as a singular complex, such that the machinery that underlies androgen-induced signal transduction and functional output is tightly linked and cannot be modified in an independent node-specific manner. A full discussion of this idea and the evidence supporting it can be found elsewhere [12], and thus is beyond the scope of this paper. Rather, we recognize that both of these ideas— *Evolutionary Potential vs. Evolutionary Constraint*—represents opposite ends of a single continuum; in other words, they are not mutually exclusive ideas [12]. Accordingly, it is likely that androgenic signaling systems do in fact impose a certain amount of constraint on the process of behavioral evolution. Unraveling precisely how (and when) this constraint is borne out in androgenic system lability promises to be a fruitful avenue of research.

Regardless of these considerations, our current aim is to assess the *Evolutionary Potential* of the androgenic system in the context of adaptive behavioral change over evolutionary time scales. As a first step toward this goal, we must explore the different molecular elements that evolution can, in theory, adjust to change how androgens impact discrete targets in the body. Canonical genomic pathways of androgenic action are the best understood (Fig. 1A), and thus offer a nice starting point for this discussion. This process begins with circulating androgens—namely, testosterone (T)—which are synthesized from cholesterol by numerous enzymes and then released (largely) from the gonads [37]. The hormone then circulates throughout the bloodstream, passing through the membrane of cellular targets. Once inside the cell, T is quickly metabolized to either dihydrotestosterone (DHT) or estradiol ($E_2$) *via* the enzymes 5α-reductase and aromatase, respectively. DHT then acts *via* intracellular androgen receptor (AR), whereas $E_2$ acts *via* intracellular estrogen receptor (ER). Both are nuclear receptors that act as ligand (hormone)-activated transcription factors, and ultimately influence patterns of gene expression [9,37]. Once activated, the receptors dimerize and translocate to the nucleus, where they recruit a variety of co-factors that mediate the process of transcription (Fig. 1A/B) [38]. At the promoter region of target genes, these newly-formed complexes bind to their respective hormone response elements—either androgen response elements (ARE) or estrogen response elements (ERE) [39]. The result is the initiation of a cellular response to circulating steroids, all made possible *via* regulating gene expression. Of course, ligand-bound AR can also interact with other transcription factors in a cell, and thus bind to response elements other than AREs [40]. In this sense, AR can behave promiscuously to induce changes in gene expression.

An alternative route of androgen action occurs through non-genomic signaling, the effects of which are typically rapid (within minutes) [41,42]. Once DHT binds AR, for example, it can operate *via* mitogen-activated protein kinase (MAPK) signaling pathways, which activate extracellular signaling-regulated kinase (ERK) [43,44]. There are a variety of ways in which this chain of events is "turned on," including through interactions with the phosphatidyl-inositol 3-kinase (PI3K)/Akt, Src, and protein kinase C (PKC) pathways [45–49]. In each of these instances, AR initiates the phosphorylation of agents that serve as upstream regulators of MAPK, thus leading to a host of functional effects that impact cellular homeostasis and proliferation [41,50]. In addition, other non-genomic effects of AR and ER signaling may occur through membrane-bound receptors. With respect to membrane-bound AR, for example, studies in different cell types suggest T activation of receptors present specifically in the plasma membrane regulate important cellular events, such as intracellular calcium concentrations and/or rapid changes to actin cytoskeleton dynamics [51,52], and many of these effects are thought to occur through direct interference with the PI3K and MAPK pathways mentioned above [53].

In theory, each molecular component involved in these signaling cascades can be subject to evolutionary modification—either in terms of expression levels within a target cell or genetic modification

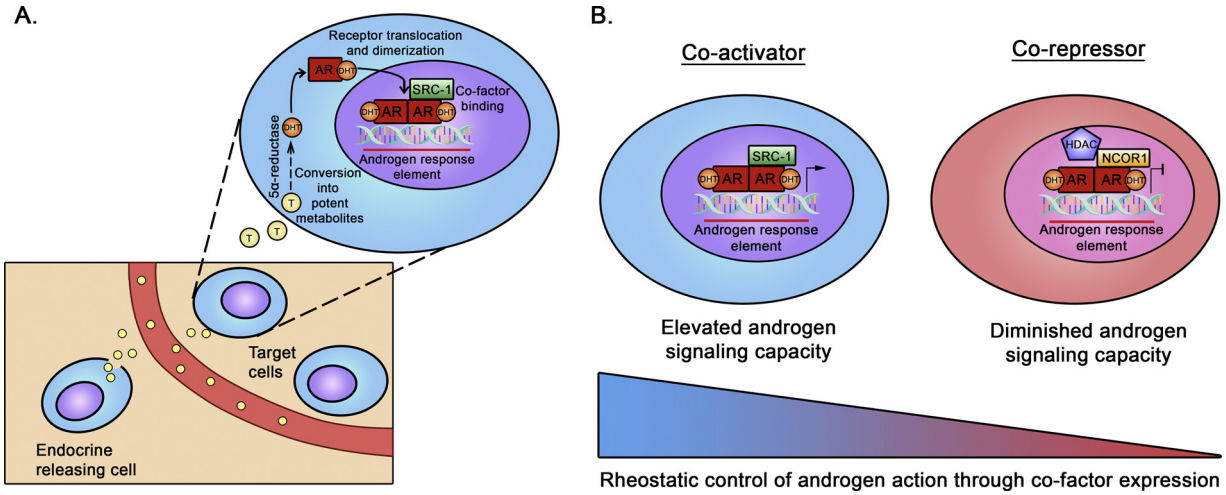


**Fig. 1.** Schematic representation of androgen signaling mechanisms and the modulatory role of co-factors. (**A**) Illustration of the release of androgens from distant endocrine cells into circulation and how these hormones exhibit their potent genomic effects on downstream gene expression in target tissues (mechanisms of non-genomic action not illustrated). (**B**) Differential expression of co-factors modulates androgen-signaling capacity. For instance, elevated co-activator expression (*e.g.* SRC-1) can augment signaling capacity by recruiting factors that enhance transcription, whereas co-repressors (*e.g.* NCOR1) recruit chromatin remodeling factors (*e.g.* histone deacetylases [HDACs]) that make the DNA inaccessible for transcription, leading to diminished signaling potential. In this sense, regulation of co-factor expression in target tissue can rheostatically modulate androgen action.

(although we expect that this first possibility is the most likely to occur, considering that many of the proteins that play a role in androgenic signaling are shared with other vital signaling mechanisms). Thus, in the following paragraphs, we will highlight the studies that have begun to explore how changes in these pathways can support the evolution of behavioral traits. We will focus specifically on genomic effects of AR in males, since these mechanisms have been studied the most in terms of their role in evolutionary process.

### 2.2. Support for the evolutionary potential hypothesis

The *Evolutionary Potential* model has rapidly accumulated support in a diverse range of taxa in recent years. Recent work in dark-eyed juncos (*Junco hyemalis*) nicely demonstrates this point. For instance, gonadal expression of two key genes that encode enzymes necessary for androgen biosynthesis [cytochrome p450 side-chain cleavage (p450scc) and cytochrome p450 17α-hydroxylase (CYP17)] is greater in the white-winged junco (*J. h. aikeni*), compared to a Carolina subspecies (*J. h. carolinensis*). Importantly, this difference in the molecular constitution of the testes is associated with differences in the ability of these two subspecies to quickly elevate circulating T levels in response to stimulation of the hypothalamic-pituitary-gonadal (HPG) axis [14,54]. As one might expect given the gene expression results, the white-winged junco can mount a significantly greater T response, potentially because its gonads contain more enzyme necessary to produce T. Furthermore, the white-winged junco is more aggressive during the breeding season, and it shows more ornamentation than its close relative. Accordingly, these findings imply that evolution optimizes bloodstream androgen levels by adjusting the mechanisms that guide gonadal synthesis to influence multiple aspects of reproductive phenotype.

Other work shows that individual- = variation in key aspects of androgenic signaling systems beyond androgenic ligands, such as AR densities in select target tissues, predicts individual differences in behavior. For example, individual male rodents and birds that express higher levels of AR in specific brain nuclei produce more aggressive behavior in sexual contexts, which can lead to important social victories that enhance reproductive success [55–58]. Likewise, researchers also document robust sex differences in the AR levels of certain tissues that are associated with the actual production of sexual signals (*e.g.*, the vocal organs of birds and frogs [59,60]). Thus, from a microevolutionary perspective, androgenic sensitivity in tissues and organs that directly control reproductive behavior seems to be linked the presence and persistence of these traits.

Yet some of the most intriguing support for the *Evolutionary Potential Hypothesis* comes from more recent work that explores differences in androgenic signaling mechanisms across multiple taxa. One example is found in a species of frog (*Staurois parvus*), where males compete at breeding sites by performing elaborate waving displays with their hind limbs. This so-called foot flag signal has co-evolved with a nearly 10-fold increase in AR expression in the thigh muscles, which generate the many of movements required to produce this behavior. Equally interesting is that these AR levels are similar to those found in the larynx, another tissue influenced by sexual selection for reproductive signaling. However, other motor tissues that similarly influence foot flag production, such as the spinal cord, show no obvious species difference in levels of AR expression [61]. Altogether, these findings imply that the evolutionary “innovation” of this gestural display is linked in part to changes in androgenic sensitivity, specifically in skeletal muscles that control the signal's output [61,62].

Work in *Anolis* lizards (anoles) similarly shows a co-evolutionary relationship between AR levels and reproductive display behavior. Males of many species court their mates and fight off other males by darting around on arboreal perches, while producing push-up and head-bob displays [63]. Species differences in rates of these displays positively predict variation in AR levels in the bicep muscle (which contributes to movements underlying a lizard push-up). At the same time, neither species body mass or muscle fiber size are correlated to AR levels [64]. These findings therefore provide intriguing support for the notion that sexual behavior and tissue-specific AR profiles can co-evolve in a functionally significant manner; however, the story is more complex. For example, the authors also report anecdotal evidence of a positive relationship between levels of circulating T and the amount of AR protein in the biceps. Because AR can auto-regulate [65,66], these differences may reflect greater levels of T in species that display more frequently. Or, this result may reflect a situation in which diversification in display behavior co-evolves with changes to both levels of androgenic ligand and receptor. Nonetheless, given that there are roughly 400 species of *Anolis* lizards that vary in terms of their display routines [63], this group of taxa promises to be a powerful model by which we can further investigate the evolution of the androgenic system.

### 2.3. Evolutionary potential in the molecular mediators of androgen action

Most of the work described in the preceding sections suggests that the mechanisms of T production and androgenic sensitivity of target tissues are the major points of variation in the androgenic signaling system. However, the mechanisms of androgenic action are complex, and thus there are many other elements within this cascade that can be shaped by evolutionary forces. This includes the co-factors that otherwise mediate how ligand-activated AR influences gene expression [38,67]. For instance, elevated co-activator expression (*e.g.* SRC-1, RPL7, CPB) can augment androgenic signaling capacity by recruiting a variety of transcription factors that impact gene expression itself [68]. At the same time, co-repressors (*e.g.* NCOR1, SRMT) can interact with steroid hormone receptors to diminish their effect on gene expression [38]. As a result of these effects, many of these co-factors are thought to act as rheostats [69], either dialing up or dialing down the effects of steroid hormones on gene expression (Fig. 1B).

Differential modulation of local co-factor expression offers an intriguing alternative route by which evolution can fine-tune hormonal control of physiology and behavior at specific sites within the brain and periphery. Support for this idea, however, is lacking; only a handful of studies show within-species sex differences in co-factor expression [70–73]. These data are nonetheless intriguing because many co-factor differences occur specifically in brain regions where steroid hormones are known to help mediate production of adaptive reproductive behavior [67,74,75]. In turn, this lends credence to the notion that co-factor abundance can evolve in response to selection for specific behavioral traits.

We present data herein that similarly suggest that co-factors can be targets of evolution. As a first step, we used PCR to amplify AR, as well as two types of co-factor (the co-activator SRC1 and co-repressor NCOR1) that are well-documented binding partners of this receptor [76,77]. We perform this analysis in a variety of tissues throughout three passerine bird species: the northern cardinal (*Cardinalis cardinalis*), white-breasted nuthatch (*Sitta carolinensis*), and blue-crowned manakin (*Lepidothrix coronata*) (Fig. 2). All individuals in this experiment were free-living males that were captured during the peak breeding season, when the gonads were enlarged and producing sperm. Importantly, the species exhibit different behavioral and life history strategies, and represent both oscine (cardinal and nuthatch) and suboscine (manakin) clades.

We found that AR, as well as both SRC1 and NCOR1, were expressed widely throughout the body of all three species (Fig. 2, see Supplemental e-Component). This included the brain, testes, and a host of other peripheral tissues that facilitate diverse physiological processes, which likely impact reproductive success. Thus, this suggests that each tissue has at least the capacity to transcribe co-factor proteins that can fine-tune local androgen action. Equally important is that expression of AR, SRC1 and NCOR1 appears to vary both across species and tissues. A subsequent analysis tested this possibility using quantitative real-time PCR (qPCR) to measure relative expression of each gene in a subset of these tissues (Fig. 3 liver, pectoralis muscle, eye, and testis). We uncovered significant variation in the expression profiles for all three genes according to both species and tissue, with significant interaction effects showing that many of the differences across the taxa occurred in a tissue-specific manner (Fig. 3). Furthermore, each gene's expression pattern was largely independent of the others', meaning that increased AR in each tissue or species did not predict patterns of either SRC1 or NCOR1 expression.

In general, we remain agnostic about the functional significance of these findings, largely because we have no way of determining how these patterns of AR and co-factor expression influence organismal performance. Indeed, there could be a host of neutral and/or selective evolutionary forces that shape the physiological profiles that comprise the androgenic signaling machinery. For example, elevated levels of AR in the cardinal and nuthatch may reflect an enhanced ability to respond to circulating androgens during the condensed breeding season in the temperate forest, compared to the tropical blue-crowned manakin. Yet, these differences may reflect a more closely shared evolutionary history between the two oscine birds (cardinal and nuthatch), relative to the

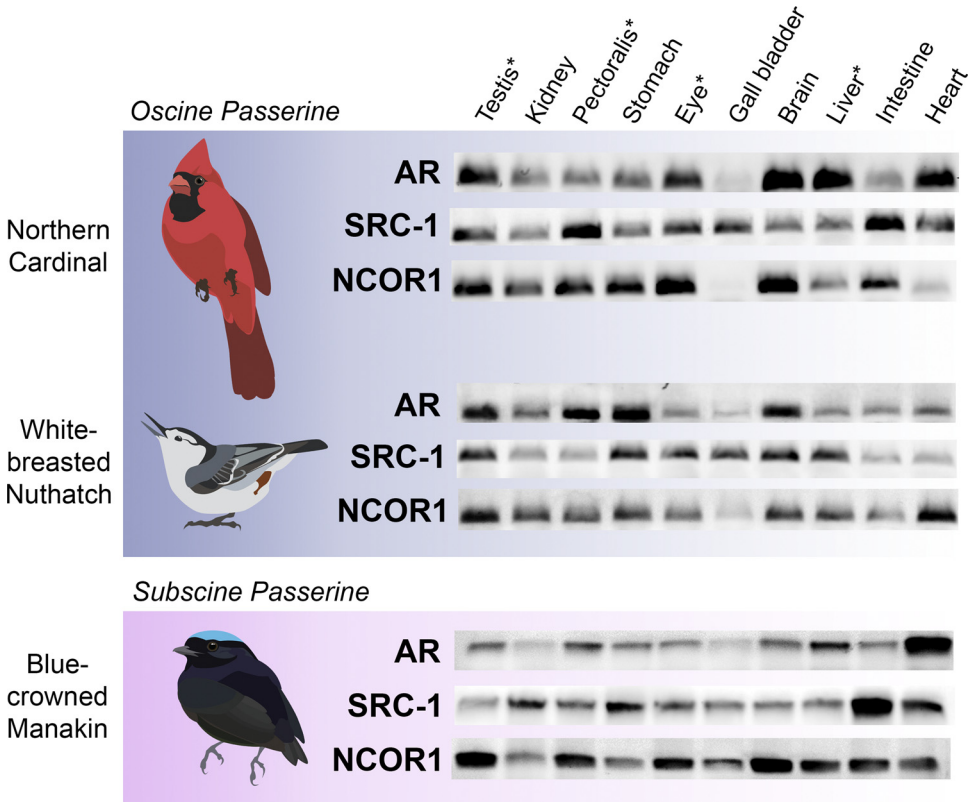


**Fig. 2.** Species comparisons of androgen receptor (AR), steroid receptor co-activator 1 (SRC-1), and nuclear co-repressor 1 (NCOR1) gene expression. Ten tissues were chosen to investigate the capacity for androgen signaling across the body. To evaluate species-level differences, we used two oscine passerines (northern cardinal and white-breasted nuthatch) and one suboscine passerine (blue-crowned manakin). Gel images show representative PCR products for each tissue run on a 1% agarose gel. Asterisks (*) indicate the four tissues used in quantitative PCR analyses.

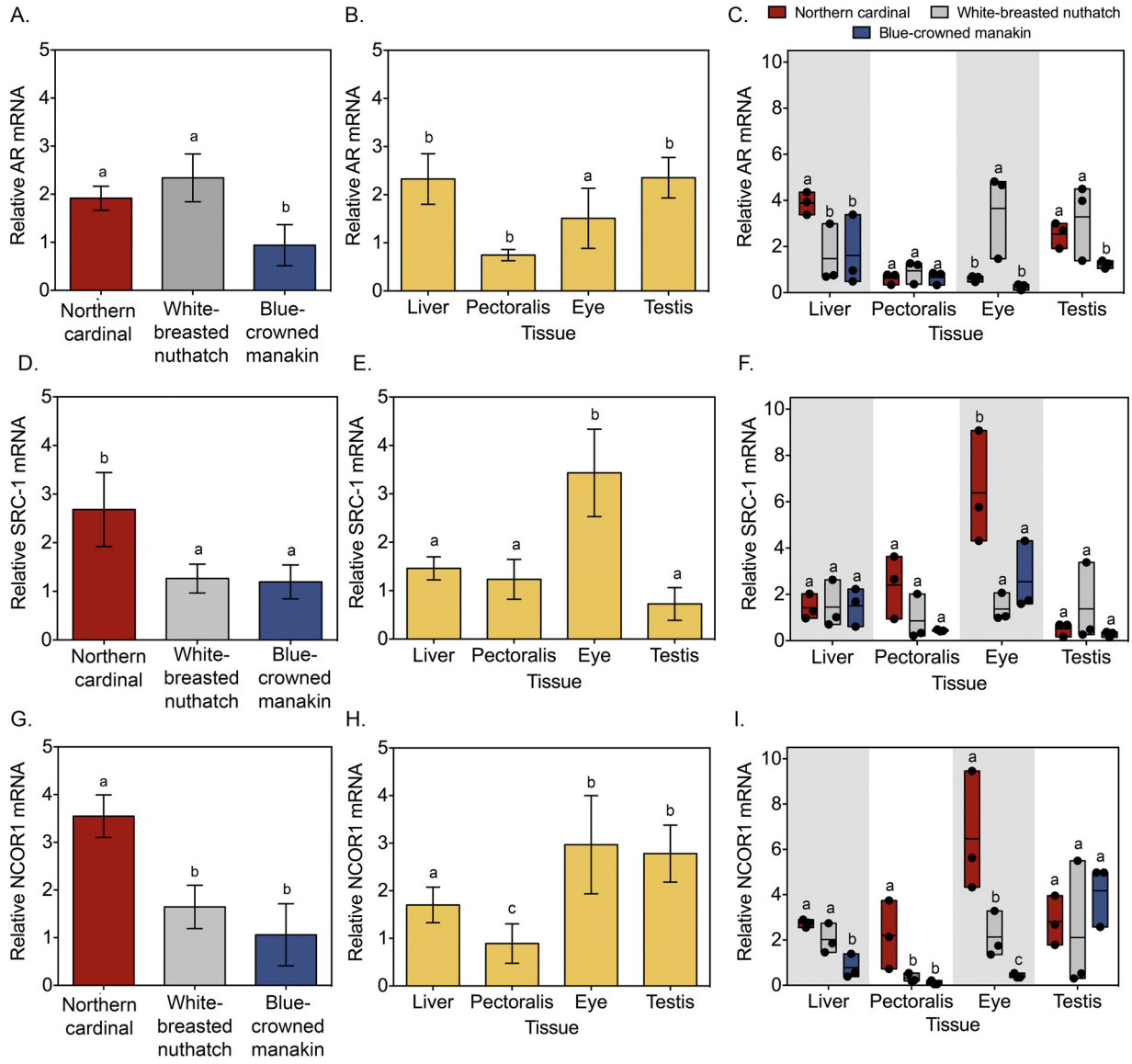


**Fig. 3.** Relative (**A–C**) androgen receptor (AR), **(D–F)** steroid receptor co-factor 1 (SRC-1), and (**G–I**) nuclear co-activator 1 (NCOR1) mRNA expression in the liver, pectoralis muscle, eye, and testis of the northern cardinal, white-breasted nuthatch, and blue-crowned manakin. Note that graphs A, D, and G represent main effects of species derived from the respective ANOVA models, whereas graphs B, E, and H represent main effects of tissue. Meanwhile, graphs C, F, and I represent mean gene expression values of the different tissues for each species. Overall, we found that AR expression differed between species (**A**; $F_{2,24} = 7.64$, $p < 0.001$), with the manakin maintaining lower levels than northern cardinal ($p < 0.01$) and white-breasted nuthatch ($p < 0.01$). Tissues also differenced in AR expression (**B**; $F_{3,24} = 7.68$, $p < 0.001$). For instance, there are higher AR levels in the testis ($p < 0.01$) and liver ($p < 0.01$) than in the pectoralis. There was also a species x tissue interaction (**C**; $F_{6,24} = 4.66$, $P < 0.01$). SRC-1 similarly illustrated species differences (**D**; $F_{2,24} = 9.47$, $p = 0.02$). The cardinal exhibited greater overall SRC-1 levels than both other species ($p < 0.01$). Although there were differences in SRC-1 expression between tissues (**E**; $F_{3,24} = 7.68$, $p < 0.001$), this effect was almost entirely driven by the high levels of co-activator expression in the eye relative to the three other tissues ($p < 0.01$). We also found that SRC-1 levels were impacted by a significant interaction (**F**; $F_{6,24} = 2.57$, $P = 0.046$). Finally, we detected species differences in NCOR1 mRNA expression (**G**; $F_{2,24} = 18.48$, $p < 0.001$). Similar to the co-factor, cardinal maintained higher NCOR1 levels than both species ($p < 0.01$). We were also identified tissue differences in NCOR1 levels (**H**; $F_{3,24} = 6.80$, $p < 0.01$). This effect was partially driven by elevated NCOR1 expression in the testis ($p < 0.01$) and eye ($p < 0.01$) relative to the pectoralis. Differences in NCOR1 levels were also driven by a species x tissue interaction (**I**; $F_{6,24} = 4.43$, $p < 0.01$).

more distantly related suboscine bird (manakin).

Perhaps the most interesting aspects of these data are the clear species differences in tissues-specific profiles of co-factor expression. In the liver, for instance, all three species are indistinguishable in terms of co-activator (SRC1) expression, but blue-crowned manakins express significantly less co-repressor (NCOR1). We see similar variation between co-activator and co-repressor expression in the pectoralis muscle and eye, implying further that the ability of each species to fine-tune androgenic action in each of these tissues using co-factors might vary from species to species. Moreover, in instances where more co-activator is expressed compared to co-repressor, the ability of androgens to exert an effect on the given target tissue may be enhanced (with the opposite true when a tissue contains more co-repressor than co-activator).

Regardless of these interpretations, it is important to note that these results do in fact support the idea that androgenic signaling systems evolve at the molecular levels. From the perspective of adaptive evolution, future work is clearly needed to uncover how similar changes to molecular machinery might ultimately support modifications to androgen-dependent behavior and physiology that augments reproductive success. Of course, this brings us to an equally important point: when

thinking about the evolution of these molecular systems, we must acknowledge the fact that not all components of the signaling pathway are likely equally as labile (both in terms of expression levels and gene evolution). In other words, certain pathways that make up the signaling machinery may be more constrained than others, rending these elements less susceptible to change over time in response to selection. We might expect such differential constrain when elements of the androgenic signaling cascade are shared with other unrelated cellular processes, which is often the case with certain transcriptional co-factors like SRC1 and NCOR1 [78–83]. Nonetheless, figuring out how and when co-factor evolution occurs to support the diversification of adaptive androgenic phenotypes promises to be an exciting new frontier in the field of evolutionary endocrinology.

## 3. A case study: the golden-collared manakin

With the framework of the *Evolutionary Potential Hypothesis* in mind, we next turn to a case study in which we have made relatively significant progress toward understanding *i*) how androgenic systems are modified to support the evolution of a complex social behavior, and *ii*) the physiological mechanisms influenced by these modifications, which ultimately allow selection for behavior to proceed. The focal species of this work is a tropical suboscine bird called the golden-collared manakin (*Manacus vitellinus*), which inhabits the rainforests of Central and South America [17]. This bird is part of the larger avian family of manakins (Pipridae), in which most genera are recognized for their remarkable courtship "dance" displays [84,85]. Although the kinematics and complexity of these physical routines vary significantly across species, they are nonetheless extraordinary in their diversity and have likely co-evolved with physiological modifications that support unorthodox performance traits [86–88]. Golden-collared manakins are no exception, as males of this species court females and compete with rivals by performing a series of high-speed gestural maneuvers [89–91]. The most spectacular of these maneuvers is called a wing-snap, which males produce by raising their opened wings above the back and quickly snapping them together (Fig. 4A). This can be repeated once (*wing-snap*) or rapidly chained together at speeds of nearly 60 snaps per second (*roll-snap*). The result is a loud mechanical sonation that resembles either a firecracker (wing-snap) or severe trill-like trains of multiple firecrackers set off in rapid succession.

Studies characterizing the kinematic basis of wing-snapping indicate that it is performed when the wings are *i*) opened and lifted above the back, and then *ii*) retracted medially until the wrists are forced to collide [92,93]. The latter movement is what generates the characteristic sound that accompanies the display. In addition, extensive field work shows that females preferentially mate with males who perform the various elements of their courtship routine faster and more frequently [94]. Thus, sexual selection by female choice drives the evolution of swift wing- and roll-snaps, likely favoring mechanisms that augment the high-speed performance necessary to produce faster displays. Indeed, examining the rapid frequency at which males can snap their wings together—nearly 60 Hz—makes this point apparent, as this frequency is 2x greater than the maximum wingbeat frequency a similar sized bird uses for powered flight [95]. This means that the golden-collared manakin's performance physiology is modified to support roll-snap production to increase its reproductive success. But which physiological attributes are specialized in this regard? The answer to this question is partially rooted in the modification of androgenic signaling mechanisms, particularly at the level of the skeletal muscular system that actuates the roll-snap display itself.

### 3.1. Skeletal muscle, androgen receptor (AR), and wing-snapping behavior

To better understand how androgen-muscle interactions are modified to support the manakin display, we must first provide a brief description of the neuromuscular basis of the wing-and roll-snap maneuvers. Studies using electromyography (EMG) nicely elucidate these mechanisms by illustrating that two major wing muscles primarily control the behavior [96]: the *supracoracoideus* (SC) and the *scapulohumeralis caudalis* (SH) (Fig. 4B). Biomechanically, the typical role of the SC is to lift the wings during powered flight, whereas the role of the SH is to act as a "strut" by rotating and retracting the wing during difference phases of flight [97,98]. For the manakin display, the SC takes on a similar task by helping to lift the extended wings into position above the back. Subsequent activation of the SH then quickly retracts the humeri [96], which causes the radii to move medially and snap together above the bird's axial mid-line. This therefore points to the SH as the main actuator of the extraordinarily rapid movement that

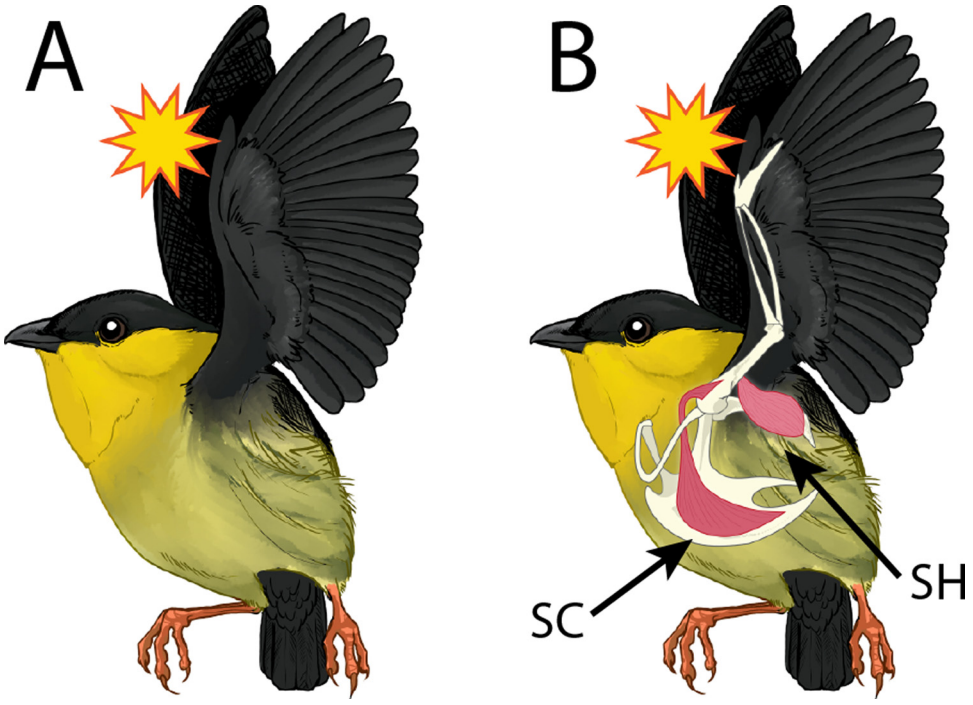


**Fig. 4.** An illustration of an (**A**) adult male golden-collared manakin (*Manacus vitellinus*) producing a roll-snap. (**B**) The same adult male in which the two main wing muscles involved in the actuation of this behavior—the *supracoracoideus* (SC) and *scapulohumeralis caudalis* (SH)—are shown. Studies using EMG demonstrate that the SC lifts the wings into position for this display, whereas the SH rapidly snaps the wings together by retracting the elevated humeri. This is believed to cause the wrists (radii) to collide above the axial mid-line.

makes up the roll-snap. Indeed, studies that assess the contraction-relaxation cycling kinetics of the manakin's muscle confirm that this is the case, showing that the SH can achieve contraction speeds that approach "superfast" levels [99]. However, neither the SC, nor the pectoralis (PEC; the main muscular engine that powers flight) exhibit such specialization in contraction-relaxation kinetics [99].

The SH's remarkable motor performance is largely the phenotypic result of specialized androgenic action in this tissue. The first line of evidence supporting this idea comes from comparative work demonstrating positive co-evolution between levels of AR expression in the wing muscles (including the SH) and motor complexity of physical dance displays across a small group of passerine birds [100]. With one of the most complex physical displays, the golden-collared manakin expresses high levels of AR in its wing muscles, compared to the other species in this analysis. Notably, this relationship was neither detected in other parts of the motor system (*e.g.*, the spinal cord), nor with respect to other steroid hormone receptors (*e.g.*, ER).

Additional studies establish that these high levels of muscle AR are vital to the production of the bird's rapid wing display. For example, if free-living males are given a peripherally selective androgen inhibitor (bicalutamide), they slow the rate at which they snap their wings together during a roll-snap [101]. Furthermore, physiological studies link these behavioral effects to SH performance by showing that AR itself increases the contraction-relaxation cycling speed of the tissue to its near-superfast levels, which otherwise drives display performance [102]. Equally interesting is that these effects occur without substantially encumbering the muscle's ability to generate force [102], which we might otherwise expect considering the trade-off between speed and strength in fast skeletal muscles [103]. Altogether, these findings suggest that sexual selection by female choice for a rapid wing display drives the evolution of increased androgen sensitivity in the muscles of the wing, including the SH. The phenotypic product of this process is increased muscle speed, tipping the SH toward rapid performance, while simultaneously easing the expected trade-offs with strength.

Exploring the functional effects of androgenic action on the SH and other wing muscles provides powerful insight into why sexual selection indirectly favors increased AR expression to enhance motor performance. Most of this work involves quantification of AR-dependent gene expression (see above), and it shows that androgenic stimulation of the SH results in up-regulation of genes that shorten muscle relaxation times and support muscular hypertrophy [104]. Other work employs transcriptomics to highlight how androgenic action re-writes the myocytic transcriptome [105]. These studies indicate that increased AR expression in manakin wing muscles confers a far more robust transcriptional response than in species with less AR in the same tissues. This effect is apparent both in terms of the number of genes that are up-regulated in response to AR action, as well as higher levels of up-regulation in those genes. Candidate genes that emerge from this analysis encode proteins that regulate myocytic fuel metabolism, contractile dynamics, and basic homeostatic processes [105]. Thus, by leveraging local expression levels of AR, evolution can modify how hormones effectuate the molecular constitution of the given target by making it more equipped to produce behavioral traits favored by selection.

Interestingly, this work also shows that androgens influence gene expression profiles in the golden-collared manakin on a tissue-specific level, operating differently in SH and PEC muscles. However, these two tissues exhibit no difference in the level of androgen receptor expression [100]. This therefore implies that levels of AR alone cannot fully account for tissue-specific differences in androgen-induced gene expression. What, then, does account for these effects? The answer is not currently known. One possibility is that tissues differ with respect to the abundance of enzymes that locally convert T to either its active form, 5α-DHT (5α-reductase), or its inactive form, 5β-DHT (5β-reductase). Yet, current research suggests that is not the case, and expression of the enzymes does not differ between the SH and PEC in a way that explains variation in the androgenic responses of each tissue [106]. Another untested possibility is that the SH and PEC differ in terms of co-factor expression (see above). For example, the SH may express higher levels of co-activators to enhance AR's effects on gene expression. Likewise, the SH may express relatively less co-repressor to otherwise suppress the diminutive effects of these transcriptional modulators on AR action. Of course, it is also important to consider the point made above–that is, these co-factors can be important mediators of androgen-*in*dependent gene expression [107–109], which means that changing their abundance too much could incur "costs" to other vital cellular processes. Thus, if evolution influences co-factor expression as we suspect it might, then key functional trade-offs must be balanced.

### 3.2. Sex differences in the manakin androgenic system

Male and female golden-collared manakins express equally high levels of AR in their wing muscles [110]. However, males also express more 5α-reductase in their wing muscles than females [106]. This suggests that, even though both sexes maintain comparable sensitivity to androgenic hormones in their wing muscles, males are better equipped to locally metabolize testosterone into the more potent ligand (DHT) that directly activates AR. Moreover, breeding females also maintain low levels of circulating androgens, adding another layer that averts the activation of muscular AR necessary for the wing-snap [111]. Notably, these "preventative mechanisms" responsible for behavioral sex differences can be overridden by administering females with T for a prolonged period of time, which increases the probability that they periodically produce wing-snaps [112].

From an evolutionary standpoint, these findings offer a number of insights into the evolution of androgenic signaling systems. The first is that strong sexual selection can drive different and often convoluted changes in cellular machinery on multiple scales. Between species, for example, there are differences in terms of muscular androgenic sensitivity (AR expression levels), whereas the sexes may diverge in terms of a tissue's ability to locally metabolize androgenic hormones. Thus, selection is only able to drive the emergence of the favored behavior—in this case, the wing-snap—through the combination of these modifications. This idea, however, brings up an important question: why do females maintain high levels of muscular AR in the first place, if this trait is only beneficial to males? We suspect that the answer lies in the fact that either *i*) expressing high levels of AR in the wing muscles is not overtly "costly," and thus is permitted to persist in light of the strong sexual selection for the trait in males, or *ii*) the evolutionarily loss of this trait is occurring slowly [see 113].

### 3.3. Adaptive genomic differences

Reconstructions of the golden-collared manakin genome provide another window into how androgenic systems might evolve. This species' genome was found to contain relatively more AREs in the promoter regions of genes, compared to another passerine species, the zebra finch [114]. These data therefore suggest the possibility that activation of AR within the manakin induces a far more potent response than in other species. Analyses of the transcriptomic response of these two species to androgenic stimulation certainly support this idea, showing that such treatment increases the regulation of significantly more genes in the manakin. If this idea bears out, then it likely means that selection can augment (or curtail) the capacity of androgenic hormones to actuate changes in animal physiology by selectively altering the presence of AREs in a variety of genes.

## 4. Conclusions

Unraveling the intricate processes that allow complex animal behavior to evolve and diversify proves to be an ongoing challenge. As time goes on, it is becoming increasingly clear that an integrative

approach to studying behavioral phenomena provides the most promising avenue to fully address these mysteries. This requires combining concepts and techniques from fields of molecular biology, genomics, physiology, behavioral ecology, and evolution to address important questions about how and why behavioral patterns change over time and among species. Work in this area must strive to explore processes of hormone action at macroevolutionary scales as well, incorporating as many species (and the "right" species) as possible and using modern comparative phylogenetic methods to understand the evolutionary processes driving novel patterns in steroid signaling systems.

The broad aim of this study was to re-cap the *Evolutionary Potential Hypothesis* and review the current empirical research that supports it. After examining this work, we provided new data to encourage deeper exploration into the cellular processes that might evolve to shape how androgens signal throughout the body. We then used case studies in the golden-collared manakin to showcase the multiple routes by which androgenic signaling systems have evolved to accommodate the emergence and production of a new behavioral trait. Indeed, work in this system has made headway in tracing the effects androgenic signaling systems (and their evolutionary modifications) to their adaptive behavioral outcomes. There are clearly more questions to pursue in these birds, as well as the many other taxa in which studies of the *Evolutionary Potential Hypothesis* are carried out. Nonetheless, experiments that seek to address the how and why steroid signaling systems evolve as a means of supporting the diversification of complex behavior traits promise to reveal novel insight to hormone-behavior relationship in the natural world.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2018.06.004.

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