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Phenotypic variation reveals sites of evolutionary constraint in the androgenic signaling pathway

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ABSTRACT

Steroid hormone systems play an important role in shaping the evolution of vertebrate sexual traits, but several aspects of this relationship remain unclear. For example, we currently know little about how steroid signaling complexes are adapted to accommodate the emergence of behavior in response to sexual selection. We use downy woodpeckers (*Dryobates pubescens*) to evaluate how the machinery underlying androgen action can evolve to accommodate this bird's main territorial signal, the drum. We focus specifically on modifications to androgenic mechanisms in the primary neck muscle that actuates the hammering movements underlying this signal. Of the signaling components we examine, we find that levels of circulating testosterone (T) and androgen receptor (AR) expression are consistently increased in a way that likely enhances androgenic regulation of drumming. By contrast, the expression of nuclear receptor co-factors—the 'molecular rheostats' of steroid action—show no such relationship in our analyses. If anything, co-factors are expressed in directions that would presumably hinder androgenic regulation of the drum. These findings therefore collectively point to T levels and AR as the more evolutionarily labile components of the androgenic system, in that they are likely more apt to change over time to support sexual selection for territorial signaling in woodpeckers. Yet the signaling elements that fine-tune AR's functional effects on the genome—namely the receptor's transcriptional co-factors—do not change in such a manner, and thus may be under tighter evolutionary constraint.

1. Introduction

The expression of reproductive traits differs tremendously among species. Sources of this variation include a wide range of morphological and behavioral traits, which influence an organism's ability to secure mates and exchange gametes (Kelley, 1988; Piersma and Drent, 2003; Taborsky, 1994). A longstanding goal of integrative evolutionary biology is therefore to uncover the physiological and molecular bases of these differences. One of the most fruitful avenues of research in this area focuses on steroid hormone action (Adkins-Regan, 2005), which shapes diverse sexual traits across the vertebrate tree of life (Adkins-Regan, 2008; Bonier and Martin, 2016; Hau et al., 2008; Ketterson et al., 2009; Zera et al., 2007). However, despite a growing body of work indicating that steroid signaling systems do in fact evolve to accommodate the adaptation of reproductive characters, many aspects of this process remain unclear.

Much of the research on steroid system evolution focuses on androgens, such as testosterone (T), and their associated mechanisms of action. The gonads are a main source of T, synthesizing it from cholesterol and releasing it into the bloodstream (Pihlajamaa et al., 2015). One of the primary ways that T exerts its effect on a target tissue is through the intracellular androgen receptor (AR), which is a ligand-activated transcription factor. After T binds to AR, this complex dimerizes, translocates to the cell nucleus, and then recruits different co-factor proteins to regulate gene expression (Pihlajamaa et al., 2015). From an evolutionary perspective, phenotypic variation in androgen-dependent sexually selected traits can result from modifications to this signaling cascade (Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; McGlothlin and Ketterson, 2008; Rosvall, 2013). Empirical support for this idea comes from a variety of studies showing (i) the abundance of enzymes that synthesize T; (ii) the levels of receptor that detect T; or (iii) that the amounts of circulating T itself can predict differences in the expression of fitness-related traits (Bentz et al., 2019; Fuxjager et al., 2015; Fuxjager et al., 2016b; Hau et al., 2008; Holmes and Wade, 2005; Kelley et al., 1989; Ketterson et al., 1992; Mangiamele et al., 2016; Marler and Moore, 1988; Rosvall et al., 2012; Rosvall et al., 2016; Schuppe et al., 2017; Veney and Wade, 2004). Yet a major limitation to this work is that it largely focuses on isolated components of

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the androgenic signaling system. Studies rarely track variation in multiple elements of steroid signaling systems at the same time (Burns et al., 2014; Kerver and Wade, 2013; Rosvall et al., 2013). This shortcoming creates a gap in our understanding of how androgenic systems can evolve as an integrated complex, and addressing this issue is important because we know that these nodes can interact with each other to influence the system's overall function (Charlier et al., 2006; McGinnis and Dreifuss, 1989; Qiu et al., 2016).

There are currently two main hypotheses that conceptualize how steroid systems might evolve. The first is the *Evolutionary Independence Hypothesis* (also called the *Evolutionary Potential Hypothesis*), which posits that the molecular components of the androgenic signaling cascade likely evolve independently of each other and in a tissue-specific manner (Hau, 2007; Ketterson et al., 2009). Accordingly, this framework implies that a given species can evolve modified reproductive traits by changing patterns of AR expression throughout the brain and/or body without modifying levels of endogenous T per se. The second major idea is captured by the *Evolutionary Integration Hypothesis* (also called the *Evolutionary Constraint Hypothesis*), which posits that T levels are the primary component of the androgenic system that evolve in response to selection (Hau, 2007; Ketterson et al., 2009). To this end, the other signaling components within the androgenic system (receptors, co-factors, etc.) are believed to change alongside shifts in T levels (Hau, 2007; Ketterson et al., 2009; McGlothlin and Ketterson, 2008). However, in reality, both ideas are likely true; thus, each of the two hypotheses may represent opposite ends of a continuum that describes how hormone systems evolve (Ketterson et al., 2009). Building on this framework, we hypothesize that components of the androgenic system that mediate *both* androgen-dependent and androgen-independent processes are more tightly constrained at an evolutionary level, compared to the components that mediate only androgen-dependent processes. Accordingly, we expect that co-factors regulating how androgens interact with the genome will experience relatively more constraint than other molecular elements within the signaling machinery (sensu Zhang et al., 2007). This is because co-factors mediate numerous non-steroidal nuclear receptor pathways (Banwell et al., 2006; Heery et al., 2001; Ramamoorthy and Cidlowski, 2013; Tremblay et al., 1999; Vella et al., 2014b) and altering their abundance can severely dysregulate cellular processes directly linked to reproduction and survival (Apostolakis et al., 2002; Auger et al., 2000; Charlier et al., 2005; Duteil et al., 2010; Mahajan et al., 2004; Mottis et al., 2013; Pérez-Schindler et al., 2012; Reily et al., 2006; Zhu et al., 2011). Tissue-specific changes to co-factor abundance therefore likely incur steep 'costs' to organismal performance that hinder or suppress fitness. By contrast, we predict that levels of AR are relatively more evolutionarily labile, since AR's sole function is to transduce androgenic signals within a given target. Modifications to AR abundance, therefore, likely have less of an effect on other androgen-independent events within a cell, theoretically reducing the relative 'costs' of such changes.

In the current study, we explore phenotypic variation in the androgenic system at multiple levels. We do this work by measuring androgenic hormone levels in circulation, as well as the expression of different genes that encode proteins important in androgenic signaling. Our analyses rely on a framework in which constraint is determined by the flexibility of hormone levels and/or transcript abundance. Past work indicates that components of a molecular system are evolutionarily constrained when their expression profiles show reduced variability or flexibility, particularly when such variability has deleterious effects on phenotypic outcome (Gilad et al., 2006; Ludwig et al., 2000; Romero et al., 2012). To this end, expression levels of genes that underlie sexually selected traits often exhibit greater variability and are highly evolvable (Connallon and Clark, 2010; Harrison et al., 2015; Pointer et al., 2013; Wyman et al., 2010; but see Ghalambor et al., 2007). Thus, components of the androgenic systems that exhibit greater phenotypic variation in relation to the expression of a sexually selected trait likely represents a more labile node within this signaling pathway. Through this lens, our multi-pronged approach to exploring variation in the androgenic systems has the power to reveal which parts of this signaling machinery are more (or less) likely to evolve under selection. Species comparisons, for example, are often used to shed light on associations between traits of interest and underlying specializations to endocrine systems (Bergeon Burns et al., 2013; Fuxjager et al., 2015; Hews et al., 2012; Mangiamele et al., 2016). Sex comparisons are similarly useful, particularly when females express "masculine" traits (Clutton-Brock, 2007; Peterson et al., 2013; Tobias et al., 2012). In these cases, correlated evolution is thought to drive the emergence of endocrine adaptation in females that are otherwise similar to their male counterparts (Muma and Weatherhead, 1989; Rosvall, 2013; Staub and De Beer, 1997). Finally, seasonal comparisons offer a practical way to elucidate the components of a hormone system that are most likely to evolve to support sexual selection. Sex steroid systems typically change between the breeding and non-breeding seasons (Canoine et al., 2007; Charlier et al., 2009; Wacker et al., 2010), and comparisons between these time periods can highlight the plastic parts of the signaling cascade.

We investigate how elements of the androgenic signaling system evolve to support sexually selected behavior in the downy woodpecker (*Dryobates pubescens*). These birds are highly territorial, initiating several androgen-dependent changes in their social behavior during the breeding season when males and females pair and cooperatively defend a home range (Kellam et al., 2006; Kellam et al., 2004). Foremost among these behavioral changes is the production of the bird's main agonistic signal: the drum. This behavior is performed by rapidly and repeatedly hammering the bill against a tree (Kilham, 1974a; Schuppe and Fuxjager, 2018). Drumming behavior is likely androgen-dependent, since its production increases at the onset of the breeding season when circulating T is elevated (Kellam et al., 2004). Furthermore, experimental work shows that exogenous administration of T to male downy woodpeckers increases mate guarding behavior and territorial patrolling (Kellam et al., 2006; Kellam et al., 2004), two phenomena directly negotiated via drumming (Dodenhoff, 2002; Jackson and Ouellet, 2002; Kilham, 1974a; Schuppe et al., 2016).

One way that androgens might mediate the drum signal is by acting on the skeletal muscles that actuate it, like the *longus colli ventralis* (LC) (Jenni, 1981; Kaiser, 1990). This muscle regulates forward neck flexion to mediate hammering movements that make up the drum (van der Leeuw et al., 2001), with additional work suggesting that the performance capacity of the LC is evolutionarily geared to support this behavior (Jenni, 1981; Kaiser, 1990; Schuppe et al., 2018). To this end, the notion that androgens might act via the LC to accommodate drumming ability and performance is consistent with numerous other studies that show increased levels of androgen sensitivity in muscles that underlie sexually selected behavioral traits (Fuxjager et al., 2013; Johnson et al., 2018; Mangiamele et al., 2016). Some of this research even illustrates that inhibiting androgenic effects on these tissues impedes the production of adaptive displays (Alward et al., 2016; Fuxjager et al., 2013).

In our study, we focus on variation in four components of the androgenic signaling system: circulating T, AR, and two transcriptional co-factors. We specifically focus on nuclear receptor co-factors, steroid receptor co-factor 1 (SRC1) and nuclear co-repressor 1 (NCOR1), that are both known to mediate AR's ability to regulate gene expression by either turning it up (SRC1) or down (NCOR1) (Bevan et al., 1999; Cheng et al., 2002). We therefore explore how these components in the androgenic system vary in the LC muscle—compared to a control muscle uninvolved in drumming called the *pectoralis* (Dial, 1992; PEC; Dial et al., 1991)—among woodpeckers and non-woodpecker birds, between male and female downy woodpeckers, and across the breeding season. In our first species comparison study, we include male downy woodpeckers, as well as red-bellied woodpeckers (*Melanerpes carolinus*) and an oscine passerine called the white-breasted nuthatch (*Sitta carolinensis*). Red-bellied woodpeckers drum much less frequently than

downy woodpeckers (Dodenhoff, 2002; Miles et al., 2018a; Wilkins and Ritchison, 1999), whereas nuthatches do not drum. Instead, the nuthatch uses its LC to actuate tapping behavior for foraging, which is kinematically similar to drumming (Dickson et al., 1979; van der Leeuw et al., 2001). Importantly, all three of these species are socially monogamous, and males actively defend territories during the breeding season (Kilham, 1968, 1972; Miles and Fuxjager, 2019; Schuppe et al., 2016). Next, for our sex comparison study, we look at the LC of male and female downy woodpeckers. Both sexes regularly drum (Kilham, 1974a; Schuppe and Fuxjager, 2018; Schuppe et al., 2016), providing an opportunity to explore whether selection for this display in females 'masculinizes' components of the androgenic systems to support this behavior (Møller et al., 2005; Rosvall, 2013). Finally, we explore which components of the androgenic system exhibit seasonal flexibility, as this should elucidate which nodes within the signaling complex are more modular and thus susceptible to evolution. We therefore compare adult male downy woodpeckers in the non-breeding season (when drumming occurs very infrequently) to the onset of the breeding season when birds drum often (Dodenhoff, 2002).

2. Materials and methods

2.1. Animals

We captured 39 downy woodpeckers (males: $n = 13$ breeding, $n = 6$ non-breeding; females: n = 13 breeding, $n = 7$ non-breeding), 7 red-bellied woodpeckers (breeding season only), and 7 white-breasted nuthatches (breeding season only) to analyze levels of circulating T. For all species, the breeding season corresponded to March through May when individuals were actively drumming or signing. Early in March, these birds start establishing breeding territories, with copulation generally occurring in April (Dodenhoff, 2002; Kilham, 1961, 1968, 1972). After clutches hatch in the beginning of May, individuals begin exhibiting parental behaviors (Dodenhoff, 2002; Kilham, 1961, 1968, 1972). In downy woodpeckers, the nonbreeding season corresponded to November through early February when drumming is used infrequently (Dodenhoff, 2002; Jackson and Ouellet, 2002).

Birds were passively caught in the woodlands of Forsyth County, North Carolina (USA) using mist nets. All appropriate federal, state, and university authorities approved of this research. We immediately euthanized a subset of these birds after collecting blood, including 17 downy woodpeckers (males: $n = 10$ in the March/April breeding season, $n = 3$ in the Nov/Dec non-breeding season; females: $n = 4$ in the March/April breeding season), 5 male red-bellied woodpeckers (breeding season only), and 7 male white-breasted nuthatches (breeding season only).

2.2. Blood sampling and testosterone measurements

After animals were caught, blood samples were rapidly collected (4.79 ± 0.45 min after capture in a mist net [mean ± SEM]) collected in heparinized capillary tubes from the brachial vein. We stored blood samples on ice in the field, and then centrifuged them each at 10,000 rpm (Eppendorf #5430R) for 10 min later that day. This allowed us to pellet the red blood cells and remove plasma for T assays. All plasma samples were stored at −80 °C until further processing.

We measured plasma T using a commercially available enzyme immunoassay (EIA) kit (Cayman #582701) according to the manufacturer's instructions. The kit has a detection limit of 3.9 pg/ml, and its antibody shows some cross reactivity with DHT (27.4%). The assay therefore measures total plasma androgens; however, circulating DHT levels are very low in most birds, such that differences in plasma T undoubtedly make up much of androgen variation (Owen-Ashley et al., 2004; Silverin et al., 2004; Steiger et al., 2006). Accordingly, we refer to plasma androgen levels as "T" to be consistent with the avian literature (Hau et al., 2000; Ryder et al., 2011). The efficacy of these kits to measure avian plasma T is well documented elsewhere and is validated for diverse avian taxa (Dickens et al., 2011; Handelsman et al., 2015; Washburn et al., 2007). We performed our own validations of this kit for downy woodpeckers, red-bellied woodpeckers, and white-breasted nuthatches. This included a detailed assessment of measurement parallelism on unextracted samples (Dickens et al., 2011; Jeffcoate, 1981; Washburn et al., 2007), which confirmed that the assay preserved linearity at various dilutions. To perform this analysis we diluted (1:10–50) pooled plasma samples in EIA buffer. We performed this validation for male, female, and non-breeding downy woodpeckers (both male and female), as well as red-bellied woodpeckers and white-breasted nuthatches. Each validation was performed separately to ensure that we could accurately detect T levels for both sexes of downy woodpeckers across the year. This also allowed us to determine the optimal dilution of plasma from which we could accurately and consistently measure T levels in plasma (1,30 in EIA buffer for all species). At this dilution factor, all samples were within bounds of the standard curve, and therefore allowed for accurate measurement of both breeding and non-breeding samples. All data were collected by running samples on two separate EIA plates, with each plate balanced for sex, season, and species. The samples were run in duplicate and had intra- and inter-assay coefficients of variation that were 9.78% and 8.45%, respectively.

2.3. Tissue Processing and cDNA synthesis

We dissected the LC and PEC muscle from each of the frozen carcasses. We then homogenized the samples in TRIzol Reagent™ with a rotor/stator homogenizer (Dremel 3000 with Tissue Tearor attachment; BioSpec Products™) set to medium speed. We extracted total RNA using a Zymo Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA), in which we included an initial phenol-chloroform separation of RNA following the manufacturer's instructions. Each RNA sample was DNase treated and reverse transcribed with SuperScript IV Reverse Transcriptase (Invitrogen) following the manufacturer's protocol, yielding a total of 1 μg of cDNA. This reaction occurred for 10 min at 55 °C, followed by 10 min at 80 °C. The quantity and integrity of cDNA was confirmed through Nanodrop (Thermofisher model: 2000) readings (1039 ng/ul ± 27 [Mean ± 1SEM]) and running samples on a 1% agarose gel, respectively.

We used PCR to establish that our genes of interest were in fact expressed in the muscle tissues of the different bird species. We therefore amplified AR, SRC1, NCOR1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts, using primers designed from highly conserved regions of the genes (Table S1). PCR reactions contained 40 ng of cDNA, 0.5 μM of forward primer, 0.5 μM of reverse primer, and OneTaq 2x Mastermix (New England Biology). Reactions were run at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 57–62 °C for 30 s, and 72 °C for 30 s. All reactions were completed with a final extension step at 72 °C for 5 min. The resulting PCR products were analyzed on a gel to ensure that the amplified fragments matched their expected size. We then purified and sequenced the PCR products (Genewiz Inc., La Jolla, CA, USA) to verify that we amplified the correct gene.

2.4. Quantitative PCR (qPCR)

We used quantitative PCR (qPCR) to measure gene expression on an Applied Biosystems 7500 Fast RealTime sequence detection system. We generated species-specific primers for AR, SRC1, NCOR1, and GAPDH (housekeeping control) genes (Table S2). Past work demonstrates the efficacy of GAPDH as an internal control for qPCR in skeletal muscle (Barber et al., 2005; Jemiolo and Trappe, 2004; Mahoney et al., 2004; Touchberry et al., 2006), as well as for studies that employ species comparisons in birds (Feng et al., 2010; Fuxjager et al., 2012). Moreover, we confirmed that downy woodpeckers exhibited no sex,

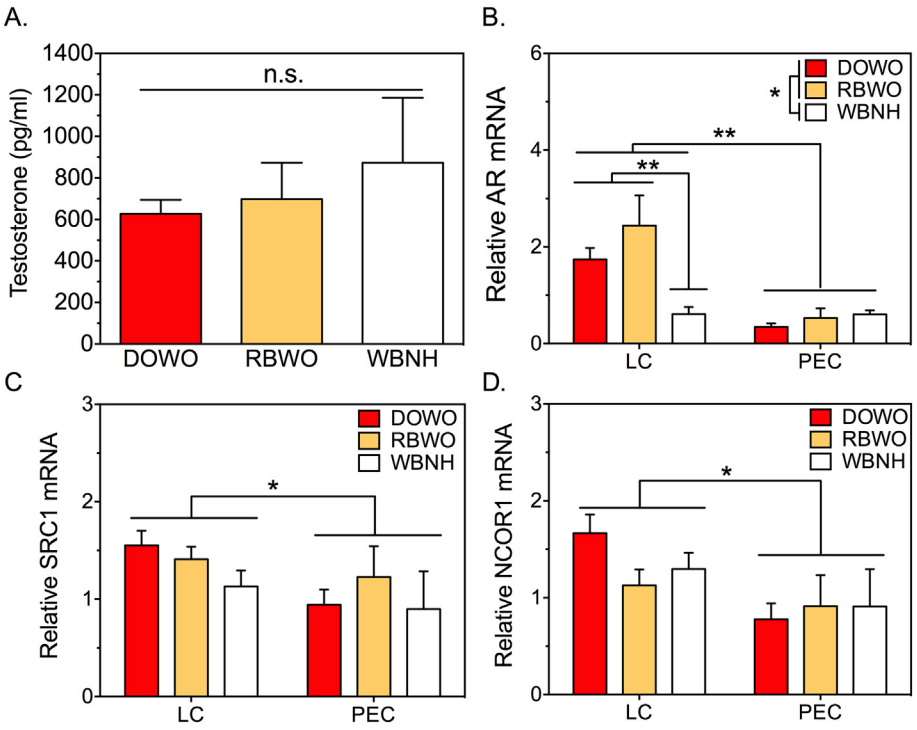

Fig. 1. Species differences in circulating testosterone (T), androgen receptor (AR) and co-factor expression in breeding (captured between March–May) adult male downy woodpeckers [DOWO (red bars); $n = 12$], red-bellied woodpeckers [RBWO (orange bars); $n = 7$], and white-breasted nuthatches [WBNH (white-bars); n = 7] during the breeding season. (**A**) Plasma levels of T across all three species; *n.s.* denotes that groups are statistically indistinguishable ($p > 0.05$). (**B-D**) Relative levels (DOWO: $n = 10$; RBWO: $n = 5$; WBNH: n = 7) of AR, steroid co-activator 1 (SRC1), and nuclear co-repressor 1 (NCOR1) in the *longus colli ventralis* (LC) and *pectoralis major* (PEC) muscles of these same species. Bars represent means ± 1SEM. Statistically significant differences between groups are denoted by asterisks (*, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seasonal, or muscular differences in GAPDH expression ($p_{min} > 0.2$). Similarly, we found no species differences in GAPDH expression in either the LC or PEC ($p_{min} > 0.2$). Altogether, this suggests that our housekeeping gene is a suitable internal control for the two skeletal muscles we examine herein.

Each reaction was performed in duplicate and contained 100 ng of template cDNA, 0.9 μM of forward and reverse primer, and SYBR green (Applied Biosystems: 4364346). For all genes, we included negative controls that consisted of all elements in the reactions described above except cDNA. We ran reactions at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The program was finished with a dissociation curve to check for contamination and ensure that the primers did not dimerize. Samples were run in duplicate, and we used the standard curve method (e.g., quantity of gene of interest/quantity of GAPDH) to determine relative expression of each gene of interest, as compared to the internal reference (GAPDH) (Fuxjager et al., 2012; Larionov et al., 2005; Schuppe et al., 2018; Taylor et al., 2010). The standard curves used in the current experiments were generated by serially diluting (1,4) pooled cDNA (e.g. LC and PEC muscle). All reaction efficiencies were between 90 and 110% (see Table S2), with negative controls always showing undetectable transcript levels.

2.5. Data analysis

All analyses were performed in R (v3.3.2), using the *lme4* package (Bates et al., 2014). Data were [log (1 + x)] transformed to achieve normality, as Q–Q plots and Shapiro-Wilk tests indicated that these transformations yielded more normally distributed data. In all analyses, significant main and interaction effects were further examined with post-hoc comparisons, using Benjamini-Hochberg corrections to account for multiple contrasts.

To investigate species differences in the androgenic system of the neck musculature, we first performed one-way analysis of variance (ANOVA) to determine whether the breeding male downy woodpeckers, red-bellied woodpeckers, and white-breasted nuthatches express differences in circulating T levels. In addition, we performed three linear mixed models (LMM) to determine how levels of AR, SRC1, and NCOR1 change between species and tissues.

Next, to examine sex differences in circulating androgens of downy woodpeckers, we used a two-way ANOVAs to compare T between sex and season (breeding season vs. non-breeding season). As before, we also used three LMMs to determine how levels of AR, SRC1, and NCOR1 differed between sexes and muscles in breeding downy woodpeckers. In these analyses, we also used individual identity as a random effect.

To first assess seasonal variation in circulating androgens of male downy woodpeckers, we performed an ANOVA to investigate how plasma T levels change across the year. To next investigate whether there are seasonal differences in AR, SRC1 and NCOR1 mRNA expression, we performed three LMMs with species and tissue as fixed factors. To account for the non-independence of gene expression levels between tissues in the same animal, we used individual identity as a random factor in each model. Finally, we performed Pearson's correlations to determine whether any element of signaling system was associated the expression of any other component of this system in the LC muscle of male downy woodpeckers. We utilized Tukey *post hoc* tests to further investigate significant main effects.

For all ANOVAs and LMMs eta squared (η^2) effect sizes were computed using the 'sjstats' package (Lüdecke, 2018). For all η^2 analyses, values above 0.25 were considered to be robust and meaningful differences between groups (Cohen, 1988). In addition, we calculated Cohen's d effect sizes for all post hoc comparisons (Cohen, 1988). Cohen's d effect sizes were interpreted based on previous criteria (Sawilowsky, 2009). Specifically, values between 0.1 and 0.49 were considered as 'small' effects; values between 0.5 and 0.8 were considered 'medium' effects; and values above 0.8 were considered 'large' effects with robust differences between groups.

3. Results and discussion

3.1. Species comparisons

We first compared elements of the androgenic systems between two species of woodpecker as well as the nuthatch. We found that circulating T in the breeding season was indistinguishable among the three species (Fig. 1A; $F_{2,23} = 0.58$, $p = 0.56$, $\eta^2 = 0.05$), but was also above

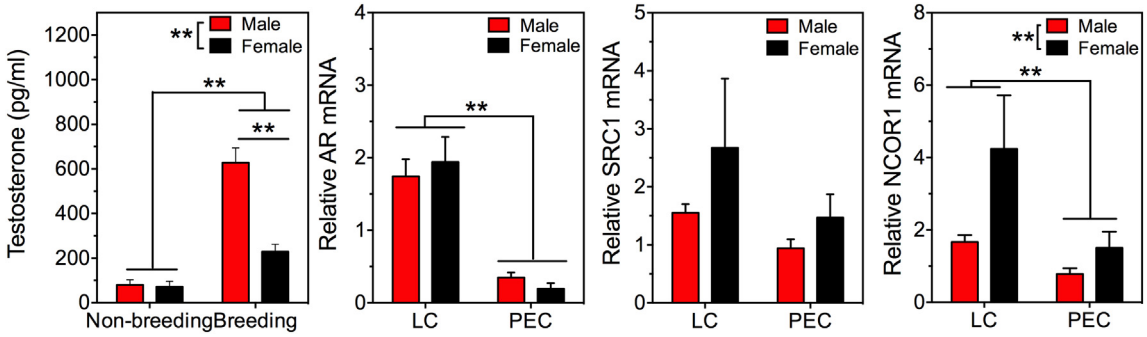

Fig. 2. Sex differences in circulating testosterone (T) and muscular androgen receptor (AR) and co-factor expression in male and female downy woodpeckers. (**A**) Plasma levels of T in individuals during non-breeding [male (red bars): $n = 6$; female (black bars): n = 7; Nov-Feb) and breeding (males: n = 12; females: $n = 14$; March–May) season. (**B-D**) Relative levels of AR, steroid co-activator 1 (SRC1), and nuclear co-repressor 1 (NCOR1) in the *longus colli ventralis* (LC) and *pectoralis major* (PEC) of both males (n = 10) and females ($n = 4$) during the breeding season. Bars represent means ± 1SEM. Statistically significant differences between groups are denoted by asterisks (*, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

basal levels. Meanwhile, we found that the LC expresses significantly more AR than the PEC muscle (Fig. 1B; $F_{1,19} = 63.77$, $p < 0.001$, $\eta^2 = 0.50$), with woodpeckers expressing higher AR levels than the nuthatch (Fig. 1B; $F_{2,19} = 4.23$, $p = 0.03$, $\eta^2 = 0.05$; downy: $p = 0.04$, Cohen's d = 0.48; red-bellied: $p = 0.01$, Cohen's d: 0.61). Our model also uncovered a significant tissue × species interaction (Fig. 1B; $F_{2,19} = 18.21$, $p < 0.001$, $\eta^2 = 0.25$), indicating that AR expression varies among species in a tissue-specific manner. *Post-hoc* analyses corroborated this finding by revealing that both woodpecker species express more AR in their LC compared to the PEC (downy woodpecker: $p < 0.001$, Cohen's d = 0.89; red-bellied woodpecker: $p < 0.001$, Cohen's d = 0.75). This difference was not present in the nuthatch, which maintains the same level of low AR expression in both muscle tissues ($p = 0.91$). Furthermore, we found that AR expression was also significantly greater in the downy ($p < 0.001$, Cohen's d = 0.9) and red-bellied ($p < 0.001$, Cohen's d = 0.77) woodpecker LC relative to that of the nuthatch.

For the co-factors, we found that SRC1 expression is significantly greater in the LC, compared to the control muscle (PEC; Fig. 1C; $F_{1,37} = 4.94$, $p = 0.032$, $\eta^2 = 0.12$). We found no differences in this transcript's expression across species (Fig. 1C; $F_{2,37} = 0.85$, $p = 0.43$, $\eta^2 = 0.04$). Moreover, we did not find a significant interaction (Fig. 1C; $F_{2,37} = 0.03$, $p = 0.97$, $\eta^2 = 0.001$). Meanwhile, we find that NOCR1 expression is significantly greater in LC than the control tissue (PEC) (Fig. 1D; $F_{1,19} = 9.44$, $p = 0.006$, $\eta^2 = 0.25$). However, NOCR1 expression did not differ across species (Fig. 1D; $F_{2,19} = 0.57$, $p = 0.57$, $\eta^2 = 0.02$), nor did it differ in response to an interaction term (Fig. 1D; $F_{2,19} = 1.15$, $p = 0.33$, $\eta^2 = 0.04$).

Overall, these data provide insight into how variation in the androgen signaling system might contribute to the expression of adaptive reproductive traits. First, we show that AR levels are especially high in the neck musculature that controls woodpecker drumming, and such specializations are absent in the nuthatch. This is consistent with a pattern of co-evolution between drumming behavior and elevated androgenic sensitivity in the muscle that actuates this display. Although other studies show a similar relationship between muscle specific AR levels and display innovations (Feng et al., 2010; Fuxjager et al., 2015; Johnson et al., 2018; Mangiamele et al., 2016; Schuppe et al., 2017; Veney and Wade, 2004), our current results build on these past studies by considering how changes to AR expression occur alongside modifications to other elements of the androgenic system. As such, we find that T levels do not differ among these species, suggesting that high T by itself is likely insufficient to explain the androgenic control of the drum. We also find no evidence of an association between levels of co-factor expression in the LC and drumming. The two co-factors are more abundant in the LC in all three species, suggesting that SRC1 and NCOR1 are not specialized in the woodpecker neck musculature to enhance androgen signaling. Altogether, these data support the notion that AR is a main component of the androgenic system that is correlated with the presence/absence of drumming behavior in woodpeckers. This supports predictions that a model of *Evolutionary Independence* accommodates the evolution of hormone-dependent traits across species (Ketterson et al., 2009).

3.2. Sex differences

Given that androgenic systems largely mediate the expression of masculine reproductive behavior, they often differ between males and females. However, female downy woodpeckers also use drums to help defend a breeding territory, and there are no detectable sex differences in the drum (Dodenhoff, 2002; Kilham, 1974b; Schuppe et al., 2018; Schuppe et al., 2016). We therefore expect correlated evolution of mechanisms that underlie drumming in females and males, including those mechanisms related to androgenic action (Ketterson et al., 2005; Rosvall, 2013; Staub and De Beer, 1997). Accordingly, we first compared circulating T levels between males and females in the non-breeding and breeding seasons. Our results show an overall effect of season, with circulating T increasing during the breeding months of March to May (Fig. 2A; $F_{1,35} = 20.40$ $p < 0.001$, $\eta^2 = 0.54$). We also find that, on average, males maintain higher levels of T than females (Fig. 2A; $F_{1,35} = 9.29$, $p = 0.004$, $\eta^2 = 0.12$). However, this effect is driven in part by a season × sex interaction (Fig. 2A; $F_{1,35} = 4.14$, $p = 0.04$, $\eta^2 = 0.04$), whereby circulating T during the breeding season is markedly elevated in males compared to female ($p = 0.001$, Cohen's d = 0.20). This sex difference disappears in the non-breeding season when animals are not actively defending territories ($p = 0.72$).

With regard to the receptor, we find that both males and females maintain higher levels of AR transcripts in the LC, compared to the PEC muscle (Fig. 2B; $F_{1,12} = 100.15$, $p < 0.001$, $\eta^2 = 0.85$). Interestingly, there is no overall sex difference in AR expression (Fig. 2B; $F_{1,12} = 0.52$, $p = 0.486$, $\eta^2 = 0.004$), nor a sex × muscle interaction (Fig. 2B; $F_{1,12} = 0.73$, $p = 0.41$,$\eta^2 = 0.005$). Thus, the capacity for androgens to mediate drumming by acting via AR in the LC is similar between male and female birds.

In examining co-factor expression, we show that SRC1 is statistically indistinguishable between males and females (Fig. 2C; $F_{1,23} = 2.34$, $p = 0.14$, $\eta^2 = 0.08$), but levels of NCOR1 were higher in females (Fig. 2C; $F_{1,12} = 8.36$, $p = 0.01$, $\eta^2 = 0.15$). Furthermore, only NCOR1 expression significantly differed between the muscles, in that it was greater in the LC compared to the PEC (Fig. 2D; $F_{1,12} = 25.76$, $p < 0.001$, $\eta^2 = 0.49$). SRC1 showed no difference in expression between muscles (Fig. 2C; $F_{1,24} = 2.63$, $p = 0.12$, $\eta^2 = 0.11$), and neither

of our models produced significant interaction terms (Fig. 2C/D; SRC1:$F_{1,24} = 0.01$, $p = 0.97$, $\eta^2 = 0.0001$; NCOR1:$F_{1,12} = 0.78$, $p = 0.39$, $\eta^2 = 0.01$). These data therefore suggest that certain components of the androgenic system evolve similarly in males and females in response to selection for drumming, while other components exhibit a sex difference. For example, both males and females express high levels of AR in the LC relative to the PEC. By contrast, seasonal profiles in circulating T differ between males and females, in that male T increases much more during the breeding season than female T. Interestingly, co-factor expression does not vary between the sexes in a manner that is consistent with drumming, since SRC1 is similar in males and females, but not different between muscles. NCOR1, on the other hand, is greater in females than in males.

Overall, these findings illustrate how presumably similar selectiion regimens can shape the endocrine mechanisms that underlie the expression of drumming in both sexes. We show that the female downy woodpecker's LC is somewhat 'masculinized' in terms of the androgenic system. These similarities may render the system functionally comparable between the sexes, whereby the seasonal T increase in females is sufficient to support drumming through action via the androgen-sensitive LC. Yet, other extrinsic or intrinsic factors might explain why some elements of this system, including T levels, differ between males and females. For example, higher T in males may be necessary to support spermatogenesis (Sharpe, 1987), while lower T in females male help them minimize costs associated with high circulating levels of the hormone (Alonso-Alvarez et al., 2007; Cox et al., 2010; Groothuis and Schwabl, 2008; Schwabl, 1993). Our data, however, do not provide insight into how females may plastically modify levels of testosterone in circulation to activate drumming in the breeding season. In other avian species where females aggressively defend territories during the breeding season, the ovaries express the steroidogenic enzymes responsible for synthesizing androgenic hormones. In fact, this expression is elevated in the breeding season, compared to other times of the year when T levels are naturally low (Bentz et al., 2019). This points to the ovaries as the site of androgen production in females, and we therefore suspect that female downy woodpeckers similarly modify circulating T levels by changing ovarian expression of these genes.

Of course, we cannot rule out the possibility that some of the sex differences we find may yield functional variation in the capacity for androgen signaling. NOCR1 levels, for instance, are greater in females (and possibly more variable), which may buffer AR-induced effects on gene expression. If anything, this result implies that females could have a greater potential to suppress nuclear receptor pathways (including AR) through elevated NCOR1 expression (Urbanucci et al., 2008). Regardless of these considerations, our findings are consistent with the idea that abundant receptor levels in the neck musculature, along with seasonal changes in T, promote the expression of drumming behavior.

3.3. Seasonal variation

Finally, we examined phenotypic variation in the androgenic system of male downy woodpeckers across the breeding season. This analysis provides a route to uncover plastic elements of the androgenic systems, which in turn may be more evolvable (Snell-Rood et al., 2010; Wund, 2012). We therefore first looked at T levels during non-breeding and breeding periods in adult male downy woodpeckers, and we found that T changed significantly over this time (Fig. 3A; $F_{4,14} = 18.36$, $p < 0.001$, $\eta^2 = 0.68$). Post-hoc tests showed that T levels were basal from November to February when birds do not engage in reproductive behavior, but T levels then quickly increase during March (vs. Nov-Dec: $p < 0.001$, Cohen's d = 0.49; vs. Jan-Feb: $p < 0.001$, Cohen's d = 0.59) and April (vs. Nov-Dec: $p < 0.001$, Cohen's d = 0.46; vs. Jan-Feb: $p < 0.001$, Cohen's d = 0.46) when males beginning drumming excessively to establish and defend territories. T levels begin to decline in May ($p = 0.02$, Cohen's d = 0.22), which corresponds to onset of hatchling provisioning. Like several avian species (Dodenhoff, 2002; Hegner and Wingfield, 1987; Khan et al., 2001; Wingfield et al., 1990), T levels in woodpeckers are known to decrease when individuals care for their offspring (Khan et al., 2001). Altogether, our results point to a close temporal association between circulating T and drumming, with levels being highest during periods of intense territorial competition (Dodenhoff, 2002). Consistent with literature across vertebrates, this suggests that T orchestrates the activation of adaptive agonistic behavior (Cavigelli and Pereira, 2000; Cooper Jr et al., 1987; Wingfield et al., 2001).

With respect to AR levels, we find no evidence of seasonal changes in either the LC or the PEC muscles (Fig. 3B; season: $F_{1,11} = 0.13$, $p = 0.73$, $\eta^2 = 0.001$; muscle × season interaction: $F_{1,11} = 0.15$, $p = 0.71$, $\eta^2 = 0.001$). However, we do find that AR is significantly higher in the LC, compared to the PEC (Fig. 3B; $F_{1,11} = 72.18$, $p < 0.001$, $\eta^2 = 0.84$). These findings are therefore consistent with a model in which selection for drumming drives the evolution of constitutively elevated androgenic sensitivity in the LC. Circulating T likely activates AR in this muscle on a seasonal basis, when drumming increases in the spring. In this sense, AR levels in the LC can remain high outside of the breeding season because there is little gonadally-derived T in the bloodstream to activate it.

We also find that both co-factors are more abundantly expressed in the LC relative to the PEC (Fig. 3C/D; SRC1: $F_{1,22} = 10.88$, $p < 0.01$, $\eta^2 = 0.24$; NOCR1: $F_{1,11} = 31.92$, $p < 0.001$, $\eta^2 = 0.65$). Expression of SRC1 significantly increases in the nonbreeding season (Fig. 3C/D; $F_{1,22} = 7.60$, $p < 0.01$, $\eta^2 = 0.19$), whereas expression of NCOR1 shows no temporal change (Fig. 3C/D; $F_{1,11} = 0.001$, $p = 0.98$, $\eta^2 = 0.001$). Neither of our models reveal significant interaction terms (Fig. 3C/D; SRC1: $F_{1,22} = 1.53$, $p = 0.23$, $\eta^2 = 0.04$; NCOR1: $F_{1,11} = 0.83$, $p = 0.38$, $\eta^2 = 0.01$). These data are therefore consistent with the notion that neither co-factor we examine changes seasonally to support androgenic modulation of drumming behavior. If this were the case, we would expect to see increases in SRC1 and decreases in NCOR1, given that these two proteins increase and decrease AR-mediated gene expression, respectively. Indeed, for SRC1, we see the opposite: its expression decreases during the time of year when drumming peaks. This result, which is also reported in the muscle that drives dewlap extension in lizards (Kerver and Wade, 2013), suggests that seasonal flexibility in SRC1 may not enhance the way that gonadal T acts via the LC to regulate drumming. At the same time, however, it is possible that this relative decrease in SRC1 during the breeding season may render a greater relative abundance of NCOR1, which could theoretically lead to greater androgenic suppression (Vella et al., 2014b). This effect may at first seem detrimental, but we suspect it is not the case; past work that shows that ligand abundance diminishes co-repressor action (Liao et al., 2003; Perissi et al., 2004; Perissi et al., 2010), implying that seasonal increases in T should inhibit NCOR1's ability to act through androgen-mediated pathways.

3.4. Correlations among components of the androgenic system

We also took this opportunity to explore correlations in the components of androgenic systems during the breeding season. The *Evolutionary Integration Hypothesis*, for example, predicts that components of the system are correlated with each other, owing to their propensity to evolve as a singular complex. Some studies certainly report that T levels can influence AR expression (Holmes and Wade, 2005; Lu et al., 1999; Menard and Harlan, 1993), but other work suggests that this effect either does not occur or only occurs in certain experimental contexts (Abdelgadir et al., 1993; Choate and Resko, 1992; Clancy et al., 1994; Rosvall et al., 2012). We find no evidence of AR autoregulation, in that individual variation in T levels fails to predict such variation in AR expression in the LC of breeding birds ($r^2 = 0.08$, $p = 0.48$). Similarly, T predicts neither SRC1 ($r^2 = 0.06$, $p = 0.53$) nor NCOR1 expression in this same muscle ($r^2 = 0.03$, $p = 0.67$). We also find no association between expression of AR in the LC and either co-

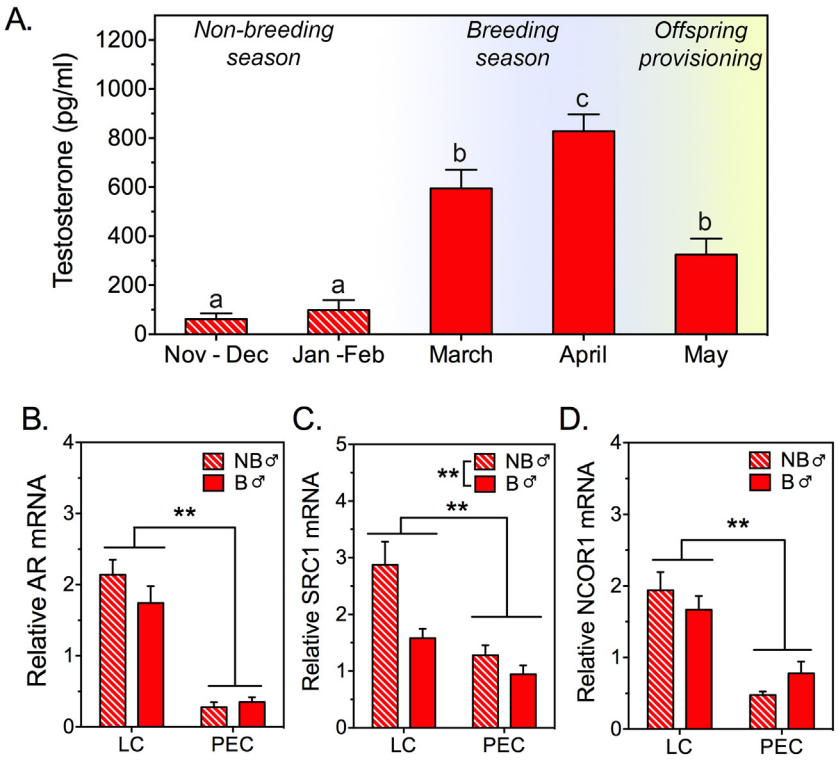

Fig. 3. Seasonal differences in circulating testosterone (T), androgen receptor (AR) and co-factor expression in adult male downy woodpeckers. (**A**) Comparison of plasma levels of T in males across the years, corresponding to the nonbreeding season (NB; red-white hatched bars) and the breeding season (B; solid red bars). Blue highlight represents periods of the breeding season in which individuals actively establish and defend territories, whereas yellow highlight represents periods of the breeding season in which individuals both defend territories and provision offspring. Sample sizes include Nov-Dec, $n = 3$; Jan-Feb n = 3; March, n = 6; April, n = 4; and May, $n = 3$. (**B-D**) Relative levels of AR, steroid co-activator 1 (SRC1), and nuclear co-repressor 1 (NCOR1) in the *longus colli ventralis* (LC) and *pectoralis major* (PEC) muscles during the NB (n = 3) and B (n = 10) seasons. Bars represent means ± 1SEM. Statistically significant differences between groups are denoted by either differences in letters atop bars or asterisks (*, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

factor (SRC1: $r^2 = 0.006$, $p = 0.83$; NCOR1: $r^2 = 0.02$, $p = 0.65$). However, we found that individual variation in SRC1 expression is positively associated with levels of NCOR1 in this same tissue ($r^2 = 0.53$, $p = 0.01$). These findings are therefore consistent with the *Evolutionary Independence Hypothesis* (Hau, 2007; Ketterson et al., 2009), in that components of androgenic signaling pathway seem to vary independently of each other. At the same time, we do uncover a positive relationship between SRC1 and NCOR1 levels, which is consistent with *Evolutionary Integration Hypothesis* (Hau, 2007; Ketterson et al., 2009). In other words, these co-factors may exist as an integrated complex within the larger androgenic signaling apparatus, whereby individuals with a greater capacity for co-activation in the LC via SRC1 also exhibit a greater capacity for co-repression via NCOR1 in the same tissue. Such a balance between these two co-factors may be advantageous in its own right, sustaining the adaptive function of other hormones important for maintaining cellular homeostasis (Vella et al., 2014a).

Interestingly, these conclusions are broadly supportive of the main points drawn from our species, sex, and seasonal comparisons. T and AR levels, for example, vary in a manner that would specifically enhance androgenic modulation of drumming via action within the LC muscle, whereas SRC1 and NCOR1 do not. Thus, *Evolutionary Independence Hypothesis* likely captures how T and AR adaptively evolve, and the *Evolutionary Integration Hypothesis* likely captures how SRC1 and NCOR1 evolve.

4. General discussion

In the current study, we assess phenotypic variation among species, between sexes, and across seasons in multiple components of the androgenic signaling machinery. We conduct this work in woodpeckers, which have evolved an androgen-dependent display known as a drum, to investigate how components of the androgenic systems correlate with the evolution of their display. In our analyses, we found that T and AR vary in a way that likely enhances drumming behavior, as opposed to the co-factors that mediate their effects on a cell. For example, the presence of drumming across species is correlated with an increase in AR levels in the neck muscle, but not with circulating T, SRC1, or NCOR1. Next, we find that both male and female downy woodpeckers experience an increase in circulating T during the breeding season (though this effect is greater in males). AR and SRC1 levels are similar between the sexes, whereas levels of NCOR1 are slightly greater in females compared to males. In adult male downy woodpeckers, we uncover seasonal changes only in circulating T; muscular expression of AR and NCOR1 is stable across the year. Interestingly, SRC1 expression decreases in the neck muscle during the breeding season, which means that it changes in a direction that is inconsistent with androgenic modulation of drumming. Finally, correlations between components of androgenic system in the LC reveal a positive association between SRC-1 and NCOR-1, but not between other molecular elements in this pathway. Altogether, this multi-level analysis points to both T and AR as components of androgenic signaling pathway that support drum performance. In this same vein, our findings suggest that SRC1 and NCOR1 fail to exhibit variation in this manner; that is, their expression patterning either does not vary, or it fails to change in a direction that would otherwise support androgenic modulation of drumming (if anything, SRC1 and NCOR1 expression shifts in directions that would theoretically suppress androgenic modulation of drumming).

Based on these results, we conclude that T and AR are relatively more evolvable than either SRC1 or NCOR1, at least with respect to the emergence of a sexually selected behavior like drumming. This idea rests on the fact that traits evolving in response to sexual selection are often marked by variation and/or flexibility in the expression of underlying genes (Connallon and Clark, 2010; Harrison et al., 2015; Pointer et al., 2013; Wyman et al., 2010), which in our case is T and AR. Notably, we do not look at genes involved in T production, but they are presumably regulated in a manner that supports plasticity in T levels (Bentz et al., 2019; Burns et al., 2014; Rosvall et al., 2016). As such, perhaps the most intriguing finding from our study is that co-factors mediating AR's functional effects on the genome are more tightly constrained evolutionarily. This makes sense with respect to the molecular biology of SRC1 or NCOR1, in that these two proteins play a role in the regulation of several androgen-independent processes (Ding et al., 1998; Kalkhoven et al., 1998; Pérez-Schindler et al., 2012; Zhu et al.,

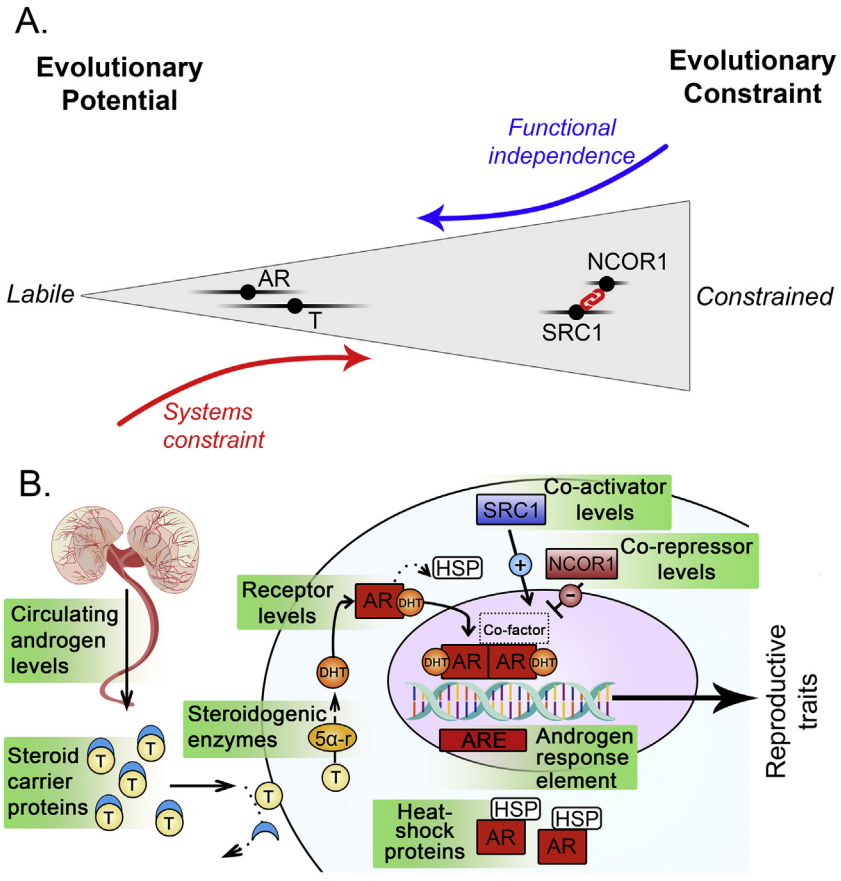

Fig. 4. (A) Schematic representation of the *System Constraint* corollary that we describe herein. Triangle represents the continuum between *Evolutionary Potential Hypothesis* and *Evolutionary Constraint Hypothesis* (Hau, 2007; Ketterson et al., 2009), with the triangle's height representing the degree to which nodes within the androgenic signaling complex are evolutionarily constrained. The left side of the triangle therefore denotes complete evolutionary lability (no constraint), whereas the right side of the triangle represents tight evolutionary constraint. The *Systems Constraint* corollary posits that the nodes of the androgenic systems fall along different points of this continuum according to their functional independence from other cell signaling events. If the component is only or primarily involved in androgenic signaling [e.g., circulating androgens like testosterone (T) and androgen receptor (AR)], then the effects of functional independence push them toward the left side of the triangle. If the component mediates other (androgen-independent) processes, then the effects of systems constraint push them toward the right. We propose that T levels and AR in the *longus colli* muscle fall on to the left side of the triangle, while both co-factors (nuclear co-repressor 1 [NCOR1] and steroid receptor co-activator 1 [SRC1]) fall to the right. Consistent with the notion that co-factors are constrained is our finding that their expression in the LC is tightly correlated (depicted by red chain linking the co-factors in the triangle (Hau, 2007; Ketterson et al., 2009). (B) Schematic representation of molecular and genetic elements (green rectangles) that make up the androgen signaling system, which in theory can evolve to influence how this pathway works. Notable elements not assessed in the current study include steroid carrier proteins, which transport lipophilic androgens in circulation; steroidogenic enzymes (e.g., 5α-reductase, 5αr), which convert androgens into metabolites like dihydrotestosterone (DHT) (Fuxjager et al., 2016b); heat-shock proteins, which stabilize AR in the cytoplasm (light blue ellipse) and allow its translocation to the nucleus (pink ellipse); and androgen response elements (ARE) located in gene promoters, which bind AR in the DNA so that it can regulate gene expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2011). Moreover, this assertion is consistent with work that demonstrates that evolutionary constraint prohibits variation in gene transcripts, particularly if the departure from an optimum broadly dysregulates cellular events or systems (Gilad et al., 2006; Ludwig et al., 2000). Thus, adjusting the expression of either SRC1 or NCOR1 to augment androgenic signaling may induce such adverse effects. Certainly, many studies show that signaling pathways within a cell "compete" for a limited amount of co-factor (Gill and Ptashne, 1988; Meyer et al., 1989; Prywes and Zhu, 1992; but see Schmidt et al., 2016), whereas other work shows that experimental reduction of co-factor expression can influence a host of cellular responses that steroid hormones normally do not affect (Banwell et al., 2006; Battaglia et al., 2010; Iannacone et al., 2002; Vella et al., 2014b). One might therefore expect that co-factor levels evolve to optima that balance these different functional demands, which in turn implies that the abundance of co-factors in a cell are tightly constrained. Our data are consistent with this notion, in that there is a strong positive correlation between SRC1 and NCOR1 in the LC of breeding downy woodpeckers. This suggests that an increase in one of these co-factors occurs in tandem with an increase in the other (As predicted by Hau, 2007; Ketterson et al., 2009).

It is equally important to point out that even if the expression of co-factors does not vary to enhance drumming, they still undoubtedly play an important role in mediating androgenic control of this behavior. We know that their presence in a target tissue is necessary to support androgenic effects of any kind. For example, experimental reduction of co-factor expression in select brain regions can suppress steroid-dependent sexual behavior (Apostolakis et al., 2002; Auger et al., 2000). Thus, although co-factor levels in the LC of woodpeckers do not vary among species, sexes, or seasons in a way that positively predicts drumming, they likely still help mediate androgenic modulation of this display. At the same time, the absence of species and tissue differences in co-factors transcript levels does not definitively rule out other ways in which these transcriptional modulators may be adaptively modified for drumming. For instance, SRC1 levels within the LC muscle may be up-regulated specifically in AR-expressing cells and down-regulated non-AR expressing cells. If so, this would add another layer of complexity to how elements of the androgen signaling system can support the evolution of novel behavioral traits. Namely, it would suggest more exquisite integration between AR and co-factors in cells whose function directly underlies behavioral performance. Addressing such an idea requires co-localization of AR and other elements of its signaling machinery that might (or might not) be specialized (Brooke et al., 2008; Nazareth et al., 1999).

With all these considerations in mind, our results provide support for a corollary to both the *Evolutionary Independence* and *Evolutionary Integration Hypotheses* that describe how androgenic systems might evolve (Hau, 2007; Ketterson et al., 2009). Namely, we propose that evolutionary constraint is differentially distributed among the components of the signaling pathway based on their involvement with other cellular processes, and we refer to this as the *Systems Constraint* corollary (Fig. 4A). Both the *Evolutionary Independence* and *Evolutionary Integration Hypotheses* allow for this idea, as they likely represent extremes of a continuum (Ketterson et al., 2009). However, neither hypothesis articulates how constraint may be laid out across the signaling pathway, or why exactly constraint might vary among its nodes. Thus, a *Systems Constraint* corollary provides a framework for addressing this issue, which is an essential step toward unlocking our understanding of how steroid systems evolve in response to selection.

Going forward, it will be important to consider how other components of the androgenic machinery may or may not evolve (Fig. 4B). In

theory, any of these components could be evolutionarily altered to fine-tune how androgens influence a specific target tissue, and there is a growing body of work that suggests that many of these components are in fact changed in this manner. For example, binding proteins that carry steroids in the bloodstream and enzymes that regulate steroid synthesis and metabolism can vary across species, sexes, and seasons (Breuner and Orchinik, 2002; Fuxjager et al., 2016b; Rosvall et al., 2016; Smith et al., 2018). More recent work even suggests that species can evolve differences in the number and location of AR response elements (ARE) in the genome, potentially altering how AR regulates gene expression (Fuxjager et al., 2016a; Fuxjager and Schuppe, 2018). Similarly, cells may differ in the abundance of chaperone molecules (e.g., heat shock proteins) that maintain unbound AR in cytoplasm, potentially regulating sensitivity to androgenic stimulation. Finally, we must also consider the potential contribution of gene mutations to the evolution of AR functionality. Past work demonstrates that even subtle changes in the AR gene can underlie variation in adaptive behavioral traits (Rajender et al., 2008; Seidman et al., 2001). Future work will need to integrate this viewpoint into the models of androgen system evolution.

A final consideration is the degree to which androgenic signaling systems are phylogenetically constrained. Here we are not able to disentangle how shared evolutionary history among the woodpeckers and other birds impacts species differences in AR expression within the neck musculature. It is indeed possible that elevated AR in the LC preceded the evolution of drumming, creating a stage on which selection could later operate to drive the emergence of the signal. Likewise, elevated AR in the neck muscles may have occurred concomitantly with selection for the drum. Regardless, woodpeckers are distantly related to nuthatches, suggesting that the effects of species' history likely play some role in the comparative results we present herein. Large-scale phylogenetic analyses that integrate multiple components of the androgenic system are needed to address this limitation. Recent work using such methods to uncover how hormone systems evolve is currently being conducted and therefore helping lead that way (Miles et al., 2018b; Vitousek et al., 2018; Vitousek et al., 2019).

5. Conclusions

In summary, we explore variability in the androgenic signaling mechanisms of the downy woodpecker LC muscle, which actuates this bird's sexual drum display. Our results suggest that certain components of the steroid system are specialized to augment androgenic regulation of this muscle (and presumably the display it controls), whereas other components of the system appear to be more constrained. This points to a *Systems Constraint* model, in which the evolutionary lability of various elements that make up this signaling cascade differ in a component-specific manner.

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Appendix A. Supplementary data

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