© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

RESEARCH ARTICLE

Specialized androgen synthesis in skeletal muscles that actuate elaborate social displays

Eric R. Schuppe[1,‡], Daniel Tobiansky[2,*], Franz Goller[3,4] and Matthew J. Fuxjager[2]

ABSTRACT

Androgens mediate the expression of many reproductive behaviors, including the elaborate displays used to navigate courtship and territorial interactions. In some vertebrates, males can produce androgen-dependent sexual behavior even when levels of testosterone are low in the bloodstream. One idea is that select tissues make their own androgens from scratch to support behavioral performance. We first studied this phenomenon in the skeletal muscles that actuate elaborate sociosexual displays in downy woodpeckers and two songbirds. We show that the woodpecker display muscle maintains elevated testosterone when the testes are regressed in the non-breeding season. Both the display muscles of woodpeckers, as well as the display muscles in the avian vocal organ (syrinx) of songbirds, express all transporters and enzymes necessary to convert cholesterol into bioactive androgens locally. In a final analysis, we broadened our study by looking for these same transporters and enzymes in mammalian muscles that operate at different speeds. Using RNA-seq data, we found that the capacity for *de novo* synthesis is only present in 'superfast' extraocular muscle. Together, our results suggest that skeletal muscle specialized to generate extraordinary twitch times and/or extremely rapid contractile speeds may depend on androgenic hormones produced locally within the muscle itself. Our study therefore uncovers an important dimension of androgenic regulation of behavior.

KEY WORDS: Steroid synthesis, Androgens, Skeletal muscle, Display, Sexual selection

INTRODUCTION

Androgens play a vital role in the regulation of male reproductive behavior, including most forms of courtship and male–male competition (Adkins-Regan, 2013). Traditionally, the testes are thought to mediate these effects by synthesizing androgenic steroids *de novo* – locally from cholesterol – and releasing them into circulation (Adkins-Regan, 2013; Blaustein and Olster, 1989; Dawson et al., 2001). However, the link between reproductive behavior and gonadal functioning is more complex than this model suggests. Individuals of several species produce androgen-dependent behavior when their testes are fully regressed, and thus only capable of making low amounts of steroid hormones (Hau et al., 2000; Wikelski et al., 2003). Thus, a major goal of organismal endocrinology is to uncover how such behavior is produced in a way that is independent of the hypothalamic–pituitary–gonadal (HPG) axis.

Extragonadal steroid synthesis is a potential mechanism by which reproductive behavior might be decoupled from testicular functioning. This phenomenon is described in diverse taxa, in which species 'Balkanize' their endocrine system by dividing it into smaller components (Schmidt et al., 2008) to enhance temporal flexibility in the programming of steroid-dependent behavior (Demas et al., 2007; Inai et al., 2003; Soma et al., 2008). This effect is often attributed to tissue-specific expression of steroidogenic enzymes, such as 3βHSD and 17βHSD. When present, these proteins can catalyze reactions leading to the local conversion of steroid hormone precursors into androgens such as testosterone (Fig. 1). Some species take this to an extreme by maintaining tissues that express all the molecular machinery necessary for synthesizing androgens *de novo* (Fig. 1). Of particular importance are translocator proteins (TSPO) and steroidogenic acute regulatory proteins (StAR), which move cholesterol from the outer to inner mitochondrial membrane. Such transportation is the rate-limiting step of steroidogenesis (Hanukoglu, 1992), and it must occur before cholesterol can be converted into the steroid hormone pregnenolone by an enzyme called cholesterol side chain cleavage (CYP11a1; Hanukoglu, 1992; Payne and Hales, 2004). Thus, if a particular tissue expresses TSPO, StAR and the enzyme CYP11a1, then it theoretically possesses the capacity for *de novo* steroid synthesis.

Other than the brain, few tissues in the body maintain the machinery needed for *de novo* steroidogenesis (Endo et al., 2008; Goetz et al., 2004; Kusakabe et al., 2009). This, however, is somewhat surprising because many facets of reproductive behavior depend on androgenic regulation of tissues outside the nervous system (Alward et al., 2016; Fuxjager et al., 2013, 2014; Regnier and Herrera, 1993). Skeletal muscle provides a good example. Androgens act on this tissue to regulate the production of certain sexual reflexes and/or elaborate display routines (Alward et al., 2016; Feng et al., 2010; Fuxjager et al., 2013; Holmes et al., 2007; Mangiamele et al., 2016; Sassoon et al., 1987; Smith et al., 2021). Androgenic effects on muscle can often be dramatic, often re-shaping muscular contraction–relaxation cycling speeds (Fuxjager et al., 2017). Androgens also help sustain muscle size, ensuring that the tissues can generate adequate force to power behavioral output (Fraysse et al., 2014; Fuxjager et al., 2017; Sassoon et al., 1987). These effects can endow individuals with novel behavioral abilities (Fuxjager et al., 2016; Fuxjager et al., 2017), but the source of these androgens may not be entirely from the gonads. Certain androgen-dependent displays, such as avian song, can be fully performed when levels of circulating androgens are almost non-detectable (Hau et al., 2000; Moore and Marler, 1987; Schlinger et al., 2008). Moreover, gonadectomized male songbirds will sing with similar

[1]Department of Neurobiology and Behavior, Cornell University, 215 Tower Road, Ithaca, NY 14850, USA. [2]Department of Ecology, Evolution, and Organismal Biology, Brown University, 171 Meeting Street, Providence, RI 02912, USA. [3]Department of Biology, University of Utah, Salt Lake City, UT 84112, USA. [4]Institute for Zoophysiology, University of Münster, Münster 48149, Germany.
*Present address: Department of Biology, St Mary's College of Maryland, St Mary's City, MD 20686, USA.

‡Author for correspondence (ericschuppe@gmail.com)

E.R.S., 0000-0001-5221-6362; M.J.F., 0000-0003-0591-6854

Received 29 October 2021; Accepted 12 May 2022

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

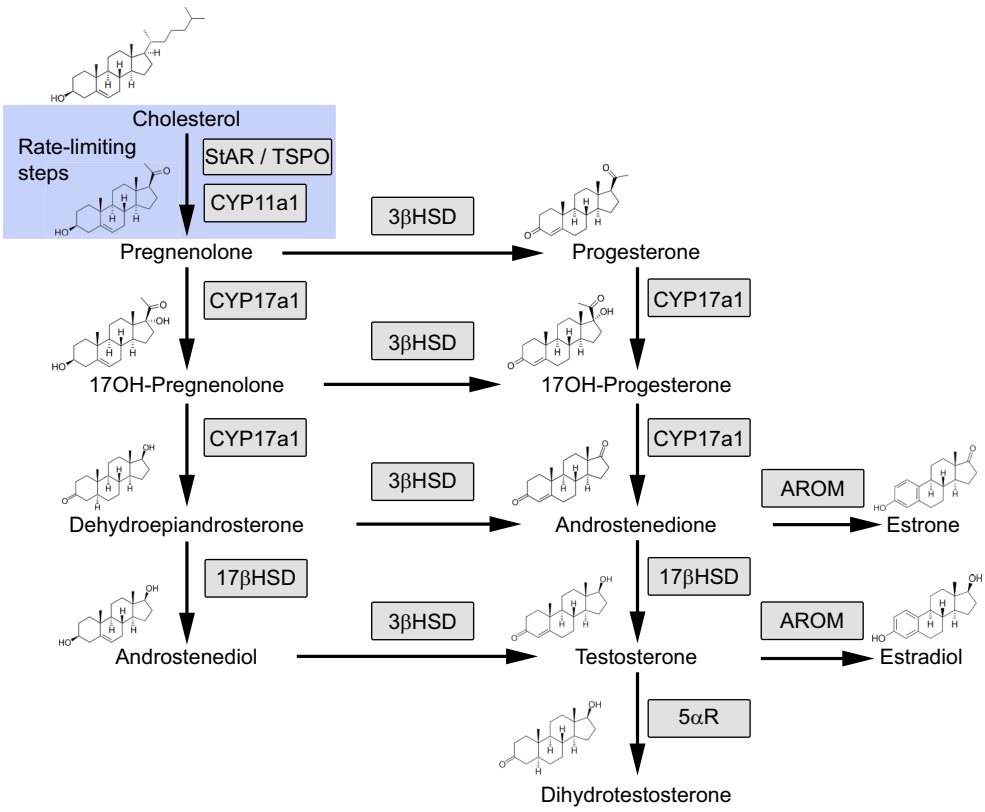

Fig. 1. Steroid biosynthetic pathway, outlining how cholesterol is converted into androgenic steroids. Transporters and enzymes involved in the rate-limiting steps of *de novo* steroid synthesis are highlighted by the blue box, reflecting the importance of initial cholesterol transport [via steroidogenic acute regulator protein (StAR) and translocator protein (TSPO)] and subsequent enzymatic conversion of cholesterol [via side chain cleavage (CYP11a1)] to pregnenolone. The other steroidogenic enzymes that metabolize the progestogens and synthesize androgens include 17-alpha-hydroxylase/17,20-lyase (CYP17a1) and 3β-hydroxysteroid dehydrogenase (3βHSD). Enzymes that further metabolize androgens include 17β-hydroxysteroid dehydrogenase (17βHSD) and 3-oxo-5α-steroid 4-dehydrogenases (5αR). Estrogen synthesis from androgenic precursors involves the enzyme aromatase (AROM; i.e. CYP19a1). Abbreviations for all transporters and enzymes are highlighted in grey rectangles.

vigor and syllable structure to intact counterparts (Alvarez-Borda and Nottebohm, 2002). Other work in *Xenopus* frogs shows that castration fails to fully demasculinize the muscles of the larynx, which regulate the structural properties of male courtship vocalizations. In effect, the lack of gonadal testosterone decreases the frequency with which males call, but the musculature is still capable of generating the rapid twitch times (Zornik and Yamaguchi, 2011) that are otherwise necessary to mediate fast click trills that attract female mates (Tobias et al., 2004). One hypothesis to explain this work is that muscle systems underlying reproductive behavior can either make their own androgens *de novo* or locally metabolize steroid precursors from other glands into androgens. We hypothesize that local synthesis and metabolism may maintain some aspects of muscle performance (e.g. speed) to allow communication to occur when androgens are low, including outside of the breeding season. Additionally, we suspect that increased gonadal synthesis and local metabolism in display muscles during periods of breeding may reinvigorate muscle performance by changing the physiological properties of this muscle, including myofibril size.

We investigated this idea in two ways. First, we assessed the capacity for androgen synthesis in skeletal muscle tissues that actuate elaborate sociosexual displays. The initial species we used for this study is the downy woodpecker (*Dryobates pubescens*). Males compete during territorial contests by drumming, or hammering their bill on trees at high speeds (Schuppe and Fuxjager, 2018; Schuppe et al., 2021). This behavior is powered by a neck muscle called the longus colli ventralis (LC; Kaiser, 1990), in which androgens likely act to support rapid drum performance (Schuppe and Fuxjager, 2019). Similar to other avian species, downy woodpeckers continue to produce these displays in the non-breeding season, albeit much less frequently (Arnold, 1975; Kilham, 1962; Kilham, 1972). Thus, we explored how local steroid synthesis may support this behavior when circulating testosterone levels are almost non-detectable (Kellam et al., 2004; Schuppe and Fuxjager, 2019). Second, we investigated two songbirds, the white-breasted nuthatch (*Sitta carolinensis*) and the zebra finch (*Taeniopygia guttata*). Males in these species court mates and compete with rivals by singing, an elaborate behavior governed by the vocal organ, or syrinx (SYR). The SYR is composed of a group of specialized muscles that sit atop the tracheo-bronchial junction, and studies show that androgens fine-tune spectral features of song by acting directly on androgen receptors expressed within these tissues (Alward et al., 2016; Veney and Wade, 2004). Previous work has shown that the songbird SYR exhibits some of the fastest twitch kinematics measured in any vertebrate muscle, generating mechanical work above 250 Hz (Elemans et al., 2004; Elemans et al., 2008). This fact, coupled with fiber-typing and molecular analyses, has led researchers to call this tissue, and other similarly specialized muscles for communication, 'superfast' muscle (Christensen et al., 2017; Mead et al., 2017; Uchida et al., 2010). In a final experiment, we used transcriptomic data to assess the capacity for androgen synthesis in a range of muscle tissues that adaptively operate at different speeds. We did this by examining two limb muscles, the extensor digitorum longus (EDL; fast twitch muscle, type 2 fibers) and the soleus (slow twitch muscle, type 1 fibers), in male mice (*Mus musculus*; Augusto et al., 2004; Soukup et al., 2002). We focused on these two muscles because they have different cycle frequencies, with the EDL operating around 10 Hz and the soleus only able to operate at half that speed under optimal conditions (James et al., 1995). We then compared the expression profiles in these muscles with the 'superfast' extraocular (EO) muscles, which actuate rapid eye saccades (Bloemink et al., 2013; Briggs and Schachat, 2000; Lennerstrand and Hanson, 1978). Emerging evidence suggests that the EO has a similar fiber type profile to the laryngeal and syringeal muscles that are specialized for

vocal communication, but the EO muscles play no known role in display behavior (Mead et al., 2017). In this way, we explored whether molecular machinery for *de novo* androgen synthesis is a property of striated muscle per se, or whether it is a feature of muscles that are specialized for rapid speeds, including display muscles (e.g. LC and SYR). Taken together, our experiments represent a broad exploration of how muscles might make their own androgenic steroids to support rapid natural animal movements.

MATERIALS AND METHODS

Animals

All appropriate federal, state and university authorities approved of the research and methods described herein (Wake Forest University IACUC no. A16-188; University of Utah IACUC no. 16-03014). We focused our study specifically on actively breeding male birds, which we captured passively using mist nets in the woodlands of Forsyth County, North Carolina (USA). In the spring breeding season (March and April), we captured a total of three male white-breasted nuthatches and four male downy woodpeckers. We conducted behavioral observations to verify that each individual defended a territory by observing dawn singing or drumming behavior. In the non-breeding season (November and December), we collected an additional three downy woodpeckers from the same free-living population. Immediately after non-breeding or breeding male downy woodpeckers were caught, blood samples were rapidly collected in heparinized capillary tubes from the brachial vein. We stored these samples on wet ice in the field, and then centrifuged them each at 14,000 ***g*** (Eppendorf no. 5430R) for 10 min later that day to separate plasma from red blood cells. All plasma samples were stored at −80°C until processing.

Also included in our study were four adult male zebra finches. These birds were individually housed in 32×23×28 cm wire cages, with newspaper lining. Individuals were in full visual and acoustic contact and were provided a mixture of red and white millet, canary seed and water *ad libitum.* Birds were supplemented with peas and corn every other day. All individuals sang in the colony and attempted to court females in nearby cages.

Nuthatches and woodpeckers were euthanized by cervical dislocation immediately upon capture, preserving the integrity of the LC muscle. In woodpeckers, animals were killed after blood samples were taken. Zebra finches were euthanized via an overdose of isoflurane. All animals were immediately flash frozen on dry ice and stored at −80°C. Later, whole (frozen) individuals were quickly dissected with a Dremel™, isolating whole brain (n=3, all species), gonads (n=3, all species), SYR muscle (n=3, nuthatch; n=4, zebra finch), LC muscle (n=4, breeding; n=3, non-breeding downy woodpecker) and PEC muscle (n=3, nuthatch; n=4, zebra finch; n=4, breeding downy woodpecker; n=3, non-breeding downy woodpecker). For each individual, we verified that gonads were enlarged to a size consistent with an actively breeding bird, and thus capable of producing and releasing testosterone (Dawson et al., 2001). In the case of woodpeckers collected in November and December, gonads were fully regressed to a size consistent with a reproductively inactive bird. Dissections occurred on dry ice, and tissues were maintained at −80°C.

Testosterone measurements

For the muscle tissue, steroids were extracted using a previously validated liquid–liquid extraction protocol refined to extract steroids from a variety of tissue types and amniote species for measurement using liquid chromatography tandem-mass spectrometry (Jalabert et al., 2021; Mohr et al., 2019; Tobiansky et al., 2020a). First, a small sample of the muscle tissue was excised from the snap frozen carcass while remaining at −15C. The excised tissue was rapidly weighed on a small EtOH-cleaned aluminum foil square using an ultra-precise scale, and the mass was recorded to nearest microgram. The tissue was then washed from the foil with 1 ml of HPLC-grade acetonitrile into a microcentrifuge tube containing five zirconium dioxide beads (1.4 mm diameter). The tissue was homogenized using a bead-mill homogenizer (Omni International Inc., Kennesaw, GA, USA) at 5 m s^{-1} for 1 min. The samples were then centrifuged at 16,100 ***g*** for 5 min at 4°C. To standardize the amount of tissue analyzed across birds and tissue types, we extracted the equivalent of 10 mg of tissue per 1 ml of acetonitrile supernatant and placed it in a MeOH-cleaned borosilicate glass culture tube (75×12 mm). To remove non-steroidal cellular matrices, we added 0.5 ml of HPLC-grade hexane to the sample, vortexed for 2 s, and centrifuged the sample at 3200 ***g*** for 2 min. The hexane was then removed and discarded. The samples were dried in the fume hood at room temperature for 48 h. Dried samples were stored at −20°C until reconstitution for analysis. Control blanks and spiked controls (50 pg of testosterone in 1 ml of acetonitrile) were concurrently processed with the samples for quality control. Dried tissue extracts and plasma samples were first resuspended in 200 µl of 5% EtOH in EIA buffer before proceeding using the manufacturer's protocol. Briefly, to assess levels of testosterone in muscle, we used a commercially available enzyme immunoassay (EIA) kit (Cayman 582701), which has been validated for all species included in this study (Schuppe and Fuxjager, 2019). All muscle samples were run on a single assay plate, with a standard curve r^2 value of 0.9967.

RNA extraction and reverse transcription

We homogenized each tissue sample in TRIzol Reagent™ using a rotor/stator homogenizer set to medium speed. We then extracted total RNA from the samples with a Zymo Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA), in which we included an initial phenol–chloroform separation of RNA as per the manufacturer's instructions. We followed this procedure by running samples on a 1% agarose gel to check their integrity and verify the absence of genomic DNA. These gels showed both a lack of gDNA contamination and RNA degradation. The latter was assessed by robust 28S and 18S ribosomal subunit bands with minimal lower molecular weight bands, indicating little RNA degradation. Finally, we measured each sample's RNA concentration using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Model 2000). Results from the nanodrop illustrated that samples were devoid of salt, phenol or protein contamination, as indicated by 260/280 and 260/230 spectrophotometry (nanodrop) readings. Finally, all sample concentrations were greater than 300 ng μl^{-1} (range: 357.8–1376.2 ng μl^{-1}).

Later, we treated RNA samples with RNase free DNase I (New England Bioscience) for 20 min at 37°C. We followed this phase with a heat inactivation step of 75°C for 10 min, which ensured that all residual gDNA was completely digested prior to cDNA synthesis. We next reverse transcribed 1 µg of RNA from each sample using SuperScript IV™ Reverse Transcriptase (Invitrogen), following manufacturer instructions. This consisted of adding oligo $d(T)_{20}$ and dNTPs to each sample, and then samples were incubated at 65°C for 5 min, after which samples were placed on wet ice for 1 min. We then added the components for the reverse transcription – including SuperScript IV buffer, DTT, RNaseOUT, SuperScript IV Reverse Transcriptase (RT) – and incubated each tube for 10 min at 55°C, followed by 10 min at 80°C. The quality and integrity of

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

samples were assessed through inspection on a 1% agarose gel and nanodrop to confirm concentrations.

PCR and sequencing

For the nuthatch and woodpecker, we used cDNA generated from either testis or display muscle (SYR or LC, respectively) to amplify transcripts for genes that encode proteins that underlie steroidogenesis (see Fig. 1 for entire list). We designed primers for these PCR reactions from either the zebra finch or downy woodpecker genomes to highly conserved regions of each gene (Table S1). All PCR reactions contained 40 ng of cDNA, 0.5 µmol l^{-1} of forward primer, 0.5 µmol l^{-1} of reverse primer and OneTaq 2× Mastermix (New England Biology). Reactions were run at 96°C for 5 min, followed by 40 cycles of 96°C for 30 s, 57°C for 30 s and 68°C for 30 s. Each reaction was completed with a final extension step at 68°C for 5 min.

The resulting PCR products were run on a 1% agarose gel to verify that we amplified a single fragment that matched the expected size (see Fig. S1). We then used a GeneJet PCR purification kit (Thermo Fisher Scientific) to purify each PCR product for sequencing through Eton Bioscience. This ensured that each amplicon was the steroidogenic enzyme gene of interest before proceeding to more quantitative measures of transcript abundance (see below). All PCR products matched the expected gene of interest. In the case of the downy woodpecker, amplicons were 100% identical to their predicted sequences in the species' available genome. In white-breasted nuthatches, sequenced PCR products fell between 95 and 100% similarity with the steroidogenic transporter and enzyme genes in other Passeriformes (i.e. zebra finch). Note that PCR products of muscular CYP11a1, StAR and CYP17a1 were amplified a second time using the same parameters described above to generate a higher concentration (> 30 ng μl^{-1}) of each product for accurate sequencing (Lorenz, 2012). Such additional amplification was never necessary to obtain robust PCR products from gonadal samples.

Real-time quantitative PCR

We used real-time quantitative PCR (qPCR) to measure relative transcript abundance of each steroidogenic transporter and enzyme (see Fig. 1) in all gonadal, brain, display muscle (SYR or LC) and non-display muscle (PEC) of the different species. All reactions were run in an Applied Biosystems QuantStudio3 machine, using either (i) species-specific primers designed from gene sequences we obtained (see above), or (ii) primers whose sequences are published elsewhere (Table S2; Mirzatoni et al., 2010). Note that primers in this latter category were for only zebra finches, and each was validated for use in extragonadal tissues. We designed qPCR primers according to previously described guidelines, using 'qPCR' settings in Primer3Plus (www.Primer3plus.com; Bustin et al., 2009; Johnson et al., 2014).

For our analyses, each transcript was relativized to the internal control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; see Table S2). Past work demonstrates that GAPDH is an appropriate house-keeping control (Schuppe and Fuxjager, 2019; Shen et al., 2010), particularly for brain, muscle and other tissues that exhibit high steroidogenic capacity (e.g. adrenals; Kim et al., 2014). To verify this, we compared GAPDH levels among tissues in each species and between breeding and non-breeding seasons in woodpeckers. In all three birds, we found no significant differences in GAPDH mRNA levels among tissues (P>0.2). Likewise, similar to previous work in downy woodpeckers (Schuppe and Fuxjager, 2019), we found no seasonal change in GAPDH mRNA levels in any tissue (P>0.2).

Our qPCR reactions included 100 ng of cDNA, 0.9 mmol l^{-1} of forward primer, 0.9 mmol l^{-1} of reverse primer and SYBR Green Master Mix (Applied Biosystems). Samples were run in duplicate, which always showed low coefficients of variation (range: 0.13–2.42%). For each gene, we included multiple negative controls. First, we set up a reaction that contained all ingredients described above, except cDNA (e.g. no template control). This allowed us to assess reactions for contamination and primer-dimer formation (Bustin et al., 2009; Johnson et al., 2014), of which we discovered none (i.e. all reactions showed no amplification). Second, to ensure that residual genomic DNA did not account for any differences we found, we included 'no-RT' controls for all genes and species. For these reactions, we included a subset of randomly chosen individuals across multiple tissues (but always including display muscle). Such reactions always showed no amplification, indicating that low gene expression in a given tissue was not a result of contamination or trace gDNA (Bustin et al., 2009; Johnson et al., 2014). Meanwhile, we also ran several positive controls for each gene. First, we used the testis as a positive tissue control, given that it abundantly expresses all of the transporters and enzymes of interest in reproductively active birds (Freking et al., 2000; London et al., 2006). Next, we used the brain as a positive control for tissue capable of local steroid synthesis (London et al., 2006; Mirzatoni et al., 2010). Thus, comparing levels in the brain and display muscle allows us to gauge whether these tissues (e.g. SYR or LC muscle) might be capable of synthesizing physiologically relevant levels of steroids.

All reaction parameters were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We added a final dissociation stage to the end of the reaction process, which consisted of 95°C for 15 s, 60°C for 30 s and finally 95°C for 15 s. Dissociation curves for each gene always showed a single peak (Fig. S1), indicating that a single amplicon was produced (Nolan et al., 2006). Before collecting the data, we determined that samples with C_t values above 35 would be considered non-detectable, because these C_t values above this threshold can be difficult to distinguish from background (Nolan et al., 2006). As such, most non-detectable samples we show occurred when 40 cycles did not result in amplification (see Table S3), with a notable exception being CYP11a1 expression in the nuthatch PEC. This sample showed amplification after 37 cycles, rendering it non-detectable according to our criteria. For other samples that exhibited C_t values in the low 30s, we confirmed that there was no amplification in the no-RT and no-template negative controls (see above); thus, these measures were not due to contamination or the presence of genomic DNA (Nolan et al., 2006).

We used the standard curve method to measure relative expression (i.e. quantity of gene of interest/quantity GAPDH). The standard curves used in all the current experiments were generated from serially diluted (1:4) pooled cDNA. Using values obtained from these standards, we calculated reaction efficiencies [$E=10^{(-1/\text{slope})}-1$] for each gene on all reaction plates. Finally, to validate that steroidogenic enzyme transcript levels reflect a functional capacity to produce steroids, we asked how gonadal *17βHSD* mRNA levels, the enzyme necessary for generating testosterone, predict with plasma levels of testosterone. As such, we reanalyzed six (non-breeding n=3; breeding n=3) downy woodpecker samples from Schuppe and Fuxjager (2019) from which we also collected steroidogenic enzyme and transporter data as part of the current. We used a linear regression to assess whether there is a relationship between circulating testosterone levels found in Schuppe and Fuxjager (2019) and gonadal *17βHSD* levels

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

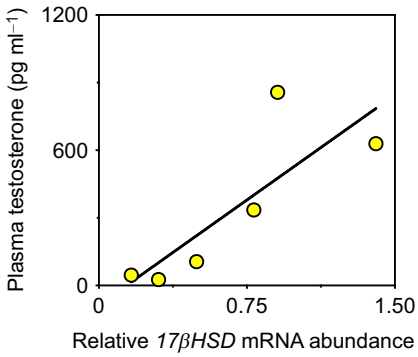

Fig. 2. Relationship between gonadal *17βHSD* transcript abundance (relative mRNA) and circulating plasma testosterone levels (re-analyzed from Schuppe and Fuxjager, 2019). Gonadal *17βHSD* positively predicted the amount of testosterone in circulation (R^2=0.8, P=0.016).

identified in the present study. These results showed that gonadal *17βHSD* transcripts positively correlated circulating testosterone (see Fig. 2).

Mouse muscle RNA-seq

Mouse skeletal muscle transcriptomic (RNA-seq) data were obtained from the open-source muscle database project (Terry et al., 2018; http://muscledb.org/). Although this dataset consists of skeletal muscles throughout the entire mouse body, we chose to focus our analysis on three more well studied muscle groups with known fiber-type composition. Specifically, we used data from extraocular muscle, soleus muscle (slow-twitch limb muscle) and extensor digitorum longus (EDL; fast-twitch limb muscle). Each muscle included six biological replicates, with each replicate including a pool of three animals. Reads were mapped and fragments per kilobase pair of exon per million (FPKM) were obtained as described in Terry and colleagues (2018).

Data analysis

We performed analyses in R (v3.3.2) after log transforming the breeding season muscular testosterone data, plasma testosterone data (Fig. S2) and steroidogenic enzyme transcript data to achieve normality and to be consistent with previous analyses that analyzed steroidogenic enzymes (Taves et al., 2015). Using Q–Q plots and Shapiro–Wilk tests, we verified that these transformations did in fact yield a more normally distributed data.

In our first analysis, we used the t.test function to run a series of *t*-tests that assessed differences in testosterone levels in the PEC and LC muscles in both the breeding and non-breeding season. We also used a *t*-test to compare seasonal changes in muscular testosterone. Next, we used the lm function to run a series of ANOVAs that compared mRNA abundance of each gene of interest across tissues for all three species. We used a false discovery rate (FDR) method to correct the models' *P*-values, thereby accounting for the total number of models run in a given species. Finally, we followed significant main effects with Benjamini–Hochberg (BH) *post hoc* comparisons to explore differences in transcript abundance among tissues. Such analyses were performed using the lsmeans package. Furthermore, we calculated an effect size (η^2) for each model using the sjstats package.

We used the t.test function to run a series of *t*-tests that compare transcript abundances between the breeding and non-breeding season in woodpeckers. We assessed the testes (positive control), as well as the LC and PEC muscles. As in the previous analyses, we also calculated effect sizes (Cohen's *d*) for all of our analyses using the lsr package.

Our mouse RNA-seq data came from Terry et al. (2018). Differential expression between tissues was determined by one-way ANOVA of FPKM values and adjusted for multiple testing as described in Terry et al. (2018).

RESULTS

Androgen levels in woodpecker drumming muscles

To test whether local steroid synthesis fuels display performance, we first looked at testosterone levels in downy woodpecker muscle across the breeding season. In breeding downy woodpeckers, testosterone levels were approximately 6× higher in the neck muscle (LC) that actuates drumming displays, compared with the PEC muscle, which is not directly involved in these displays (t_5=2.48, P=0.04; Fig. 3A). Muscular testosterone significantly declines in the non-breeding season (t_{12}=3.54, P<0.001), mirroring previously identified changes in circulating testosterone (Schuppe and Fuxjager, 2019; also see Discussion). Nonetheless, the LC maintains significantly higher levels than the PEC during the non-breeding season, when these same birds had fully regressed testes (t_5=2.92, P=0.04; Fig. 3A). Importantly, whereas 66% of PEC samples were at or near the limit of quantification of our testosterone assay in non-breeding birds, all LC testosterone samples were well above this detection limit in the same individuals.

De novo androgen synthesis in the woodpecker neck musculature

We next explored the idea that the LC maintains testosterone levels year-round by expressing transporters and enzymatic machinery necessary for local androgen synthesis. Using real-time relative quantitative PCR (qPCR), we compared relative expression of androgenic mRNA transcripts among the: (i) gonads, which are the main site of androgen synthesis; (ii) brain, which is a known site of extra-gonadal androgen synthesis in birds (London and Schlinger, 2007; London et al., 2006; Mirzatoni et al., 2010); (iii) LC muscle; and (iv) PEC muscle of male animals in breeding condition. Analyses showed significant variation in the expression of all gene transcripts across these four tissues (Fig. 3B, Table 1). *Post hoc* tests revealed that each of these transcripts was more abundantly expressed in the LC compared with the PEC. The one exception was 17-α-hydroxylase/17,20-lyase (*CYP17a1*), which showed expression levels that were statistically indistinguishable between the two muscles (Fig. 3B, Table 1). These analyses also showed that transcript levels in the LC and PEC were significantly lower than both the brain and gonad. However, expression of StAR was a notable exception, in that it was equally expressed in both the LC and brain. Our results therefore point to the LC as a site for not only androgen metabolism, but also potentially *de novo* androgen synthesis.

In a subsequent analysis, we examined how steroid biosynthesis transcripts in the gonad and muscle vary between the breeding and non-breeding season. We found significant seasonal variation in transcript levels of nearly all transporters and enzymes expressed in the gonads, with drops in expression occurring during the non-breeding season when the gonads are fully regressed (Fig. 3C, Table 2). By contrast, we found few changes in the expression of these transporters and enzymes in the LC and PEC (Fig. 3C, Table 2).

De novo androgen synthesis in the songbird syrinx

Using two oscine songbirds (white-breasted nuthatches and zebra finches), we investigated whether superfast SYR muscles, which regulate phonation, also express all the genes that are needed to

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

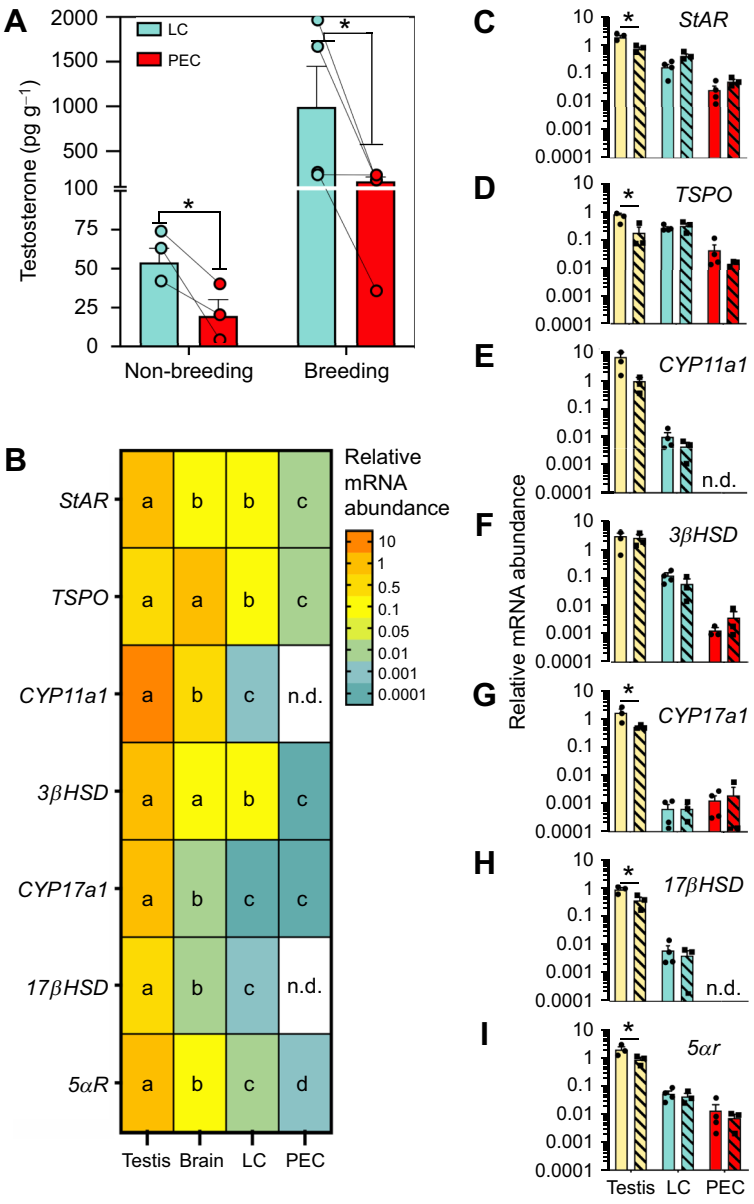

Fig. 3. Expression of muscular testosterone and steroidogenic capacity across muscular and previously identified sites of *de novo* steroid synthesis in male downy woodpeckers across seasons. (A) Mean±1 s.e.m. levels of testosterone in the longus colli ventralis (LC; woodpecker display muscle; light blue) or pectoralis (PEC; non-display muscle) in the breeding season (n=4) or non-breeding season (n=3). Each circle represents an individual, with lines connecting LC and PEC samples in the same bird. (B) Heat map illustrating mean relative mRNA abundance (gene interest/GAPDH) for all the transporters and enzymes necessary for *de novo* androgen synthesis. Letters denote significant ANOVA *post hoc* differences across tissues for each gene. (C–I) Seasonal differences in the testis, LC and PEC of breeding (n=4, solid bars) and non-breeding (n=3, hashed bars) male downy woodpeckers. Bars represent mean±1 s.e.m. relative mRNA abundance (gene interest/GAPDH). In all bar graphs, asterisks denote significant differences (P<0.05) between the breeding and non-breeding season. n.d., non-detectable transcripts.

make androgens *de novo*. Indeed, we found expression of all these genes in syringeal muscles. Transcript abundance, however, significantly differed among tissues (Fig. 4A,B, Table 3). The one exception was zebra finch *TSPO*, which was expressed at the same level in all four tissues. Further *post hoc* analyses of the main effects indicated that the gonads showed the highest levels of transcript expression for each gene. Interestingly, unlike those in the woodpecker LC muscle, these analyses also revealed that the SYR muscle expressed transcripts for these genes at levels similar to the brain, which is a well-known site of extragonadal *de novo* steroid synthesis (London and Schlinger, 2007; London et al., 2006; Mirzatoni et al., 2010). Transcript expression levels in the SYR were significantly greater than in the PEC (Fig. 4A,B, Table 3). Only *CYP17a1* in zebra finches showed an expression profile in the SYR that was lower than in the brain and equal to the PEC. Altogether, these data suggest that the SYR maintains the machinery for *de novo* androgen synthesis similar to the avian brain.

Table 1. Summary of ANOVAs used to compare gene expression among tissues for breeding male downy woodpeckers

	Downy woodpecker		
Gene of interest	$F_{3,9}$	P	Effect size
StAR	28.31	<0.001	0.90
TSPO	20.96	<0.001	0.87
CYP11a1	57.18	<0.001	0.95
3βHSD	114.89	<0.001	0.97
CYP17a1	47.92	<0.001	0.94
17βHSD	86.15	<0.001	0.96
5αR	81.83	<0.001	0.96

All significant effects were followed with BH *post hoc* test, as indicated in Fig. 3. Measures of η^2 were used to assess effect size, with values above 0.25 reflecting 'strong' effects (Cohen, 1988).

Molecular machinery for *de novo* steroid synthesis in mammalian extraocular muscles

We also examined whether the capacity for *de novo* steroid synthesis is specific to androgen-sensitive display muscles, or whether it is likely a more general feature of muscles specialized to operate at

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

Table 2. Summary of *t*-tests used to compare steroidogenic transporter and enzyme mRNA abundance between the breeding season and non-breeding season in male downy woodpecker testes, longus colli (LC) muscle and pectoralis (PEC) muscle

	Testis			LC muscle			PEC muscle		
Gene of interest	t_4	*P*	Effect size	t_5	*P*	Effect size	t_5	*P*	Effect size
StAR	4.77	<0.01	3.89	−2.39	0.06	1.82	−1.47	0.20	1.12
TSPO	5.57	<0.01	4.54	−0.48	0.65	0.37	1.22	0.27	0.94
CYP11a1	2.56	0.056	2.16	1.28	0.25	0.98	n.d.	n.d.	n.d.
3βHSD	0.23	0.82	0.20	1.61	0.17	1.22	−1.32	0.24	1.01
CYP17a1	3.11	0.036	2.53	−0.28	0.79	0.22	0.64	0.55	0.37
17βHSD	3.39	0.02	2.77	1.03	0.35	0.78	n.d.	n.d.	n.d.
5αR	3.15	0.034	2.57	0.55	0.61	0.42	−0.45	0.67	0.34

Note that n.d. denotes transcripts that were not detectable in the given tissue. Measures of Cohen's *d* were used to assess effect size, with values above 0.8 reflecting 'strong' effects (Cohen, 1988).

rapid speeds. We focused on the EDL (fast twitch muscle, type 2 fibers), soleus (slow twitch muscle, type 1 fibers), as well as EO muscles that are thought to operate at or near 'superfast' speeds (Bloemink et al., 2013; Briggs and Schachat, 2000; Lennerstrand and Hanson, 1978). Interestingly, the rate limiting transporters and enzymes were detected in all muscle types (Fig. 4C). Although the transporters (e.g. StAR and TSPO) exhibited little tissue differentiation (Fig. 4C, Table 4), we still found significant tissue differences in these genes that encode key transport proteins. Extraocular muscles have ∼10× greater *CYP11a1* transcripts, compared with slow twitch soleus muscle. Extraocular muscle also has ∼5× greater expression compared with a fast-twitch EDL muscle (Fig. 4C). Finally, other than the extraocular muscle, there was no detectable mRNA expression of *CYP17a1* or *17βHSD* in soleus or EDL muscles. Together, these results suggest that 'superfast' extraocular muscles have the molecular machinery for *de novo* synthesis, and in many cases, expression is significantly enriched in muscles that must operate at extraordinary speeds relative to muscles that operate at slower speeds.

DISCUSSION

One way that androgens can mediate elaborate reproductive displays is by acting on skeletal muscles (Alward et al., 2016; Fuxjager et al., 2013; Fuxjager et al., 2014; Regnier and Herrera, 1993). Our study uncovers an important new dimension to this process, in that we illustrate how these same muscles may produce their own androgens *de novo*. Several lines of evidence support this idea. First, the woodpecker neck muscle (LC) appears to maintain higher testosterone levels year-round, compared with muscles not directly involved in drum displays (PEC). Next, we found that the woodpecker LC, as well as the songbird SYR, expresses all transporters and enzymes necessary for transporting and converting cholesterol into bioactive androgens. This includes transcripts for *StAR*, *TSPO* and *CYP11a1*, which are typically absent from extragonadal tissue but underlie the rate-limiting steps of steroid biosynthesis (Bentz et al., 2019; Endo et al., 2008; Goetz et al., 2004; Kusakabe et al., 2009). Moreover, we show that nearly all these transcripts are more abundant in display muscle, compared with non-display muscles. These display muscles even seem to maintain transcript levels that are similar to those in the brain, a major extragonadal site for *de novo* steroid biosynthesis (London and Schlinger, 2007; London et al., 2006; Matsunaga et al., 2004; Newman et al., 2008; Remage-Healey et al., 2008; Taves et al., 2010; Tobiansky et al., 2018). Importantly, display muscles also maintain higher levels of *17βHSD* and *5αR* throughout the year compared with a muscle not involved in display. This result, of course, also means that display muscles can convert steroid precursors, such as DHEA, to androgens (e.g. testosterone and

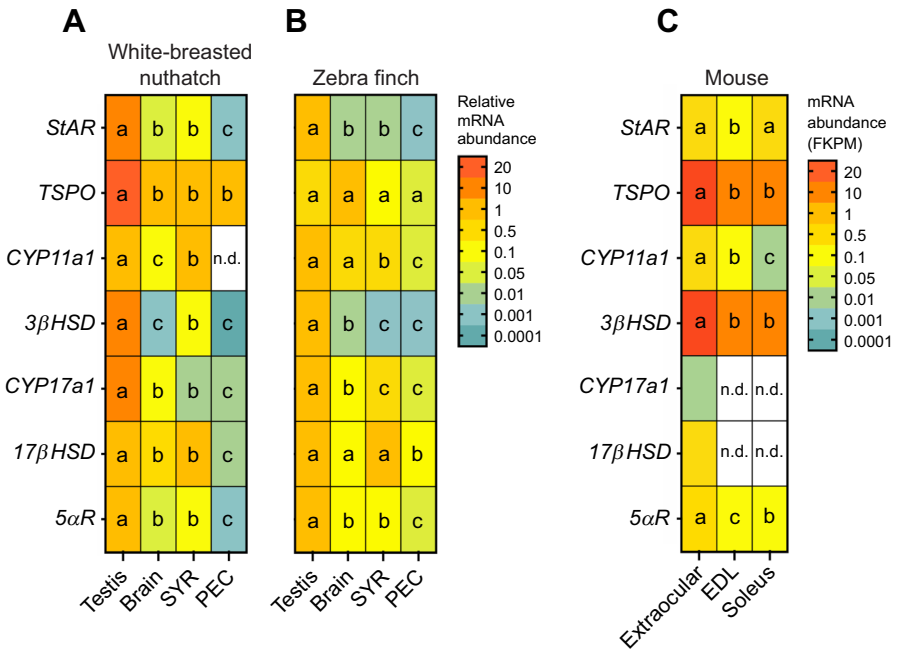

Fig. 4. Assessment of steroidogenic capacity across in superfast display (zebra finch, *n*=4; white-breasted nuthatch, *n*=3) and extraocular muscles in mouse (*n*=6). (A,B) Heatmaps illustrate mean gene expression of steroidogenic enzymes in the testis, brain, pectoralis muscle (PEC; non-display muscle) and syrinx (SYR; display muscle) muscle of the male zebra finch and white-breasted nuthatch obtained through quantitative PCR. (C) Heatmaps illustrate mean normalized transcript count (fragments per kilobase million; FKPM) of each transporter and enzyme involved in steroid synthesis in extraocular (superfast muscle), extensor digitorum longus (EDL; fast muscle) and soleus (slow muscle) of muscle. Data for mouse were obtained through publicly available mouse RNA-seq data (Terry et al., 2018). Differences in letters denote significant ANOVA *post hoc* differences ($P<0.05$) between tissues. n.d., non-detectable transcripts.

Journal of Experimental Biology (2022) 225, jeb243730. doi:10.1242/jeb.243730

Table 3. Summary of ANOVAs used to compare gene expression among tissues for the two songbird species

Gene of interest	White-breasted nuthatch			Zebra finch		
	$F_{3,8}$	*P*	Effect size	$F_{3,9}$	*P*	Effect size
StAR	17.05	<0.001	0.86	25.01	<0.001	0.89
TSPO	13.11	<0.001	0.83	0.77	0.54	0.20
CYP11a1	47.05	<0.001	0.93	11.42	<0.01	0.79
3βHSD	83.54	<0.001	0.96	73.93	<0.001	0.96
CYP17a1	12.39	<0.01	0.82	30.49	<0.001	0.91
17βHSD	163.48	<0.001	0.93	8.34	<0.01	0.71
5αR	45.29	<0.001	0.94	16.35	<0.001	0.83

All significant effects were followed with BH *post hoc* test, as indicated in Fig. 4. Measures of η^2 were used to assess effect size, with values above 0.25 reflecting 'strong' effects (Cohen, 1988).

dihydrotestosterone). In a final analysis, we suggest that the complete molecular machinery for *de novo* synthesis is similarly found in mammalian 'superfast' extraocular muscles. Local androgen synthesis in muscular tissues might therefore not only arise in the context of rapid display movements, but also more generally to power the performance of some of the fastest muscles in the vertebrate body.

One of the main functions of extragonadal steroid synthesis is to support steroid-dependent behavior during times in which gonads are regressed and functionally quiescent (Demas et al., 2007; Soma et al., 2008). Our data are consistent with this viewpoint, as the woodpecker LC maintains elevated testosterone levels compared with the PEC across the year, as well as the molecular machinery likely needed to sustain this effect. Thus, if androgenic modulation of this muscle is a necessary component to actuate drumming (Schuppe and Fuxjager, 2019), then these birds are likely able to exhibit this behavior outside of the breeding season when circulating testosterone is very low (Demas et al., 2007; Schmidt et al., 2008). Indeed, downy woodpeckers can drum outside of the breeding season, but they do so much less frequently than during the breeding season (Dodenhoff, 2002; Kilham, 1962). What, then, might androgens do to the LC to support year-round (but less frequent) drumming behavior? The answer to this question is not clear, but we suspect that it involves the preservation of muscular performance attributes that are necessary for effective communication and/or advertisement. Studies in manakin birds, for example, suggest that androgens mediate muscular twitch speeds so that individuals can produce rapid gestural displays (Fuxjager et al., 2012, 2013, 2017; Tobiansky et al., 2020b). Other work in these birds suggest that androgens might also maintain muscular hypertrophy (Luine et al., 1980), which is thought to help sustain strength that could otherwise be lost by way of an increase in muscular speed (Fuxjager et al., 2017; Sakakibara et al., 2021; Yin et al., 2020). Building on this idea, we note the LC sequesters high levels of testosterone during the breeding season, when the gonads also produce this hormone. How elevated testosterone in this tissue might further influence its performance abilities is not clear, but one possibility is that testosterone activates a series of other modifications to the muscle that support frequent drum production. Otherwise, high circulating androgen presumably acts in the brain to enhance the motivational underpinnings of drumming.

Table 4. Summary of ANOVAs used to compare RNA-seq transcript abundance (FKPM) among the three mouse skeletal muscles that operate at different speeds

Gene of interest	Mouse	
	$F_{2,15}$	*P*
StAR	41.29	<0.001
TSPO	138.30	<0.001
CYP11a1	86.86	<0.001
3βHSD	583.4	<0.001
CYP17a1	Only detectable in extraocular	–
17βHSD	Only detectable in extraocular	–
5αR	92.64	<0.001

All significant effects were followed with BH *post hoc* test, as indicated in Fig. 4.

Many of these same principles should hold true for zebra finches and white-breasted nuthatches, if the SYR maintains elevated expression of the steroidogenic machinery throughout the year. Recent work suggests that androgenic modulation of the SYR itself is critical to effective singing; for example, blocking androgen receptor (AR) in this tissue specifically slows trill rates and increases the time intervals between notes (Alward et al., 2016). Thus, by producing its own androgens, the SYR may preserve its ability to sustain effective signaling when circulating testosterone is low. Both zebra finches and white-breasted nuthatches do display outside of their respective periods of breeding (Dunn and Zann, 1996; Kilham, 1972), and the spectral characteristics of their song during this time are likely unchanged (Alvarez-Borda and Nottebohm, 2002).

A few important caveats to our conclusions should be discussed. It is crucial to discuss our current findings in the context of past work by Schuppe and Fuxjager (2019), which shows that male downy woodpeckers maintain plasma testosterone levels of ≈75 pg ml^{-1} in November and December (non-breeding season). These values are slightly higher than the testosterone levels we currently report in the LC during the same months. At first, this difference might seem counterintuitive, as one would presumably expect plasma testosterone to set the minimum level of this hormone in a tissue such as skeletal muscle. However, this is not likely the case. In fact, if it were, then we would expect the PEC to similarly mirror plasma in terms of testosterone levels, but we do not see this result. Rather, the PEC shows little to no testosterone, with most samples from this tissue registering at the limit of detection of our assay. Muscles therefore appear capable of buffering testosterone levels, and likely making their own. Furthermore, we find similar discord between muscular and plasma testosterone in the breeding season, but this time it occurs in the opposite direction. In other words, the LC maintains testosterone levels (≈1000 pg g^{-1}) that are higher than previously reported levels of plasma testosterone (≈600–850 pg ml^{-1}). The LC also shows higher levels of testosterone than the PEC in the breeding season, which again contains testosterone levels (≈150 pg g^{-1}) that appear much lower than plasma levels. While these findings collectively suggest that the LC is capable of local synthesis and metabolism of steroids, it is also clear that this muscle sequesters large amounts of androgens in the breeding season. Despite evidence of sequestration, LC testosterone levels are often near or above levels in in plasma. This typically suggests that a tissue is capable of local synthesis. Nonetheless, it remains possible that rates of sequestration might differ between the LC and PEC muscles. Thus, future research is needed to fully evaluate the amount of local synthesis that each of these muscles can perform.

We must also recognize that if machinery for *de novo* androgen synthesis is present in muscle, then machinery for androgen metabolism is also in place. This means that muscle tissues, such as songbird SYR and woodpecker LC, can likely still synthesize

androgens from circulating androgenic precursors such as dehydroepiandrosterone (DHEA) or androstenedione (see Fig. 1). Many studies show that local conversion of DHEA to androgens and estrogens underlies steroidal regulation of important reproductive behavior when circulating testosterone is low or basal (Demas et al., 2007). Thus, it would not be surprising if such mechanisms were also relevant to maintain elevated testosterone levels in the LC muscle. Moreover, this ability to synthesize and/or metabolize androgens differs among species in the PEC muscle. Although our findings show that zebra finch PEC appears capable of making certain steroids and metabolizing them (Fig. 4), the nuthatch PEC and mouse limb muscles appear only capable of the latter. Meanwhile, the woodpecker PEC seems only capable of metabolizing testosterone into dihydrotestosterone. Musculoskeletal systems therefore presumably vary within and among species in terms of their capacity for synthesizing and metabolizing steroid hormones.

Many of the ideas we outline above are predicated on the ability of transcripts for steroidogenic transporters and enzymes to reflect a tissue's functional capacity to make bioactive steroids. This assumption, however, has its limitations that must be considered. For instance, some studies have suggested that transcript levels do not always correspond to protein levels (Liu et al., 2016; Suarez and Moyes, 2012). We suspect that such effects do not entirely account for our current results, as other work demonstrates that mRNA levels of many steroidogenic enzymes do in fact accurately predict differences in circulating steroid levels (Geslin and Auperin, 2004; Komar et al., 2001; Price et al., 2004). Our data point to this latter effect by demonstrating that gonadal transcript abundance significantly declines in the non-breeding season when circulating testosterone is low (<100 ng μl^{-1}; Schuppe and Fuxjager, 2019). Furthermore, we also show that transcript abundance of gonadal *17βHSD* and *5αR* (the enzymes responsible for making androgens) predicts seasonal variation in plasma testosterone (Fig. 2). Additional support for a link between mRNA levels and steroidogenic capacity comes from our results that show similar gene expression profiles in display muscle and the brain. The brain is a well-known site of steroidogenesis in birds and other taxa (London and Schlinger, 2007; London et al., 2006; Mirzatoni et al., 2010; Taves et al., 2015; Tobiansky et al., 2018), and thus we expect that both the brain and display muscles maintain a comparable ability to make androgens *de novo*. This idea is further supported by work in rat muscle that demonstrates transcript levels of *3βHSD* and *17βHSD* are expressed at levels that are similar to the brain (see Sato and Iemitsu, 2018). Additionally, injecting different amounts of DHEA leads to increasing amounts of both testosterone and dihydrotestosterone (Sato et al., 2008). Together, this suggests muscle transcript levels can predict a functional capacity to generate steroids.

We also note that transcript abundance in the brain and display muscle of breeding birds is minimal compared with in the testes (1–5%). It is important to mention, however, that such low levels of steroidogenic machinery are still capable of producing fully functional steroids. In regions of the brain that maintain the steroidogenic machinery, steroid levels often exceed levels in the blood (Jalabert et al., 2021; Tobiansky et al., 2018). This suggests that even low transcript levels can result in high concentrations of bioactive steroids locally. These neural derived steroids (e.g. testosterone or estradiol) are hypothesized to have functional effects on motivated behaviors (Tobiansky et al., 2018). Work on cardiomyocytes further illustrates this point by demonstrating that these cells contain similar (low) levels of the transporters and enzymes needed to make steroids (Kayes-Wandover and White, 2000; Young et al., 2001), but are fully capable of doing so nonetheless.

Conclusions

Here, we report for the first time that skeletal muscle involved in the production of elaborate sexual displays as well as non-display muscles that operate at superfast speeds maintain the molecular machinery needed to create androgen steroids *de novo*. This implies that androgenic regulation of these display muscles can occur autonomously from the peripheral sex organs. Meanwhile, we show that this display muscle likely expresses steroidogenic machinery at levels that match those found in the brain, one of the few established extragonadal steroidogenic organs in the vertebrate body. Thus, the muscle's capacity for steroidogenesis is likely significant, and fuels local regulation similar to that found in the central nervous system. These data support the hypothesis that local steroid regulation in muscles closes the motor control loop, and thus maintains year-round stereotyped behavioral output. In this way, muscular androgen synthesis can temporally and spatially free the processes by which hormones regulate behaviour.

Acknowledgements

We thank Johnny Petersen, Emma Vivlamore and Ryan Walsh for field and laboratory assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.R.S., M.J.F.; Methodology: E.R.S., D.T.; Formal analysis: E.R.S., D.T.; Investigation: E.R.S., D.T., M.J.F.; Resources: F.G., M.J.F.; Data curation: E.R.S., D.T.; Writing - original draft: E.R.S., D.T., F.G., M.J.F.; Writing - review & editing: E.R.S., D.T., F.G., M.J.F.; Supervision: F.G., M.J.F.; Project administration: M.J.F.; Funding acquisition: M.J.F.

Funding

This work was funded by National Science Foundation grant IOS-1947472 (to M.J.F.), and institutional funds from Brown University.

Data availability

Data are available from figshare: https://doi.org/10.6084/m9.figshare.c.6024812.v1. Mouse RNAseq data came from Terry et al. (2018), and are available under accession code GSE100505. Analyzed data are available on http://muscledb.org.

References

Adkins-Regan, E. (2013). *Hormones and Animal Social Behavior*. Princeton University Press.

Alvarez-Borda, B. and Nottebohm, F. (2002). Gonads and singing play separate, additive roles in new neuron recruitment in adult canary brain. *J. Neurosci* **22**, 8684-8690. doi:10.1523/JNEUROSCI.22-19-08684.2002

Alward, B. A., Madison, F. N., Gravley, W. T. and Ball, G. F. (2016). Antagonism of syringeal androgen receptors reduces the quality of female-preferred male song in canaries. *Anim. Behav.* **119**, 201-212. doi:10.1016/j.anbehav.2016.07.010

Arnold, A. P. (1975). The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (*Poephila guttata*). *J. Exp. Zool* **191**, 309-326. doi:10.1002/jez.1401910302

Augusto, V., Padovani, C. R. and Campos, G. E. R. (2004). Skeletal muscle fiber types in C57BL6J mice. *Brazilian J. Morphol. Sci.* 21.

Bentz, A. B., Dossey, E. K. and Rosvall, K. A. (2019). Tissue-specific gene regulation corresponds with seasonal plasticity in female testosterone. *Gen. Comp. Endocrinol.* **270**, 26-34. doi:10.1016/j.ygcen.2018.10.001

Blaustein, J. D. and Olster, D. H. (1989). Gonadal steroid hormone receptors and social behaviors. In *Molecular and Cellular Basis of Social Behavior in Vertebrates* (ed. J. Balthazart), pp. 31-104. Springer.

Bloemink, M. J., Deacon, J. C., Resnicow, D. I., Leinwand, L. A. and Geeves, M. A. (2013). The superfast Human extraocular myosin is kinetically distinct from the fast skeletal IIa, IIb, and IId isoforms. *J. Biol. Chem* **288**, 27469-27479. doi:10.1074/jbc.M113.488130

Briggs, M. M. and Schachat, F. (2000). Early specialization of the superfast myosin in extraocular and laryngeal muscles. *J. Exp. Biol* **203**, 2485-2494. doi:10.1242/jeb.203.16.2485

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L. et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611-622. doi:10.1373/clinchem.2008.112797

Christensen, L. A., Allred, L. M., Goller, F., Meyers, R. A. (2017). Is sexual dimorphism in singing behavior related to syringeal muscle composition? *Auk* **134**, 710-720. doi:10.1642/AUK-17-3.1

Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*, 2nd edn. Routledge. doi:10.4324/9780203771587

Dawson, A., King, V. M., Bentley, G. E. and Ball, G. F. (2001). Photoperiodic control of seasonality in birds. *J. Biol. Rhythms* **16**, 365-380. doi:10.1177/074873001129002079

Demas, G. E., Cooper, M. A., Albers, H. E. and Soma, K. K. (2007). Novel mechanisms underlying neuroendocrine regulation of aggression: a synthesis of rodent, avian, and primate studies. In *Handbook of Neurochemistry and Molecular Neurobiology: Behavioral Neurochemistry, Neuroendocrinology and Molecular Neurobiology* (ed. A. Lajtha and J. D. Blaustein), pp. 337-372. Springer.

Dodenhoff, D. (2002). An analysis of acoustic communication within the social system of downy woodpeckers (*Picoides pubescens*). PhD dissertation, The Ohio State University.

Dunn, A. M. and Zann, R. A. (1996). Undirected song in wild zebra finch flocks: contexts and effects of mate removal. *Ethology* **102**, 529-539. doi:10.1111/j.1439-0310.1996.tb01145.x

Elemans, C. P. H., Spierts, I. L. Y., Müller, U. K., Van Leeuwen, J. L. and Goller, F. (2004). Superfast muscles control dove's trill. *Nature* **431**, 146. doi:10.1038/431146a

Elemans, C. P. H., Mead, A. F., Rome, L. C. and Goller, F. (2008). Superfast vocal muscles control song production in songbirds. *PLoS One* **3**, e2581. doi:10.1371/journal.pone.0002581

Endo, D., Kanaho, Y. I. and Park, M. K. (2008). Expression of sex steroid hormone-related genes in the embryo of the leopard gecko. *Gen. Comp. Endocrinol.* **155**, 70-78. doi:10.1016/j.ygcen.2007.04.010

Feng, N. Y., Katz, A., Day, L. B., Barske, J. and Schlinger, B. A. (2010). Limb muscles are androgen targets in an acrobatic tropical bird. *Endocrinology* **151**, 1042-1049. doi:10.1210/en.2009-0901

Fraysse, B., Vignaud, A., Fane, B., Schuh, M., Butler-Browne, G., Metzger, D. and Ferry, A. (2014). Acute effect of androgens on maximal force-generating capacity and electrically evoked calcium transient in mouse skeletal muscles. *Steroids* **87**, 6-11. doi:10.1016/j.steroids.2014.05.005

Freking, F., Nazairians, T. and Schlinger, B. A. (2000). The expression of the sex steroid-synthesizing enzymes CYP11A1, 3β-HSD, CYP17, and CYP19 in gonads and adrenals of adult and developing zebra finches. *Gen. Comp. Endocrinol* **119**, 140-151. doi:10.1006/gcen.2000.7503

Fuxjager, M. J., Barske, J., Du, S., Day, L. B. and Schlinger, B. A. (2012). Androgens regulate gene expression in avian skeletal muscles. *PLoS One* **7**, e51482. doi:10.1371/journal.pone.0051482

Fuxjager, M. J., Longpre, K. M., Chew, J. G., Fusani, L. and Schlinger, B. A. (2013). Peripheral androgen receptors sustain the acrobatics and fine motor skill of elaborate male courtship. *Endocrinology* **154**, 3168-3177. doi:10.1210/en.2013-1302

Fuxjager, M. J., Heston, J. B. and Schlinger, B. A. (2014). Peripheral androgen action helps modulate vocal production in a suboscine passerine. *Auk* **131**, 327-334. doi:10.1642/AUK-13-252.1

Fuxjager, M. J., Lee, J. H., Chan, T. M., Bahn, J. H., Chew, J. G., Xiao, X. and Schlinger, B. A. (2016). Research resource: hormones, genes, and athleticism: effect of androgens on the avian muscular transcriptome. *Mol. Endocrinol.* **30**, 254-271. doi:10.1210/me.2015-1270

Fuxjager, M. J., Miles, M. C., Goller, F., Petersen, J. and Yancey, J. (2017). Androgens support male acrobatic courtship behavior by enhancing muscle speed and easing the severity of its tradeoff with force. *Endocrinology* **158**, 4038-4046. doi:10.1210/en.2017-00599

Geslin, M. and Auperin, B. (2004). Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **135**, 70-80. doi:10.1016/S0016-6480(03)00283-1

Goetz, F. W., Norberg, B., McCauley, L. A. R. and Iliev, D. B. (2004). Characterization of the cod (*Gadus morhua*) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **137**, 351-362. doi:10.1016/j.cbpc.2003.12.006

Hanukoglu, I. (1992). Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. *J. Steroid Biochem. Mol. Biol.* **43**, 779-804. doi:10.1016/0960-0760(92)90307-5

Hau, M., Wikelski, M., Soma, K. K. and Wingfield, J. C. (2000). Testosterone and year-round territorial aggression in a tropical bird. *Gen. Comp. Endocrinol.* **117**, 20-33. doi:10.1006/gcen.1999.7390

Holmes, M. M., Bartrem, C. L. and Wade, J. (2007). Androgen dependent seasonal changes in muscle fiber type in the dewlap neuromuscular system of green anoles. *Physiol. Behav.* **91**, 601-608. doi:10.1016/j.physbeh.2007.03.022

Inai, Y., Nagai, K., Ukena, K., Oishi, T. and Tsutsui, K. (2003). Seasonal changes in neurosteroid concentrations in the amphibian brain and environmental factors regulating their changes. *Brain Res.* **959**, 214-225. doi:10.1016/S0006-8993(02)03745-9

Jalabert, C., Ma, C. and Soma, K. K. (2021). Profiling of systemic and brain steroids in male songbirds: seasonal changes in neurosteroids. *J. Neuroendocrinol* **33**, e12922. doi:10.1111/jne.12922

James, R. S., Altringham, J. D. and Goldspink, D. F. (1995). The mechanical properties of fast and slow skeletal muscles of the mouse in relation to their locomotory function. *J. Exp. Biol* **198**, 491-502. doi:10.1242/jeb.198.2.491

Johnson, G., Nour, A. A., Nolan, T., Huggett, J. and Bustin, S. (2014). Minimum information necessary for quantitative real-time PCR experiments. *Methods Mol. Biol.* **1160**, 4-17.

Kaiser, M. (1990). Untersuchungen zur Biomechanik des Balztrommelns der Specte (Picidae). *Beitr. Vogelkd* **36**, 129-159.

Kayes-Wandover, K. M. and White, P. C. (2000). Steroidogenic enzyme gene expression in the human heart 1. *J. Clin. Endocrinol. Metab.* **85**, 2519-2525.

Kellam, J. S., Wingfield, J. C. and Lucas, J. R. (2004). Nonbreeding season pairing behavior and the annual cycle of testosterone in male and female downy woodpeckers, *Picoides pubescens*. *Horm. Behav* **46**, 703-714. doi:10.1016/j.yhbeh.2004.06.014

Kilham, L. (1962). Reproductive behavior of downy woodpeckers. *Condor* **64**, 126-133. doi:10.2307/1365481

Kilham, L. (1972). Reproductive behavior of white-breasted nuthatches II. Courtship. *Auk* **89**, 115-129. doi:10.2307/4084063

Kim, H. J., Na, J. I., Min, B. W., Na, J. Y., Lee, K. H., Lee, J. H., Lee, Y. J., Kim, H. S. and Park, J. T. (2014). Evaluation of protein expression in housekeeping genes across multiple tissues in rats. *Korean J. Pathol.* **48**, 193-200. doi:10.4132/KoreanJPathol.2014.48.3.193

Komar, C. M., Berndtson, A. K., Evans, A. C. O. and Fortune, J. E. (2001). Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for P450 aromatase and P450 17α-hydroxylase, in bovine preovulatory follicles. *Biol. Reprod.* **64**, 1797-1805. doi:10.1095/biolreprod64.6.1797

Kusakabe, M., Zuccarelli, M. D., Nakamura, I. and Young, G. (2009). Steroidogenic acute regulatory protein in white sturgeon (*Acipenser transmontanus*): cDNA cloning, sites of expression and transcript abundance in corticosteroidogenic tissue after an acute stressor. *Gen. Comp. Endocrinol.* **162**, 233-240. doi:10.1016/j.ygcen.2009.02.007

Lennerstrand, G. and Hanson, J. (1978). The postnatal development of the inferior oblique muscle of the cat: I. Isometric twitch and tetanic properties: I. Isometric twitch and tetanic properties. *Acta Physiol. Scand.* **103**, 132-143. doi:10.1111/j.1748-1716.1978.tb06200.x

Liu, Y., Beyer, A. and Aebersold, R. (2016). On the dependency of cellular protein levels on mRNA abundance. *Cell* **165**, 535-550. doi:10.1016/j.cell.2016.03.01

London, S. E. and Schlinger, B. A. (2007). Steroidogenic enzymes along the ventricular proliferative zone in the developing songbird brain. *J. Comp. Neurol.* **502**, 507-521. doi:10.1002/cne.21335

London, S. E., Monks, D. A., Wade, J. and Schlinger, B. A. (2006). Widespread capacity for steroid synthesis in the avian brain and song system. *Endocrinology* **147**, 5975-5987. doi:10.1210/en.2006-0154

Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J. Vis. Exp.* **63**, e3998. doi:10.3791/3998

Luine, V., Nottebohm, F., Harding, C. and McEwen, B. S. (1980). Androgen affects cholinergic enzymes in syringeal motor neurons and muscle. *Brain Res.* **192**, 89-107. doi:10.1016/0006-8993(80)91011-2

Mangiamele, L. A., Fuxjager, M. J., Schuppe, E. R., Taylor, R. S., Hödl, W. and Preininger, D. (2016). Increased androgenic sensitivity in the hind limb muscular system marks the evolution of a derived gestural display. *Proc. Natl. Acad. Sci. USA* **113**, 5664-5669. doi:10.1073/pnas.1603329113

Matsunaga, M., Ukena, K., Baulieu, E. E. and Tsutsui, K. (2004). 7α-Hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system. *Proc. Natl. Acad. Sci. USA* **101**, 17282-17287. doi:10.1073/pnas.0407176101

Mead, A. F., Osinalde, N., Ørtenblad, N., Nielsen, J., Brewer, J., Vellema, M., Adam, I., Scharff, C., Song, Y., Frandsen, U. et al. (2017). Fundamental constraints in synchronous muscle limit superfast motor control in vertebrates. *eLife* **6**, e29425. doi:10.7554/eLife.29425

Mirzatoni, A., Spence, R. D., Naranjo, K. C., Saldanha, C. J. and Schlinger, B. A. (2010). Injury-induced regulation of steroidogenic gene expression in the cerebellum. *J. Neurotrauma* **27**, 1875-1882. doi:10.1089/neu.2010.1330

Mohr, M. A., Wong, A. M., Tomm, R. J., Soma, K. K. and Micevych, P. E. (2019). Pubertal development of estradiol-induced hypothalamic progesterone synthesis. *Horm. Behav* **111**, 110-113. doi:10.1016/j.yhbeh.2018.12.007

Moore, M. C. and Marler, C. A. (1987). Effects of testosterone manipulations on nonbreeding season territorial aggression in free-living male lizards, *Sceloporus jarrovi*. *Gen. Comp. Endocrinol.* **65**, 225-232. doi:10.1016/0016-6480(87)90170-5

Newman, A. E. M., Pradhan, D. S. and Soma, K. K. (2008). Dehydroepiandrosterone and corticosterone are regulated by season and acute

stress in a wild songbird: jugular versus brachial plasma. *Endocrinology* **149**, 2537-2545. doi:10.1210/en.2007-1363

Nolan, T., Hands, R. E. and Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* **1**, 1559-1582. doi:10.1038/nprot.2006.236

Payne, A. H. and Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr. Rev.* **25**, 947-970. doi:10.1210/er.2003-0030

Price, C., Cooke, G. and Sanford, L. (2004). Influence of season and low-level oestradiol immunoneutralization on episodic LH and testosterone secretion and testicular steroidogenic enzymes and steroidogenic acute regulatory protein in the adult ram. *Reproduction* 118, 251-262.

Regnier, M. and Herrera, A. A. (1993). Changes in contractile properties by androgen hormones in sexually dimorphic muscles of male frogs (*Xenopus laevis*). *J. Physiol.* **461**, 565-581. doi:10.1113/jphysiol.1993.sp019529

Remage-Healey, L., Maidment, N. T. and Schlinger, B. A. (2008). Forebrain steroid levels fluctuate rapidly during social interactions. *Nat. Neurosci.* **11**, 1327-1334. doi:10.1038/nn.2200

Sakakibara, I., Yanagihara, Y., Himori, K., Yamada, T., Sakai, H., Sawada, Y., Takahashi, H., Saeki, N., Hirakawa, H., Yokoyama, A. et al. (2021). Myofiber androgen receptor increases muscle strength mediated by a skeletal muscle splicing variant of Mylk4. *iScience* **24**, 102303. doi:10.1016/j.isci.2021.102303

Sassoon, D. A., Gray, G. E. and Kelley, D. B. (1987). Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. *J. Neurosci.* **7**, 3198-3206. doi:10.1523/JNEUROSCI.07-10-03198.1987

Sato, K. and Iemitsu, M. (2018). The role of dehydroepiandrosterone (DHEA) in skeletal muscle. *Vitam. Horm.* **108**, 205-221. doi:10.1016/bs.vh.2018.03.002

Sato, K., Iemitsu, M., Aizawa, K. and Ajisaka, R. (2008). Testosterone and DHEA activate the glucose metabolism-related signaling pathway in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **294**, E961-E968. doi:10.1152/ajpendo.00678.2007

Schlinger, B. A., Day, L. B. and Fusani, L. (2008). Behavior, natural history and neuroendocrinology of a tropical bird. *Gen. Comp. Endocrinol.* **157**, 254-258. doi:10.1016/j.ygcen.2008.05.015

Schmidt, K. L., Pradhan, D. S., Shah, A. H., Charlier, T. D., Chin, E. H. and Soma, K. K. (2008). Neurosteroids, immunosteroids, and the Balkanization of endocrinology. *Gen. Comp. Endocrinol.* **157**, 266-274. doi:10.1016/j.ygcen.2008.03.025

Schuppe, E. R. and Fuxjager, M. J. (2018). High-speed displays encoding motor skill trigger elevated territorial aggression in downy woodpeckers. *Funct. Ecol.* **32**, 450-460. doi:10.1111/1365-2435.13010

Schuppe, E. R. and Fuxjager, M. J. (2019). Phenotypic variation reveals sites of evolutionary constraint in the androgenic signaling pathway. *Horm. Behav.* **115**, 104538. doi:10.1016/j.yhbeh.2019.06.002

Schuppe, E. R., Rutter, A. R., Roberts, T. J. and Fuxjager, M. J. (2021). Evolutionary and biomechanical basis of drumming behavior in woodpeckers. *Front. Ecol. Evol.* 9, 649146. doi:10.3389/fevo.2021.649146

Shen, Y., Li, Y., Ye, F., Wang, F., Lu, W. and Xie, X. (2010). Identification of suitable reference genes for measurement of gene expression in human cervical tissues. *Anal. Biochem.* **405**, 224-229. doi:10.1016/j.ab.2010.06.029

Smith, S. M., Eigerman, A. R., Lecure, K. M., Kironde, E., Privett-Mendoza, A. G., Fuxjager, M. J., Preininger, D. and Mangiamele, L. A. (2021). Androgen receptor modulates multimodal displays in the bornean rock frog (*Staurois parvus*). *Integr. Comp. Biol.* **61**, 221-230. doi:10.1093/icb/icab042

Soma, K. K., Scotti, M. A. L., Newman, A. E. M., Charlier, T. D. and Demas, G. E. (2008). Novel mechanisms for neuroendocrine regulation of aggression. *Front. Neuroendocrinol.* **29**, 476-489. doi:10.1016/j.yfrne.2007.12.003

Soukup, T., Zachařová, G. and Smerdu, V. (2002). Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem.* **104**, 399-405. doi:10.1078/0065-1281-00660

Suarez, R. K. and Moyes, C. D. (2012). Metabolism in the age of 'omes'. *J. Exp. Biol.* **215**, 2351-2357.

Taves, M. D., Schmidt, K. L., Ruhr, I. M., Kapusta, K., Prior, N. H. and Soma, K. K. (2010). Steroid concentrations in plasma, whole blood and brain: Effects of saline perfusion to remove blood contamination from brain. *PLoS One* **5**, e15727. doi:10.1371/journal.pone.0015727

Taves, M. D., Plumb, A. W., Sandkam, B. A., Ma, C., Van Der Gugten, J. G., Holmes, D. T., Close, D. A., Abraham, N. and Soma, K. K. (2015). Steroid profiling reveals widespread local regulation of glucocorticoid levels during mouse development. *Endocrinology* **156**, 511-522. doi:10.1210/en.2013-1606

Terry, E. E., Zhang, X., Hoffmann, C., Hughes, L. D., Lewis, S. A., Li, J., Wallace, M. J., Riley, L. A., Douglas, C. M., Gutierrez-Monreal, M. A. et al. (2018). Transcriptional profiling reveals extraordinary diversity among skeletal muscle tissues. *Elife* **7**, e34613. doi:10.7554/eLife.34613

Tobiansky, D. J., Korol, A. M., Ma, C., Hamden, J. E., Jalabert, C., Tomm, R. J. and Soma, K. K. (2018). Testosterone and corticosterone in the mesocorticolimbic system of male rats: Effects of gonadectomy and caloric restriction. *Endocrinology* **159**, 450-464. doi:10.1210/en.2017-00704

Tobiansky, D. J., Kachkovski, G. V., Enos, R. T., Schmidt, K. L., Angela Murphy, E. and Soma, K. K. (2020a). Sucrose consumption alters steroid and dopamine signalling in the female rat brain. *J. Endocrinol* **245**, 231-246. doi:10.1530/JOE-19-0386

Tobiansky, D. J., Miles, M. C., Goller, F. and Fuxjager, M. J. (2020b). Androgenic modulation of extraordinary muscle speed creates a performance trade-off with endurance. *J. Exp. Biol* **223**, jeb222984. doi:10.1242/jeb.222984

Tobias, M. L., Barnard, C., O'Hagan, R., Horng, S. H., Rand, M. and Kelley, D. B. (2004). Vocal communication between male Xenopus laevis. *Anim. Behav* **67**, 353-365. doi:10.1016/j.anbehav.2003.03.016

Uchida, A. M., Meyers, R. A., Cooper, B. G. and Goller, F. (2010). Fibre architecture and song activation rates of syringeal muscles are not lateralized in the European starling. *J. Exp. Biol.* **213**, 1069-1078. doi:10.1242/jeb.038885

Veney, S. L. and Wade, J. (2004). Steroid receptors in the adult zebra finch syrinx: a sex difference in androgen receptor mRNA, minimal expression of estrogen receptor α and aromatase. *Gen. Comp. Endocrinol.* **136**, 192-199. doi:10.1016/j.ygcen.2003.12.017

Wikelski, M., Hau, M., Robinson, W. D. and Wingfield, J. C. (2003). Reproductive seasonality of seven neotropical passerine species. *Condor* **105**, 683-695. doi:10.1093/condor/105.4.683

Yin, L., Lu, L., Lin, X. and Wang, X. (2020). Crucial role of androgen receptor in resistance and endurance trainings-induced muscle hypertrophy through IGF-1/IGF-1R-PI3K/Akt-mTOR pathway. *Nutr. Metab.* **17**, 26. doi:10.1186/s12986-020-00446-y

Young, M. J., Clyne, C. D., Cole, T. J. and Funder, J. W. (2001). Cardiac steroidogenesis in the normal and failing heart. *J. Clin. Endocrinol. Metab.* **86**, 5121-5126. doi:10.1210/jcem.86.11.7925

Zornik, E. and Yamaguchi, A. (2011). Vocal pathway degradation in gonadectomized *Xenopus laevis* adults. *J. Neurophysiol.* **105**, 601-614. doi:10.1152/jn.00883.2010