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ORIGINAL PAPER

Preparing to migrate: expression of androgen signaling molecules and insulin-like growth factor-1 in skeletal muscles of Gambel's white-crowned sparrows

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Abstract

Migratory birds, including Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), exhibit profound modifications of skeletal muscles prior to migration, notably hypertrophy of the pectoralis muscle required for powered flight. Muscle growth may be influenced by anabolic effects of androgens; however, prior to spring departure, circulating androgens are low in sparrows. A seasonal increase in local androgen signaling may occur within muscle to promote remodeling. We measured morphological parameters, plasma and tissue levels of testosterone, as well as mRNA expression levels of androgen receptor, 5α-reductase (converts testosterone to 5α-dihydrotestosterone), and the androgen-dependent myotrophic factor insulin-like growth factor-1. We studied the pectoralis muscle as well as the gastrocnemius (leg) muscle of male sparrows across three stages on the wintering grounds: winter (February), pre-nuptial molt (March), and pre-departure (April). Testosterone levels were low, but detectable, in plasma and muscles at all three stages. Androgen receptor mRNA and 5α-reductase Type 1 mRNA increased at pre-departure, but did so in both muscles. Notably, mRNA levels of insulin-like growth factor-1, an androgen-dependent gene critical for muscle remodeling, increased at pre-departure in the pectoralis but decreased in the gastrocnemius. Taken together, these data suggest a site-specific molecular basis for muscle remodeling that may serve to enable long-distance flight.

Keywords 5α-Reductase · Androgen receptor · Life-history stages · Songbird · Testosterone

Abbreviations

5α-DHT	5α-Dihydrotestosterone
AR	Androgen receptor
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
IGF-1	Insulin-like growth factor-1, IGF-1
T	Testosterone

Introduction

Birds that migrate annually to and from distant breeding grounds display phenotypic flexibility in physiology, behavior and morphology to meet the demands of current conditions while preparing for the subsequent life history stage (Ramenofsky and Wingfield 2007; Piersma and van Gils 2010; Price et al. 2011). As birds prepare for migration, they become hyperphagic, increase body mass, and fat deposition (King and Farner 1963; Dolnik and Blyumental 1967; Ramenofsky 1990; Driedzic et al. 1993). Additionally, the pectoralis major and minor, breast muscles necessary for generating the force for propelling the wings during downstroke of avian flight (Edwards 1886), undergo hypertrophy in some species, as well as enhanced lipid and glycogen storage (Fry et al. 1972; Driedzic et al. 1993; Jehl 1997;

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Bauchinger and Biebach 2006). Ultrastructural studies have revealed muscle hypertrophy is associated with increased diameter of muscle fibers in several species during the migratory stages, as well as increased mitochondria (Marsh 1984; Gaunt et al. 1990; Evans et al. 1992) (A. Campion and M. Ramenofsky, in prep). Together, these changes can provide increased fuel, aerobic capacity, and power to support migratory flight as well as thermogenesis (Bauchinger and Biebach 2006; Vézina et al. 2007).

The regulation of pre-migratory hyperphagia and fattening is well studied in several species and is initiated by the increased daylengths of spring in conjunction with endogenous rhythms to activate central neuropeptidergic and endocrine systems (Lofts and Marshall 1960; Dolnik and Blyumental 1967; Gwinner 1990; Wingfield 2008; Ramenofsky et al. 2012; Cornelius et al. 2013; Ramenofsky and Németh 2014). However, the mechanisms regulating pectoralis muscle remodeling in migrants remain enigmatic (Fry et al. 1972; Evans et al. 1992; Price et al. 2011). It is possible that muscle hypertrophy is initiated by the photoperiodic mechanisms regulating hyperphagia and fattening, or it may be induced in response to the increased body mass and wing loading as suggested for some species (Evans et al. 1992; Driedzic et al. 1993; Battley and Piersma 1997). Alternatively, increased flight activity or exercise during the pre-migratory period may promote flight muscle remodeling (Gaunt et al. 1990; Price et al. 2011). However, red knots (*Calidris canutus*) exhibit flight muscle remodeling during the pre-migratory period even when confined to cages that prohibit extensive movements (Dietz et al. 1999; Vézina et al. 2007). Thus, the mechanisms of flight muscle remodeling in migrants are not well understood, but an investigation thereof offers a potentially informative platform for further understanding the regulatory pathways preparing this major tissue for migration.

Given the roles of hormones in pre-migratory hyperphagia and fattening, it is possible that hormones also regulate flight muscle remodeling. Anabolic effects of androgens on skeletal muscle are well known (Herbst and Bhasin 2004; Dubois et al. 2011). Moreover, power training (or endurance exercise) in humans increases muscle androgen concentrations, as well as 5α-reductase and androgen receptor (AR) protein levels (Feng et al. 2010; Fuxjager et al. 2013, 2016b). In rodents, acute exercise training increases skeletal muscle levels of 5α-DHT, dehydroepiandrosterone, and testosterone (T) (Aizawa et al. 2010). Levels of AR may be especially important to regulate tissue sensitivity to androgens. In the bluebanded goby (*Lythrypnus dalli*), male-specific courtship behavior is correlated with AR expression in the supracarinalis muscle (Schuppe et al. 2017). In the golden collared manakin *(Manacus vittelinus)*, acrobatic courtship behavior is activated by T (Fusani et al. 2007; Fuxjager et al. 2013), and AR and 5α-reductase expression are elevated in limb muscles associated with wing movement (Feng et al. 2010; Fuxjager et al. 2016b). Across diverse avian species, skeletal muscle AR levels correlate with the physical complexity of male courtship (Fuxjager et al. 2015). Although these experiments demonstrate a link between androgen signaling, muscle size and activity, there has been no demonstration of seasonal, migration-related changes in androgen signaling in muscles.

In skeletal muscle, AR regulates multiple myotrophic and metabolic processes (Fuxjager et al. 2013). In manakin skeletal muscles, for example, androgens upregulate genes associated with muscle hypertrophy, Ca^{2+} availability, and fuel transport and metabolism (Fuxjager et al. 2012, 2016a) including the gene for insulin-like growth factor-1 (IGF-1), a myokine that stimulates growth and regeneration of skeletal muscles (Velloso 2008). Skeletal muscle expression of IGF-1 increases in photo-stimulated white-throated sparrows (*Zonotrichia albicollis*) expressing migratory behavior or *Zugunruhe*, as well as in repeatedly exercised European starlings (*Sturnus vulgaris*) (Price et al. 2011). Thus, changes in androgen synthesis, action or IGF-1 concentration may prepare muscles to support flight performance, but a link between androgen action and flight muscle remodeling has not been reported in free-living birds. One caveat is that systemic androgen levels do not always reflect local androgen synthesis or action within tissues (Baulieu and Robel 1998; Schmidt et al. 2008; Pradhan et al. 2015), suggesting that tissue androgen metabolism and sensitivity are important. This is particularly relevant prior to spring migration in sparrows, when circulating androgen levels are low, relative to levels measured upon arrival on the breeding grounds (Wingfield and Farner 1978). Conceivably, local androgen synthesis or signaling within flight muscles prior to departure may promote remodeling in the face of low systemic androgen levels.

To test this idea, we measured circulating and muscle levels of T, as well as mRNA levels of AR, 5α-reductase and IGF-1 in the pectoralis muscle of male sparrows. We also examined the gastrocnemius muscle, a leg muscle of significant importance for terrestrial locomotion in birds (Marsh 1984; Biewener 2011; Velten et al. 2016). We sampled male sparrows across three distinct stages on wintering grounds as birds progressed from wintering condition through preparatory stages for pre-departure: (1) Winter (February): birds in flocks showing some fattening but no muscle hypertrophy, (2) Pre-nuptial molt (March): birds showing extensive molt on crown and body but no fattening or muscle hypertrophy, and (3) Pre-departure (April): birds completed pre-nuptial molt, showing body mass increase, fattening and muscle hypertrophy but no departure flight detected. We predicted that T levels and/or expression levels of genes that promote androgen action or muscle remodeling would be elevated

just prior to migration in the pectoralis but less so, if at all, in the gastrocnemius.

Materials and methods

Animals

We captured sparrows between 06:00–11:00 AM in seed-baited potter traps on their wintering grounds in Yolo County, California, (38°33′N, 121°44′W). Captures were made during three stages, winter (February 4–7; 6 first-year males settled in winter flocks); pre-nuptial molt (March 17, 18; 6 adult males showing body molt and replacing juvenile brown crown for adult black and white feathers); and spring pre-departure (April 10–13, 6 adult males with molt completed, increased body mass, fat scores and pectoralis muscle profiles). The dates for the pre-departure sampling were specifically chosen as they preceded recorded departure of radio-tracked sparrows from these sites by at least 1 week (Németh and Ramenofsky, in preparation). Through extensive fieldwork (2007–2015) we found that the three stages of collection (4–7 February, 17–18 March, and 10–13 April) are outside of the windows of observed migratory movements through our sites in spring.

Sample collection

Immediately after capture, measures of body mass, wing and tarsus length were taken and birds were visually assessed for fat score, and flight muscle profile, and stage of molt (Ramenofsky et al. 2017). Fat score was determined by observing the presence of lipid in the coelomic cavity and chorico-clavicular fossa with values that ranged from 0, no observable fat, to 5, bulging fat deposits (Ramenofsky et al. 2008). Both scores were summed for statistical analyses. Flight muscle size was assessed from visual scores of pectoralis muscle profile based on four classes ranging from 0 to 3. Specifically, a score of 0 presents a sharp protruding edge of the keel to the touch and an extreme concave muscle mass, typical of emaciated birds. Score of 1 assigned when bird still has a prominent edge of the keel but the muscle is beginning to enlarge slightly. For score 2, the muscle mass is convex in shape but the edge of the keel edge remains evident to the touch, and 3, the pectoralis rises above the keel that is now embedded within the bulging muscle (Velten et al. 2016). Pre-nuptial molt was scored on three body regions: crown, back (including nape, back and rump) and abdomen (including throat, breast, abdomen and flanks). Each region was given a score between 0 and 3 depending on the extent of molting feathers: 0—no molt; 1—light molt (1–15% area); 2—moderate molt (16–50% area) and 3—heavy molt (51–100% area). Scores of each region were summed to generate a total molt score for each sampling period (Ramenofsky and Németh 2014). For consistency, scoring of all observed measures were assessed and recorded by MR.

Within 5–7 min of capture, after all the live body measurements were completed, birds were sacrificed via rapid decapitation, and trunk blood was collected and kept at 4 °C until the plasma was separated. Immediately following blood collection, the brain, pectoralis and gastrocnemius muscles and testes were rapidly and completely dissected and mass was measured using an electronic balance to the nearest 0.001 g. Approximately 100 mg was dissected from the right pectoralis for steroid and molecular analyses. Testes were measured to the nearest 0.1 mm while recording the length and width at the widest point of each testis. All tissues were quickly flash frozen on dry ice. Testicular volume was later calculated using the formula of $4/3\ \pi\ a^2b$, where a is the radius of the testis at its widest point and b is half the long axis. Plasma samples, right gastrocnemius and the smaller portion of the right pectoralis were shipped on dry ice to the University of British Columbia for T measurement. All the remaining tissues were shipped to UCLA on dry ice for mRNA measurement.

Testosterone measurement

We extracted T from all samples (plasma, gastrocnemius muscle, pectoral muscle) using solid-phase extraction with C18 filled sorbent columns (Taves 2011; Bailey et al. 2013). For muscle tissues, samples were weighed (~ 100 mg) and homogenized in ice-cold water:methanol (1:5, v/v) (Fokidis et al. 2013; Bailey et al. 2013). We diluted a portion of the supernatant from each sample (corresponding to 8 mg of muscle) with 10 mL deionized water before loading onto a C18 column. For plasma samples, we diluted 10 µL with 10 mL deionized water. Before sample loading, we primed C18 columns with 3 mL HPLC-grade methanol and equilibrated with 10 mL deionized water. Then, to remove interfering substances, we washed the loaded columns with 10 mL 40% HPLC-grade methanol. Using 5 mL 90% HPLC-grade methanol, we eluted T and dried samples at 40 °C in a vacuum centrifuge.

We measured T with a modified double-antibody ^{125}I-Testosterone RIA (07189102; MP Biomedicals) (Maddison et al. 2012; Overk et al. 2013; Heimovics et al. 2016). Dried extracts were re-suspended with assay diluent containing 1% absolute ethanol. To calculate T recovery, we spiked aliquots of plasma or muscle samples with a known amount of T, and compared T amounts in spiked and un-spiked samples. Samples were corrected for recovery using the average recovery values.

Table 1 PCR primers utilized in this study

Gene	Primer direction	Sequence (5′–3′)	T_m (°C)
5α-reductase type 1	Forward	TTTCACTTTTGTGGTTAGCACTTC	61
	Reverse	TGGATAGTCTTCAAATTTCTCAA	
5α-reductase type 2	Forward	CCTTTCTTCACTAGAGGCAGACC	62
	Reverse	TGGATAGTCCGTAAATGTCTTGAG	
Androgen receptor	Forward	TGACGTGTGGGAGCTGCAAA	61
	Reverse	GGCCATCCACTAATAATAC	
Insulin-like growth factor-1	Forward	TCCTACATCCATTTCTTCTACCTTG	56
	Reverse	ACATTCATTCTTCATTCTTGTGGAT	

mRNA measurement

For measurement of mRNA, we homogenized all tissue samples for 30–50 s at medium speed using a rator/stator homogenizer (Dremel) (Fuxjager et al. 2016b). We then extracted total RNA from each sample, using 1 mL TRIzol® Reagent (Invitrogen, Carlsbad, CA), according to manufacturer's directions, and determined RNA concentrations and quality (260/280 range 1.85–2.02) using a Nanodrop system (Thermo Scientific). To prepare cDNA, we reverse transcribed RNA (1 µg for AR, 1.5 µg for 5α-reductase Type 1, 5α-reductase Type 2 and IGF-1) using Superscript III Reverse Transcriptase (Invitrogen). For all four genes (AR, 5α-reductase Type 1, 5α-reductase Type 2, and IGF-1) we used degenerate primers from zebra finches, *Taeniopygia guttata* to amplify the cDNA via PCR amplification (Table 1), to verify the presence of the genes in a subset of sparrow samples of testis, hypothalamus, gastrocnemius, and pectoralis muscle tissues. For verification, we ran the resultant PCR products on a gel to determine the size of the amplified fragment which was then sequenced (Genewiz Inc., La Jolla, CA, USA). Because the sparrow genome is not annotated, a BLAST for AR verified 98% similarity of the sequence against the closely related white-throated sparrow (*Zonotrichia albicollis*). For each, 5α-reductase Type 1 and 5α-reductase Type 2, the resultant sequences revealed a strong PCR signal for hypothalamus and testis. In muscle, there was a strong signal for 5α-reductase Type 1, but a very weak signal for 5α-reductase Type 2. The sequences were 78–84% similar to 5α-reductases in other species and Primer 3 was used to design more specific qPCR primers for sparrows (Table 2). For IGF-1, the sparrow amplified PCR product was 99% similar to *Z. albicollis* and 96% similar to zebra finch.

The detailed procedure for quantitative PCR (qPCR) used in this study has been explained in (Fuxjager et al. 2016b; Eaton et al. 2018; Rensel et al. 2018), which we used with modifications. All the qPCR reactions were performed using the SYBR Green Master Mix kit (Applied Biosystems, Inc., Foster City, CA) in the ABI 7300 sequence detection system. To determine the most optimal primer concentration for the qPCR reactions, we tested primer concentrations of 0.05 µM, 0.3 µM, and 18 µM on a subset of randomly selected individuals which were assayed in triplicate. We added pooled zebra finch testes cDNA as a positive control. For 5α-reductase Types 1 and 2, we randomly selected three individuals and used hypothalamus, pectoralis, and gastrocnemius samples (not pooled, 1:2 dilution) for these validations. Only 5α-reductase Type 1 was successfully amplified, with the greatest amplification seen in the hypothalamus, and lower levels in both the muscles. Given the absence of 5α-reductase Type 2 amplification, we conclude that this isoform is only expressed at low levels, if at all, in these skeletal muscles and no further discussion of this isoform will follow. For AR, we used pooled cDNA (1:10 dilution) from sparrow testis, gastrocnemius, and pectoralis muscle samples for validations. For IGF-1, we applied similar validation procedures to the pectoralis muscle (1:2 cDNA

Table 2 qPCR primers utilized in this study

Gene	Primer Direction	Sequence (5′–3′)
5α-reductase type 1	Forward	GAGGAATGTTTGAGTGTGTCAG
	Reverse	TTCAAATTCTCAAGGTACCA
Androgen receptor	Forward	ATGAGTACCGCATGCACAAA
	Reverse	AACTCCTGGGGTGTGATCTG
Insulin-like growth factor-1	Forward	AACCAGTTCTGTTGCTGCTG
	Reverse	AAAGCCTCTGTCTCCACACAC
Glyceraldehyde-3-phosphate dehydrogenase	Forward	TGACCTGCCGTCTGGAAAA
	Reverse	CCATCAGCAGCAGCCTTCA

dilution). During validation and primer optimization studies, we checked for reliability and repeatability in dissociation curves and CT values at each primer concentration and for each tissue type tested in triplicate. We used "reliable" to indicate that the variation within the values was small, such that the results were accurate, and "repeatable" to indicate that each time a measurement was taken, it had approximately the same value. We chose the 0.3 μM concentration to be most optimal for all the primers (Table 2).

We assayed real time qPCR reactions for each muscle type (gastrocnemius and pectoralis) on a separate plate and each individual in duplicate. We investigated one primer of interest (AR, 5α-reductase Type 1, or IGF-1) per reaction plate, along with an internal control reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 0.3 μM) for each sample. The standard curves for each plate were only used to verify primer efficiency and reproducibility compared to the validations and not used to interpolate unknown values. We generated standard curves using serially diluted cDNA from pooled tissues from each reach reaction plate. Given the low expression of 5α-reductase Type 1 in muscle, we also added hypothalamic cDNA to the pool, to ensure the sensitivity of the curve, such that the values fell within the dynamic range of the curve. All reaction efficiencies were between 90 and 110%, were reproducible across assays, and we visually inspected each dissociation curve to ensure the absence of contamination. We also confirmed that as in the validations, the efficiencies of the primers were consistent and the efficiencies between the target gene and reference gene were within ~5% of each other. For each qPCR assay, we generated CT values and determined relative expression against GAPDH; we calculated ∂CT using the following formula: $[1000 \times (2^{-[\text{CT gene of interest} - \text{CT GAPDH}]})]$. We verified that GAPDH mRNA expression did not differ among the sampling stages in this study.

Statistical analyses

All data were tested initially for normal distributions. Those with normal distributions showing homogeneity of variances were tested using Multivariate General Linear Models across the three stages. Post hoc analyses were conducted first by Fisher's least significant difference further assessed with a Bonferroni correction. Both molt and muscle profile scores failed to conform to normal distributions and were analyzed with Kruskal–Wallis followed by Mann–Whitney tests with Bonferroni corrections. Across winter, molt, and pre-departure, we used linear regression analysis to determine the relationship between morphological parameters (1) body mass and mass of muscles (2) body mass and fat score (3) body mass and visual muscle profile (4) between physiological parameters. For these analyses, we adjusted the α levels for the number of comparisons made for each independent variable (n) using the Bonferroni correction α/n; where $\alpha = 0.05$ and n = number of comparisons. We used one-way ANOVAs to compare differences in morphological characteristics, T levels, and mRNA expression of all three genes (5α-reductase Type 1, AR and IGF-1) in each muscle across the three life-history stages. We used Tukey's Multiple Comparison Test for post hoc analyses. In cases where the normality test or Bartlett's test for equal variances were not satisfied, we log transformed the data. We analyzed data using either SPSS Statistics Software (Version 22.0, IBM, Chicago) or GraphPad Prism 7.0 for Macintosh and data are represented as mean ± SEM.

Results

Changes in morphology across life-history stages

Importantly, pectoralis muscle profile was significantly greater at pre-departure than in winter or pre-nuptial molt ($\chi^2 = 9.18$, $p < 0.01$) (Table 3). Fat score and body mass were also greater at pre-departure ($F = 19.62$, $p < 0.0001$, $F = 4.36$, $p = 0.03$, respectively) (Table 3). As expected, molt was observed only in March ($F = 17.21$, $p = 0.00$), and testis volume was greatest at pre-departure ($F = 27.46$, $p < 0.0001$).

Body mass was significantly associated with fat score ($r^2 = 0.48$, $p = 0.0014$), pectoralis muscle profile ($r^2 = 0.50$, $p = 0.001$), and pectoralis muscle mass ($r^2 = 0.40$, $p = 0.005$), but not gastrocnemius muscle mass ($r^2 = 0.135$, $p = 0.134$). Additionally, fat score was positively correlated with pectoralis mass ($r^2 = 0.468$, $p = 0.043$), but not gastrocnemius mass ($r^2 = 0.272$, $p = 0.259$).

Systemic testosterone levels increased, but local testosterone levels did not change

Testosterone was detected in plasma of all birds throughout the stages, albeit at very low levels (< 0.1 ng/mL). Even at such low levels, however, there was a significant change in plasma T levels across stages (Fig. 1a; $F_{2,15} = 7.71$, $p = 0.005$), such that at pre-departure, plasma T was significantly elevated compared to winter ($q = 4.66$, $p < 0.05$) and pre-nuptial molt ($q = 4.95$, $p < 0.05$), but there was no difference between winter and pre-nuptial molt ($q = 0.29$, $p > 0.05$). In contrast, local T levels did not differ across seasons in either muscle (Fig. 1b, c; gastrocnemius, $F_{2,15} = 2.11$, $p = 0.156$; pectoralis, $F_{2,15} = 0.60$, $p = 0.56$). At all stages, plasma T levels were lower than local T levels in both muscles ($p < 0.05$).

Table 3 Changes in morphology (mean ± SEM) during different life-history stages in free-living male white-crowned sparrows ($n = 6$ at each stage)

Morphology	Winter	Pre-nuptial Molt	Pre-departure	F statistic or χ^2	p
Fat score	3.67 ± 0.49	3.5 ± 0.50	7.17 ± 0.98	19.62	< **0.0001**
Body mass (g)	27.40 ± 1.21	27.92 ± 0.47	31.42 ± 1.32	4.36	**0.03**
Wing length (mm)	75.80 ± 0.33	75.80 ± 0.30	76.70 ± 0.76	1.05	0.38
Pectoralis muscle profile	2.00 ± 0.00	2.30 ± 0.02	2.80 ± 0.20	9.18	**0.01**
Tarsus length (mm)	26.32 ± 0.43	26.03 ± 0.26	26.08 ± 0.28	0.21	0.81
Molt stage	0	5.67 ± 0.80	0	17.21	**0.000**
Gastrocnemius muscle mass (g)	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	2.09	0.156
Pectoralis muscle mass (g)	3.67 ± 0.28	3.74 ± 0.16	3.64 ± 0.11	0.23	0.801
Testis volume (mm^3)	0.81 ± 0.13	1.53 ± 0.25	3.60 ± 0.38	27.46	< **0.0001**

Underscore indicates significant difference between the stages

Bold font denotes statistical significance

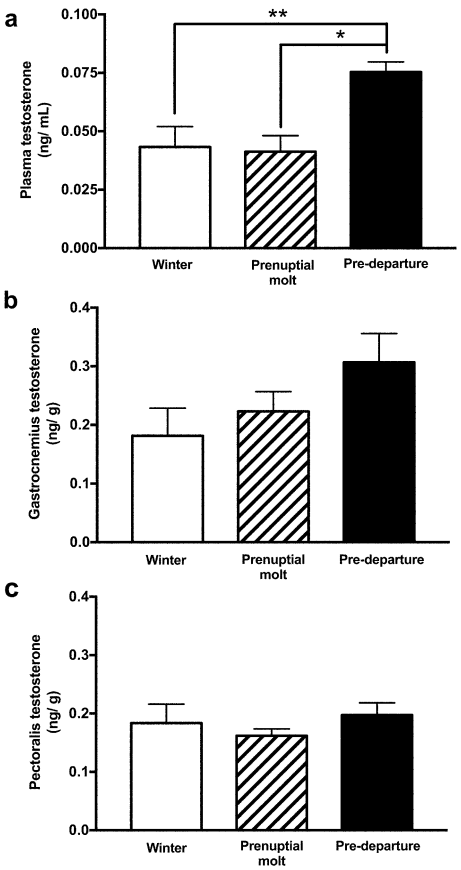

Fig. 1 Systemic and local levels of testosterone (T) in free-living male white-crowned sparrows sampled during life history stages leading up to pre-nuptial migration. **a** Plasma T was significantly elevated at pre-departure compared to both winter and pre-nuptial molt stages. There were no differences in T levels in (**b**) Gastrocnemius muscle or (**c**) Pectoralis muscle. $n = 6$ per stage, $^*p < 0.05$, $^{**}p < 0.01$

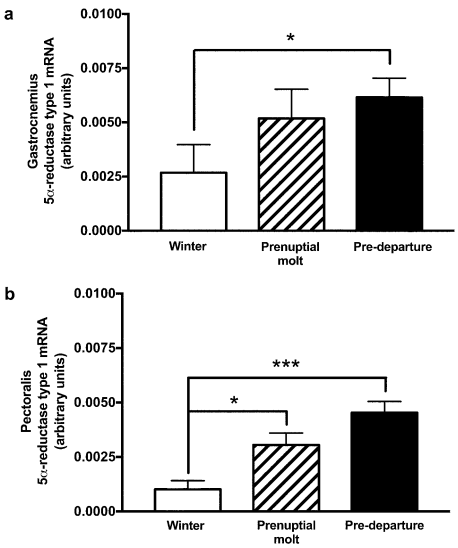

Fig. 2 Relative expression patterns of the enzyme 5α-reductase Type 1 mRNA in free-living male white-crowned sparrows sampled during life history stages leading up to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. **a** Gastrocnemius 5α-reductase Type 1 mRNA was elevated during pre-departure compared to winter and **b** Pectoralis 5α-reductase Type 1 mRNA in winter was lower than both pre-nuptial molt and pre-departure. $n = 6$ per stage, $^*p < 0.05$, $^{***}p < 0.001$

5α-reductase type 1 mRNA expression increased in both muscles

In the gastrocnemius muscle, expression of 5α-reductase Type 1 mRNA varied significantly with stage (Fig. 2a, $F_{2,15} = 5.60$, $p = 0.0153$). While the expression at pre-departure

was significantly elevated, ~2.4x compared to winter ($q = 4.47$, $p < 0.05$), it was not different from pre-nuptial molt ($q = 0.88$, $p > 0.05$). Although the pre-nuptial values approached those of pre-departure, there was no statistical difference with measurements of winter ($q = 3.59$, $p > 0.05$). In the pectoralis muscle, the mRNA expression of 5α-reductase Type 1 mRNA varied significantly by stage (Fig. 2b, $F_{2,15} = 12.91$, $p = 0.0005$) such that at pre-departure values exceeded those of winter ($q = 7.156$, $p < 0.001$), but not pre-nuptial molt ($q = 3.01$, $p > 0.05$). Expression of 5α-reductase Type 1 was also elevated at pre-nuptial molt over winter ($q = 4.15$, $p < 0.05$).

Androgen receptor mRNA expression increased in both muscles

In the gastrocnemius, measurements of mRNA expression of AR varied significantly with stage (Fig. 3a, $F_{2,15} = 6.426$, $p = 0.0096$). Expression, however, was similar between that of winter and pre-nuptial molt ($q = 2.36$, $p > 0.05$), but increased at pre-departure over those of winter ($q = 5.07$, $p < 0.01$). No changes were observed between pre-nuptial molt and pre-departure ($q = 2.71$, $p > 0.05$). In the pectoralis muscle, expression varied significantly with stage (Fig. 3b, $F_{2,15} = 28.20$, $p < 0.0001$). The pre-departure expression increased over that of winter ($q = 10.15$, $p < 0.001$) as well as pre-nuptial molt ($F_{2,15} = 7.776$, $p < 0.001$). There was no difference in AR mRNA expression between the winter and pre-nuptial molt stages ($q = 2.376$, $p > 0.05$).

Opposite expression patterns of insulin-like growth factor-1 mRNA in muscles

Measurements of mRNA expression of IGF-1 in both muscles (Fig. 4) varied significantly with stage in both gastrocnemius ($F_{2,15} = 10.48$, $p = 0.0014$) and pectoralis ($F_{2,15} = 3.82$, $p = 0.046$) muscles (Fig. 4). Interestingly, in the gastrocnemius, IGF-1 was not detected at pre-departure, and thus was significantly lower than either winter ($q = 5.57$, $p = 0.0035$) or pre-nuptial molt ($q = 5.65$, $p = 0.0032$). There was no difference in expression between winter and molt stages ($q = 0.07$, $p = 0.99$). By contrast, in the pectoralis, values of mRNA expression at pre-departure almost doubled compared to winter ($q = 3.87$, $p = 0.04$), but did not change from pre-nuptial molt stage ($q = 1.48$, $p = 0.56$). No significant change was detected between winter and pre-nuptial molt ($q = 2.39$, $p = 0.24$).

Correlation analyses across the morphological and molecular measurements

Plasma T levels were not associated with any of the variables tested (Table 4). Further, T levels in the gastrocnemius

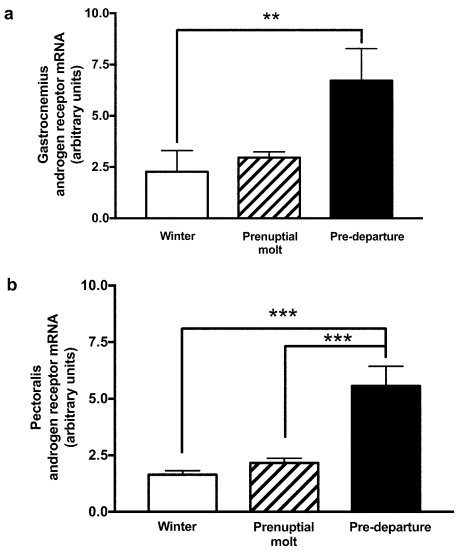

Fig. 3 Relative expression patterns of androgen receptor (AR) mRNA in free-living male white-crowned sparrows sampled during life history stages leading up to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. **a** Gastrocnemius AR mRNA was elevated during pre-departure compared to winter and **b** Pectoralis AR mRNA in pre-departure was higher than both winter and pre-nuptial molt. $n = 6$ per stage, $^{**}p < 0.01$, $^{***}p < 0.001$

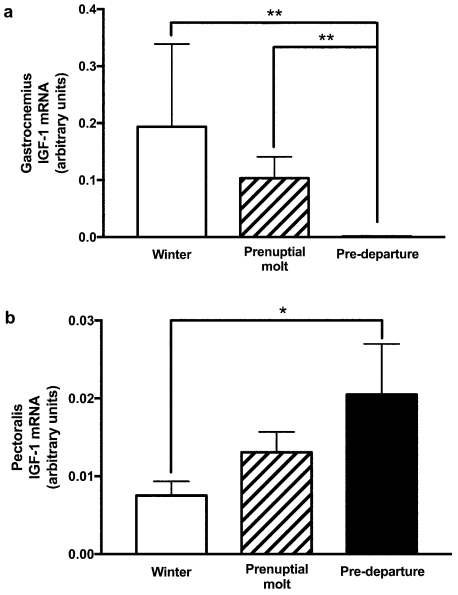

Fig. 4 Relative expression patterns of insulin-like growth factor-1 (IGF-1) mRNA in free-living male white-crowned sparrows sampled during life history stages leading up to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. **a** Gastrocnemius IGF-1 mRNA was reduced compared to both winter and pre-nuptial molt and **b** Pectoralis IGF-1 mRNA was elevated during pre-departure compared to winter. $n = 6$ per stage, $^{*}p < 0.05$

Table 4 Relationships between androgen signaling machinery measured in free-living adult male white-crowned sparrows captured during three life-history stages ($n = 18$)

Physiological relationships	r^2	$F_{1, 16}$	p
Plasma T versus			$\alpha = 0.005$
Gastrocnemius T	0.12	2.22	0.16
Gastrocnemius AR	0.22	4.5	0.04
Gastrocnemius 5α-reductase Type 1	0.18	3.42	0.08
Gastrocnemius IGF-1	0.006	0.1	0.76
Pectoralis T	< 0.01	0.10	0.76
Pectoralis AR	0.30	6.89	0.02
Pectoralis 5α-reductase Type 1	0.09	1.66	0.22
Pectoralis IGF-1	0.06	0.96	0.34
L. Testis volume	0.35	8.65	0.009
Gastrocnemius T versus			$\alpha = 0.017$
Gastrocnemius AR	0.37	9.38	**0.007**
Gastrocnemius 5α-reductase Type 1	0.04	0.72	0.41
Gastrocnemius IGF-1	0.08	0.08	0.73
Pectoralis T versus			$\alpha = 0.017$
Pectoralis AR	0.04	0.75	0.40
Pectoralis 5α-reductase Type 1	0.02	0.40	0.53
Pectoralis IGF-1	0.49	0.03	0.50
Gastrocnemius AR versus			$\alpha = 0.025$
Gastrocnemius 5α-reductase Type 1	0.30	6.91	**0.02**
Gastrocnemius IGF-1	1.42	0.08	0.25
Pectoralis AR versus			$\alpha = 0.025$
Pectoralis 5α-reductase Type 1	0.40	10.69	**0.005**
Pectoralis IGF-1	13.18	0.45	**0.002**
			$\alpha = 0.05$
Gastrocnemius 5α-reductase Type 1 versus IGF-1	0.71	0.04	0.41
Pectoralis 5α-reductase Type 1 versus IGF-1	4.34	0.21	**0.05**

Bonferroni correction applied and revised α value stated for each category of comparisons

Bold font denotes statistical significance

muscle were correlated with AR mRNA expression, but not with 5α-reductase Type 1 mRNA expression. By contrast, T levels in the pectoralis muscle were not related to AR or 5α-reductase Type 1 mRNA expression. In both muscles, AR and 5α-reductase Type 1 expression levels were positively correlated. Finally, only in the pectoralis, both AR and 5α-reductase Type 1 were positively correlated with IGF-1 mRNA expression.

Discussion

Across many species, specific skeletal muscles undergo pre-migratory modifications for enhanced contractile strength, lipid deposition, oxidative capacity, and thermogenesis (Fry et al. 1972; Dietz et al. 1999; Vézina et al. 2007; Price et al. 2011). Our results for a long-distance migrant, white-crowned sparrow, reveal multiple changes in gene expression of skeletal muscles as they prepare for spring migration. Increased expression of AR and 5α-reductase, together with small but significant increases in circulating T, are possible signs that androgens contribute to some of the muscle remodeling in preparation for migration. Moreover, divergent expression patterns of IGF-1 in the pectoralis and gastrocnemius suggest an important role for this androgen-sensitive myokine in muscle remodeling. The relationship between androgen-signaling and IGF-1 remains to be elucidated. Nevertheless, these data highlight molecular pathways that have not previously been well-explored and may be instrumental for preparing the pectoralis muscle for long-distance flight.

As has been described previously (Marsh 1984; Ramenofsky 1990; Boswell et al. 1995; Bauchinger et al. 2005; Vézina et al. 2007), several of our anatomical and physiological measures confirm that the birds under study were undergoing preparation for migration. They completed pre-nuptial molt while also increasing fat deposition, body mass and enlargement of flight muscle profiles. Interestingly, circulating levels of T were slightly elevated at pre-departure, relative to winter and molt, and

testis volume increased across the stages, indications of the onset of testicular recrudescence (Blanchard 1941). Although plasma titers of T were significantly increased pre-departure, the levels in male sparrows upon arrival at high latitude breeding sites are at least 40 times greater (Wingfield and Farner 1978). This raises the question as to whether the modest elevation in circulating T is biologically active. We measured expression levels of a gene important for muscle growth, IGF-1, which can be considered as one index of androgen action that can influence androgen-dependent regulation in avian skeletal muscle (Fuxjager et al. 2012) and perhaps other species (Grinspoon et al. 1996; Chambon et al. 2010). Whereas expression of this gene increased in the pectoralis, a possible sign that androgens were indeed providing transcriptional regulation, we found that IGF-1 mRNA decreased in the gastrocnemius despite the gastrocnemius also showing increased expression of AR and 5α-reductase. Thus, it is possible that (a) androgens impact IGF-1 expression in opposing ways in different muscles, (b) androgens acted solely on the pectoralis and not on the gastrocnemius, or (c) IGF-1 is regulated independently of androgens in both muscles. Studies of AR-dependent action in two muscles of manakins and zebra finches provide evidence for striking differences in androgen modulation of skeletal muscle gene expression (Fuxjager et al. 2016a, b) suggesting that IGF-1 might experience different transcriptional regulation between the pectoralis and gastrocnemius. More work is needed to resolve this interesting set of observations and relevant questions. Nevertheless, our results do not exclude the possibility that low levels of circulating androgens were functional in these muscles.

Tissues can influence the efficacy of a circulating hormone by locally converting that hormone into more potent metabolites and/or by increasing their expression of hormone receptor concentrations. This has been observed several times across seasons and life-history stages of several wild birds and mammals (Soma et al. 1999, 2003; Canoine et al. 2007; Fraley et al. 2010; Pradhan et al. 2010; Boonstra et al. 2014). Notably, we detected expression of both AR and 5α-reductase in both the pectoralis and gastrocnemius muscles, pointing to these skeletal muscles in sparrows as targets of androgen action. Moreover, expression levels of both AR and 5α-reductase increased in both muscles during pre-departure phases, evidence that the conversion of T into 5α-DHT and subsequent binding to AR are important events in these muscles prior to migration. There is increasing evidence for androgenic control of skeletal muscle phenotypes (Kadi 2008; Carson and Manolagas 2015) including in birds (Fuxjager et al. 2012, 2016a, 2017) and these results suggest adding preparation for migration to that expanding list.

Although plasma levels of T changed across these winter and pre-migratory phases, local levels of T in muscle remained constant. A likely reason for this discrepancy is that cells metabolized T into active or inactive metabolites. We provide evidence here for 5α-reductase in these skeletal muscles, but birds also express the androgen inactivating enzyme 5β-reductase in most tissues (Schlinger and Brenowitz 2017). Thus, these enzymes may very well alter local T concentrations in skeletal muscle such that they differ from that found in the circulation.

In light of our findings the question remains as to mechanisms regulating muscle hypertrophy. As proposed previously, the possible and non-mutually exclusive candidates are the photoperiodic induction of endocrine secretion associated with hyperphagia and fattening and/or the more functional roles of increased mass and exercise. Given our results showing increased IGF-1 expression in the pectoralis initiated before onset of intense activity of migratory flight offers support for previous observations of photostimulated white-throated sparrows expressing *Zugunruhe* and repeatedly exercised starlings (Price et al. 2011). Although it is unclear why no seasonal alterations of IGF-1 expression were found during winter, spring or autumn migration in free-living white-throated sparrows, such results emphasize the need for further investigations comparing both free-living and captive birds under various conditions of flight.

Conclusion

This study reports a seasonal increase in systemic and local skeletal muscle androgen signaling in sparrows as they prepare for migration. Our data suggest that the local up-regulation of androgenic machinery in flight muscle, but not the gastrocnemius muscles of the leg, may contribute to increased endurance and power for flight once birds depart for spring migration. The myotrophic factor IGF-1, whether regulated by androgens or not, might also exert a functional role. It is important to note that flight muscle hypertrophy in white-crowned sparrows also occurs prior to autumn departure from the breeding grounds at a time when circulating levels of androgen appear to be basal (Ramenofsky and Németh 2014). Moreover, female white crowned sparrows also migrate and undergo similar morphological migratory preparations. Females have been shown to secrete low levels of T in spring (Schwabl and Farner 1989) that may promote flight muscle hypertrophy without stimulating other androgenic traits. In autumn, it is possible that both sexes secrete very low levels of gonadal or adrenal T and show enhanced androgenic and IGF-1 signaling in flight muscles, which collectively prepare individuals to migrate. However, to date little is known of the physiological mechanisms regulating autumn migration. Nevertheless, our findings provide

a blueprint for further studies into the role of androgen signaling in skeletal muscle of migratory birds.

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Compliance with ethical standards

Conflict of interest Authors have no competing or conflict of interests.

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