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Phenotypic flexibility of glucocorticoid signaling in skeletal muscles of a songbird preparing to migrate

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ABSTRACT

Glucocorticoids are commonly associated with responses to stress, but other important functions include homeostatic regulation, energy metabolism and tissue remodeling. At low circulating levels, glucocorticoids bind to high-affinity mineralocorticoid receptors (MR) to activate tissue repair and homeostasis (anabolic pathways), whereas at elevated levels, glucocorticoids bind to glucocorticoid receptors (GR) to activate catabolic pathways. Long distance migrations, such as those performed by Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), require modification of anatomy, physiology and behavior. Plasma corticosterone (CORT) increases in association with impending departure and flight and may promote muscle-specific anabolic states. To test this idea, we explored glucocorticoid signaling in the pectoralis (flight) and gastrocnemius (leg) muscles of male sparrows on the wintering grounds at three stages leading up to spring departure: winter (February), pre-nuptial molt (March), and pre-departure (April). CORT was detected in plasma and in both muscles, but measures of CORT signaling differed across muscles and stages. Expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) Type 2 (inactivates CORT) increased in the pectoralis at pre-departure, whereas 11β-HSD Type 1 (regenerates CORT) did not change. Neither of the two 11β-HSD isoforms was detectable in the gastrocnemius. Expression of MR, but not GR, was elevated in the pectoralis at pre-departure, while only GR expression was elevated at pre-nuptial molt in gastrocnemius. These data suggest that anabolic functions predominate in the pectoralis only while catabolic activity is undetected in either muscle at pre-departure.

1. Introduction

Hormonal control of behavior requires activation of motivational and motor systems in the central nervous system and the creation of appropriate physiological states, especially of striated muscle, to enable behavior (Schlinger et al., 2018). Migration, the seasonal movement of organisms, is widespread across taxa and allows individuals to exploit seasonal peaks in resources (Alerstam et al., 2003; Dingle 2014). For many birds, seasonal migrations occur in spring and autumn, accomplishing passage to and from breeding grounds (Cornelius et al., 2013; Newton, 2008). Birds prepare for migratory flight well before the actual departure (Price et al., 2010; Ramenofsky and Wingfield, 2007). For migrants, this means considerable phenotypic flexibility in physiology, behavior and morphology for fuel storage, enhanced power, and endurance (Jenni and Jenni-Eiermann, 1998; Price et al., 2011).

Preparations for migration include hyperphagia, increased body weight, and fat deposition (Dolnik and Blyumental, 1967; Driedzic et al., 1993; King and Farner, 1963; Ramenofsky, 1990; Price et al., 2010). In addition, flight muscles show hypertrophy, changes in muscle fiber size and composition, and greater lipid and glycogen stores (Driedzic et al., 1993; Fry et al., 1972; Gaunt et al., 1990; Marsh, 1984; Price et al., 2011; Velten et al., 2016). Specifically in *Zonotrichia leucophrys gambelii* (hereafter, sparrows) levels of several anabolic signaling molecules (testosterone, 5α-reductase, and androgen

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receptor) increase at pre-departure in both pectoralis and gastrocnemius, while levels of insulin-like growth factor, an androgen-dependent gene, increases only in the pectoralis (Pradhan et al., 2019). This raises the question of whether other signaling molecules such as those involved in glucocorticoid (GC) signaling also exhibit phenotypic flexibility at pre-departure, given that once migratory flight commences, catabolic processes regulated by GCs provide the fuels required to sustain movement (Bauchinger and Biebach, 2001; Jenni and Jenni-Eiermann, 1998; Landys et al., 2004; Salewski et al., 2009).

Glucocorticoids facilitate transitions between life history stages (Ramenofsky and Wingfield, 2017; Wada, 2008; Crespi et al. 2013) and may be integral in promoting the metabolic functions within organs, namely skeletal muscles required for avian migratory flight. Baseline GC secretion functions homeostatically to maintain energy balance throughout predictable life history stages (Orchinik, 1998; Landys et al., 2006). More pronounced GC secretion occurs acutely in response to unpredictable or stress-induced stimuli (Sapolsky, 2000; Romero and Wingfield, 2015). At baseline concentrations, GCs bind to the high-affinity mineralocorticoid receptor (MR) to influence, in general, osmoregulatory and metabolic functions; whereas at higher (stress-induced) concentrations, GCs binds to the lower affinity glucocorticoid receptor (GR) to promote metabolic mechanisms to cope with heightened energy demand (Breuner and Orchinik, 2001; Landys et al., 2004; Seckl and Chapman, 1997). The abundance and transcriptional actions of these receptors vary greatly across target tissues (Landys et al., 2006; Lattin et al., 2012; Romero and Wingfield, 2015), and as a result, adrenal GC secretion can impact tissues in highly divergent ways and might be instrumental in the transition from winter residency to migratory flight.

The breadth of tissue-specific corticosterone (CORT) effects on tissues and downstream metabolic pathways are also influenced by CORT metabolism within tissues (Krause et al., 2015; Rensel and Schlinger, 2016; Schmidt et al., 2008; Schmidt and Soma, 2008). Local expression of 11β-hydroxysteroid dehydrogenases (11β-HSD) regulate CORT availability to bind receptors by locally metabolizing or regenerating CORT (Wyrwoll et al., 2011; Yang and Yu, 1994). The enzyme 11β-HSD Type 1 increases local CORT levels via regeneration of 11-dehydrocorticosterone to CORT (Taves et al., 2015). In contrast, 11β-HSD Type 2 metabolizes CORT to 11-dehydrocorticosterone, which binds poorly to MR and GR (Edwards et al., 1988; Funder et al., 1988; Krause et al., 2015; Rensel et al., 2018). Thus, tissue-specific responses to CORT are regulated by the concentration of CORT in blood and expressed levels of CORT metabolic enzymes and receptors.

In this study we examined the role of GC signaling molecules in preparing skeletal muscles for migration because the mechanisms involved in this crucial life history stage are not fully understood. In dark-eyed juncos (*Junco hyemalis*) and mammals, administration of GCs or aldosterone, a GR and MR agonist, promotes muscle atrophy (Gray et al. 1990; Baehr et al., 2011; Chadwick et al., 2015), protein metabolism (Chadwick et al., 2015; Kuo et al., 2013), as well as glucose uptake and utilization (Kuo et al., 2013). Corticosterone signaling is also regulated in muscle by environmental inputs; for example, in house sparrows (*Passer domesticus*) exposed to chronic stress, GR and MR mRNA levels increase in the pectoralis, but not gastrocnemius. These data suggest that CORT has distinct influence on individual muscles, likely promoting appropriate physiological responses to environmental demands (Lattin and Romero, 2014). Whether such changes in CORT signaling occur in skeletal muscle prior to migration is unknown. Thus, we hypothesize that flight and non-flight skeletal muscles in birds may be targets of GCs as they transition from winter to preparation for spring departure and migratory flight.

As a test, we sampled male sparrows across three distinct stages as they progressed from wintering condition through preparation for spring departure, 1) winter (February) with birds in flocks showing some fattening but no muscle hypertrophy, 2) pre-nuptial molt (March) with birds showing extensive molt on crown and body but no fattening or muscle hypertrophy, and 3) pre-departure (April) with birds having completed pre-nuptial molt, showing increased body mass, fattening and muscle hypertrophy, but departure flights not detected. To determine systemic and local GC levels, we first measured plasma and tissue CORT levels. Second, to examine the potential for the local regulation of CORT levels, we measured levels of 11β-HSD Types 1 and 2 mRNA. Third, to examine the downstream pathway through which CORT might influence muscle composition and/or function, we measured mRNA levels of GR and MR. We examined two muscles, the forelimb pectoralis, the primary flight muscle, and the hindlimb gastrocnemius, a muscle crucial for terrestrial locomotion. If CORT activates anabolic pathways that promote muscle remodeling prior to onset of migratory flight, we predict that CORT titers and/or mRNA levels of genes including MR, l11β-HSD Type 1 that promote GC signaling would be elevated in the pectoralis but not the gastrocnemius, during the pre-departure stage. Alternatively, if CORT promotes catabolic pathways, CORT and/or mRNA levels of genes including GR might be decreased in the pectoralis but increased in the gastrocnemius during pre-departure as birds anticipate onset of flight when terrestrial movements will be limited.

2. Methods

2.1. Subjects

Timing and sampling procedures for the 18 birds used in this study are described in (Pradhan et al., 2019). Specifically, birds were captured between 06:00–11:00 AM in seed-baited potter traps on their wintering grounds in Yolo County, California (33°33′N, 121°44′W). Captures were made during three stages in 2015, winter (February 4–7; 1 adult male and 5 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 pre-departure (April 10–13, 6 adult males with molt completed, increased body weight, fat scores, and a visual score of pectoralis size, all features observed in birds at the stage of preparation for departure). During the winter stage, relatively few adults visited the traps in a timely manner thus we resorted to catching first year birds. However, by prenuptial molt all birds had developed adult plumage (black crowns) and were categorized as adults. Given that all birds migrate and on a similar schedule, it is likely that comparable mechanisms regulate the phenotypic changes in first year and adult birds. The dates for the pre-departure sampling period were specifically chosen as they preceded the departure date of the majority of radio-tracked sparrows from our local sites by at least one week (Lisovski et al., 2019; Pradhan et al., 2019). All applicable international, national, and University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol #17144) guidelines were followed and conducted under the scientific permits of M. Ramenofsky issued by the California Department of Fish and Wildlife (#11024) and US Fish and Wildlife (# MB11826A-5).

2.2. Sample collection and morphometric measurements

Immediately after capture, morphological measurements were performed to verify life history stages (methods and data reported in Pradhan et al., 2019). First, wing length (mm) was measured to identify males using the flattened wing cutoff of >74 mm (Fugle and Rothstein, 1985). Next, body mass, fat score, muscle profile and condition of molt were assessed to confirm life history stage (Pradhan et

al., 2019). Birds were then anesthetized with 8–10% Isoflurane (Baxter Laboratories, Ill.) and euthanized via rapid decapitation. Trunk blood was collected (within 5–8 min) and kept at 4 °C until centrifugation to separate plasma from cells. Next, entire left and right pectoralis and gastrocnemius muscles were removed and weighed to the nearest 0.01 g on an Entris Analytical Balance, 120 g (Sartoirus Laboratory Equipment, NY). The mid-belly of the right pectoralis was subdivided into two samples; a small portion (100 mg) from a more anterior area and a larger posterior sample, were collected, weighed, and then flash frozen on dry ice. Along with plasma samples, we shipped the smaller portion of the right pectoralis and the entire right gastrocnemius to University of British Columbia for determinations of plasma and muscle CORT via radioimmunoassay. We also immediately flash froze the larger portion of the right pectoralis, entire left gastrocnemius from all individuals, and whole brain, liver, kidney, and testes from a subset of three individuals that were then sent to UCLA on dry ice.

2.3. Corticosterone measurement

We extracted CORT from all samples (plasma, gastrocnemius muscle, pectoral muscle) using solid-phase extraction with C18-filled sorbent columns (Bailey et al., 2013; Newman et al., 2008; Taves et al., 2011) as part of a previous study (Pradhan et al., 2019). Approximately 70–100 mg of pectoral muscle was cut and weighed and the entire sample of gastrocnemius (~70 mg) was homogenized in ice-cold water:methanol (1:5 v/v) as reported in (Bailey et al., 2013; Eaton et al., 2018; Fokidis et al., 2013; Pradhan et al., 2019). After homogenization, samples were centrifuged, supernatant was collected, and all protein eliminated. After using a fraction of the supernatant, the rest of the supernatant was stored at −20 °C. After a period of 8 months, we took a fraction of the supernatant from each muscle sample (corresponding to 10 mg of muscle) and added 10 mL deionized water before loading onto a C18 column. For serum samples, we diluted 5 μL with 10 mL deionized water. Before sample loading, we primed C18 columns with 3 mL HPLC-grade methanol and equilibrated columns with 10 mL deionized water. After loading samples, we removed interfering substances, by washing columns with 10 mL 40% HPLC-grade methanol. CORT was eluted with 5 mL 90% HPLC-grade methanol and all samples were dried in a vacuum centrifuge at 60 °C (ThermoElectron SPD111V) and stored at −20 °C until assayed.

Dried extracts were reconstituted in steroid diluent containing 5% absolute ethanol and measured CORT with a Corticosterone Double Antibody Radioimmunoassay kit (MP Biomedicals, cat. 07120103). We analyzed the samples in duplicate following the instructions of the manufacturer, with slight modifications in the protocol to increase assay sensitivity as described previously (Newman et al., 2008; Taves et al., 2010). We analyzed recovery samples in parallel, during the solid-phase extraction process and corrected all values for recovery (CORT in plasma, 93.6%; CORT in muscle 96.2%) (Fokidis et al., 2013). The detection limit for the assay was 1.5 pg/tube.

2.4. mRNA measurement

We measured mRNA as (Eaton et al., 2018; Fuxjager et al., 2016; Pradhan et al., 2019), with slight modifications. Each sample of muscle tissue was cut on dry ice into an approximately 10 mg subsample from a random part of the muscle and immediately transferred into 1 mL TRIzol® Reagent (Invitrogen, Carlsbad, CA) at 4 °C. Briefly, we homogenized all tissue samples for 30–50 s at medium speed using a rator/stator homogenizer (Dremel). We extracted total RNA from each sample, according to manufacturer's directions, and RNA concentrations and quality were determined (260/280 range 1.85 to 2.02) using a Nanodrop system (Thermo Scientific). To prepare cDNA, we reverse transcribed 1.5 μg RNA using Superscript III Reverse Transcriptase (Invitrogen). For 11β-HSD Type 1, and 11β-HSD Type 2, we used degenerate primers from zebra finches (*Taeniopygia guttata*) to amplify the cDNA via PCR amplification (Table 1). For 11β-HSD Type 1, we used liver and pectoralis from a subset of sparrows, whereas for 11β-HSD Type 2, we used kidney and pectoralis from a subset of sparrows. The final PCR reaction volume was 21 μL and was initiated at 95 °C for 5 min, with mixing 17.6 μL sterile water, 2.5 μL 10× KCl buffer (0.04 μM final concentration), 0.25 μL of each forward and reverse primer (1 μM final concentration) and 0.375 μL dNTPs (0.18 μM final concentration) to 100 ng cDNA sample mix from the different tissues. Next, we added 2.9 μL sterile water and 0.3 μL DNA Taq Polymerase (Invitrogen; 0.07 U/μL final concentration) to each sample and continued the following thermocycle program 37 times: 60 s at 72 °C, 30 s at 95 °C, and 45 s at a temperature gradient of 56–64 °C, and ended with 10 min at 72 °C. We verified bands by running the PCR products through gel electrophoresis and compared them to a ladder. The samples that yielded a band at the expected bp length (11β-HSD Type 1, T_m 1 = 62 °C; bp = 200; and 11β-HSD Type 2, T_m = 58.6 °C; bp = 500) were sequenced (Genewiz Inc., La Jolla, CA, USA). The white-crowned sparrow genome is not annotated, however both 11β-HSD Types 1 and 2 were 99% similar to the closely related white-throated sparrow (*Zonotrichia albicollis*). For each gene, we then designed specific qPCR primers using Primer3 Plus.

To verify candidate primers and to determine optimal primer concentrations, we used three different primer concentrations (0.05 μM, 0.3 μM, and 18 μM) on a subset of randomly selected individuals from the study and assayed in triplicate. We used a pool of gastrocnemius and pectoralis tissues for GR (1:10 dilution) and MR (1:5 dilution). We used liver and pectoralis for 11β-HSD Type 1 and kidney and pectoralis for 11β-HSD Type 2 at three different dilutions from three sparrows: 1:2, 1:5, and 1:10 dilutions. For liver and kidney, we used 1:10 dilutions. For all four genes, primers (Table 1) generated reliable and repeatable dissociation curves and C_t values at a concentration of 0.3 μM, but at different cDNA dilutions (GR, 1:10; MR and 11β-HSD Type 2, 1:5; 11β-HSD Type 1, 1:2).

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 (GR, MR, 11β-HSD Type 1, or 11β-HSD Type 2) per reaction plate, along with an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 0.3 μM) for each sample (Table 1). Using serially diluted cDNA from pooled tissues from each reach reaction plate, we generated a standard curve. To ensure the sensitivity of the curve for 11β-HSD Type 1, we included in the pool additional liver cDNA and for 11β-HSD Type 2, we included kidney cDNA, both known to express these respective genes, to ensure the sensitivity of the curve. All reaction efficiencies were between 90 and 110% with no evidence for contamination in the melt curves, for example a single curve and absence of primer dimers. For each qPCR assay, we generated C_t values and determined relative levels against GAPDH; we calculated ∂C_t using the following formula: $[1000 \times (2^{-[C_t \text{ gene of interest} - C_t \text{ GAPDH}]})]$. We verified that levels of GAPDH mRNA did not differ among the sampling stages in this study.

Table 1
Primers utilized in this study.

	Gene	Primer Direction	Sequence (5′ – 3′)
PCR primers	11β-hydroxysteroid dehydrogenase Type 1	Forward	AGACCAACTTCGTCAGCTACGTG
		Reverse	GTTGACCTTGTCTATGATGATTTCC
	11β-hydroxysteroid dehydrogenase Type 2	Forward	CACTTGGACATTATGGGCTTTCG
		Reverse	CATGTTGTCCCTGTTTTCTAGTAGC
qPCR primers	Glucocorticoid receptor	Forward	TGCAGTACTCCTGGATGTTCC
		Reverse	GAGCATGTGTTTGCATTGTTC
	Mineralocorticoid receptor	Forward	AAGAGTCGGCCAAACATCCTTGTTCT
		Reverse	AAGAAACGGGTGGTCCTAAAATCCCAG
	11β-hydroxysteroid dehydrogenase Type 1	Forward	ATCCATAGCGCGGGTAAAATTGC
		Reverse	GTGTTGATGTAGCCCAGGATG
	11β-hydroxysteroid dehydrogenase Type 2	Forward	AACTCACACAGGAACCACACC
		Reverse	GGAGGAAGGATCAATGGAAGAG
	Glyceraldehyde-3-phosphate dehydrogenase	Forward	TGACCTGCCGTCTGGAAAA
		Reverse	CCATCAGCAGCAGCCTTCA

2.5. Statistical analyses

2.5.1. Body condition

We used IBM SPSS 26 for Macintosh for the body condition analysis and SAS (v 9.3) for life history and model selection analyses. As birds prepare for spring departure, body mass and pectoral muscle mass as well as fat scores increase (see Pradhan et al., 2019) that, in turn, may contribute to variation of gene mRNA expression. To assess body condition for each bird at each stage, a Principal Components Analysis (PCA) of morphometrics including $\log_{10}$ transformed measures of body mass, fat score, and pectoralis muscle mass was conducted to collapse the data into one variable for body condition. Component 1 explained 70.6% of the variation using the three variables with each loading over 79.8% or greater. Comparison of PCA eigenvalues (body condition) across the 3 stages (winter, pre-nuptial molt, and pre-departure) was analyzed using general linear models (GLM) followed by Fischer's least squares difference tests (LSD) for post hoc analyses. For all cases where the normal distributions or Bartlett's Tests for equal variances were not satisfied, we $\log_{10}$-transformed the variables. Partial eta squared is indicated by ηp^2.

2.5.2. mRNA variation

One-way ANOVA was used to compare differences in CORT levels and mRNA of all four genes (GR, MR, 11β-HSD Type 1, and11β-HSD Type 2) in each muscle across the three life-history stages followed by Tukey's Multiple Comparison Test for *post-hoc* analyses (PROC MIXED). We ln-transformed all of the variables to improve the distribution of the data. For all pair-wise comparisons, Cohen's d effect size values (hereafter, d) were calculated using $\text{mean}_{\text{stage1}} - \text{mean}_{\text{stage2}} / ((\text{std}_{\text{stage1}} + \text{std}_{\text{stage2}})/2)$ of ln-transformed values (PROC MEANS). All data are represented as back-transformed estimates and their 95% confidence intervals. Because many of these variables are interrelated, we also explored differences in the relationships of all CORT and gene expression variables across our three life-history stages using a Discriminant Function Analysis (R v. 3.5.3, packages: ggord, klaR, MASS). Here we included ln-transformed values of all CORT and gene expression variables to improve distributions and ensure a similar scale across all variables. We also conducted a MANOVA to show if separation between the stages was statistically different (R packages: MVN, goftest, nlme).

2.5.3. General linear models, model selection, and averaging

We conducted multiple linear regression, model selection, and averaging in PROC GLMSELECT (SAS v. 9.3) comparing plasma CORT (*y*, or response variable) to pectoralis levels of CORT and gene expression (GR, MR, 11β-HSD Type 1, and 11β-HSD Type 2) (*x*, or explanatory variables). We also conducted model selection on pectoralis CORT and compared it to pectoralis mRNA of GR, MR, 11β-HSD Type 1, and 11β-HSD Type 2. Model selection and model averaging allowed us to simplify the interpretation of the top regression models using the bootstrapped model with the highest frequency score (relativized by the number of bootstraps) to calculate model weight (π_i) (ModelAverage, selection = stepwise (AICc), nsample = 10,000 iterations, Subset (best = 1) (Burnham and Anderson, 2002; Cohen, 2006). The top model represents the set of explanatory variables that best fit the response variable and has the highest model weight. We then conducted multiple linear regressions on all of the final models (PROC GLM), which we present here. We also ran a series of linear regressions to assess how pectoralis or gastrocnemius gene expression co-varied. Specif-

ically, we compared 1) pectoralis MR to GR, 2) gastrocnemius CORT to gene expression of MR and GR, and 3) gastrocnemius MR to GR.

We ensured that we met model assumptions (normal distribution and equal variance) for each analysis. We also determined that the variables were not highly correlated in order to avoid inflating model variance (i.e., variance inflation factor, VIF ≥ 10; Pearson $r \geq 0.7$). To approximate normal distributions and to improve the fit of the models, we transformed all continuous variables to their natural logs (ln) with the exception of pectoralis and gastrocnemius MR which did not require transformation. We present back-transformed parameter estimates and 95% confidence intervals (CI) in the results. Because many of these variables were ln- and then back-transformed (hereafter, BT), they cannot be interpreted in their original units but instead should be interpreted as a fold-change.

To avoid the potential of statistically redundant variables inflating the model variance due to a high correlation between plasma CORT and pectoralis MR (Pearson $r = 0.75$, Spearman $r = 0.68$) or gastrocnemius GR and MR (Pearson $r = 0.61$, Spearman $r = 0.72$), we compared body condition to pectoralis and gastrocnemius mRNA expression that included the genes encoding GR, MR and receptors for 11β-HSD Types 1 and 2 in pectoralis. We also included plasma CORT in the models.

3. Results

3.1. Differences in body condition across stages

The body condition of sparrows based on the loadings from the 1st PCA axis of mass, fat score and pectoralis mass differed significantly across the three stages ($F_{2,17} = 3.9$, $p = 0.043$; $\eta p^2 = 0.343$) with pre-departure values exceeding those of either winter or pre-nuptial molt respectively ($p = 0.025$, $d = 0.381$; $p = 0.032$, $d = 0.674$). No differences existed between winter and pre-nuptial molt stages ($p = 0.991$, $d = 0.06$).

3.2. CORT significantly increased at pre-departure

We measured CORT levels in trunk plasma and the two skeletal muscle tissues during the three stages prior to spring departure (Fig. 1). Given that capture and handling times were >3 min across all birds (see Methods), the values are not considered baseline, but a response to capture stress (Lynn and Porter, 2008; Romero and Reed, 2005; Wingfield et al., 1994). Capture and handling times were recorded for each individual across all stages: winter (5.28 ± 0.32 min, mean ± SEM; range, 4–6 min), pre-nuptial molt (6.94 ± 0.30 min, 6–8 min), and pre-departure (7.42 ± 0.29 min, 6–8 min). Because plasma CORT levels are known to increase with handling duration, we tested whether there was a relationship between the time required to obtain a blood sample, circulating plasma CORT and difference across the stages. We found that neither plasma CORT, after accounting for stage that the effect of minutes to capture and the interaction between these two variables was not significant (stage: $F_{2,12} = 0.26$, $p = 0.777$; mins to capture: $F_{2,12} = 0.82$, $p = 0.382$; stage x mins to capture $F_{2,12} = 0.43$, $p = 0.662$). Because the relationship between CORT and handling duration by stage was not significant, we did not consider it a significant source of variation within the models; thus, we focus our further analyses on stage and exclude capture-handling time from the models. CORT levels significantly increased across the life history stages in plasma ($F_{2,15} = 4.80$, $p = 0.025$, $\eta p^2 = 0.35$) and in pectoralis muscle ($F_{2,15} = 5.00$, $p = 0.022$, $\eta p^2 = 0.36$), but not in gastrocnemius muscle ($F_{2,15} = 2.9$, $p = 0.086$, $\eta p^2 = 0.24$) (Fig. 1). At pre-departure, plasma CORT levels were significantly higher compared to Winter (lsmeans difference BT est. = 1.68; BT 95% CI: 1.02–2.75, $p = 0.040$, Cohen's $d = 1.38$) and pre-nuptial molt (1.65, CI: 1.01–2.71, $p = 0.046$, $d = 1.53$), but there was no difference in plasma CORT between winter and pre-nuptial molt stages (0.99, CI = 0.60–1.62, $p = 0.99$, $d = -0.04$). CORT levels in the pectoralis almost doubled at pre-departure compared to winter (1.71, CI = 1.06–2.68; $p = 0.011$, $d = 1.69$), but the pre-nuptial molt stage was not different from winter (1.59, CI = 0.98–2.57, $p = 0.596$, $d = 1.55$) or pre-departure (0.93, CI = 0.57–1.50, $p = 0.912$, $d = 0.19$). Overall, CORT levels in the plasma were 5 times greater than in tissues.

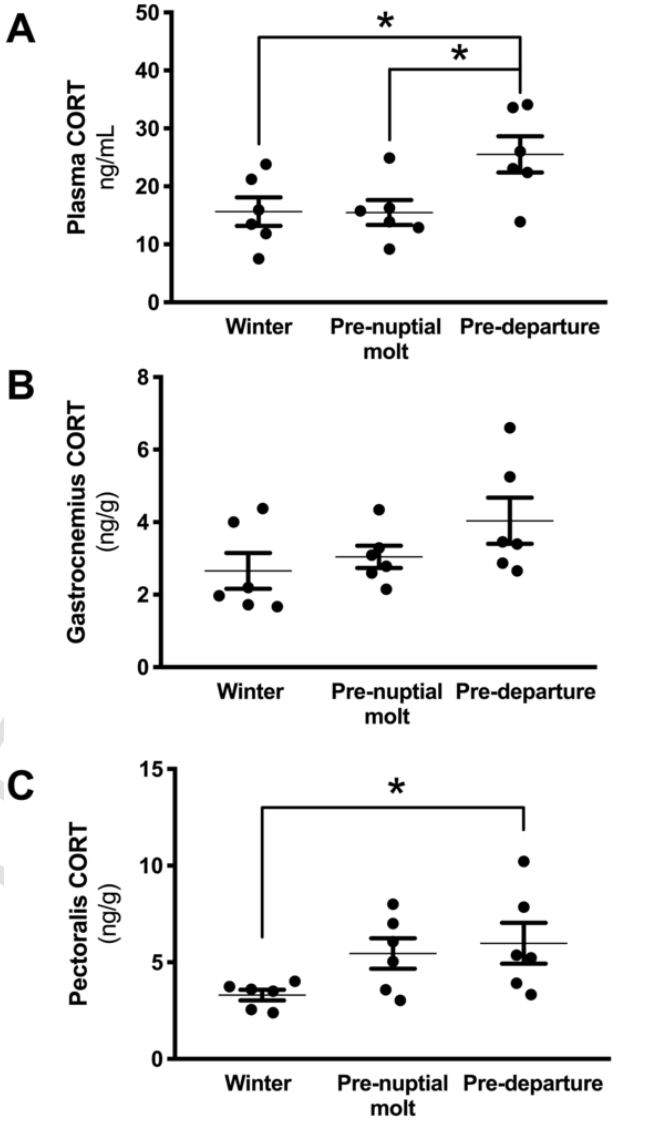

Fig. 1. Systemic and local levels of corticosterone (CORT) in free-living male white-crowned sparrows sampled during life history stages prior to pre-nuptial migration. (A) Plasma CORT was significantly elevated at pre-departure compared to both winter and pre-nuptial molt stages. (B) Gastrocnemius muscle CORT did not differ across the stages. (C) Pectoralis muscle CORT was significantly elevated at pre-departure compared to both winter and pre-nuptial molt stages. n = 6 per stage, *$p < 0.05$.

3.3. mRNA variation

3.3.1. Levels of 11β-HSD Type 1 and 2 mRNA

Notably, 11β-HSD Type 1 and 2 were undetected in the gastrocnemius muscle whereas both enzymes were expressed in the pectoralis but at differing concentrations (Fig. 2). There was no difference in

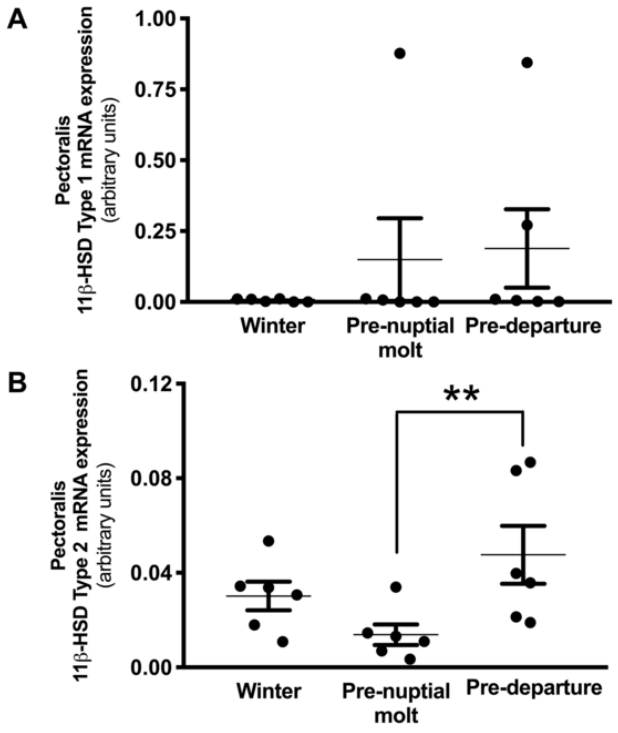

Fig. 2. Relative mRNA levels of enzymes that regenerate and deactivate CORT in pectoralis muscle of free-living male white-crowned sparrows sampled during life history stages prior to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. (A) 11β-HSD Type 1 mRNA did not differ across the stages. (B) 11β-HSD Type 2 was as elevated at pre-departure compared to pre-nuptial molt. These transcripts were not detected in the gastrocnemius muscle. n = 6 per stage, **p < 0.01.

11β-HSD Type 1 mRNA across life history stages in the pectoralis ($F_{2, 15} = 1.10$, $p = 0.371$, $\eta p^2 = 0.11$). However, there was a significant increase in 11β-HSD Type 2 mRNA in the pectoralis across the life history stages ($F_{2, 15} = 7.20$, $p = 0.006$, $\eta p^2 = 0.45$), with levels 3.66 fold higher at pre-departure compared to pre-nuptial molt (3.66, CI = 1.48–9.08, $p = 0.005$, $d = 1.84$). No differences were detected between winter and pre-nuptial molt (2.45, CI = 1.0–6.07, $p = 0.054$, $d = 1.35$) or between winter and pre-departure (0.67, CI = 0.27–1.66, $p = 0.498$, $d = -0.67$).

3.3.2. Levels of MR and GR mRNA

Muscle MR mRNA levels are depicted in Fig. 3 with GR mRNA levels in Fig. 4. In gastrocnemius muscle, there was no significant difference in mRNA levels across stages for MR ($F_{2, 15} = 0.60$, $p = 0.57$, $\eta p^2 = 0.06$). There was a significant difference in gastrocnemius GR ($F_{2, 15} = 4.10$, $p = 0.039$, $\eta p^2 = 0.31$), which was driven by the increased GR levels at pre-nuptial molt (lsmeans difference pre-nuptial molt vs. winter: 2.47, CI = 1.08–5.67, $p = 0.032$ $d = 1.75$; pre-nuptial molt vs. pre-departure: (1.43, CI = 0.62–3.28, $p = 0.520$, $d = 0.98$), pre-departure vs. winter: (1.73, CI = 0.75–3.97, $p = 0.2318$, $d = 0.77$). Interestingly, in pectoralis muscle, MR mRNA levels were significantly different across the stages ($F_{2, 15} = 11.20$, $p = 0.001$, $\eta p^2 = 0.25$), whereas GR mRNA levels did not vary ($F_{2, 15} = 1.70$, $p = 0.210$, $\eta p^2 = 0.16$). Pectoralis MR mRNA levels increased at pre-departure compared to both winter (6.13, CI = 1.65–22.7, $p = 0.007$, $d = 1.33$) and pre-nuptial molt (9.47, CI = 2.55–35.15, $p = 0.001$, $d = 1.71$), but MR mRNA levels did not differ between winter and pre-nuptial molt ($p = 0.671$, $d = 0.22$).

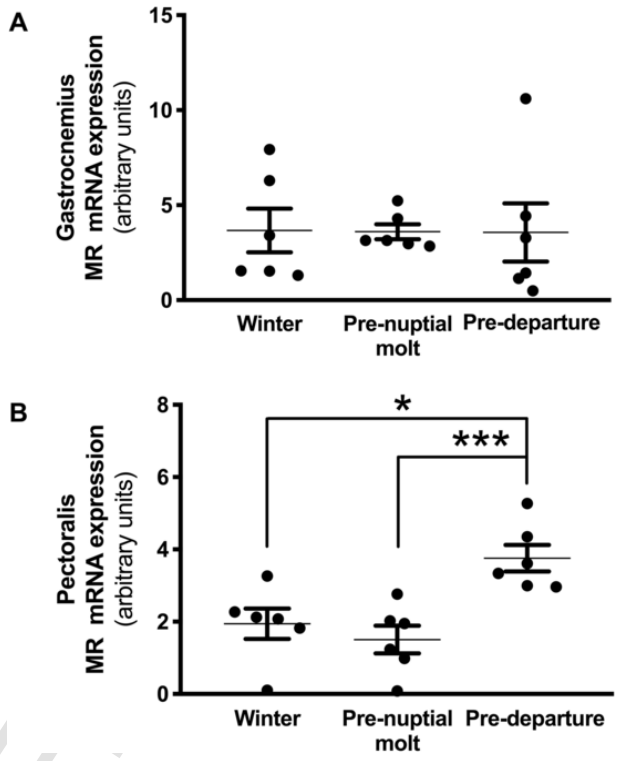

Fig. 3. Relative mRNA levels of mineralocorticoid receptor (MR) in free-living male white-crowned sparrows sampled during life history stages prior to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. (A) There was no difference in gastrocnemius MR mRNA levels. (B) Pectoralis MR mRNA was higher at pre-departure was higher than both winter and molt. n = 6 per stage, *p < 0.05, ***p < 0.001.

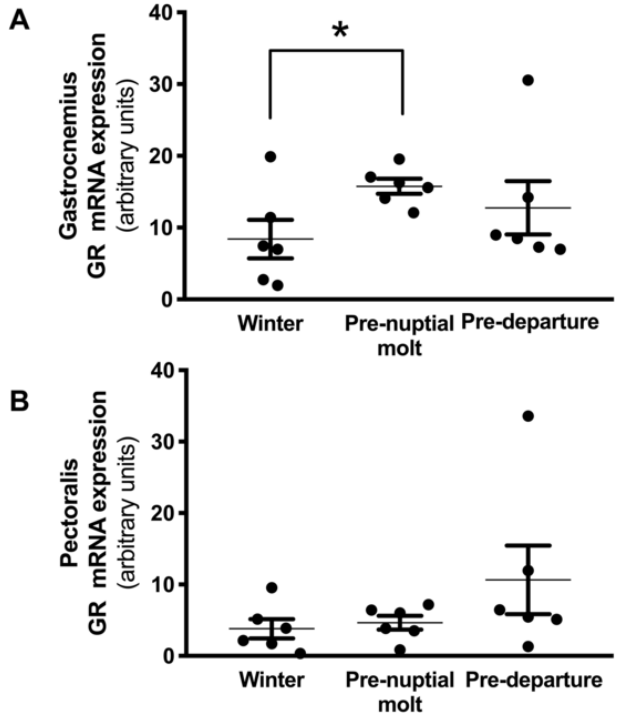

Fig. 4. Relative levels of glucocorticoid receptor (GR) mRNA in the gastrocnemius (A) and pectoralis (B) muscles of free-living male white-crowned sparrows sampled during three life history stages prior to pre-nuptial migration. The values represent the ∂CT values corrected with the reference gene, GAPDH. There was a significant increase in GR at molt in the gastrocnemius, but not in the pectoralis muscles. n = 6 per stage, *p < 0.05.

3.3.3. Discriminant function analysis

We found a significant difference between life-history stages in CORT and gene expression variables (Wilk's lambda p = 0.046). The first axis (LD1) explained 70.59% of the separation between stages while the second axis (LD2) explained 29.41%. While there is some overlap between the stages, there are clear differences between winter, pre-nuptial molt, and pre-departure stages (Fig. 5). The strength of the relationships between the stages and variables can be seen in the direction and length of the arrows with longer arrows indicating a stronger effect.

3.4. Model selection and averaging: associations across physiological parameters

We found a significant, positive relationship between plasma CORT levels and pectoralis MR and a suggestive positive relationship with pectoralis CORT ($\pi_i = 0.20$, $F_{2,\ 15} = 13.90$, $p < 0.001$, pectoralis CORT BT est. = 1.40, CI = 0.96–2.03, p = 0.076, pectoralis MR est. = 0.19, CI = 0.08–0.30, p = 0.003). We also found a significant, positive association between pectoralis CORT levels and pectoralis GR ($\pi_i = 0.41$, $F_{1,\ 16} = 9.80$, p = 0.006, GR BT est. = 1.27, 95% CI = 1.08–1.50, p = 0.006). When we assessed how physiological parameters co-varied in the gastrocnemius, we found no significant association between gastrocnemius CORT levels and MR ($F_{1,16} = 0.20$, p = 0.628) and gastrocnemius CORT levels and GR ($F_{1,\ 16} = 1.00$, p = 0.327). When we compared gastrocnemius MR versus GR, we found a significant, positive association ($F_{1,\ 16} = 9.40$, p = 0.008, est. = 0.07, CI = 0.02–0.11) (Table 2).

3.5. Physiological parameters versus body composition

We found that the plasma CORT, muscle CORT, and gene expression of MR, GR, 11β-HSD Type 1, and 11β-HSD Type 2 were not related to body condition (plasma CORT, $F_{1,16} = 2.50$, p = 0.134; pectoralis, CORT: $F_{1,16} = 2.1$, p = 0.169; GR, $F_{1,16} = 0.9$, p = 0.346; MR: $F_{1,16} = 1.7$, p = 0.205; 11β-HSD Type 1, $F_{1,16} = 0.2$, p = 0.682; 11β-HSD Type 2, $F_{1,16} = 3.2$, p = 0.095; gastrocnemius: CORT, $F_{1,16} = 3.4$, p = 0.085; GR, $F_{1,16} = 1.1$, p = 0.315; MR, $F_{1,16} = 1.9$, p = 0.185.

4. Discussion

Multiple physiological pathways likely undergo realignments as organisms transition between life-history stages and are especially important in birds as they prepare for and perform migratory flights that require a balance of anabolic and catabolic processes across multiple tissues. In an attempt to better assess the likelihood of CORT signaling prior to migration, our study examined: 1) plasma CORT levels at three stages prior to migration, 2) skeletal muscle CORT levels, 3) genes that encode enzymes that process CORT and 4) genes that encode the receptors on which CORT acts. Our results offer support for the hypothesis that skeletal muscle and in particular the pectoralis is a target of GC as sparrows prepare for migratory flight. Our results provide a window into likely modifications in CORT signaling experienced by muscles in preparation for migration. Skeletal muscles of the fore- and hind-limbs express both GR and MR, showing they are likely sensitive to both baseline and elevated CORT levels. Despite some similarities, these skeletal muscles also differ in crucial ways that impact how CORT may function. In the pectoralis, levels of CORT and MR increased at pre-departure, suggesting that CORT activation of MR is especially important as flight muscles prepare for long-distance migration. Similar changes were not observed in the gastrocnemius muscle suggesting for at least

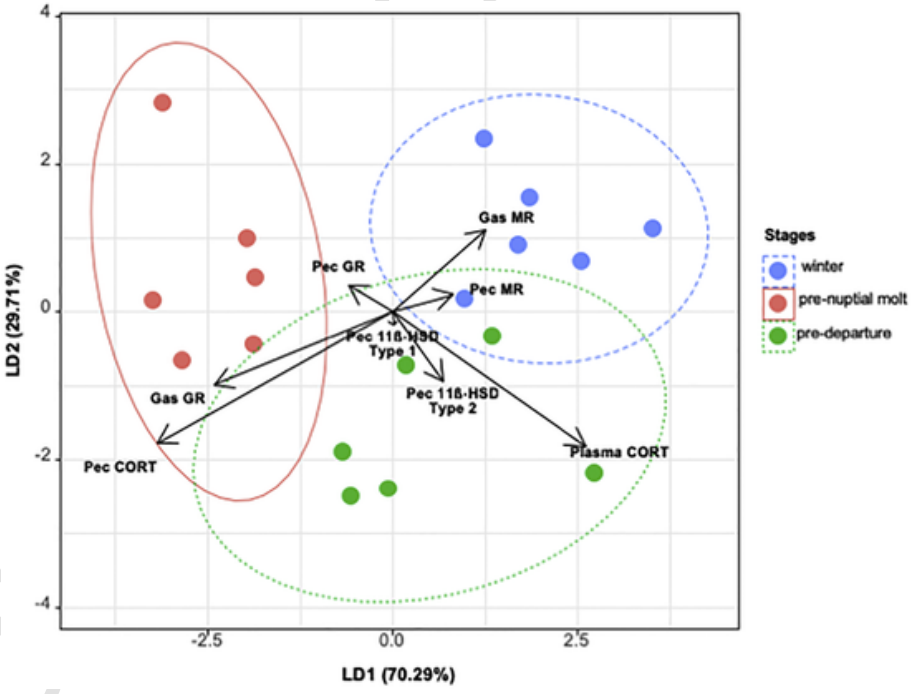

Fig. 5. Discriminant function analysis of corticosterone (plasma, pectoralis, and gastrocnemius), and mRNA expression of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and 11β-HSD Type 1 and 2 in the gastrocnemius and pectoralis muscles of free-living male white-crowned sparrows sampled during three life history stages prior to pre-nuptial migration. The axes explained 70.59% (LD1) and 29.41% (LD2) of the separation between stages. Stages are indicated by blue (winter), red (pre-nuptial molt), and green (pre-departure). There is clear separation between stages. The strength of the relationships among variables and by stage can be seen in the direction and length of the arrows. Longer arrows indicate a stronger effect. Direction of the arrows indicates the values of the model coefficients. n = 6 per stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
A summary of differences between life-history stages in plasma CORT, pectoralis and gastrocnemius CORT and gene expression of 11β-HSD Type 1 and 2, GR, and MR). Statistically significant differences (Tukey adjusted $p < 0.05$) are indicated by an asterisk (*) whereas non-significance is indicated by a dash (–).

Tissue	Variable	Stage Differences		
		winter vs. molt	winter vs. pre-departure	molt vs. pre-departure
Plasma	CO	–	*	*
Pectoralis	CORT	–	*	
	1β-HSD Type 1	–	–	–
	11β-HSD Type 2	–	–	*
	GR	–	–	–
	MR	–	*	*
Gastrocnemius	CORT	–	–	–
	GR	*	–	–
	MR	–	–	–

these 3 stages MR activation may not change in this muscle. This difference is especially striking in that we detected transcripts encoding enzymes that both synthesize and de-activate CORT only in the pectoralis. In the same birds, we did not find differences in androgen signaling molecules in pectoralis versus gastrocnemius (Pradhan et al., 2019). However, IGF-1 levels do change in the pectoral muscles of these sparrows in parallel with the overall findings of this study. Although mRNA levels may not always reflect active protein levels (Medina et al., 2013), these expression patterns suggest that prior to migration, the regulation of CORT is of greater importance in the pectoralis than in the gastrocnemius, a subject considered more fully below.

4.1. Plasma CORT significantly increased at pre-departure

Following the winter months, sparrows undergo a molt and then begin preparations for spring departure by becoming hyperphagic, a process regulated by photoperiod, testosterone, and possibly CORT (Landys et al., 2004; Ramenofsky and Németh, 2014). Under natural conditions, the hyperphagia leads to increased body mass, fat deposits, and muscle hypertrophy all to support long distance flight (Cornelius et al., 2013; Lindström and Piersma, 1993; Marsh, 1984; McWilliams et al., 2004; Ramenofsky, 1990; Ramenofsky and Németh, 2014). In the field, sparrows exhibit such morphological changes over a period of 20–25 days after daylength surpasses the vernal equinox of 12L:12D (Donham et al., 1983; Follett et al., 1974; King and Farner, 1963). The timing of these morphological changes along with our current results of the enzymes and receptors, offers a deeper understanding regulating of potential mechanisms of CORT signaling at a local level. Plasma CORT levels were elevated just prior to departure, a similar pattern observed in other migrants (Cornelius et al., 2013; Eikenaar et al., 2017, 2018; Landys et al., 2004). Although our data are correlational, the increase in CORT at pre-departure in this and other studies suggest an anticipatory role by GCs for coping with the impending and unpredictable events incurred during migratory flight (Donham et al., 1983; Follett et al., 1974; King and Farner, 1963; Romero and Wingfield, 2015).

4.2. Skeletal muscle CORT

Although we found that systemic CORT levels were relatively higher in plasma than in skeletal muscle, levels in plasma and pectoralis were related to one another; whereas, levels in plasma and gastrocnemius were not. Other distinctions between the muscle types were identified. CORT levels were elevated in the pectoralis only at pre-departure. Given that the gastrocnemius expressed little if any 11β-HSD Type 1 or 2, we thought that CORT levels would fluctuate in this muscle in parallel with levels in plasma. By contrast we would expect CORT levels in the pectoralis might differ from plasma due to the presence of both CORT-metabolic enzymes. Perhaps 11β-HSD Type 1 regenerated CORT in the pectoralis (and not the gastrocnemius) to produce the life-history stage differences we detected. These differences across muscle types require further investigation but warrant a word of caution for over generalizations about effects of circulating hormones on specific tissues when tissue metabolism and binding properties are not fully considered as discussed elsewhere (Wingfield, 2018).

4.3. Elevated potential for CORT deactivation during pre-departure in pectoralis

A hallmark of many endocrine systems is that circulating hormones are modified by enzyme activity in a tissue-specific manner to produce appropriate signaling (Balthazart and Ball, 2013; Wilson, 2001). This may also be the case for signaling by GCs. An increase in expression of 11β-HSD Type 1 may reflect a state of increased CORT activation but an increase in 11β-HSD Type 2 would reduce such activation. Thus, if CORT has catabolic effects on muscle then increased 11β-HSD Type 1 would promote such actions, whereas increased 11β-HSD Type 2 would limit these effects. With this in mind, our results raise a number of questions. Why might 11β-HSD Type 2 increase in the pectoralis prior to pre-departure? Our data suggests that 11β-HSD Type 2 in the pectoralis might reduce GC signaling (binding to MR and/or GR) when CORT is elevated at pre-departure, possibly to limit catabolic actions such as muscle atrophy through proteolysis and the inhibition of protein synthesis (Kuo et al., 2013). Inactivation of free CORT by 11β-HSD Type 2 enzyme might also liberate MR binding sites for occupancy by aldosterone or 11-deoxycorticosterone (Rusvai and Náray-Fejes-Toth, 1993; Taves et al., 2015; Yang and Yu, 1994). The capacity for binding of MR by aldosterone likely regulates osmoregulatory balance and homeostasis (Carsia, 2014), both conditions that would be advantageous for muscle once flight begins. Future studies might assess aldosterone binding in skeletal muscle of birds prior to migration.

Given that 11β-HSD Type 1 did not differ across the stages in the pectoralis and was undetectable in the gastrocnemius suggests that this enzyme plays little role in muscle during periods prior to migration. Overall, our findings suggest that expression of 11β-HSD Type 2 likely helps establish the appropriate flight muscle phenotype required for long-distance migration. The pectoralis-specific responses – increased local CORT, MR, and 11β-HSD Type 2 expression – point to the fact that GC-signaling in flight muscle plays a crucial anabolic role in preparing birds for endurance flight of migration and offers support for Prediction 1.

4.4. CORT sensitivity in skeletal muscle at pre-departure

In the pectoralis, MR mRNA doubled at preparation for departure compared to both winter and molt. Mineralocorticoid Receptor regulates a diversity of physiological functions in numerous tissues (Joëls

et al., 2008; Pascual-Le Tallec and Lombès, 2005) and elevated expression, perhaps enhanced by increased plasma CORT levels, may activate the high affinity receptors within the flight muscle before migratory flight begins. Mineralocorticoid Receptor is known to influence membrane excitability as well as ion and fluid transport in neuromuscular systems (Chadwick et al., 2015; Gomez-Sanchez and Gomez-Sanchez, 2014) and may serve similar functions in the pectoralis. Whereas an increased expression of MR likely reflects an enhanced role of this receptor in the pectoralis prior to migratory departure while levels of GR are more difficult to interpret. While MR was invariant in the gastrocnemius, GR expression was elevated only at molt. Without an indication of anticipatory preparation for impending flight, we find little support for Prediction 2. However, the large variance, low sample and small effect sizes of gastrocnemius GR may obscure a potential difference between the stages; therefore we cannot confidently reject Prediction 2. Thus, we surmise that the predominant effect of the GC signaling promotes an anabolic state in the flight muscle in preparation for impending migratory flight.

5. Conclusions

Spring migration requires synchronization of behavior, physiology, and morphology with environmental cues to effectively prepare, depart, and arrive at distant sites to breed in a timely manner and reproduce successfully (Woodworth et al., 2016; Dingle 2014). Phenotypic flexibility observed in migrants preparing for flight departure include multiple physiological pathways to enhance fuel and power. Our data indicate that prior to migratory flight, CORT may function via MR in the pectoralis to promote a migratory readiness while 11β-HSD Type 2 acts to limit the catabolic actions of CORT via GR. Taken together this work reveals potential pathways by which GCs could function to regulate the phenotypic flexibility expressed by birds anticipating migratory departure, and whether these changes exist for females or birds preparing for autumn migration remains to be tested.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2019.104586.

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