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# Gene expression of sex steroid metabolizing enzymes and receptors in the skeletal muscle of migrant and resident subspecies of white-crowned sparrow (*Zonotrichia leucophrys*)

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**Abstract**

Circulating sex steroid concentrations vary dramatically across the year in seasonally breeding animals. The ability of circulating sex steroids to effect muscle function can be modulated by changes in intracellular expression of steroid metabolizing enzymes (e.g., 5α-reductase type 2 and aromatase) and receptors. Together, these combined changes in plasma hormones, metabolizing enzymes and receptors allow for seasonally appropriate changes in skeletal muscle function. We tested the hypothesis that gene expression of sex steroid metabolizing enzymes and receptors would vary seasonally in skeletal muscle and these changes would differ between a migrant and resident life history strategy. We quantified annual changes in plasma testosterone and gene expression in pectoralis and gastrocnemius skeletal muscles using quantitative polymerase chain reaction (qPCR) in free-living migrant (*Zonotrichia leucophrys gambelii*) and resident (*Z. l. nuttalli*) subspecies of white-crowned sparrow during breeding, pre-basic molt, and wintering life history stages. Pectoralis muscle profile was largest in migrants during breeding, while residents maintained large muscle profiles year-round. Circulating testosterone peaked during breeding in both subspecies. Pectoralis muscle androgen receptor mRNA expression was lower in females of both subspecies during breeding. Estrogen receptor-α expression was higher in the pectoralis muscle, but not gastrocnemius, of residents throughout the annual cycle when compared to migrants. Pectoralis aromatase expression was higher in resident males compared to migrant males. No differences were observed for 5α-reductase 2. Between these two subspecies, patterns of plasma testosterone and androgen receptors appear to be conserved, however estrogen receptor gene expression appears to have diverged.

## Introduction

Skeletal muscle is a plastic tissue that can vary dramatically is size, which is regulated by a series of hormonal and paracrine signaling mechanisms as well as resistance exercise (Bodine and Baar 2012, Velders, Schleipen et al. 2012, Velders and Diel 2013). Sex steroids play a crucial role in regulating changes in skeletal muscle morphology and physiology (Velders and Diel 2013). Androgen receptor (AR) and estrogen receptor-α (ERα) are known for their anabolic effects on muscle that result in hypertrophy through the expression of growth-promoting genes (Velders, Schleipen et al. 2012, Serra, Tangherlini et al. 2013) and increase in force generation (Velders and Diel 2013). Intracellular regulatory enzymes can alter the signaling efficiency of sex steroids. For instance, the enzyme 5α-reductase locally converts testosterone to 5α-dihydrotestosterone (5α-DHT), which is the preferred ligand of the androgen receptor (Fuxjager, Barske et al. 2012) thereby increasing localized androgen signaling. In addition, testosterone can also be metabolized to estradiol by aromatase, allowing signaling via the estrogen receptor (Trainor, Kyomen et al. 2006). Thus, changes in steroid metabolizing enzyme represent an important mechanism for fine tuning steroid signaling in target tissues, including skeletal muscle.

For many seasonally breeding animals, sex steroids are very low or undetectable during the non-breeding season and are elevated during breeding following the activation of the reproductive axes (Wingfield, Hegner et al. 1990). This dynamic change in circulating sex steroids between the breeding and nonbreeding seasons provides a mechanism to affect

muscle through androgen and estrogen dependent signaling pathways. In particular, breeding is a time when skeletal muscle function and recovery from elevated levels of exercise is vital for fighting for dominance, defending resources, and attracting mates. For example, enhanced AR, but not ER mRNA expression, was positively correlated with display flight performance in golden-collard manakins (*Manacus vitellinus*; (Fuxjager, Eaton et al. 2015) and displays in Anolis lizards (Johnson, Kircher et al. 2018) during breeding. Furthering this thought, animals breeding at higher latitudes, or that have fewer broods, tend to have higher circulating levels of sex steroids compared with animals breeding at lower latitudes with more broods (Hau, Gill et al. 2008, Hau, Ricklefs et al. 2010). Animals at different latitudes are exposed to unique environmental and evolutionary pressures that provide a model for understanding unique patterns of hormones and gene expression. Knockout studies in rodents provide potential evolutionary insights into the functional significance of modulating levels of androgen and estrogen receptors. Muscle specific AR gene knockout study in male rodents has demonstrated reduced skeletal muscle mass, reduced force production from fast twitch fibers, and increased fatigue resistance in slow twitch fibers (Yeh, Tsai et al. 2002, MacLean, Chiu et al. 2008). Conversely, ERα skeletal muscle knockout mice showed reductions in peak force generation and reduced fatigue resistance (Collins, Mader et al. 2018). Interestingly, ERα knockout mice did not differ from wildtype in muscle fiber type abundance; however, there was a reduction in their cross sectional area (Collins, Mader et al. 2018). Estrogen signaling ameliorates post-exercise muscle damage and inflammation while also enhancing skeletal muscle repair (Enns, Iqbal et al. 2008, Velders, Schleipen et al. 2012). Thus, seasonally appropriate modulation of steroid receptors provides a mechanism for regulating skeletal muscle in terms of hypertrophy,

fiber composition, and fatigue resistance. Changes in gene expression for AR and ER across the annual cycle may be critical for migration, territoriality, flight performance, and competition for mates or resources during breeding, and thermogenesis during periods of cold weather.

Currently, little is known about how skeletal muscle expression of sex steroid metabolizing enzymes and receptors change across the annual cycle in free-living birds. We investigated circulating testosterone and gene expression in skeletal muscle of two subspecies of white-crowned sparrow during breeding, pre-basic molt, and wintering stages of their annual cycle. Over evolutionary time, Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*) evolved a non-migratory life history strategy while Gambel's white-crowned sparrows (*Z.l. gambelii*) retained their migratory life history strategy. (Weckstein, Zink et al. 2001). In addition, hormone profiles appear to have diverged with residents having lower circulating hormones during breeding (Krause, Németh et al. 2016, Wingfield 2020, Krause, Németh et al. 2021, Perez, Krause et al. 2021). We collected pectoralis muscle because it is critical for flight and gastrocnemius muscle which is important for terrestrial locomotion. These two muscles were chosen because of their functional differences, thus the flight muscle would be expected to alter gene expression on an annual basis in the migrant, while the gastrocnemius muscle would be expected to have more stable gene expression, but we note that foraging demands may change seasonally which could also impact gene expression in the gastrocnemius. We aimed to understand life history dependent changes in gene expression of AR, ERα, aromatase and 5α-reductase 2 that would affect the tissue sensitivity to circulating levels of androgens and estrogens. For both subspecies, we hypothesized that plasma testosterone profiles and the expression of steroid metabolizing enzymes and receptors would change seasonally, and these

changes would be muscle group dependent. We predicted that a seasonal increase in AR, ER, aromatase, and 5 alpha reductase gene expression could occur during periods of low circulating testosterone to maintain tissue sensitivity. Alternatively, we predicted that gene expression could be increased during periods of high circulating testosterone to bolster sex steroid signaling in skeletal muscle to affect cellular physiology and muscle profile (visual inspection of muscle size). Tissues were not collected during the migratory period because they were collected as part of another study. However, this study allowed us to assess if there were signatures of selection on endocrine traits associated with migratory tendency that persists even into non-migratory life history stages. We also hypothesized that the expression of genes within estrogen and androgen pathways would have diverged over evolutionary time between migrants and residents due to the distinct life history strategies, environmental conditions, and migratory traits (e.g. seasonal pectoralis muscle profile). We predicted that skeletal muscle AR, ER, aromatase and 5 alpha reductase expression would be higher in migrants, promoting increased skeletal muscle size. Furthermore, we predicted that seasonal changes in regulatory enzymes and receptors would be greater in the pectoralis compared to the gastrocnemius, due to the transient demands of migration and territory defense as compared to the relatively constant year-round demands of terrestrial locomotion.

## Materials and methods

### *Study species and field sites*

Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*; hereafter migrants) are long distance migrants that winter in the American Southwest and Northern Mexico

(Blanchard and Erickson 1949). Wintering birds are found in flocks varying from several to hundreds of individuals and they depart their wintering grounds in mid-April to northern breeding grounds in tundra and taiga habitats in Canada and Alaska (Lisovski, Németh et al. 2019). In a typical year, males arrive on the breeding grounds several days before females in mid- to late-May which is immediately followed by a brief territorial phase (Boelman, Krause et al. 2017). Clutch initiation is highly synchronous, often occurring over 7-10 days for the local population, with the first egg being laid in late May to early June (Boelman, Krause et al. 2017). Collection in 2016, was characterized by early snowmelt with an average clutch initiation date of Jun 1$^{st}$ (Boelman, Krause et al. 2017, Chmura, Krause et al. 2018, Krause, Pérez et al. 2018). However, a snowstorm on May 24 and 25$^{th}$ may have skewed our estimate to a later date since only few nests had been found prior to the storm. Due to the shortness of the Arctic summer, the migrants are single brooded and have a brief opportunity to re-nest if first clutch failure occurs (Chmura, Krause et al. 2018). Pre-basic molt is typically initiated in early to mid-July and completed by late-August prior to autumn migration (Morton, King et al. 1969, Chmura, Krause et al. 2020).

Nuttall's white-crowned sparrow (*Zonotrichia leucophrys nuttalli*; hereafter residents) reside along coastal California and do not migrate (Baker and Mewaldt 1979). They are most often found within several hundred meters from where they hatched and have home ranges averaging 6-7 hectares (Baker and Mewaldt 1979). Wintering behavior differs between individuals. Some individuals remain on territories year-round, while others form mixed flocks - typically consisting of juveniles and adult females. Because the California spring and summer is long, residents are able to raise 2 to 3 broods between late-March and late-July (Mewaldt and

King 1977). The transition from wintering to breeding is often asynchronous across the population, since they lack the temporal constraints of having to migrate (Mewaldt and King 1977). At the time of sampling some individuals were completing their first clutch while others were initiating their second clutch. It is common for males to take over parental care while females initiate the second clutch (Baker, Mewaldt et al. 1981). The spring season during which the samples were collected was typical in terms of weather and the timing of breeding. Similarly to the migrants, pre-basic molt is initiated in July and terminates in August or September in the residents depending on the individual (Mewaldt and King 1977).

During breeding and molt, birds were caught with Japanese mist nets in conjunction with playback of previously recorded conspecific songs. During winter sampling, seed-baited walk-in potter traps were used in addition to mist nets. Traps and mist nets were continuously monitored to determine the elapsed time in seconds from initial capture to tissue and blood sampling. Body mass was measured to the nearest 0.1 g with a Pesola scale. Pectoralis muscle profile was visually scored on a scale from 0 – muscle extremely concave (emaciated), 1 – concave muscle, 2 – flat muscle, and 3 convex muscle (bulging)(Bairlein and Simons 1995, Krause, Chmura et al. 2015). Fat deposits were also visually scored from 0 (none) to 5 (bulging) for the furcular and abdominal deposits (Kaiser 1993). Presence of molt was determined by checking both wing and body feathers for either feather loss or new growth described by Morton, King et al. (1969). All work was approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC) under protocol 19758. Permits were obtained from the United States Fish and Wildlife Service - Federal MB90026B-0, California State SC13449, and Point Reyes National Parks PORE-00092.

*Tissue and blood collections in field*

Residents were collected at Point Reyes National Seashore (N 38°04”, W 122°53’) and migrants were collected in Davis, CA, (N 38° 33’, W 121°44’) during winter, and near Toolik Research Station on the North Slope of Alaska (N 68°38’, W 149°36’) during breeding and pre-basic molt. Migrant and resident white-crowned sparrows were collected during winter (December 8-9 for residents and December 6-7 for migrants), breeding (April 21-22 for residents and May 28-29 for migrants), and pre-basic molt (August 9-10 for residents and July 17-21 for migrants). The sample size are as follows: breeding migrants 10M and 8F and residents 9M and 7F, pre-basic molt migrants 10M and 8F and residents 8M and 8F, and for winter: migrants 9M and 9F and residents 9M and 7F. When the birds were captured, an initial blood sample was collected within three minutes of capture by venipuncture of the alar vein with a 26 gauge needle and collected into heparinized glass microcapillary tubes (VWR: 15401-56). The bird was quickly sedated with isoflurane and euthanized (3 min 20 s ± 52 s). After euthanasia, the left pectoralis and left gastrocnemius muscles were dissected, fresh frozen on dry ice, wrapped in aluminum foil into labelled plastic bags and kept frozen on dry ice until they were stored in a -80$^{o}$C freezer upon returning to the laboratory. Samples were later shipped on dry ice to the Roslin Institute, University of Edinburgh, UK where they were stored at -80$^{o}$C until qPCR analyses. Blood samples were centrifuged at 15,000 g for 5 minutes, the plasma was aspirated using a Hamilton syringe, transferred to a microcentrifuge tube, and kept frozen at -30$^{o}$C until radioimmunoassay for total androgens was conducted.

*Androgen Assay*

Androgen (testosterone) was measured by a radioimmunoassay following Wingfield and Farner (1978). Plasma volumes were measured with a Hamilton syringe and ranged from 35-125 µl. Approximately, 2000 CPM of tritiated testosterone (Perkin Elmer NET370250UC, Waltham, MA USA) was added to each sample prior to extraction to estimate recovery efficiency. Endogenous and radiolabeled testosterone were extracted from the samples using 4 ml of diethyl ether (296082; Sigma Aldrich, St. Louis, MO USA). Next, the samples were dried in a water bath at 35°C under a stream of nitrogen gas, and then reconstituted using 550 µl of phosphate buffered saline with gelatin. The samples were aliquoted into 200 µl duplicate assay tubes and 100 µl recoveries in scintillation vials. 4 ml of scintillation fluid (Ultima Gold: 6013329; Perkin Elmer, Waltham, MA USA) were added to each scintillation vial and were counted for percent recoveries. Duplicate assay tubes each received 100 µL of tritiated testosterone (~10K CPM) and 100 µl testosterone antibody (20R-TR018w, Fitzgerald Antibodies, North Acton, MA USA). Steroids bound by antibody were separated from unbound by the addition of 500 µl of dextran coated charcoal followed by centrifugation at 3000 g for 10 minutes at 4°C. The supernatant was decanted into scintillation vials and combined with 4 ml of scintillation fluid. Samples were placed on a Beckman 6500 liquid scintillation counter and counted for 5 min. or within 2% accuracy. The testosterone values were adjusted using the corresponding recovery percentage estimate concentration in the plasma sample. Mean recoveries were 78.12 ± 9.19 % and intra-assay (calculated using C.V. between duplicates) and inter-assay variations were 4.86% and 8.06%, respectively. The detection limit of the assays was 10.56 ± 2.56 pg per tube (~0.9 ng/ml per tube). The antibody has high specificity for

testosterone, but also has 40% cross reactivity with DHT. Therefore, testosterone and DHT are being reported, but will be collectively referred to as androgens.

*Preparation of cDNA*

Pectoralis and gastrocnemius muscle from each bird was dissected out into 100 ± 20 mg tissue pieces into labeled 2 ml tubes and homogenized using Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG) in conjunction with 1000 µl of trizol. Total RNA was extracted from 250 µl of tissue homogenate using Zymo Research Direct-zol 96 well plates (R2057) with the optional DNA digestion step per the manufacturer's instructions. Total RNA for pectoralis and gastrocnemius muscles were found to have an average yield of 327 ± 129, and 206 ± 79 (mean ± SD) ηg/µl with an average 260/280 ratio of 2.06 ± 0.02, and 2.09 ± 0.02, respectively. cDNA was produced by reverse transcribing 750 ng of RNA per sample using the High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's guidelines and the final volume was adjusted to 110 µl per sample using ultra-pure (Milli-Q) water.

*Quantitative polymerase chain reaction (qPCR)*

All assays used Brilliant III Ultra-fast SYBR Green qPCR Master mix (Agilent Technologies 60083) which was read on an Agilent Mx3005p qPCR system and processed with MxPro software (Agilent Technologies) as previously described by Reid, Wilson et al. (2017). In brief, a 20 µl total reaction volume per sample was generated by combining 10 µL SYBR Green, 8 µL cDNA, 0.4 µl 20 µM forward primer, 0.4 µL 20 µM reverse primer, 0.3 µL 1/500 dilution of ROX reference dye solution, and 0.9 µL Milli-Q $H_20$. Each assay used the following thermal conditions: 50 °C; 120 s, 95 °C; 120 s, (40 cycles of 95 °C; 15 s, 60 °C; 30 s), then 95 °C; 60 s, 60

°C; 30 s, 95 °C; 15 s. Apparent reaction efficiencies were determined by analyses of the standard curves and ranged between 92.4-96.8%.

Multiple primer combinations were tested for each gene using the previously mentioned thermal conditions for SYBR reactions and with Fast start PCR (Roche) using a standard PCR thermal conditions (95 °C; 240 s, (40 cycles of 95 °C; 30 s, 58 °C; 30 s, 72 °C; 30 s), then 72 °C; 420 s). Products from both PCR and qPCR reactions were visualized using 2% agarose gel electrophoresis. The primer combination for each gene was selected based on the amplicons that yielded highest amplifying reaction that lacked visible primer dimers or infidelity signals. We tested 5 alpha reductase type 1, and type 2, but we only achieved significant amplification for type 2. Once primers were selected (see Table 1), amplicons were isolated from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen 28704). The purified cDNA was used to generate standard curves of known concentration by quantification on a Nanodrop and then a serial dilution was conducted from an aliquot of the stock amplicon solution. All genes were normalized to the reference genes YWHAZ and NDUFA using a geometric mean due to reliability in previous avian studies (Reid, Wilson et al. 2017, Krause, Pérez et al. 2021). Amplicons were sent for sequencing for AR, ERα, aromatase and 5α-reductase 2 which were found to have sequence homologies with white-throated sparrow, *Zonotrichia albicollis,* of 94, 100, 97.5, and 100%, respectively. An additional set of supra-amplicon primers were created so that the cDNA could be amplified upstream of the forward primer and downstream of the reverse primer. These supra-amplicons were sent for sequencing and to verify that single nucleotide polymorphisms were not present between subspecies which could affect qPCR reaction efficiency.

*Statistical Analyses*

Statistical analyses were performed in R statistical analysis software versions 4.1.1 (R Core Development Team 2018). Muscle profile and plasma androgens were analyzed using factorial type III analysis of variance (ANOVA) using the Satterthwaite's approach with the main effects of life history stage, subspecies, and their interaction. AR, ER, aromatase and 5α-reductase type 2 data were analyzed using a blocked factorial type III ANOVA using the Satterthwaite's approach with the main effects of life history stage, subspecies, sex and their interaction and rtPCR plate was included as a blocking variable. Since there was low detection with aromatase and DHT, TOBIT models were run using the VGAM package (Yee 2010). Since the zeroes were often nearly evenly distributed across groups the TOBIT models did not detect any significant differences compared to using ANVOA and therefore ANOVA analyses were used throughout. All post hoc tests were performed using Tukey's Honestly Significant Difference (HSD) test using the emmeans package (Lenth, Singmann et al. 2018). In order to determine if stage for each group could be distinctly predicted by the combination of expressed genes, a multivariate analysis of variance (MANOVA) was used to determine differences between groups (subspecies within life history stage) based on gene expression of aromatase, AR, and ERα. 5α-reductase 2 was excluded from the MANOVA and DFA analysis because of the high rate of zeros in the data set. Inclusion or exclusion of the 5α-reductase 2 yielded the same response, however we removed remove 5α-reductase 2 from the analysis to simplify the final model. Following a significant MANOVA, discriminate function analyses were performed in R using the package MASS (Ripley, Venables et al. 2013).

**Results**

300 *Pectoralis muscle profile score*

301 Pectoralis muscle profile was affected by the main effects of sex ($F_{1,2801}$=43.14, P<0.001)
302 and life history stage ($F_{1,201}$=6.63, P=0.001) and the interactions of life history stage and
303 subspecies ($F_{2,201}$=3.97, P=0.01), and sex and life history stage ($F_{2,201}$=6.36, P=0.001; Fig. 1a).
304 Pectoralis muscle profile changed seasonally in migrants and was largest during breeding.
305 Resident muscle profile did not change across the annual cycle. Muscle size was smaller in
306 migrants compared to residents during molt and winter. During breeding, males of both
307 subspecies, had a larger pectoralis muscle profiles than females.

308 *Plasma androgens*

309 Plasma androgens were affected by the main effects of sex (Fig.1b; $F_{1,90}$=167.50,
310 P<0.001) and the interactions of life history stage and sex ($F_{1,90}$=54.64, P<0.001). No other
311 model term was significant (Fig. 1b). Both sexes had elevated levels of androgens during
312 breeding compared to pre-basic molt and wintering stages. Androgen levels were higher in
313 males during breeding for both subspecies compared to females, but sex-based differences
314 were absent during molt and wintering stages.

315 *Pectoralis Gene mRNA Expression*

316 AR, ERα, and aromatase, but not 5α-reductase 2, mRNA expression were affected by
317 one or more of the main effects of subspecies and life history stage, and in some instance the
318 interactions (Table 2; Fig 2). AR mRNA was not different between subspecies (Fig. 2a). Migrants
319 and residents had the greatest expression of AR mRNA during the winter months compared to
320 molt and breeding. To further explore the effects of season on AR expression, which appeared
321 to have sex specific effects, we conducted two additional statistical tests for each sex. These

additional models, indicated that AR mRNA gene expression varied by the main effect of stage for the females ($F_{2,40}$=3.40, P=0.04), but not males ($F_{2,47}$=1.31, P=0.27) and the interactions of stage and subspecies were not significant for females ($F_{1,40}$=0.08, P=0.45) or for males ($F_{2,48}$=0.50, P=0.60). ERα mRNA expression was higher in residents compared to migrants. The ERα mRNA interaction term was driven by resident females who had higher expression during breeding compared to molt or winter (Fig. 2b). Aromatase mRNA expression did not change seasonally, but there was a significant sex by subspecies interaction (Fig. 2d). Male, but not female, residents had higher expression of aromatase compared to male and female migrants. To further explore aromatase, we analyzed the data using simplified models that contained either migrant or resident white crowned sparrows. In the model for residents, there was significant stage by sex interaction ($F_{2,36}$=3.26, P=0.05) for aromatase expression. Male resident birds had higher aromatase mRNA expression in breeding compared to winter while female aromatase expression remained unchanged across the annual cycle (Fig. 2d). Both aromatase and 5α-reductase 2 mRNA expression had relatively low levels of detection with many 5α-reductase-2 samples being undetectable (Fig. 2c).

*Gastrocnemius Gene mRNA Expression:*

Gastrocnemius AR, aromatase, or 5α-reductase-2 expression, were not affected by any parameter in the statistical model (Table 2, Fig. 3). However, ERα was significantly affected by the interactions of subspecies and life history stage and the interaction of sex and life history stage. Post hoc analysis indicated that ERα mRNA expression in female migrants was lower during breeding compared to breeding female residents and breeding migrant males (Fig 3b).

*Pearson correlations:*

There was a correlation between ERα and AR mRNA for resident pectoralis (r=0.49, P=0.003, Fig. 4a) and gastrocnemius muscle (r=0.29, P=0.04, Fig. 4b) and nearly a significant positive relationship for migrants pectoralis (r=0.25, P=0.06, Fig. 4a), but not for gastrocnemius (r=0.22, P=0.11; Fig 4b). No correlations were found for ERα and aromatase or AR and 5α-reductase.

*MANOVA of gene expression by tissue:*

MANOVA analysis comparing migrants to residents indicated significant differences between life history stages for pectoralis ($F_{5,88}$=8.6, Wilks=0.44, P<0.001; Fig. 5a), but not gastrocnemius ($F_{5,94}$=1.05, Wilks=0.84, P=0.38; Fig. 5b) muscle.

MANOVA analysis comparing pectoralis and gastrocnemius muscle gene expression across stages of the annual cycle indicated a significant difference for residents ($F_{5,86}$=1.05, Wilks=5.66, P<0.001; Fig. 5c) and migrants ($F_{5,96}$=1.05, Wilks=1.71, P=0.02; Fig. 5d)

*Discriminant function analysis (DFA) of gene expression by tissue*

DFA analyses between subspecies for pectoralis and gastrocnemius muscle indicated that the first axis (LD1) explained 94.66 and 80.78 % of the separation between stages while the second axis (LD2) explained 3.16 and 13.36%, respectively (Fig. 5a & b). The vectors AR and ERα are in the opposite directions on Fig. 4a largely attributed to the consistent patterns observed in each respective gene.

DFA analysis between muscle groups within a subspecies indicated that for residents the LD1 explained 91.53 of the separation while the LD2 explained just 5.51% of the variance. For migrants, the LD1 explained 59.5% while LD2 explained 23.7%. There was greater separation observed for the residents and while minimal separation was observed for the migrants.

## Discussion

Migrant and resident white-crowned sparrows diverged from a migratory ancestor 50,000 years ago (Weckstein, Zink et al. 2001). Our data suggest that genes associated with sex steroid signaling in pectoralis, but not gastrocnemius muscle, have undergone divergence over evolutionary time according to our MANOVA analyses. These results can be largely explained by differences in pectoralis mRNA expression of ERα and aromatase between subspecies. Surprisingly, no differences were observed in androgen or 5α-reductase 2 gene expression between migrants and residents . DFA analysis showed clear separation in gene expression patterns between the gastrocnemius and pectoralis muscle in residents, but this relationship was far less distinct in migrants. Both migrants and residents had the highest circulating androgens during breeding, and lowest AR mRNA expression during breeding and molt, when compared to the wintering stage. However, the seasonal differences in AR mRNA expression were driven by females and not males. These pectoralis gene expression findings suggest a dynamic regulation of androgen signaling, while estrogen signaling pathways appear to be more fixed across the annual cycle, at least at our sampling points. The gastrocnemius muscle did not show the same robust differences in gene expression between migrants and residents. Thus, changes in pectoralis gene expression may be related to the use of the pectoralis in flight compared to the leg muscle used for terrestrial locomotion. In considering the discussion below it is important to note that both subspecies have very similar breeding, foraging, and social behavior with the only major behavioral divergence being associated with migration and winter territoriality and length of the breeding season. Thus, differences in gene expression likely

388 originate from the selective pressures of either migratory or sedentary life history strategies.
389 Additionally, environmental conditions that they are exposed to at given times of year may also
390 apply selective pressures.

391 *Plasma Androgens*

392 Plasma androgen concentrations were measured to assess the potential exposure of the
393 tissues to either high or low circulating concentrations of hormones which could then be
394 subsequently modified by steroid enzymes and bound by a steroid receptor. Based on the
395 collected data, it is unknown if circulating androgen concentrations reflect intracellular
396 concentrations, but steroids readily cross the cell membranes by diffusion. We anticipate that
397 plasma levels should provide a reasonable indication of intracellular androgen levels at the
398 target cell (Giorgi 1980). Counter to our prediction, and previous work that found that birds at
399 higher latitudes have higher circulating testosterone (Hau, Gill et al. 2008), we did not detect a
400 difference in androgen concentrations between subspecies. Bird behavior in the Arctic in 2016
401 was atypical as birds were less active than normal in terms of singing and other behaviors
402 related to breeding (Krause personal observation). It is possible that this was a consequence of
403 reduced androgen concentrations in migrants and also our relatively small sample size. An
404 analysis of a larger data set from our long-term population studies of white-crowned sparrows,
405 showed that migrants of both sexes have higher circulating testosterone concentrations
406 compared to residents during the breeding season (Wingfield 2020); in the current data set
407 plasma testosterone levels in the migrants were lower than expected. In addition, we have
408 unpublished data indicating that injection of gonadotropin-releasing hormone (GnRH) into male
409 white-crowned sparrows produced higher circulating androgens in migrants compared to

residents when controlling for life history stage (Krause et al. in prep). Although the current small data set does not match our prediction, our larger data sets generally indicate that androgen concentrations are higher in migrants compared to residents consistent with the published literature.

AR and 5α-reductase 2 mRNA expression

Our data showed that AR mRNA expression appears to be conserved across subspecies despite dramatic differences in migratory strategy, life history differences, and their environmental conditions. Parallel changes in AR mRNA expression were observed between subspecies, although this effect was largely driven by females which had lower AR expression during breeding and molt compared to winter. No subspecies differences were observed. The reduction of pectoralis AR mRNA during breeding is contrasted by observations in the brain which have consistently shown increased AR, aromatase, 5α-reductase 2, and/or ERα mRNA expression during breeding compared to winter in song sparrows (*Melospiza melodia*), Lapland longspurs (*Calcarius lapponicus*), Gambel's white-crowned sparrows, and black redstarts (*Phoenicurus ochruros*) suggesting enhancement of expression for genes associated with the steroid signaling pathways (Soma, Bindra et al. 1999, Soma, Schlinger et al. 2003, Canoine, Fusani et al. 2007, Fraley, Steiner et al. 2010, Wacker, Wingfield et al. 2010, Apfelbeck, Mortega et al. 2013). Upregulation of specific sex steroid signaling components in the brain may be critical for regulating reproductive physiology and behavior during breeding. In contrast to the brain, down regulation of AR in skeletal muscle may be necessary for controlling muscle size by regulating the degree of hypertrophy that can occur during periods of high circulating androgens. These results highlight that genes associated with sex steroid signaling appear to be

differentially regulated by tissue type, to appropriately alter sensitivity to androgen-based signaling across the annual cycle.

Based on the data obtained in this study, circulating androgens increased by 26-fold from winter to breeding. Whereas AR mRNA expression changed 1-fold from breeding to winter. These data suggest that the increase in plasma androgens was not directly offset by the reduction in AR mRNA expression. The presumed net effect would be increased androgen signaling in skeletal muscle, but measurement of intracellular levels of androgens is necessary to draw definitive conclusions. Evidence from Gambel's white-crowned sparrows collected during the transition from wintering to migration (Pradhan, Ma et al. 2019) found no parallel changes between plasma levels of testosterone and intracellular levels. However, it is important to note that androgen levels in that study were very low, in contrast to this current study. It is possible that the seasonal peak in plasma testosterone is sufficient to alter gene expression and further enhancement of AR mRNA expression is either not beneficial during our sampling points or could be detrimental due to overactivation of the androgen pathway. Induction of androgen signaling by elevated plasma androgens may be sufficient to explain the increase in pectoralis muscle profile and fiber size in migrants during breeding (Ramenofsky et al, in prep). Note, the same does not hold true for residents who maintained large pectoralis muscle size across the annual cycle despite changes in AR expression, suggesting perhaps that these differences may be estrogen dependent (see discussion below).

It was surprising to discover that there were no changes in the expression of the 5α-reductase 2 gene which metabolizes testosterone to $5\alpha\text{–DHT}$. However, our current data set does not capture the transition points associated with the preparation for, or during migration.

For instance, a previous study on the same migrant white-crowned sparrows (*Z. l. gambelii*), showed that AR and 5α-reductase 1 in both pectoralis and gastrocnemius were higher in individuals that were preparing for spring migratory departure when compared to the wintering and pre-alternate molt stages (Pradhan, Ma et al. 2019). It is critical to point out that the elevation in AR and 5α-reductase 1, during spring migratory departure, may be necessary to sensitize the tissue during a period in which circulating levels of androgens are very low. The timing of tissue sampling may be critical for observing seasonal appropriate changes in androgen or estrogen signaling pathways as they temporally align with either muscle hypertrophy or atrophy. Changes in 5α-reductase may be dependent upon the isoform. We did not measure type 1 in this due to poor primer amplification. Counter to our hypothesis, AR and 5α-reductase 2 mRNA expression did not differ between subspecies which suggests that androgen signaling has not been under evolutionary selection despite environmental and life history differences. This is interesting as the androgen pathway would be the most obvious regulator of phenotypic differences in skeletal muscle between subspecies with a resident and migrant strategy.

Studies in domestic chickens and golden manakins have shown the direct effects of androgens on pectoralis muscle gene expression (Chen, Huang et al. 2010, Fuxjager, Barske et al. 2012). Clearly androgens could have a potential role in controlling seasonal changes in muscle size as well as enhancing flight performance. Muscle twitch speed has been positively correlated with AR mRNA expression in golden manakins (Tobiansky, Miles et al. 2020), but interestingly many tropical birds have low concentrations of androgens year round (Day, Fusani et al. 2007). Increased AR mRNA expression, as opposed to marked change in circulating

androgens observed in temperate and arctic birds, may be necessary in equatorial birds to affect androgen signaling and adjust muscle function. Comparative studies routinely find similar patterns between AR expression and flight display performance in birds and leg displays in lizards (Fuxjager, Eaton et al. 2015, Johnson, Kircher et al. 2018). Breeding is an aerobically active period as individuals compete for mates, mate guard, and provide parental care (Wingfield, Krause et al. 2015). Thus, steroidogenic signaling would be expected to be enhanced at this time of year.

*Estrogen and aromatase mRNA expression*

In the pectoralis muscle, increased aromatase mRNA expression in male residents compared to male migrants, and consistently higher ERα expression in residents compared to migrants, regardless of sex, suggests that residents may be more sensitive to estrogenic signaling. There is evidence to suggest that aromatase expression is positively correlated with aromatase activity (Díaz-Cruz, Shapiro et al. 2005) which could likely affect local estrogen signaling in white-crowned sparrows. The enhanced role of estrogenic signaling in controlling skeletal muscle mass, recovery from exercise, ability to metabolize fats, and preventing damage from stress or exercise are conserved across taxa (MacRae, Mahon et al. 2006). Given the importance of long-distance flight it would be expected that migrants would have greater signaling of both estrogen and androgen pathways. Migration encompasses approximately 21% of the annual cycle (Lisovski, Németh et al. 2019), so it is puzzling that evolution has selected for consistently lower estrogen receptor expression throughout the annual cycle in migrants compared to residents. A previous study in golden manakins did not detect any performance correlates with ERα expression (Fuxjager, Eaton et al. 2015). This raises the question of whether

498 a relative balance is always maintained between estrogenic and androgenic pathways that is
499 critical for muscle size, performance, and maintenance.

500 Our unpublished data show that residents have larger cross-sectional areas of muscle
501 fibers compared to migrants during post-breeding, molt, and winter stages of the annual cycle
502 (Ramenofsky et al. in review). Cross sectional area of muscle is reduced in animals with ERα and
503 AR knockout compared to wildtype controls (MacLean, Chiu et al. 2008, Callewaert, Venken et
504 al. 2009, Collins, Mader et al. 2018). These data may suggest in white-crowned sparrows that
505 overall higher ERα expression may be important for controlling cross sectional area of muscle
506 fibers. Migrants have been shown to have smaller cross-sectional areas which may be
507 important for nutrient delivery by reducing the diffusion distance between the capillaries and
508 cellular machinery (Lundgren and Kiessling 1985, Lundgren and Kiessling 1988). Whether this
509 potential mechanism is a consequence of increased ERα mRNA expression or the change in the
510 ratio between AR and ER must be tested empirically. AR is involved in controlling the fiber type
511 composition with AR knockout rodents showing higher prevalence of fast oxidative myosin
512 compared to slow oxidative myosin protein levels (Altuwaijri, Kun Lee et al. 2004). However,
513 migrant white-crowned sparrows only express fast oxidative fibers. In addition, they express a
514 unique myosin heavy chain isoform from winter through preparatory stage of spring migration
515 (Velten, Welch et al. 2016). At this time, we do not know the composition of fiber types in the
516 resident subspecies. It is possible that other fiber types exist due to their resident lifestyle.

517 Both AR and ERα control muscle size in mammals, birds, and fish, but AR is traditionally
518 thought to have a more dominant role (MacRae, Mahon et al. 2006, McFarland, Pesall et al.
519 2013, Yue, Zhao et al. 2018, Schuppe, Miles et al. 2020). Interestingly, AR and ER mRNA

expression were significantly positively correlated in residents but not in migrants although a positive trend was observed. It could be that the balance between estrogen and androgen signaling pathways is critical for regulating the morphology and physiology of skeletal muscle. Residents did not undergo seasonal changes in their pectoralis muscle profile, which could possibly be linked to the effects of higher year-round estrogen signaling. Residents are territorial throughout the year so maintenance of larger muscle size maybe beneficial for territorial defense (Blanchard 1936). This observation suggests that future studies should aim to investigate further estrogen signaling in muscle since our current understanding, especially in birds, is limited.

**Conclusion**

Differences in gene expression associated with sex steroid signaling between migrant and resident subspecies of white-crowned sparrow was observed. Additional studies are needed to investigate the functional significance of enhanced estrogen signaling in residents compared to migrants. Furthering this, additional studies in free-living animals will provide better understanding of whether this is a common finding or one that is unique to migratory birds in general or even white-crowned sparrow subspecies. Extending beyond this study, we do not fully appreciate total androgens and estrogens, their relative ratios and their relationship with intracellular receptors and the enzymes that can modify steroids. Other components of the steroid hormone signaling pathways, not investigated here, may also be involved. Do birds with lower plasma hormones generally have higher enzyme and receptor abundance or vice versa? Future studies can begin to remove the vail of mystery around this important question in environmental endocrinology.

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**Figure and Table Legends**

**Table 1.** Primers utilized in the study.

**Table 2.** A linear mixed effects model investigating the effects of life history (LH) stage, sex, subspecies and their interactions on expression of pectoralis and gastrocnemius muscle mRNA of genes involved in sex steroid signaling in migrant and resident, male and female white-crowned sparrows. Plate was included as a blocking variable to account for variation across qPCR plates.

**Figure 1.** Seasonal changes in pectoralis muscle profile and (B) plasma testosterone (ng/ml) in male and female migrant and resident white-crowned sparrows. Letters denote significant differences between life history stages within a subspecies (migrant or resident). Daggers (†) indicate significant differences between migrants and residents within life history stage. All values represent means + S.E.M.

**Figure 2.** Seasonal changes in mRNA expression measured in pectoralis muscle of migrant and resident white-crowned sparrows for (A) androgen receptor (AR), (B) estrogen receptor alpha

(ERα), (C) 5α-Reductase 2, and (D) aromatase during breeding (BR), pre-basic molt (PBM) and winter (WI) stages. Letters denote significant differences between life history stages within a subspecies (Capital letters for migrants, lower case for residents). Daggers (†) indicate significant differences between migrants and residents within life history stage. All values represent means + S.E.M.

**Figure 3.** Seasonal changes in mRNA expression measured in gastrocnemius muscle of migrant and resident white-crowned sparrows for (A) androgen receptor (AR), (B) estrogen receptor alpha (ERα), (C) 5α- Reductase 2 , and (D) aromatase during breeding (BR), pre-basic molt (PBM) and winter (WI) stages. Letters denote significant differences between life history stages within a subspecies (migrant or resident). A lack of statistical significance is indicated by N.S. Daggers (†) indicate significant differences between migrants and residents within life history stage. All values represent means + S.E.M.

**Figure 4.** Pearson correlations between androgen receptor and estrogen receptor mRNA expression in A) pectoralis and B) gastrocnemius muscles of migrant and resident subspecies of white-crowned sparrows. There was a significant relationship between the genes in the pectoralis muscle for both subspecies but for the gastrocnemius muscle this relationship was positive for residents, only.

**Figure 5.** Discriminant function analysis of mRNA expression of androgen receptor (AR), estrogen receptor alpha (ERα), and aromatase in (A) pectoralis and (B) gastrocnemius muscle of free-living resident (open circles) and migrant (filled circles) white-crowned sparrows sampled during winter (green), breeding (black), and pre-basic molt (red). Discriminant function analysis of mRNA gene expression between gastrocnemius (filled circles) and pectoralis (open circles) muscles in (C) resident (open circles) and (D) migrant (filled circles) white-crowned sparrows sampled during winter (blue), breeding (grey), and pre-basic molt (red-orange). Sex differences were not included in the analysis.

784 Table 1

| Gene | Accession number | Forward sequence | Reverse sequence |
|---|---|---|---|
| 5α-reductase Type 2 (5RED2) | XM_014271093.2 | CCTTTCTTCACTAGAGGCAGACC | TGGATAGTCCGTAAATGTCTTGAG |
| Androgen Receptor (AR) | XM_026794270.1 | GGTCAAATGGGCAAAGGCTC | CCACCCCATAGCAAACACCA |
| Aromatase (AROM) | XM_005483351.3 | CCACCGTGCCCATACTCATC | TTGCAGGCATTCCCTACTCC |
| Estrogen Receptor alpha (ESR1) | XM_026794125.1 | AAACGCCAAAGAGAGGAGCA | ACTTGTCCAAAGGGTGGGAG |
| NDUFA1 | XM_005479464.3 | ATGTGGTACGAGATCCTGCC | TTCTCCAGACCCTTGGACAC |
| YHWAZ | XM_005484436.3 | GTGGAGCAATCACAACAGGC | GCGTGCGTCTTTGTATGACTC |

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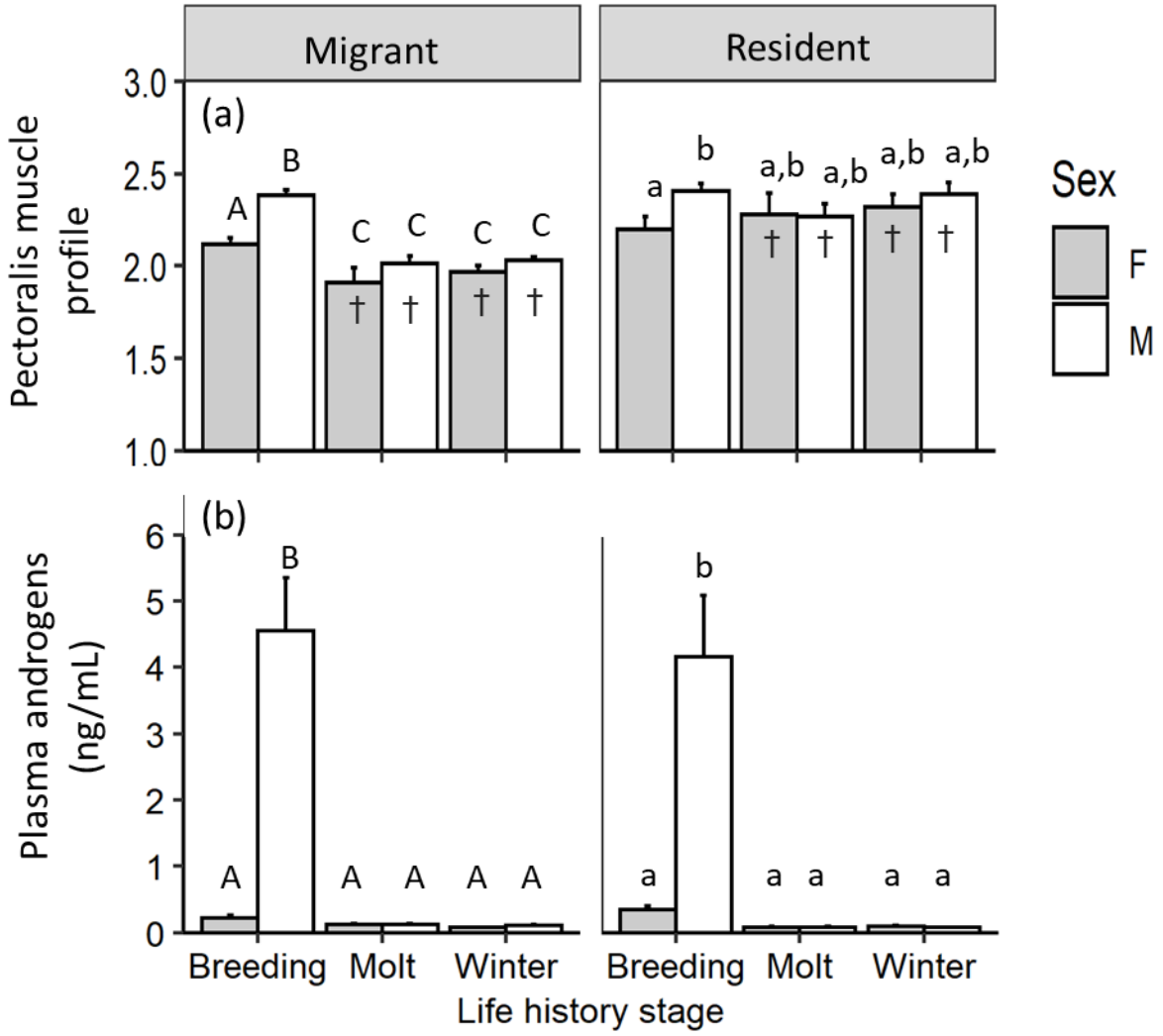
Table 2

| | Pectoralis Androgen Receptor (AR) | | | Pectoralis Estrogen Receptor α (ERα) | | | Pectoralis Aromatase (AROM) | | | Pectoralis 5α-Reductase 2 (5RED) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* |
| LH Stage | **2,89** | **3.77** | **0.027** | 2,89 | 0.71 | 0.49 | 2,75 | 0.40 | 0.67 | 2,84 | 1.1677 | 0.32 |
| Subspecies | 1,89 | 1.18 | 0.28 | **1,89** | **13.17** | **0.0005** | 1,75 | 0.70 | 0.41 | 1,84 | 0.0954 | 0.76 |
| Sex | 1,89 | 3.21 | 0.08 | 1,89 | 0.98 | 0.32 | 1,75 | 0.07 | 0.79 | 1,84 | 0.001 | 0.97 |
| Plate | **1,89** | **16.88** | **0.0001** | **1,89** | **7.64** | **0.007** | 1,75 | 0.73 | 0.39 | **1,84** | **8.0373** | **0.01** |
| LH Stage*Subspecies | 2,89 | 0.88 | 0.42 | **2,89** | **3.40** | **0.04** | 1,75 | 0.97 | 0.38 | 2,84 | 1.1393 | 0.32 |
| LH Stage*Sex | 2,89 | 2.38 | 0.10 | 2,89 | 1.72 | 0.19 | 2,75 | 0.21 | 0.81 | 2,84 | 0.1204 | 0.89 |
| Subspecies*Sex | 1,89 | 0.79 | 0.38 | 1,89 | 1.52 | 0.22 | **1,75** | **4.58** | **0.04** | 1,84 | 0.0492 | 0.83 |
| LH Stage*Subspecies*Sex | 2,89 | 1.22 | 0.30 | **2,89** | **3.43** | **0.04** | 2,75 | 0.71 | 0.49 | 2,84 | 0.0334 | 0.97 |

| | Gastrocnemius Androgen Receptor (AR) | | | Gastrocnemius Estrogen Receptor α (ERα) | | | Gastrocnemius Aromatase (AROM) | | | Gastrocnemius 5α-Reductase 2 (5RED) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* | *D.F.* | *F* | *P* |
| LH Stage | 2,86 | 2.81 | 0.07 | **2,86** | **3.20** | **0.05** | 2,83 | 1.62 | 0.20 | 2,83 | 0.59 | 0.56 |
| Subspecies | 1,86 | 0.24 | 0.62 | **1,86** | **9.72** | **0.002** | 1,83 | 0.09 | 0.76 | 1,83 | 0.03 | 0.87 |
| Sex | 1,86 | 2.14 | 0.15 | **1,86** | **7.96** | **0.01** | 1,83 | 0.19 | 0.67 | 1,83 | 0.10 | 0.75 |
| Plate | **1,86** | **57.85** | **<0.001** | 1,86 | 1.35 | 0.25 | 1,83 | 1.46 | 0.23 | **1,83** | **10.16** | **0.002** |
| LH Stage*Subspecies | 2,86 | 0.08 | 0.92 | 2,86 | 1.52 | 0.22 | 2,83 | 0.20 | 0.82 | 2,83 | 0.15 | 0.86 |
| LH Stage*Sex | 2,86 | 2.06 | 0.13 | 2,86 | 2.95 | 0.06 | 2,83 | 1.95 | 0.15 | 2,83 | 0.37 | 0.69 |
| Subspecies*Sex | 1,86 | 0.34 | 0.56 | **1,86** | **9.03** | **0.003** | 1,83 | 0.37 | 0.55 | 1,83 | 0.01 | 0.93 |
| LH Stage*Subspecies*Sex | 2,86 | 0.86 | 0.43 | 2,86 | 2.56 | 0.08 | 2,83 | 0.38 | 0.69 | 2,83 | 0.10 | 0.91 |

825 Figure 1

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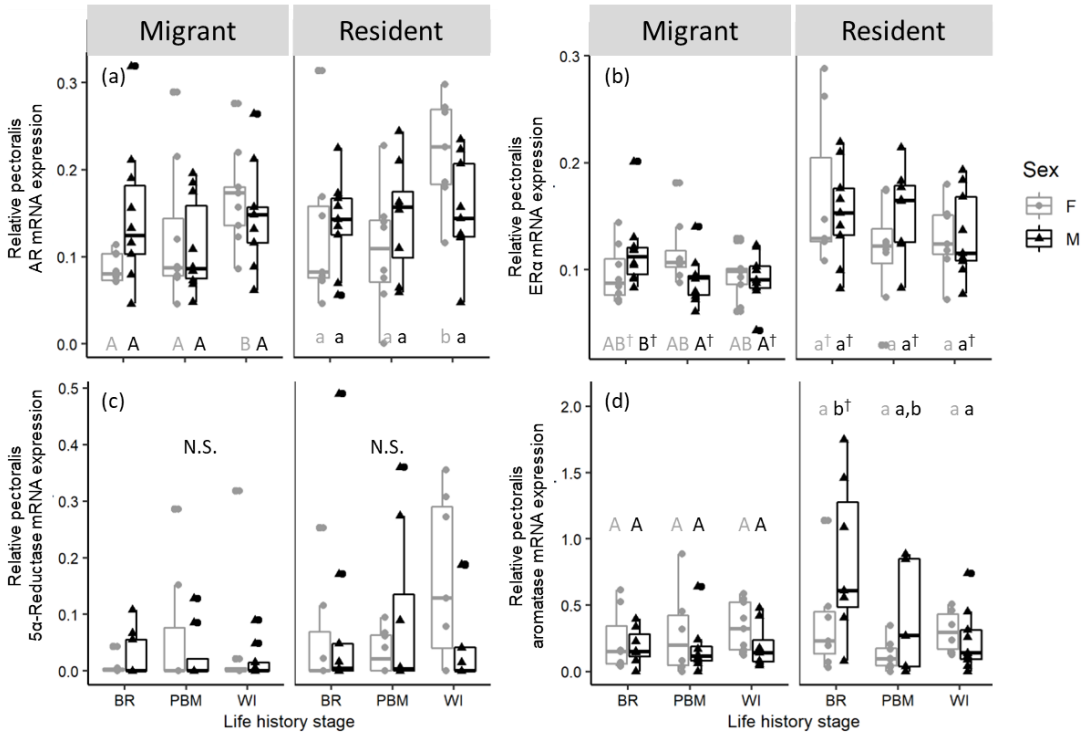
827 Figure 2


Migrant
Resident
(a)
(b)
(c)
(d)
Relative pectoralis AR mRNA expression
Relative pectoralis ERα mRNA expression
Relative pectoralis 5α-Reductase mRNA expression
Relative pectoralis aromatase mRNA expression
N.S.
BR
PBM
WI
Life history stage
Sex
F
M

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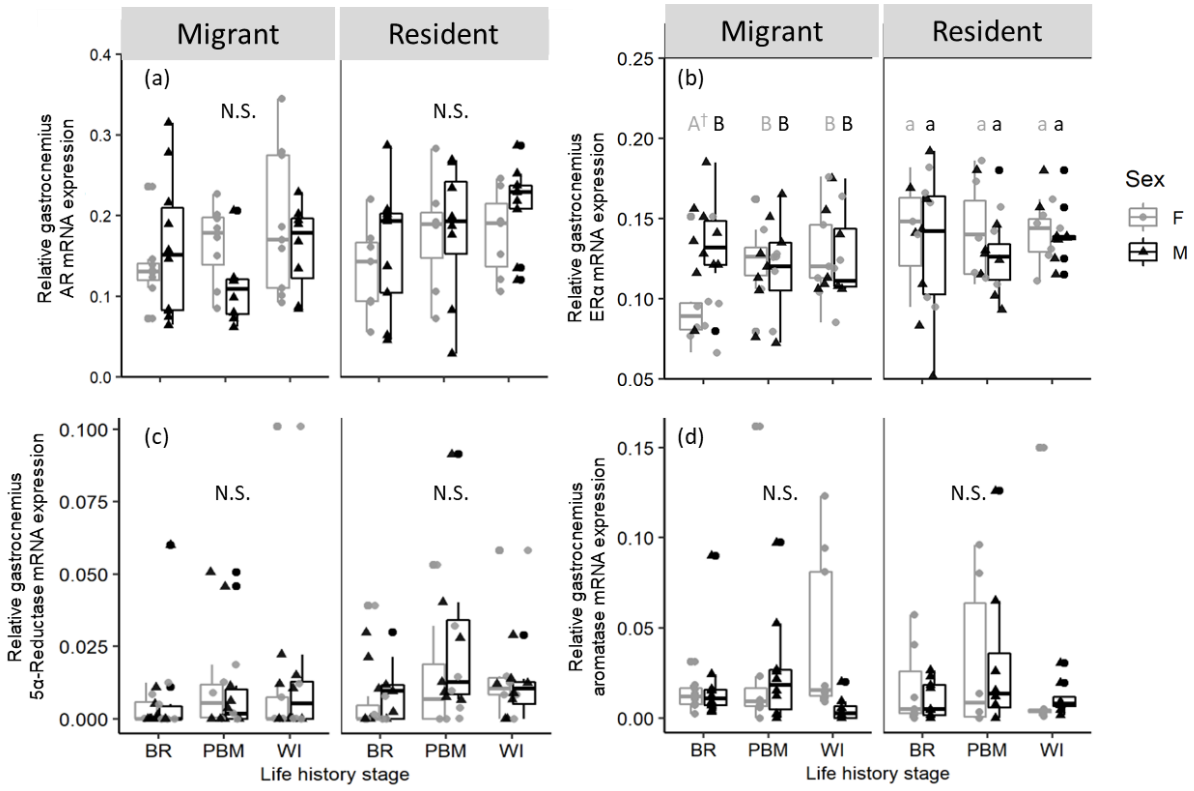
830 Figure 3

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