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Relationship between reproductive hormones and migration distance in a polygynous songbird, the Red-winged Blackbird (*Agelaius phoeniceus*)

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Abstract

Many bird species migrate to southern overwintering locations to avoid harsh conditions at their breeding grounds, but at the cost of an energetically demanding migration that may delay their spring reproductive development. Previous work on the relationship between migration distance and reproductive readiness has primarily focused on early season baseline testosterone in both males and females. However, for females, testosterone alone may not be the appropriate measurement of reproductive development. Estradiol, a metabolite of testosterone that is essential for breeding behaviors and reproduction, should also be measured. Furthermore, baseline testosterone varies throughout the day and may change due to social interactions that occurred prior to sampling. Injection of gonadotropin-releasing hormone (GnRH) elicits an individual's maximum potential testosterone production, minimizing daily and social variation. We explored relationships between migration distance and reproductive status after arrival to the breeding ground in Red-winged Blackbirds (*Agelaius phoeniceus*). We predicted that individuals that travel a shorter distance will have higher levels of reproductive hormones upon arrival given they are able to invest less in migration and more in reproduction. This is important because individuals that breed earlier often have higher reproductive success. In females, we measured baseline estradiol and testosterone. In males, we assessed baseline and GnRH-induced testosterone. Hormone values were related to migration distance, estimated by stable isotope analysis of claw samples collected before breeding began in eastern North Dakota. We found that males with shorter inferred migration distances have higher baseline testosterone upon arrival. However, inferred migration distance was not correlated with GnRH-induced testosterone. Female inferred migration distance was not correlated with baseline testosterone, but it was correlated with baseline estradiol. Females with higher testosterone had lower estradiol, suggesting that testosterone in females is not a reliable indicator of estradiol levels, thus readiness to breed. Our observations suggest that baseline hormone levels were related to migration distance, but baseline testosterone alone may not provide a complete assessment of a male or female's preparedness to breed following spring migration.

Keywords Overwinter latitude · Carry-over effect · HPG activation · Hydrogen stable isotopes · Reproductive phenology · Declining species

Zusammenfassung

Beziehung zwischen Fortpflanzungshormonen und Zugstrecke bei einem polygynen Singvogel, dem Rotflügelstärling (*Agelaius phoeniceus*).

Viele Vogelarten ziehen in südliche Überwinterungsgebiete, um den rauen Bedingungen in ihren Brutgebieten zu entgehen, allerdings um den Preis eines energieaufwendigen Zuges, der ihre Fortpflanzung im Frühjahr verzögern kann. Frühere

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Arbeiten über den Zusammenhang zwischen Zugstrecke und Fortpflanzungsbereitschaft konzentrierten sich in erster Linie auf den Testosteron-Basiswert von Männchen und Weibchen zu Beginn der Saison. Aber bei Weibchen ist Testosteron allein möglicherweise nicht das geeignete Maß für die Entwicklung der Fortpflanzung. Östradiol, ein Testosteron-Metabolit, der für das Brutverhalten und die Fortpflanzung wichtig ist, sollte ebenfalls gemessen werden. Außerdem schwankt der Testosteron-Basiswert im Laufe des Tages und kann sich aufgrund von sozialen Interaktionen kurz vor der Probenentnahme ändern. Durch die Injektion von Gonadotropin-Releasing-Hormon (GnRH) wird die für ein Einzeltier maximal mögliche Testosteronproduktion ausgelöst, wobei die täglichen und sozialen Schwankungen minimiert werden. Wir untersuchten den Zusammenhang zwischen der Zugstrecke und dem Fortpflanzungsstatus vom Rotflügelstärling (*Agelaius phoeniceus*) nach der Ankunft im Brutgebiet und sagten voraus, dass Individuen, die eine kürzere Strecke zurücklegen, bei ihrer Ankunft einen höheren Gehalt an Fortpflanzungshormonen aufweisen, da sie weniger in den Zug und mehr in die Fortpflanzung investieren können. Dies ist deshalb wichtig, weil Tiere, die sich früher fortpflanzen, oft einen höheren Fortpflanzungserfolg haben. Bei den Weibchen bestimmten wir den Basiswert von Östradiol und Testosteron, bei den Männchen den Basiswert und das GnRH-induzierte Testosteron. Die Hormonwerte wurden zur Zugstrecke in Relation gesetzt, die anhand der Analyse stabiler Isotope von Krallenproben, gesammelt vor dem Brutbeginn im östlichen North Dakota, geschätzt wurde. Wir stellen fest, dass der Basiswert des Testosterons bei Männchen mit kürzeren Zugstrecken bei der Ankunft auf einem höheren Niveau lag. Aber die ermittelte Zugstrecke korrelierte nicht mit dem GnRH-induzierten Testosteron. Bei den Weibchen korrelierte die Zugstrecke nicht mit dem Testosteron-Basiswert, wohl aber mit dem Basiswert des Östradiols. Weibchen mit einem höheren Testosteronspiegel wiesen einen niedrigeren Östradiolspiegel auf, was darauf hindeutet, dass Testosteron bei weiblichen Tieren kein zuverlässiger Indikator für den Östradiolspiegel und damit für die Bereitschaft zur Fortpflanzung ist. Unsere Ergebnisse deuten darauf hin, dass die Hormon-Basiswerte mit der Zugstrecke zusammenhängen, der Testosteron-Basiswert allein aber vermutlich keinen vollständigen Aufschluss über die Brutbereitschaft eines Männchens oder Weibchens direkt nach dem Frühjahrzug gibt.

Introduction

Migratory birds leave the breeding grounds in the fall and travel to latitudes that provide food (Newton and Dale 1996) and reduce the threat of potentially lethally cold temperatures (Duff et al. 2011). They must then traverse long distances in the spring to return to their breeding location. However, there is often significant variation in overwinter locations and migration distances observed within species (Robbins et al. 2014) and among individuals that breed together (Finch et al. 2017; Ketterson and Nolan, 1983). It has often been observed that younger, smaller birds, or females of sexually dimorphic species migrate farther south due to bioenergetic constraints or social subordination, especially in territorial songbirds (Dolbeer 1982; Ketterson and Nolan 1983).

With bird populations in steep decline (Rosenberg et al. 2019), there is a vital need to understand how life events, such as migration, influence key physiological processes and mechanisms that regulate reproduction and survival in subsequent seasons (Fudickar and Ketterson 2018). Relatively few studies on physiology and reproduction have incorporated how processes occurring in one season affect processes in another season of the annual cycle; understanding these carry-over effects is crucial for conserving species (Marra et al. 2015). Research on carry-over effects on migratory songbirds has been particularly difficult, owing to the great distances individuals often move throughout the year, and the limited ability to track individuals. Assessment of

stable isotopes, which are incorporated from environmental sources into inert tissues (e.g., claw tips), allows researchers to estimate an individual's relative wintering latitude and investigate how overwinter location and spring migration distance influences the subsequent breeding season (Hobson 1999). Yet, research relating estimates of overwinter location and migration distance with reproductive physiology (i.e., readiness to breed) upon arrival to the breeding grounds remains sparse (cf. Lymburner et al. 2016; Covino et al. 2017, 2018).

In songbirds, individuals of a breeding population that initiate breeding early often have higher reproductive success than members of the population starting later in the season with various explanations including habitat quality, food availability, parasite and predation pressure, or parental investment (reviewed in Verhulst and Nilsson 2008). Preparation for breeding requires an activation of the hypothalamic–pituitary–gonadal axis (HPG axis) and gonadal development. In songbirds, the processes leading from the regressed gonadal state in the winter to full reproductive development each spring do not happen quickly. It takes approximately 1 month for a male songbird to regrow his testes from a regressed state (Ramenofsky et al. 2012). While male songbirds are more reliant on photoperiod for gonadal development, female songbirds ultimately determine the timing of breeding and undergo rapid reproductive development in response to cues on the breeding ground such as food availability (Ball and Ketterson 2008; Caro et al. 2009). Given the importance

of testosterone, and its metabolite estradiol, for breeding behaviors and reproduction (Adkins-Regan 2005; Ketterson et al. 2005; Rosvall 2013a, b; Wingfield et al. 2001), these hormones have been used as indicators of the reproductive development of birds traveling toward and arriving at their breeding site in the spring.

Increased testosterone on the wintering grounds may stimulate and support migration (Tonra et al. 2011, 2013), but individuals wait until arriving at the breeding ground to reach full reproductive maturity (Covino et al. 2017). A bird cannot be fully prepared to breed while migrating because migration and reproduction are both energetically demanding (Ramenofsky 2011). However, although they will not arrive fully prepared to breed, a shorter migration distance may allow an individual to devote more resources to reproduction than an individual traveling a longer distance. A recent study of Song Sparrows (*Melospiza melodia*) showed that migratory distance during spring migration was negatively correlated to baseline testosterone levels upon arrival to the breeding ground in both males and females (Lymburner et al. 2016). A single blood sample measuring baseline testosterone, however, may not be fully capable of comparing reproductive development across individuals because circulating levels of sex steroid hormones fluctuate on daily endogenous rhythms (Guchhait and Haldar 1999; Needham et al. 2017) and in response to social interactions (Wingfield 1985; Wingfield et al. 1990, 2011). Often researchers cannot control for social interactions, or lack thereof, that may influence baseline testosterone levels prior to capture.

Administering an injection of gonadotropin-releasing hormone (GnRH) and measuring the subsequent testosterone peak may be a useful method to investigate relationships between migratory distance and reproductive physiology upon arrival to the breeding ground. GnRH injections stimulate the reproductive endocrine axis causing downstream production of testosterone to show the maximum amount of testosterone that a bird can produce at the time of sampling and minimize the variations arising from daily and social influences (Apfelbeck and Goymann 2011; Devries et al. 2011; Jawor et al. 2006; Needham et al. 2017). However, when sampling small songbirds, researchers will need to consider if they can obtain both a baseline and postGnRH-injection sample given the limited allowable blood sample volume (e.g., Graham et al. 2019).

While both males and females show variation in the amount of testosterone they produce throughout the year, circulating testosterone regulates traits differently in both sexes (Goymann and Wingfield 2014). Previously, there has been a significant male bias in studies on the mechanisms of reproduction in birds, but to understand reproductive timing researchers must look at both sexes (Kimmit 2020). Due to

physiological differences between sexes, such as the role of circulating testosterone, both males and females should be considered to avoid a sex bias (Caro 2012). In our study, given the role of testosterone in territory defense and mate attraction, we chose to measure baseline and post-GnRH testosterone in males (Beletsky et al. 1989; Enstrom et al. 1997). In females, we chose to measure estradiol in addition to testosterone. This understudied yet important hormone for female reproduction peaks when yolky follicles are developing (Williams et al. 2004), and it is necessary for female copulation solicitation and the auditory processing of song (Remage-Healey et al. 2010; Searcy and Capp 1997). Therefore, assessing female estradiol levels in addition to baseline testosterone after arrival to the breeding grounds is likely to inform female readiness to breed. Due to limitations on blood volume that can safely be obtained from females, we were unable to assess GnRH-induced hormones simultaneously with baseline testosterone and estradiol. Additional knowledge on GnRH-induced testosterone levels in males and baseline estradiol levels in females will allow us to build on our knowledge of how baseline levels of testosterone relate to reproductive readiness in each sex.

Here, we investigate the hypothesis that migration, an energetically costly life-history stage, and the distance an individual must migrate influences reproductive hormones after arrival to the breeding grounds in Red-winged Blackbirds (*Agelaius phoeniceus*). Relative migration distance was estimated using hydrogen stable isotopes in claws. In males, we assess relationships between estimated migration distances and baseline and GnRH-induced testosterone levels. In females, we assess relationships between estimated migration distance with baseline estradiol and testosterone levels. Based on previous banding and geo-logger data (Dolbeer 1982; Stonefish et al. 2021), we predicted that female blackbirds would overwinter farther south and have a longer spring migration. Furthermore, within sexes, we hypothesized that individuals migrating a shorter distance will have elevated reproductive hormones upon arrival, because they will be investing fewer resources in migration than those migrating a longer distance. We also predicted that migration distance in males will be more strongly related to GnRH-induced testosterone than with baseline testosterone because GnRH-induced testosterone values will not be biased by time of day or social interactions. In females, we hypothesized that baseline testosterone and estradiol will both be correlated with migration distance due to the importance of both hormones in female breeding behaviors, aggressive interactions, and egg laying. Similarly, we predicted that females traveling a shorter distance will also lay eggs earlier. Finally, we predicted that baseline testosterone and estradiol in females will be correlated as estradiol is a metabolite of testosterone.

Methods

Study site and capture

We studied a population of Red-winged Blackbirds at the Alice Waterfowl Production Area (46°46'36" N, 97°30'31" W) near Alice, North Dakota USA during the spring and summer of 2018 and 2019. This site has ample, consistent, wetland habitat for Red-winged Blackbird breeding with large areas of cattail (*Typha* spp.). The Red-winged Blackbird is polygynous and protandrous with males arriving to the breeding site before females (Cristol et al. 1999). We captured individuals early in the breeding season prior to clutch initiation in mist nets and walk-in traps baited with cracked corn. The walk-in traps were placed on the ground or elevated above the water in cattails. We monitored mist nets every 20 min and walk-in traps every 30–45 min and placed a unique combination of a USGS aluminum band and three color bands for identification on each captured bird. Researchers recorded mass, tarsus, and wing cord measurements for all birds. Our analysis only included known breeders or individuals captured multiple times at our study site, indicating that they spend the summer at this location ($n=28$ females, $n=31$ males).

Estimation of winter location

We took a 2–3 mm claw sample from the middle toe of the bird's right foot. The University of Regina processed the claw samples. For $\delta^2\text{H}$ analysis, we washed and dried claw material and weighed c. 0.35 mg into silver capsules. The samples were reduced to H_2 by reaction with Cr under helium flow at 1030 °C with a Eurovector 3000 (Milan, Italy—<http://www.eurovector.it>) elemental analyzer. We measured the resultant H_2 gas for $\delta^2\text{H}$ in an Isoprime (Crewe, UK) continuous flow stable isotope mass spectrometer and corrected for H exchange using the comparative equilibration technique of Wassenaar and Hobson (2003) and two keratin calibrated standards: Caribou Hoof Standard (EC1, $\delta^2\text{H} = -197$ per mil) and Kudu Horn Standard (EC2, $\delta^2\text{H} = -54.1$ per mil). All measurements are reported in δ -notation as the non-exchangeable claw H component in parts per thousand relative to the Vienna Standard Mean Ocean Water (VSMOW)–Standard Light Antarctic Precipitation (SLAP) scale. Measurement error was estimated to be ± 2 per mil based on replicate within-run measurements of standards.

Claw samples provided a stable hydrogen isotope ratio ($\delta^2\text{H}$). These ratios vary in the United States from the southeast to the northwest portion of the country (Taylor 1974) and provided an estimation of the relative over-winter latitude where the claw was grown (Gagnon and

Hobson 2009). We do not assign a specific winter location to the individuals because claws continually grow during migration and incorporate new isotopic signatures (Mazerolle and Hobson 2005) and variation exists for individuals from a given site (Hobson et al. 2014). However, we feel confident comparing relative migration distance between $\delta^2\text{H}$ values, given that a study of five passerine species estimated that claw samples provide information on the premigratory habitat for at least 3–4 weeks after arrival (Bearhop et al. 2003) and a study in Song Sparrows found that their claw tips represented tissue grown 3 months prior to sampling (Kelly et al. 2016). Additionally, we compared $\delta^2\text{H}$ values and spring migration distance estimated from five GPS tags deployed at our study site, and these were correlated in our birds ($t=3.25$, $df=1,3$, adjusted $R^2=0.71$, $P=0.047$, $n=5$; see Supplemental Material).

Blood sampling and GnRH injections

We took a blood sample from the brachial (wing) vein of each individual using heparinized capillary tubes. The average time from capture to baseline blood sample was 13.31 min, and the average time from capture to GnRH blood sample was 51.44 min. Given the limited volume that we can safely take from a ~40–50 g songbird and the large amount of plasma required for the estradiol assay, we were able to measure baseline estradiol and testosterone in females but not GnRH-induced levels. For females, we took a 350 μL blood sample after capture to measure testosterone and estradiol in both years. For males, we took a 200 μL baseline sample for testosterone in 2018 and a 200 μL baseline and 200 μL post-GnRH sample to measure testosterone 30 min after the GnRH injection in 2019. We injected GnRH diluted in PBS into the pectoral muscle at a dose of 2 mg/kg. Researchers collected all baseline samples between 06:30 and 11:10 AM CDT. We stored samples on ice in the field and separated the plasma using an Eppendorf™ Centrifuge 5430R in the lab upon returning that same day. We separated plasma from the red blood cells and stored it in an Eppendorf™ tube at -20 °C until assayed.

Testosterone enzyme immunoassay

We measured testosterone using a testosterone ELISA kit (Enzo Life Sciences ADI-900-065) following the manufacturer's instructions. Briefly, we extracted 30 μL of plasma two times with ether. The extracted sample was then dried with N_2 gas at 25°C and reconstituted overnight with 300 μL of assay buffer. We followed the assay kit instructions and all samples were run in duplicate (100 μL per well). Plates

had intra-plate variation of 0.00, 8.66, 9.49, 9.56, 9.68, and 10.55%, and inter-plate variation was 15.94%. Samples with undetectable values were assigned the lowest value detectable by the kit which was 5.67 pg/mL (2 females and 0 males). We excluded samples that had a duplicate % CV value > 10% (3 males).

17 β -Estradiol enzyme immunoassay

We measured estradiol levels using a 17 β -estradiol high sensitivity ELISA (enzyme-linked immunosorbent assay) kit (Enzo Life Sciences ADI-900-174) following the manufacturer's instructions. Briefly, we extracted 100 μ L of plasma three times with ether. The extracted sample was then dried with N₂ gas at 25 °C and reconstituted overnight with 250 μ L of assay buffer. We ran all samples in duplicate (100 μ L per well). The estradiol plates had an intra-plate variation of 4.35 and 10.64%, and inter-plate variation was 7.86%. One sample with an undetectable value was assigned the lowest value detectable by the kit which was 14 pg/mL. We excluded samples that had a duplicate % CV value > 10% (3 females). This extraction protocol using songbird plasma has been shown to have high efficiencies (~90%) (Ronald et al. 2017; Rosvall et al. 2013; Voigt and Leitner 2013), and both the testosterone and estradiol assays have been validated in a wide range of species, including another Icterid species (Ronald et al. 2017; Wilcoxon et al. 2015).

Clutch initiation observations

We searched cattail-dominated wetlands for nests in the spring and summer of 2018 and 2019 and observed nests throughout the breeding season to investigate the relationship between reproductive timing and migration distance during the annual cycle. Once a nest was located, we checked it every 3 days to determine clutch initiation, assuming females lay one egg per day until clutch completion and that incubation lasts for approximately 12 days (Martin 1995; Muma 1986). From the 17 nests used in our analysis, we found 14 before clutch initiation, two with eggs before clutch completion, and one after clutch completion. We identified parents using the leg band color combination of the male and female that defended the nest as an observer approached.

Statistical analyses

We used R version 3.5.2 (R Core Team, 2018) to perform all statistical analyses. We log-transformed all hormone values to meet assumptions of a normal distribution. Fisher's F test was used to test for equal variance among groups. We felt that it was more appropriate to run two separate models given difference in the variance in male testosterone

vs female baseline testosterone and the small sample size. We ran a Levene's Test for Homogeneity of Variance for untransformed values ($P < 0.001$) and log-transformed values ($P < 0.001$), which showed that male and female testosterone levels are quite different in their variance. We ran a linear model to determine if males and females were traveling different distances in the spring with $\delta^2\text{H}$ as our dependent variable and sex as our independent variable. For males, we used separate linear models to examine the relationship between baseline testosterone or GnRH-induced testosterone with $\delta^2\text{H}$ values (i.e., migration distance estimate) and included capture date and year. Year was only included in baseline testosterone models as GnRH-induced samples were only collected in 2019. We ran linear models for females to examine the relationship among the dependent variables of pre-breeding hormone levels, baseline estradiol or testosterone, with the independent variables of $\delta^2\text{H}$ values, capture date, and year. To examine the relationship between baseline testosterone and its metabolite estradiol in females, we ran a linear model with estradiol as the dependent variable and testosterone and capture date as the independent variables. We ran three linear models to examine the relationship between the dependent variable of clutch initiation date and the independent variables of $\delta^2\text{H}$, estradiol or testosterone for females in their respective models. The time between removing the bird from the trap and blood sampling was not a significant variable, so we did not include it in our models on the relationship of reproductive hormones and stable isotope values. Bird ID was not included in linear models as a random effect because there were only four males and two females with $\delta^2\text{H}$ values in both 2018 and 2019. For the linear models, the results of the summary function in R version 3.5.2 (R Core Team 2018) are reported.

Results

Capture of Red-winged Blackbirds

In 2018, we captured and sampled males between 28 April and 15 May (median = 07 May) and females between 11 and 22 May (median = 18 May). In 2019, we captured and sampled males between 07 April and 23 May (median = 05 May) and females between 26 April and 23 May (median = 20 May). From individuals with stable isotope data, female mass ranged from 41.5 to 50.0 g ($\bar{x} = 44.9 \pm 0.5$ SE g, $n = 25$) and male mass ranged from 60.5 to 76.5 g ($\bar{x} = 69.5 \pm 0.8$ SE g, $n = 27$). Female tarsus ranged from 24.1 to 27.3 mm ($\bar{x} = 25.97 \pm 0.15$ SE mm) and male tarsus ranged from 27.5 to 30.9 mm ($\bar{x} = 29.40 \pm 0.15$ SE mm). Female wing cord ranged from 100 to 108 mm ($\bar{x} = 105.04 \pm 0.39$ SE mm) and male wing cord ranged from 120 to 136 mm

($\bar{x} = 127.48 \pm 0.63$ SE mm). The earliest clutch initiation from our sampled birds was 22 May in 2018 and 23 May in 2019. All male blackbirds included in the hormone analyses were after-second year (ASY) territory holders, and all females included in the hormone analyses were at least 1 year old and aged as after-hatch-year (AHY).

Hydrogen stable isotopes

We collected claw tissue for stable isotopes from 25 birds in 2018 (10 females and 15 males) and 34 birds in 2019 (18 females and 16 males). Females with both claw samples and hormone samples had a mean $\delta^2\text{H}$ value of -33.9 ± 2.5 SE and ranged from -51 to -6 . Males with both claw samples and hormone samples had a mean $\delta^2\text{H}$ value of -56.7 ± 1.6 SE and ranged from -45 to -72 . A linear model revealed that males traveled a significantly shorter distance than females as estimated by the $\delta^2\text{H}$ values ($t = -7.06$, $df = 1,57$, adjusted $R^2 = 0.46$, $P < 0.001$, $n = 59$, Fig. 1).

Relationship between sex steroids and migration distance

Male baseline testosterone ranged from 0.10 to 4.51 ng/mL ($\bar{x} = 1.18 \pm 0.24$ SE ng/mL) and GnRH-induced testosterone ranged from 0.52 to 10.12 ng/mL ($\bar{x} = 6.20 \pm 0.68$ SE ng/mL). Males with $\delta^2\text{H}$ values indicating a shorter distance traveled had higher baseline testosterone ($t = -2.662$, $df = 1,14$, $P = 0.02$, $n = 18$; Fig. 2a). There was no significant relationship between male post-GnRH testosterone and $\delta^2\text{H}$ values ($t = 0.726$, $df = 1,12$, $P = 0.48$, $n = 15$; Fig. 2b). Day of capture was significantly positively related with male post-GnRH testosterone ($t = 2.310$, $df = 1,12$, $P = 0.04$) but not with baseline testosterone ($t = 0.813$, $df = 1,14$, $P = 0.43$); males captured later in the season had higher GnRH-induced

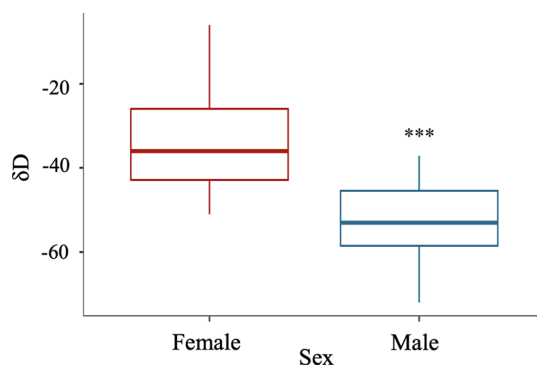


Fig. 1 Female and male Red-winged Blackbird (*Agelaius phoeniceus*) hydrogen stable isotope values ($\delta^2\text{H}$) from claw tissues collected at the breeding grounds in eastern North Dakota upon spring arrival from April to May in 2018–2019. Females traveled significantly farther than males as denoted by asterisk *** $P < 0.001$

levels than earlier-captured males. Individuals that traveled from a more northern relative wintering latitude had a smaller difference between maximum testosterone output and baseline levels ($t = 2.55$, $df = 1,10$, $P = 0.03$, $n = 13$; Fig. 2c).

Female baseline testosterone ranged from 0.06 to 0.27 ng/mL ($\bar{x} = 0.13 \pm 0.01$ SE ng/mL). Estradiol ranged from 35.0 to 355.0 pg/mL ($\bar{x} = 144.1 \pm 21.7$ SE pg/mL). In females, we observed no relationship between $\delta^2\text{H}$ values and baseline testosterone ($t = -0.24$, $df = 1,21$, $P = 0.81$, $n = 25$; Fig. 3a), but it was correlated with baseline estradiol, showing that females with a shorter migration distance have higher levels of estradiol at the time of sampling ($t = 2.23$, $df = 1,11$,

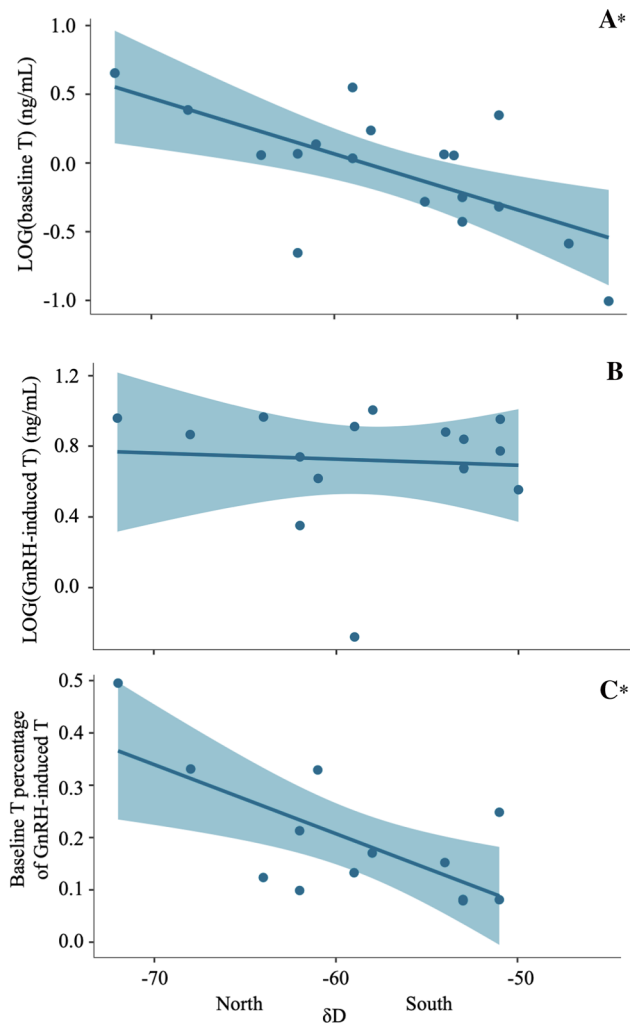


Fig. 2 **A** Log of baseline testosterone (ng/mL)*, **B** log of GnRH-induced testosterone (ng/mL), and **C** baseline testosterone (ng/mL) percentage of GnRH-induced testosterone (ng/mL)* as a function of the relative wintering latitude (i.e., migration distance) for male Red-winged Blackbirds (*Agelaius phoeniceus*). We collected blood for hormone analyses and a claw tissue for stable isotope analyses ($\delta^2\text{H}$) at the breeding grounds in eastern North Dakota upon spring arrival from April to May in 2018–2019; * $P < 0.05$

$P = 0.048$, $n = 15$; Fig. 3b). Year was significant in both models with $P < 0.01$ ($t = 3.138$, $df = 1,21$) for testosterone and $P < 0.001$ ($t = -4.725$, $df = 1,12$) for estradiol. In 2018, females had lower testosterone ($\bar{x} = 0.10 \pm 0.01$ SE ng/mL) but higher estradiol ($\bar{x} = 212.20 \pm 37.37$ pg/mL), and in 2019, females had higher testosterone ($\bar{x} = 0.15 \pm 0.01$ ng/mL) and lower estradiol ($\bar{x} = 98.69 \pm 12.27$ pg/mL). In our study population, the first laid egg was observed on 12 May 2018 and 23 May 2019. We observed no relationship between female $\delta^2\text{H}$ values and lay date ($t = 1.59$, adjusted $R^2 = 0.09$, $df = 1,15$, $P = 0.13$, $n = 17$, see supplemental Fig. 1), estradiol ($t = 0.65$, adjusted $R^2 = -0.07$, $df = 1,8$, $P = 0.54$, $n = 10$), or testosterone ($t = 0.15$, adjusted $R^2 = -0.07$, $df = 1,14$, $P = 0.88$, $n = 16$). We observed a negative relationship between baseline levels of testosterone and estradiol ($t = -2.821$, $df = 1,11$, $P = 0.02$, $n = 14$; Fig. 4); individuals with elevated estradiol had lower circulating levels of testosterone.

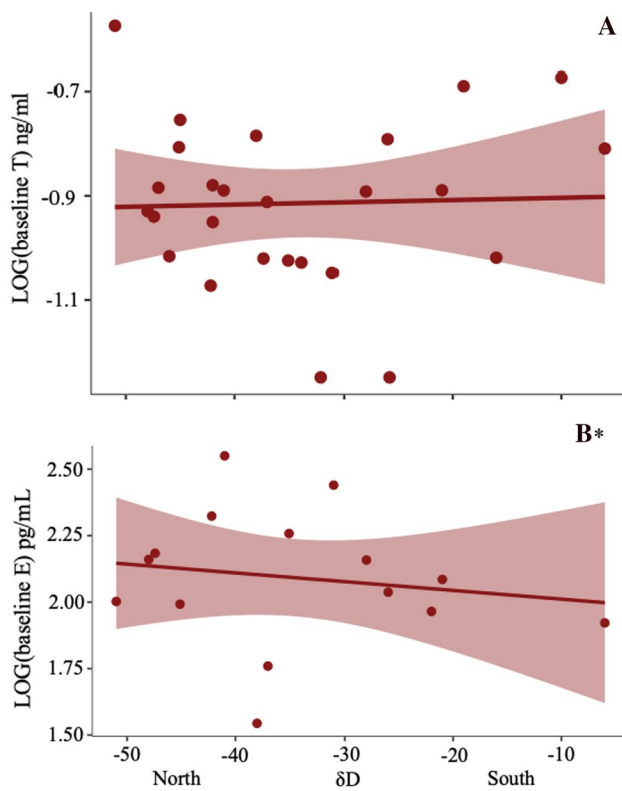


Fig. 3 **A** Log of baseline testosterone (ng/mL); **B** log of baseline estradiol (pg/mL)* as a function of the relative wintering latitude (i.e., migration distance) for female Red-winged Blackbirds (*Agelaius phoeniceus*). We collected blood for hormone analyses and a claw tissue for stable isotope analyses ($\delta^2\text{H}$) at the breeding grounds in eastern North Dakota upon spring arrival from April to May in 2018–2019; * $P < 0.05$

Discussion

We hypothesized that individuals migrating a shorter distance would arrive with more developed gonads capable of producing higher levels of reproductive hormones, suggesting earlier onset of reproductive readiness than conspecifics within their sex that migrated a longer distance. We did find, in both males and females, that baseline hormone levels were related to migration distance. In males, we observed that baseline testosterone was elevated in shorter distance migrants. In females, we found that baseline estradiol was elevated in shorter distance migrants, but there was no relationship between migration distance and baseline testosterone. In females, we found that baseline estradiol was elevated in shorter distance migrants, but there was no relationship between migration distance and baseline testosterone. Lay date was not associated with migration distance or reproductive hormones in females.

Not surprisingly, our stable isotope analysis confirmed that females overwintered farther from the breeding grounds than males. This difference in relative wintering latitude and migration distance for males and females could be due to sexually dimorphic size differences in this species (Dolbeer 1982). Male Red-winged Blackbirds weigh approximately 50% more than females (Yasukawa and Searcy 2019) and the larger body size may provide benefits for heat conservation (Smith et al. 1995). Males may also stay closer to the breeding grounds while overwintering to arrive early and claim the best territories as seen in Dark-eyed Juncos (*Junco hyemalis*) (Ketterson and Nolan 1976). Since Red-winged Blackbirds are a polygynous species, a high-quality territory that can support multiple females greatly increases a male's reproductive success (Beletsky et al. 1992). This is likely why males are overwintering farther north than females.

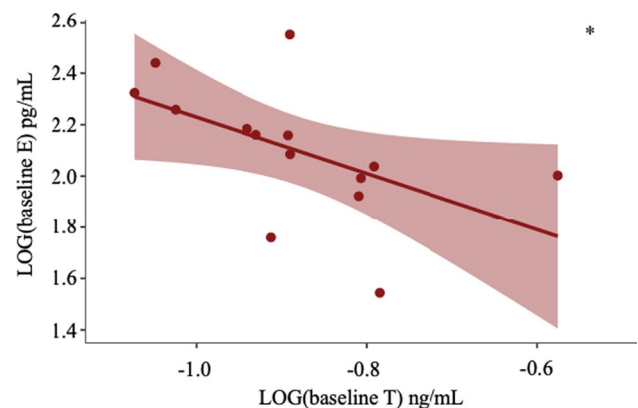


Fig. 4 Log of baseline estradiol (pg/mL)* as a function of the log of baseline testosterone (ng/mL) for female Red-winged Blackbirds (*Agelaius phoeniceus*). We collected blood for hormone analyses at the breeding grounds in eastern North Dakota upon spring arrival from April to May in 2018 and 2019; * $P < 0.05$

Our finding of higher baseline androgens in males with a shorter migration was similar to what is observed in a socially monogamous songbird, the Song Sparrow (*Melospiza melodia*; Lymburner et al. 2016). Dark-eyed Junco males captured on their overwintering grounds and housed under natural photoperiods have larger testes in early spring if they were overwintering closer to their breeding grounds (based on feather stable isotopes) compared with males that were overwintering farther from their breeding grounds (Fudickar et al. 2016; Singh et al. 2021). Earlier reproductive development in short-distance migrants is consistent with our observation of elevated testosterone in the birds that overwintered closer to their breeding grounds where they were sampled.

Migration distance in female Red-winged Blackbirds was not correlated with baseline testosterone, but it was correlated with baseline estradiol. This is in contrast to a previous study conducted over several years indicating that female Song Sparrows migrating a shorter distance have increased testosterone levels upon arriving to the breeding grounds (Lymburner et al. 2016). If blackbirds, similar to other female songbirds, do not reach full breeding development until after they arrive to the breeding grounds (Covino et al. 2018), it may be difficult to relate migration distance with testosterone levels because they may all be at a consistently low level. On the contrary, our testosterone levels could be near peak levels for females in this population. Red-winged blackbird females in a more southern breeding location had their highest testosterone levels soon after they arrived to the breeding grounds (Cristol and Johnsen 1994), and the levels we report are within the range reported from this more southerly population. However, without knowledge of the ovarian development in these females, a second blood sample later in the breeding season, or a post-GnRH blood sample it is difficult to know how these hormone values relate to a female's maximum levels. It is also possible that baseline testosterone and estradiol levels in females and baseline testosterone levels in males could be lower due to time spent in traps, as acute stress has been shown to decrease testosterone (Deviche et al. 2012). Although, effects of capture and handling time, similar to the length of time reported in the current manuscript, caused only marginal effects on baseline (and GnRH-induced) testosterone in another songbird species (Jawor et al. 2006).

Interestingly, while males that traveled a shorter distance (i.e., overwintered closer to breeding grounds) had elevated baseline testosterone compared with longer distance migrants, there was no relationship between estimated migration distance and GnRH-induced testosterone levels. This lack of relationship was unexpected given that GnRH induces the testes to secrete maximum testosterone levels, removing potential variation from timing of sampling or social encounters prior to capture. Furthermore,

GnRH-induced testosterone has been found to be repeatable and has been related to reproductive traits of interest (McGlothlin et al. 2008; Needham et al. 2017). However, the males migrating a shorter distance had a smaller increase in the GnRH-induced testosterone levels over their baseline testosterone levels when compared with males that migrated a longer distance. These results suggest that shorter distance males may have been secreting baseline testosterone close to their maximum physiological level, while longer distant migrants were secreting testosterone at a level lower than which they were physiologically capable of producing. To arrive early and claim the best territories for attracting females, some males overwinter closer to the breeding grounds than others (Ketterson and Nolan 1976). The challenge hypothesis posits that variation in baseline male testosterone is influenced by social interactions to guard a mate or defend a territory (Wingfield et al. 1990). Goymann et al. (2019) proposed that variation in testosterone levels may be more related to interactions between males and reproductively active females than male–male interactions. In support of this, the majority of our male Red-winged Blackbird samples were collected while females were arriving to territories (see supplemental Fig. 2). Unfortunately, our study did not collect data on territory disputes or quality to confirm or refute these hypotheses. Future studies should measure both baseline and GnRH-induced testosterone to better understand relationships between migration distance, territorial behavior upon arrival, and the maximum level of hormones that an individual can produce.

Although some studies, such as Lymburner et al. (2016), have measured female testosterone, we are not aware of any previous studies using female estradiol levels to investigate the relationship between migratory distance and reproduction in wild songbirds at their breeding ground. This is surprising given estradiol's role in breeding behaviors and reproduction in females and the fact that the relationship between testosterone and estradiol in wild birds is not completely clear. One study observed that captive Dark-eyed Junco females with higher estradiol levels also had higher testosterone levels in pooled, post-GnRH plasma samples (Rosvall et al. 2013), but another study of free-ranging individuals of the same species did not show a relationship between estradiol and testosterone in post-GnRH blood samples for individuals (Graham et al. 2019). Our study analyzed baseline hormone samples and found that there was a negative relationship between testosterone and estradiol. This indicates that baseline testosterone alone may not be a reliable indicator of baseline estradiol levels, HPG activity, or gonadal development prior to breeding. Our observation of a negative relationship between estradiol and testosterone may be a result of more testosterone being converted to estradiol by the enzyme aromatase, leading to lower concentrations of testosterone and higher estradiol in the blood

plasma at the time of sampling. Females in other polygynous species have been shown to elevate estradiol in response to a territorial intrusion (Pärn et al. 2008). It is possible that the females in our study were similarly converting testosterone to estradiol in response to social interactions, as these samples were taken while females were settling on territories. The relationship between testosterone and estradiol is complicated and will require further research of both baseline and post-GnRH testosterone and estradiol levels. It will be necessary to more fully examine whether testosterone, estradiol, or the levels of both are needed to address questions about reproductive preparedness in females and timing of breeding.

Lay date was not correlated with baseline testosterone, estradiol, or migration distance. This may have been due to the within species social interactions previously mentioned, or it could be due to interactions among species following a prescribed burn. In 2019, a controlled burn occurred on 14 May at the Alice Waterfowl Production area primarily north of our monitored territories. This burn destroyed cattail used by breeding Red-winged Blackbirds, Yellow-headed Blackbirds (*Xanthocephalus xanthocephalus*), and Marsh Wrens (*Cistothorus palustris*) and likely delayed nest building or forced females to move and build away from our study site, limiting our sample size of nests found for individuals also sampled for stable isotopes. Marsh Wrens are known to break eggs of Red-winged Blackbirds in eastern North Dakota (Sawin et al. 2003) and Yellow-headed Blackbirds are known to displace Red-winged Blackbirds to wetland edge territories (Orians and Willson 1964). Yellow-headed Blackbirds and Marsh Wrens were found invading Red-winged Blackbird territories after being displaced by the prescribed burn in 2019. To support this hypothesis, there is a stronger relationship for relative migration distance and lay date for 2018 ($t=2.59$, adjusted $R^2=0.49$, $df=1,5$, $P=0.049$, $n=7$) than for 2019 ($t=0.79$, adjusted $R^2=-0.05$, $df=1,8$, $P=0.46$, $n=9$). The change in environment less than 2 weeks before the first Red-winged Blackbird nest of the season may have influenced reproduction that year and obscured the relationship between migration distance, reproductive hormones, and lay date in our study. Future work (in non-burn years) is likely to clarify any relationship that may exist between overwintering distance from breeding groups and seasonal clutch initiation.

Due to the difficulty of tracking passerines during migration and throughout the year, there is still much to be learned about how even small differences in spring migration distance may influence the birds upon their return to the breeding grounds. In our study, we utilized $\delta^2\text{H}$ to estimate overwinter latitude, and it should be noted that there can be variation of 9–12 per mil (SD) in $\delta^2\text{H}$ values for a given site (Hobson et al. 2014). Individual variation arises due to stable isotope samples being a composite of individual

movements over a given period (Werner et al. 2016). For this study, the claw samples collected at the breeding site were representative of the overwintering and spring migration locations relative to the breeding ground. Other studies of passerines with similar ranges in $\delta^2\text{H}$ values to the samples in our study have also found that spring migration distance is related with reproduction (Dale and Leonard 2011; Lymburner et al. 2016). Dale and Leonard (2011) found that male Ipswich Sparrows (*Passerculus sandwichensis princeps*) wintering farther north were in better condition, established territories earlier, and tended to produce nestlings of higher condition than their counterparts to the south.

Our findings indicate that, in a short-distance migrant such as a Red-winged Blackbird, a few hundred kilometers difference in overwinter location may make a difference in how prepared these individuals are to breed. It has been noted that males that overwinter farther north are more dominant and capture territories earlier than males that winter farther south, due to the shorter distance that they need to travel in the spring (Ketterson and Nolan 1976; Dolbeer 1982). However, more accurate knowledge of the weather conditions at the breeding grounds may be the main advantage of a shorter migration distance. Climate change is causing many species to decrease their migration distance as the warmer winter temperatures allow them to stay at more northern latitudes (Visser et al. 2009). This is advantageous because short-distance migrant species can use meteorological cues to more appropriately time their arrival than long-distance migrant species (Butler 2003). Future studies that develop claw isoscapes for $\delta^2\text{H}$ based on continental patterns of $\delta^2\text{H}$ in precipitation could inform overwintering latitudes and movements of migratory blackbirds (Werner et al. 2016). Additionally, studies that incorporate variation in claw $\delta^2\text{H}$ at a given site will be able to provide valuable information on movement patterns such as natal dispersal or overwinter locations (Rushing et al. 2015). Alternatively, tracking technologies such as GPS tags or automated radio telemetry tracking will be valuable to quantify the exact distance that each bird has traveled and associate that with reproduction.

Our study sampled both sexes and demonstrated that migration distance may influence reproductive hormones and HPG axis function (fully activated in short-distant males) upon arrival to the breeding grounds. Males and females use different cues to time reproduction (Ball and Ketterson 2008) and for too long researchers have viewed female hormones as complicated (Shansky 2019). It is crucial that future physiological studies include both males and females and incorporate measures of reproductive readiness beyond baseline testosterone (Caro 2012). There is still much to be learned about the factors that influence reproductive preparation in migrating songbirds, and a variety of HPG

axis activity measurements will be required to understand the influence of spring migration distance on reproduction.

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Author contributions ME, TG, and PK contributed to the experimental design and data analysis. ME, EM-V, and TG conducted the field work. BW analyzed stable isotopes in claw samples. All authors contributed to the writing of this manuscript.

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Availability of data and material code availability Data and R code are available upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The data collected on this project was approved by the North Dakota State University Institutional Animal Care and Use Committee, North Dakota Game and Fish Department, the United States Geological Survey Bird Banding Laboratory, the US Fish and Wildlife Service in Valley City, and the USDA-APHIS-WS NWRC. Experiments comply with the current laws of the country in which they were performed.

Consent to participate NA.

Consent for publication All authors gave approval for publication.

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