NATURAL SCIENCES

# Breeding Latitude and Annual Cycle Timing in a Songbird

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## ABSTRACT

In spring, songbirds undergo physiological changes such as migratory fattening and gonadal recrudescence in response to increasing day length. Past research suggests that the day length required to initiate physiological changes, known as the photoperiodic threshold, can vary by breeding latitude. In this study, we explored whether migrants breeding at higher latitudes require longer days in spring before physiological changes occur (i.e., whether breeding latitude of origin predicts photoperiodic threshold). We caught and housed male migrant and resident dark-eyed juncos (*Junco hyemalis*) in an indoor aviary. Photoperiod was increased incrementally from nine to sixteen hours over fourteen weeks. During each photocycle, morphological measurements of mass, subcutaneous body fat, and cloacal protuberance were measured as indicators of migratory and reproductive condition. Stable isotope signatures of hydrogen were used to estimate breeding latitude as an index of migratory distance. Our results show that migrants and residents differed in physiological changes, as migrants accumulated more subcutaneous fat, increased body mass, and displayed a significant delay in gonadal recrudescence relative to residents. Additionally, individuals breeding at higher latitudes deposited fat at a faster rate than individuals breeding at lower latitudes. These results supported our hypothesis that migratory strategy and breeding latitude may predict differences in photoperiodic threshold for both migratory and reproductive timing. Our findings contribute to the understanding of regulation of timing in annual cycles and improve predictions of how species might respond to changing environments.

**KEYWORDS:** *migration, reproduction, annual cycle timing, reproduction physiology, climate change, phenology, migration physiology, photoperiod, photoperiodic threshold*

## INTRODUCTION AND BACKGROUND

Many animals exhibit a consistent annual cycle of changes in physiology and activity, referred to as seasonality (Chemineu, Malpaux, Brillard, & Fostier, 2007). Timing of reproduction is synchronized with the phenology of the surrounding environment to ensure optimal exploitation of natural resource pulses. Seasonally breeding animals that undergo an annual gonadal recrudescence and regression use both a primary predictive cue and supplementary cues to initiate and regulate timing of reproductive development. The change in day length, or photoperiod, is the initial predictive cue for many animals, as it initiates and regulates the reproductive cycle (Bronson & Heideman, 1994). Supplementary cues, such as temperature, food availability, and precipitation, can fine-tune the cycle's progress (Bronson & Heideman, 1994).

Many birds have a unique annual cycle because it not only includes a reproductive and non-reproductive state, but also a period of migration and molt, which are all energetically expensive chapters of the annual cycle. Because each of the chapters is so energy -intensive, timing of energy expenditure is crucial. Birds, like many other animals, use change in photoperiod as the initial predictive cue in the regulation of an optimally timed annual cycle (Dawson, 2001; Rowan, 1926). Birds change in sensitivity to photoperiod throughout the annual cycle, with periods of photosensitivity, photostimulation, and photorefractoriness (Kumar et al., 2010). Photosensitivity refers to responsiveness to changes in day length, while photorefractoriness refers to unresponsiveness to changes in day length. A critical day length initiates the photostimulated phase, when physiological changes, such as migratory fattening and gonadal recrudescence, are induced (Kumar et al., 2010). This critical day length is known as the photoperiodic threshold, which differs among species and even among populations of the same species (Robinson & Follett, 1982). The photoperiodic threshold is a valuable measure because it indicates the shift to a new chapter of the annual cycle (i.e., migration, reproduction, molt). In some species, individuals or populations do not migrate, allowing for more flexibility in the timing of reproduction. Therefore, reproduction is not constrained by the necessity to migrate in non-migratory, or resident, populations (Chemineu et al., 2007).

During reproduction in birds, a critical change in photoperiod activates the hypothalamic-pituitary-gonadal (HPG) axis, or endocrine reproductive axis (Dawson, 2001). This axis regulates the growth of gonads, which produce testosterone and sperm in males. The cloacal protuberance (CP) is the primary sperm storage structure for male birds that grows with the swelling of vas deferens tubules. Cloacal protuberance size is a reliable index of reproductive condition (Wolfson, 1952), and its volume can be easily calculated with the formula for volume of a cylinder with measurements of CP height and width.

Cues that drive the timing of reproduction (i.e., photoperiod) and act on the HPG axis also influence migratory timing (Ramenofsky, Cornelius, & Helm, 2012). Whereas CP is a reliable indicator of reproductive timing, fat deposition is a reliable indicator of migratory timing (Clark, 1979). Subcutaneous body fat varies seasonally throughout the annual cycle of migratory birds, in which fat deposition occurs prior to migration and wanes at the initiation of the breeding season (Clark, 1979). Prior to migration, birds experience temporary hyperphagia, leading to a notable increase in body mass and fat deposition. Fat is the primary energy source for migration; therefore, subcutaneous body fat is a good measure of timing of migration (Jenni & Jenni-Eiermann, 1998).

Previous studies have investigated the significance of non-photic supplementary cues, such as temperature (Perfito, Meddle, Tramontin, Sharp, & Wingfield, 2005; Wingfield, 1985), food availability (Hau, Wikelski, & Wingfield, 2000; Kumar, Singh, Misra, & Malik, 2001), social interaction (Dawson & Sharp, 2007), and the endogenous circannual rhythm (Gwinner, 2003; Wingfield, 1993). Until recently, implications of migratory strategy on annual cycle progress did not receive much attention (Fudickar, Grieves, Atwell, Stricker, & Ketterson, 2016; Ramenofsky, Campion, Pérez, Krause, & Németh, 2017), and there is still much to be explored in this area. Even less is known about how breeding latitude interacts with the timing of the annual cycle (Silverin, Massa, Stokkan, 1993), especially in consideration of seasonally sympatric populations.

Some populations receive the same photoperioidic and supplementary cues but differ in migratory strategy and migratory distance. Investigating these populations provides a terrific

opportunity to better understand the timing of reproductive phenology. In some special cases, populations that differ in migratory strategy (i.e., migrants and residents), overlap during part of the year in a distribution called seasonal sympatry (Winker, 2010). In dark-eyed juncos, migrants overwinter in sympatry with residents before departing on spring migration. These migrants breed in the boreal forest of Canada and extreme northern U.S. with a fairly extensive latitudinal breeding range. Migrants can also differ greatly in their breeding latitude, even when found at the same wintering site. Therefore, this provides an opportunity to determine how photoperiodic thresholds vary among individuals that differ in migratory strategy and migratory distance (n.b. migratory distance was measured as breeding latitude), as all individuals receive the same photoperiodic cues prior to migration. We hypothesized that migratory strategy and breeding latitude would predict differences in photoperiodic threshold for timing of migration and reproduction.

To address this hypothesis, we studied the dark-eyed junco (*Junco hyemalis*), a common north-temperate songbird that exhibits seasonal sympatry. Populations of migrants and residents are found in sympatry in Virginia during the overwintering season (i.e., October to April) prior to spring migration. Although migrants and residents share similar conditions throughout the nonbreeding season, behavioral and physiological differences between the populations can be observed by early spring. During spring, residents initiate breeding by defending breeding territories and growing CPs, while migrants prepare instead for migration by increasing fat (Nolan Jr. et al., 2002).

In this study, we asked how both migratory strategy and breeding latitude interact with migratory and reproductive phenology in migratory and resident populations of dark-eyed juncos. We quantified differences in cycles by measuring physiological traits associated with migration and reproduction as birds undergo an increasing photoperiodic treatment from short days of 9L (nine hours of light) to long days of 16L. Measurements included a subcutaneous body fat score, body mass, and cloacal protuberance size. We distinguished breeding latitude using stable isotopes of hydrogen in wing feathers. Stable isotopes H-1 (protium) and H-2 (deuterium) are variants of the hydrogen molecule whose ratios can be measured by mass spectrometry for isotopic ratios. These molecular variants are found in water, with isotopic signatures that vary predictably by latitude. Birds incorporate stable isotopes into their feather keratin in ratios specific to a location by ingestion of local organic resources. We predicted that individuals with higher breeding latitude would deposit more pre-migratory body fat and exhibit later onset of gonadal recrudescence than both residents and individuals migrating to lower latitudes. Differential responses to the initial predictive cue have potential to cause asynchrony in breeding phenology among populations, which would reinforce population divergence. We will be better able to predict and prepare for the effects of environmental change, including climate change, on populations with a greater understanding of annual cycle timing physiology.

# MATERIALS AND METHODS

## *Study Species*

Dark-eyed juncos (*Junco hyemalis*) are common songbirds that breed in temperate coniferous and mixed forests across Canada, Alaska, and high altitude regions of the eastern and western United States. In most recent phylogeny, there are approximately five distinct groups of *Junco hyemalis,* of which the slate-colored dark-eyed junco includes a migratory subspecies (*Junco hyemalis hyemalis*), hereafter 'migrants' or 'migratory population', and a non-migratory subspecies (*Junco hyemalis carolinensis*), hereafter 'residents' or 'sedentary population' (Nolan Jr. et al., 2002).

Migratory populations breed north in Alaska and Canada and migrate south in the fall to overwintering grounds in the eastern United States, including the Appalachian Mountains. A resident population breeds in the Appalachian Mountains, remaining at the breeding grounds year-round. During the nonbreeding season, there is extensive overlap between migrants and residents, establishing a seasonally sympatric distribution of the populations. Seasonal sympatry occurs when populations live and interact in the same geographic location on a seasonal basis. Both migratory and resident populations are found within the same habitat, as they can be found foraging in mixed flocks in the winter (Nolan Jr. et al., 2002).

## *Bird Capture and Housing*

Male juncos (15 resident and 49 migrant) were captured between November 10 and December 14, 2017. Residents and migrants were caught at Mountain Lake Biological Station in Giles County, VA (MLBS; 37°22' N, 80°32' W) and the surrounding Jefferson National Forest using regularly baited and continually monitored mist nets and walk-in Potter traps. Note that 'resident' does not imply birds that were resident at the common garden study site in Bloomington, IN. Additional migrants were captured near Bloomington, IN in rural areas and on Indiana University Research and Teaching Preserve sites.

At locations of seasonal sympatry in Virginia, we distinguished migrant and resident individuals based on bill coloration and other morphological indicators. Residents had a characteristic blue-gray bill color, whereas migrants had a pink bill color (***Image 1***) (Hamel, 1979). We confirmed classification using stable isotopes in feathers.

Only males were used in this experiment because females did not exhibit reliable, easily measured indicators of reproductive condition. Birds were sexed at capture using right wing chord length and plumage differences. Males were determined by a wing length of 80 mm or more, while individuals that did not meet this cutoff were released. Males typically had darker hood plumage coloration than did females, and this characteristic helped confirm our sex designations (Pyle, 1997).

Indiana-captured migrants and Virginia-captured migrants and residents were transported by car to Kent Farm Research and Teaching Preserve in Bloomington, IN and housed in an outdoor aviary under the same conditions until December 14, 2017. On December 15, 2017, all individuals were moved to climate-controlled (16 °C) free flight indoor aviary rooms (6.4 x 3.2 m and 5.7 x 5.5 m),

***Image 1.***
*Blue-gray bill coloration in a resident (*J. h. carolinensis*, pictured left) and pink bill coloration in a migrant (*J. h. hyemalis*; pictured right).*

where they experienced nine hours of artificial light for one month prior to sampling. On January 18, 2018, all birds were moved to individual cages (61 x 46 x 46 cm and 46 x 46 x 46 cm) that were organized across three rooms for the duration of the experiment (ten weeks). Migrant and resident groups were randomly assigned to the three rooms. The multi-room design allowed for a feasible sampling schedule.

Birds were provided with fresh food and water three days per week (M, W, F) for *ad libitum* consumption. Food and water were provided in elevated plastic containers and checked daily. Birds were fed a 2:1 mixture of white millet and sunflower chips, orange slices, and a soft diet of ground puppy chow, hard-boiled eggs, and carrots. Fresh water was mixed with powdered *Nekton-S Multivitamin for Birds*. On April 26, 2018, all birds were returned to free-flying condition in an indoor aviary to be held for future studies.

## Experimental Design

In order to determine differences in physiological and morphological responses to photoperiodic cues by residents and migrants, we artificially regulated changes in day length. We refrained from incorporating a simulation of civil twilight, as birds might include twilight as a photoperiodic signal (Dawson, 2015). Photoperiod was increased every twelve days from January 18, 2018 to May 6, 2018 in the following schedule: 9 Hours Light (L):15 Hours Dark (D), 10L:14D, 11L:13D, 11⅓L:12⅔ D, 11⅔L:12⅓D, 12L:12D, 12⅓L:11⅔D, 12⅔L:11⅓D, 13L:11D, 15L:9D, 16L:8D. The focus on photoperiods surrounding the spring equinox at 12L was designed to provide better resolution of the potential photoperiodic threshold for migration, as most migrants migrate northward during this time period. We will refer to each photoperiod schedule as the number of hours of light. We completed sampling of morphological measurements after a seven-day light entrainment period following each photoperiodic change.

## Morphological Measurements

Experimental parameters included morphological measurements of subcutaneous fat, body mass, and cloacal protuberance volume. These parameters were measured for all individuals every twelve days.

***Image 2.***
*(Left) Fat score 1: trace lining of fat within furcular cavity. (Right) Fat score 5: fat bulging from both furcular cavity and abdomen.*

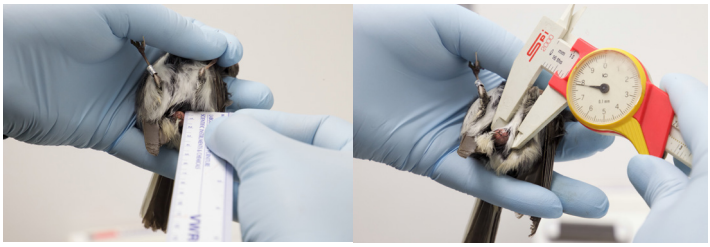

***Image 3.***
*(Left) Measurement of CP height using ruler. (Right) Measurement of CP width using calipers.*

Subcutaneous body fat—hereafter 'fat score' or 'fat deposition'—was visually measured on a scale from 0-5: (0) no fat, (1) trace lining of fat in either furcular cavity or abdomen (***Image 2L***), (2) trace lining in both furculum and abdomen/full lining in either furculum or abdomen, (3) full lining in both furculum and abdomen, (4) bulging in either furculum or abdomen, or (5) bulging in both furculum and abdomen (***Image 2R***).

Body mass was measured using a sheer sock and Pesola spring scale (0-50 g) tared to zero. Body mass was rounded to the nearest half-gram.

CP height was measured using a ruler (***Image 3L***), while width was measured using calipers (***Image 3R***). Different measuring tools were used to ensure that measurements were made with the instrument flush against the CP structure. A CP volume estimate was calculated using the formula for the volume of a cylinder: $V=\pi \cdot r^2 \cdot h$. CP volume will hereafter be referred to as 'CP size'.

## Feather Stable Hydrogen Isotopes

The most distal secondary feather of the right wing was collected from each individual at the time of capture for analysis of hydrogen stable isotope ratios ($\delta^2H$). Feather samples were stored in individual coin envelopes. After feathers were collected, oils and particulates were removed using a 2:1 chloroform-methanol mixture. Feathers were air-dried under a fume hood.

The distal end of each feather was cut, weighed to 0.5 mg, and placed into a 3 x 5-mm silver capsule. Capsules were plated and mailed to the US Geological Survey Stable Isotope Lab in Denver, CO. Hydrogen isotope ratios were measured using established methods of mass spectrometry (Fudickar et al., 2016; Wundner, Jehl, & Stricker, 2012). The $\delta^2H$ ratios were reported in parts per mil notation (‰) with respect to Vienna Standard Mean Oceanic Water (VSMOW) using internal water standards.

## Statistical Analysis

To quantify the effects of population, photoperiod, and the interaction between population and photoperiod on recurring measures of subcutaneous fat score, body mass, and CP volume, we used two-way ANOVA tests (alpha level set at 0.05). To analyze significance of both inter- and intra-population variation of morphological measurements, we performed two-way ANOVA Bonferroni post-hoc multiple comparison tests. To test if mean differences in CP volume or fat score were associated with breeding latitude, we examined potential relationships between morphological measurements and hydrogen isotope values using Pearson correlation and linear regression. Differences in mean hydrogen isotope ratios between migrants and residents were determined using a one-sample t-test of population means. We performed all statistical analyses using Prism GraphPad. All reported p-values are two-tailed. All reported tests are two-way ANOVA unless otherwise annotated.

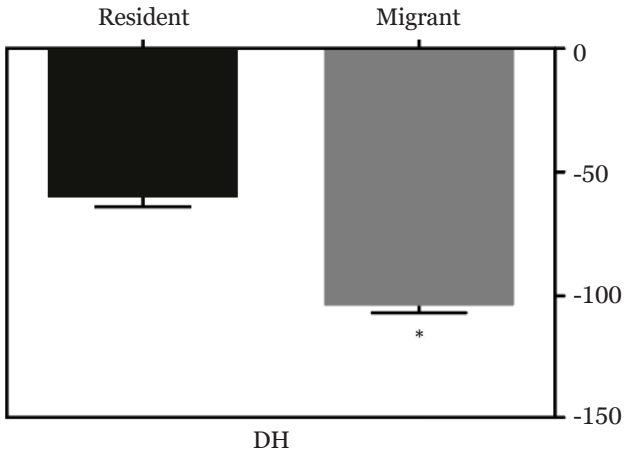


***Figure 1.***
*Migrant feathers have a significantly lower δ²H ratio than resident feathers ($F_{48, 14}$ = 1.292, P < 0.0001; t-test). Data are expressed as mean ± SEM. *p < 0.05 vs. resident, t-test.*

# RESULTS

## *Stable Hydrogen Isotopes*

Mean hydrogen isotope values (δ²H) were significantly lower in migrants than in residents ($F_{48, 14}$=1.292, P < 0.0001; t-test; migrant mean = -104.9‰; resident mean = -58.8‰; ***Figure 1***). Migrant δ²H values also covered a larger range than did those of residents (δ²H migrant range: -33‰ to -141‰, SEM=2.9; δ²H resident range: -37‰ to -109‰, SEM=4.6).

## *Fat Score*

Migrants had significantly greater fat scores than residents and exhibited fat scores that increased significantly within the time frame of the experiment, whereas residents had fat scores that remained fairly constant throughout the study (***Figure 2a***). The interaction between population and photoperiod had a statistically significant effect on fat scores ($F_{10, 653}$= 15.30, P < 0.001), as did the main effects of population and photoperiod ($F_{1, 653}$= 138.4, P < 0.001; $F_{10, 653}$=17.61, P < 0.001). Fat scores in migrants were significantly higher at 12L than at 11.7L ($t_{653}$=5.143, SE=0.2147; two-way ANOVA Bonferroni multiple comparisons).

There was also a significant relationship between breeding latitude and fat score among the migrant population at photoperiods of or exceeding 12L (***Table 1; Figures 3b-c***), which is the photoperiod at which fat deposition increased significantly among migrants (***Figure 2a***). However, there were no significant correlations between breeding latitude and fat scores among migrants at all photoperiods less than 12L (***Table 1; Figure 3a***).

## *Body Mass*

At the start of the experiment, residents had a significantly higher mean body mass than did migrants ($t_{653}$=5.461, SE=0.5043; two-way ANOVA Bonferroni multiple comparison test). Body mass of residents did not notably change throughout the experiment, while that of migrants significantly increased after 11.7L, and remained significantly higher through 13L (12L: $t_{653}$=4.640, SE=0.3480; 12.3-13L: $t_{653}$=4.677, SE=0.5069; two-way ANOVA Bonferroni multiple comparisons; ***Figure 2b***). The interaction effect of photoperiod and population on body mass was statistically significant ($F_{10, 654}$ = 14.06, P < 0.001), as was the main effect of photoperiod ($F_{10, 654}$= 10.38, P < 0.001). There was not a statistically significant relationship between population and body mass ($F_{1, 654}$= 2.216, P = 0.1371). Migrants started with a lower mean mass than residents and ended with a higher mean mass.

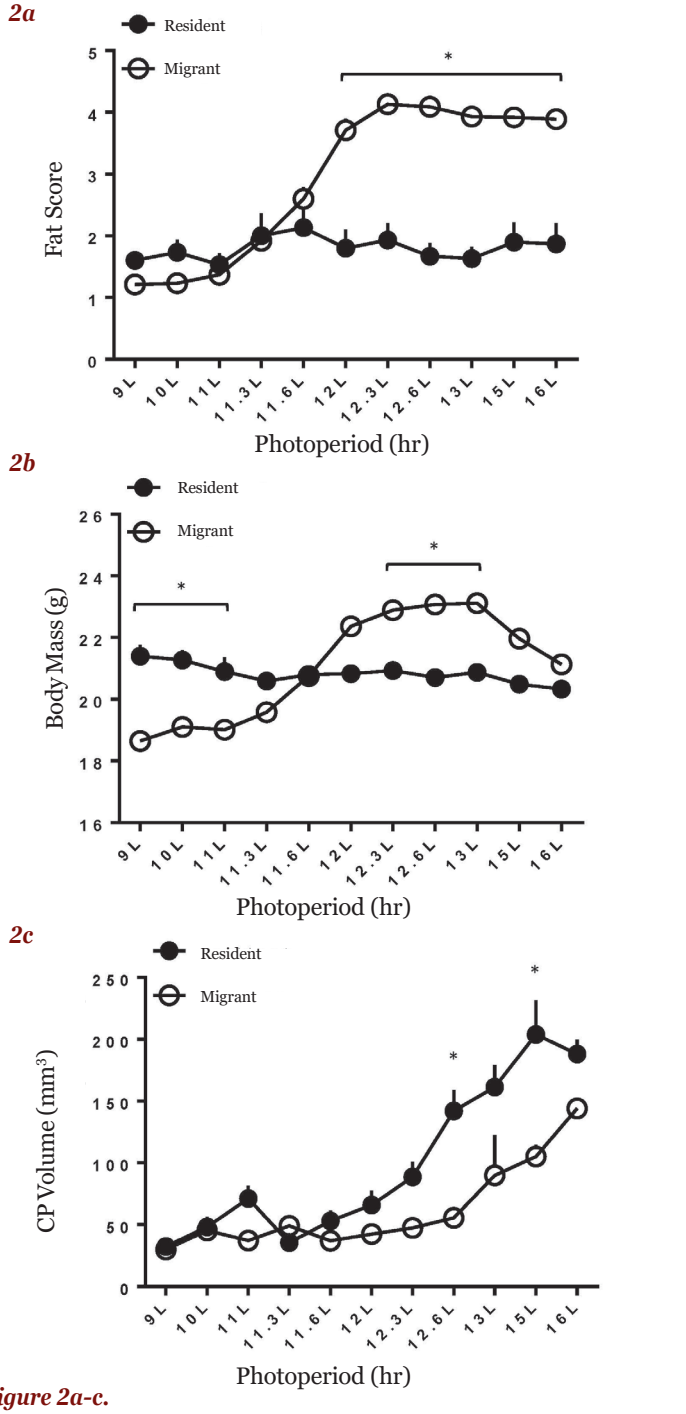


***Figure 2a-c.***
**Figure 2a:** *Fat scores of migrants increased throughout the experiment, while those of residents did not.* **Figure 2b:** *Body mass fluctuated over time in migrants, while it remained fairly consistent in residents. Migrants started with a lower mean mass than residents and ended with a higher mean mass.* **Figure 2c:** *CP growth rate differed between migrants and residents, with residents having a faster rate. For all figures, data are expressed as mean ± SEM. *p < 0.05 vs. resident, two-way ANOVA followed by Bonferroni post hoc.*

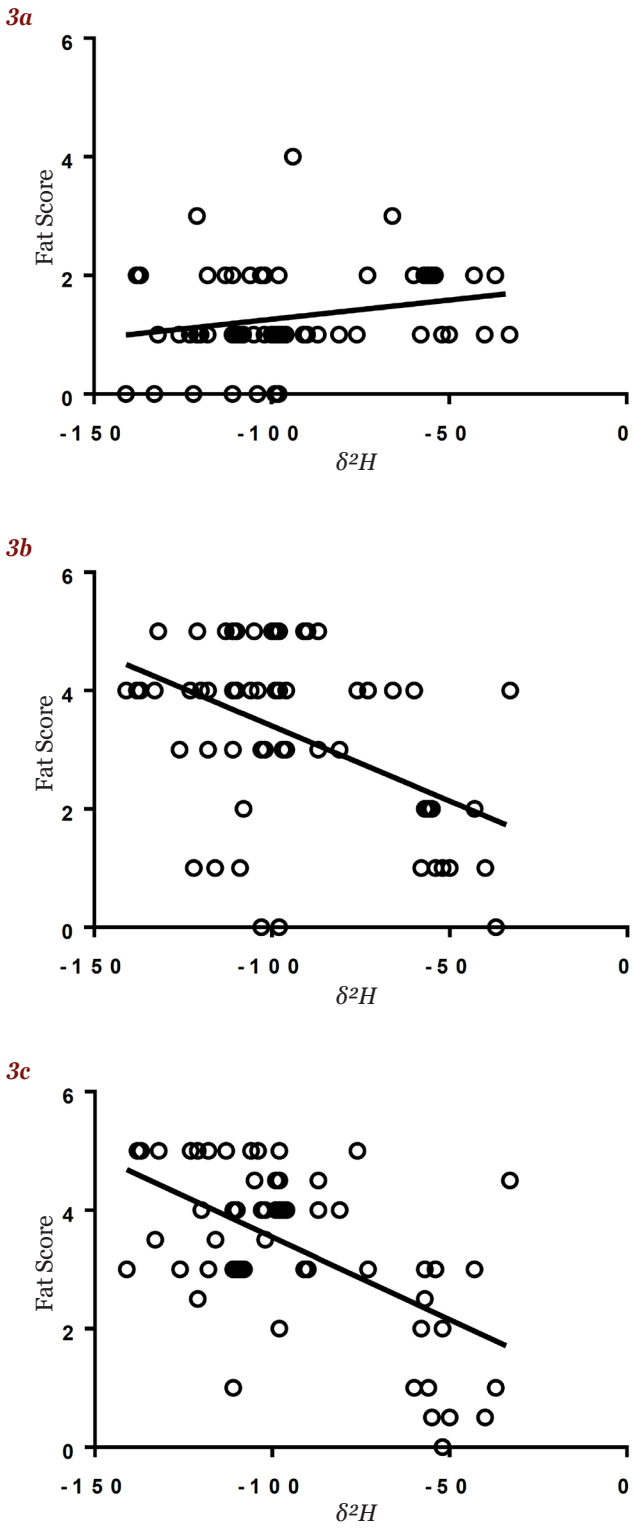


*Figure 3a-c.*
*δ²H and fat score in migrants experiencing different photoperiods: (a) start of experiment at 9L, r²=0.054, P=0.066; (b) middle of experiment at 12L, r²=0.221, P=0.001; and (c) end of experiment at 16L, r²=0.331, P<0.001. A significant relationship was found between δ²H and fat score from 12L through 16L.*

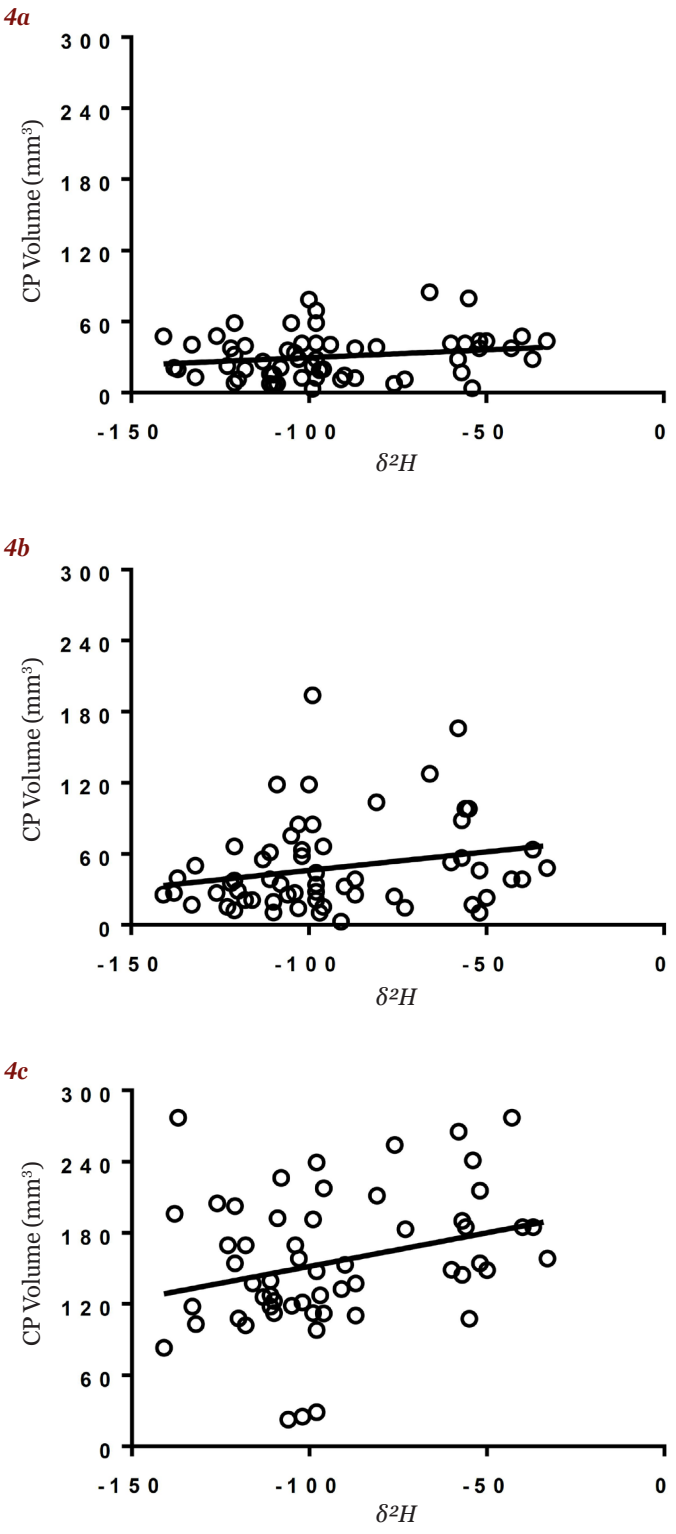


*Figure 4a-c.*
*δ²H and CP volume/size in migrants experiencing different photoperiods: (a) start of experiment at 9L, r² =0.027, P=0.267; (b) middle of experiment at 12L, r² =0.047, P=0.139; and (c) end of experiment at 16L, r² = 0.005, P=0.655. No significant relationship was found between δ²H and CP size.*

*Table 1.*
*Relationship between δ²H (i.e., breeding latitude) and fat score at each of the eleven photoperiods in migrants*

| Photoperiod | P-value | $r^2$ |
|---|---|---|
| 9L | 0.066 | 0.054 |
| 10L | 0.065 | 0.055 |
| 11L | 0.277 | 0.020 |
| 11.3L | 0.851 | 0.001 |
| 11.7L | 0.450 | 0.009 |
| 12L | 0.001*** | 0.221 |
| 12.3L | <0.001**** | 0.303 |
| 12.7L | <0.001**** | 0.393 |
| 13L | <0.001**** | 0.387 |
| 15L | <0.001**** | 0.326 |
| 16L | <0.001**** | 0.331 |

*Table 2.*
*Relationship between δ²H (i.e., breeding latitude) and CP size at each of the eleven photoperiods in migrants*

| Photoperiod | P-value | $r^2$ |
|---|---|---|
| 9L | 0.0267 | 0.027 |
| 10L | 0.141 | 0.046 |
| 11L | 0.018* | 0.115 |
| 11.3L | 0.955 | 0.000 |
| 11.7L | 0.169 | 0.041 |
| 12L | 0.139 | 0.047 |
| 12.3L | 0.001** | 0.200 |
| 12.7L | 0.457 | 0.013 |
| 13L | 0.107 | 0.061 |
| 15L | 0.501 | 0.011 |
| 16L | 0.655 | 0.005 |

## Cloacal Protuberance Size

Both migrant and resident CP size increased throughout the experiment, with CP size growing faster among residents (***Figure 2c***). There was a significant interaction effect of population and photoperiod on CP size ($F_{10, 653}$= 3.030, P < 0.0009), and the main effects of population and photoperiod were also significant ($F_{1, 653}$= 35.24, P < 0.001; $F_{10, 653}$= 21.22, P < 0.001).

No consistent relationship was found between breeding latitude (δ²H) and CP size among the migrants (***Figures 4a-c***). Of the eleven photoperiods, CP size varied significantly with breeding latitude in only two (***Table 2***).

# DISCUSSION

Our results showed that migrants and residents differed in physiological changes as a function of photoperiod, as migrants accumulated more subcutaneous fat, increased body mass, and displayed a significant delay in gonadal recrudescence relative to residents. Additionally, individuals breeding at higher latitudes deposited fat at a faster rate than did individuals breeding at lower latitudes, as there was a positive relationship between fat scores and δ²H beginning at 12L. These results supported our hypothesis that migratory strategy and breeding latitude may each predict differences in photoperiodic threshold for both migratory and reproductive timing. However, breeding latitude did not predict CP size as expected, which opposed our hypothesis that breeding latitude affects the photoperiodic threshold for gonadal recrudescence. Although our results indicated that breeding latitude is not associated with reproductive phenology (i.e., CP size), we instead found a strong relationship between breeding latitude and migratory phenology (i.e., fat deposition).

## Hydrogen

As expected, mean hydrogen isotope values were significantly lower in migrants than in residents, reflecting the fact that migrants molt and grow feathers on their breeding grounds (i.e., at higher latitudes than residents) (Hobson & Wassenaar, 1996). The range of migrant δ²H values was larger than that of residents, indicating that migrants breed over a broader latitudinal band (Wundner et al., 2012).

## Body Fat Deposition and Migratory Phenology

We used fat score as an indicator of migratory phenology, as fat deposition occurs prior to migration and wanes at the onset of the breeding season (Clark, 1979). Migrants accumulated more body fat than did residents during the experiment. Both groups were exposed to the same photoperiodic treatment and were provided with food *ad lib*. Therefore, individuals were likely allocating energy differently and responding differently to photoperiodic cues based on migratory strategy. Migrants did not experience significant changes in consecutive fat scores except at 12L—a point that we considered the migrant photoperiodic threshold for induction of changes associated with migration (i.e., fat deposition). At this point, migrants experienced pre-migratory hyperphagia, resulting in a dramatic increase in fat deposition (Odum, 1960). The results agreed with our predictions that migrants would gain more body fat in preparation for an energy-intensive spring migration, while residents would not undergo fat deposition since they do not migrate. Therefore, we found migratory strategy to be a reliable predictor of photoperiodic threshold for phenological changes associated with migration.

In order to determine a greater resolution of differences in photoperiodic threshold among migrants, we investigated the relationship between body fat accumulation and breeding latitude. We found that δ²H ratios and fat scores significantly covaried after 12L (***Table 1***), suggesting that individuals breeding at higher latitudes accrued body fat at a faster rate than did residents and individuals breeding at lower latitudes. Migration is energy-intensive, and fat is utilized as the primary energy source during migration (Jenni & Jenni-Eiermann, 1998; Odum, Connell, & Stoddard, 1961). Individuals traveling to higher breeding latitudes migrate greater distances; therefore, they likely prepare for migration earlier and utilize more fat energy in the process.

Fat scores and δ²H did not significantly covary prior to 12L (***Table 2***). Migrants might not need to gain as much fat until reaching a photoperiod closer to their time of departure. Variation among fat scores was higher in the second half of the experiment (average SEM: 9L to 11.7L=0.11; 12L to 16L=0.16). Migrants likely did not increase their rate of fat deposition until they reached a critical day length that is longer than the winter day lengths of the first half of the experiment (i.e., closer to the vernal equinox). We concluded that both migratory strategy and breeding latitude were predictors of photoperiodic threshold for physiological changes associated with migratory phenology.

## Mean Body Mass

Despite exposure to the same environmental conditions, migrants had lower mean body mass than did residents at the start of the experiment (9L) and had a higher mean body mass at the end of the experiment (16L). Body mass significantly increased among migrants at 12L. This change in body mass was linked to the photoperiod in which migrants also exhibited significantly higher fat scores, further confirming the prospective photoperiodic threshold for physiological changes associated with migratory timing. In contrast, residents did not change their average body mass throughout the experiment. These results supported the prediction that migrants would exhibit an increase in body mass and body fat deposition in preparation for migration, while residents would not increase mass because they do not accumulate pre-migratory fat.

We expected body mass to covary with subcutaneous fat deposition; however, this was not the case. The differences between body mass and fat deposition might be explained by the lack of body condition analysis. Body mass and condition together likely provide a stronger, more cohesive complement to body fat scores.

## Cloacal Protuberance Size and Reproductive Phenology

Both migrant and resident CP size increased throughout the experiment, with residents exhibiting faster CP growth. This supported our prediction that CP growth rate would be slower in migrants; migration is energetically demanding and therefore constrains reproductive timing (Ramenofsky et al., 2012). Although both populations grew CPs steadily, there were no statistically significant increases in CP growth between consecutive increases in photoperiod among migrants or residents. Therefore, the photoperiodic threshold for induction of CP growth remained unclear.

We investigated the relationship between CP size and breeding latitude to test breeding latitude as a predictor of reproductive timing. CP size did not consistently covary with $\delta^2H$. Since CP size is highly correlated with testis size (Fudickar et al., 2016), it was a strong indicator of reproductive condition. We deduced that breeding latitude was not a strong predictor of a photoperiodic threshold for reproductive phenology. These results differ from those of a previous common garden study on migrant and resident dark-eyed juncos (Fudickar et al., 2016). In this study, testis mass was found to significantly covary with $\delta^2H$ at approximately 12.7L (Ramenofsky et al., 2012). However, at a similar photoperiod, we found that CP size did not covary with $\delta^2H$. Fudickar et al. (2016) collected testis samples on March 31 and April 1 at approximately 12.7L, while we completed 12.7L sampling on April 13; therefore, endogenous annual cycle clocks did not differ enough to suggest that we would have had such different findings. Differences in findings might be explained by inconsistency in migratory distance in the sample sizes of each study. More research is necessary to determine whether breeding latitude predicts timing of reproductive development in migratory songbirds.

## Conclusions

Our results reinforce known differences between strategies of migrant and resident populations. Our results demonstrate a classic energy life-history trade-off between migration and reproduction. As seasonal animals, migrant birds experience three energy-intensive chapters in their annual cycle—migration, reproduction, and molt. These chapters are so energetically expensive that they cannot fully overlap without costs. Carry-over effects, costs of reproduction and migration, and trade-offs are integrated among life-history stages (McGlothlin et al., 2010; Wilson, 2012). The amount of energy expended during each life history stage of the annual cycle varies greatly within and between species (Williams & Vézina, 2001). In this study, we find that a differential response to the initial predictive cue, photoperiod, resulted in differences in timing of annual cycles between populations. Migration constrained reproduction in the migrant population, as the rate of CP growth was slower in migrants than in residents and residents initiated breeding activity before the majority of the migrants. Residents did not experience the constraint of migration, and their rate of CP growth was significantly faster. The migrant population must undergo physiological changes associated with migration prior to expending energy when entering full reproductive condition. These differences in timing foster divergence between populations, since breeding phenology is not synchronized.

Our results also suggested that breeding latitude can predict migratory phenology but not necessarily reproductive phenology. We found that breeding latitude was a poor predictor of reproductive timing, which is not consistent with past research. This difference in results might be better explained with a more comprehensive understanding of how endogenous cycles affect reproductive timing. Alternatively, inconsistent results might be due to differences in variation of breeding latitude of populations. New technology, such as the use of high-resolution geolocators, will be helpful in future studies in more accurately measuring breeding latitude and migratory distance to determine how these factors affect phenology. Future studies may help explain our contradictory results with an improved experimental design that incorporates greater photoperiodic resolution from 13L to 15L, more precisely identifying the photoperiodic threshold for reproductive timing. Future studies are also urged to examine initial predictive cues and supplementary cues together by focusing on interactions between the non-photic endogenous circannual rhythm, photoperiod, and breeding latitude. This focus will help us to better understand the dynamics of how endogenous clocks interact with initial predictors in the design of reproductive and migratory phenology.

Many birds are considered indicator species of environmental health, as they are highly sensitive to resource abundance, temperature, precipitation, etc. (Carey, 2009; Németh, Bonier, & MacDougall-Shackleton, 2013). As climate change is experienced, phenological misalignments have arisen that create asynchronies in migration and reproduction alongside gaps between food resource pulses and energy-intensive annual cycle chapters (Cohen, Lajeunesse, & Rohr, 2018; Visser & Both, 2005). Timing of breeding and migration were found to be flexible in some populations but not others in adaptation to changing environments (Visser, Caro, Oers, Schaper, & Helm, 2010). Therefore, it is important to investigate phenology of birds and other sensitive animals because they act as indicator species of not only environmental health but also environmental change. The better we understand the mechanisms and physiology that contribute to annual cycle phenology, the better we are able to predict and prepare for the effects of environmental change on populations. Our findings contribute to the understanding of regulation of timing in annual cycles and improve predictions of how species will respond to changing environments.

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### *Ethics Statement*

This study was in compliance with BIACUC protocol #15-026-19 and conducted under the US Fish and Wildlife migratory bird scientific collection permit MB093279.

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