REPRODUCTIVE TIMING IN A CHANGING WORLD: UNDERSTANDING MECHANISMS AND COSTS

ASSOCIATED WITH REPRODUCTIVE TIMING DECISIONS

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The Supervisory Committee certifies that this ***disquisition*** complies with North

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ABSTRACT

Organisms in temperate zones time reproduction to occur when conditions are optimal for raising offspring. However, individuals of many taxa vary in precise timing of breeding. The mechanisms underlying the existing variation are not well understood, particularly in females. I hypothesized that individual variation in daily (i.e., circadian) rhythms, which are highly conserved across taxa, are related to variation in reproductive timing. By measuring activity onset in two species of songbirds, we found that females beginning their day earlier also breed earlier. I further asked if sex-steroid hormones were related to onset of daily activity in free-living individuals. My results show that maximal levels of estradiol are correlated with onset of activity in free-living females. This suggests that circadian rhythms may influence much of the variation seen in timing of breeding and individual physiology influences circadian behavior. In addition to mechanisms underlying reproductive timing, I also investigated potential selective pressures that could act on timing decisions. Early breeding females often produce more and larger offspring, however, most of the population breeds later than this optimum, suggesting there are costs associated with early reproduction. To determine if early breeding females are better able to handle an additional energetic challenge, I injected incubating females with a mild antigen and monitored nest survival in early and late breeders. The immune challenge caused a significant increase in nest failure compared to controls and success did not differ between early and late breeders. Next, using a long-term dataset I asked whether females who breed early experience accelerated rates of aging via telomere loss. I found that early breeding females experience higher telomere attrition compared to females breeding later. Annual change in telomere length was not related to reproductive output, but females experiencing cooler temperatures during egg laying and incubation (i.e., laying earlier in the season) had higher telomere attrition. With telomere loss and length being important determinants of lifespan and longevity, higher telomere attrition in early breeders may be linked with reduced overwinter survival. A better understanding of mechanisms and costs will help determine how populations will adjust to, or suffer from, a changing climate.

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CHAPTER 1: GENERAL INTRODUCTION

Background

Seasonal bouts of reproduction have been observed in vertebrate species in temperate-zones and are thought to have been shaped through selection (Baker 1938). The physiological and behavioral changes that organisms must undergo to prepare for seasonal breeding have been found to be predominantly regulated by photoperiod (Tauber and Tauber 1976; Thomas and Vince-Prue 1996; Dawson et al. 2001; Hau 2001; Dawson 2003). Supplementary cues, such as temperature or food availability, allow for year to year variation to adjust timing of reproduction to optimal breeding conditions (Schoech 1996; Rubenstein and Wikelski 2003; Nilsson and Källander 2006; Post and Forchhammer 2008; Shine and Brown 2008). Studies of temperate breeding vertebrates have found that early-breeding individuals generally have higher reproductive success compared to individuals that breed later in the season (Perrins 1970; Bourdon and Brinks 1982; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004), but individual variation around the optima is observed. The mechanisms underlying individual variation in timing of breeding and the costs associated with early reproduction remain unclear.

Extensive research in seasonal breeders has determined that initial activation of the reproductive system is regulated by photoperiod (Dawson 2003). Predictable changes in photoperiod across the year cues organisms to physiologically prepare for reproduction during appropriate environmental conditions in temperate breeding zones (Dawson et al. 2001; Dawson 2008). The combination of appropriate photoperiod, combined with other supplementary cues, activates gonadal growth through the hypothalamic-pituitary-gonadal (HPG) axis, a neuroendocrine pathway that integrates internal and external signals at the level of the hypothalamus (Figure 1)(Adkins-Regan 2008). The hypothalamus releases gonadotropin releasing hormone (GnRH) to elicit secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. LH then causes release of the sex-steroid hormones testosterone (T) and estradiol (E_2) from the gonads (Adkins-Regan 2008; Wingfield 2012). T and E_2 must then bind with the receptors of target tissues to prepare for and maintain reproduction (Ball and Ketterson 2008).

Figure 1. Photoperiod and other supplementary cues stimulate production of 1) GnRH from the hypothalamus. GnRH acts on the pituitary, resulting in production of 2) LH and FSH. These gonadotropins cause the gonads to produce 3) T and E_2, which 4) bind to receptors on target tissues. Photo from Needham (2017).

While the role of the HPG axis is well understood, individual variation in timing of reproduction still occurs (Perrins 1970). While there is significant variation in hormone output levels at the level of both the pituitary and the gonad, it is unclear how variation in these levels pre-breeding relate to individual reproductive timing decisions (Chastel et al. 2003; Williams 2012a; Caro et al. 2013; Greives et al. 2016). In addition, timing of reproductive activation is more thoroughly studied in males and less is known about how females differ in their reproductive response to environmental cues (Ball and Ketterson 2008). An abundance of research has been conducted in males; however, physiology of females is less well understood, even though they ultimately dictate when offspring are born (Caro et al. 2009; Zucker and Beery 2010; Beery and Zucker 2011; Caro 2012). Thus, this work focuses solely upon females, though future work in males could help separate important differences between the sexes in the wild.

My dissertation addresses two aims with respect to individual variation in timing of breeding. The first is to identify potential interactions between endogenous rhythms and reproductive timing behavior and physiology in free-living organisms. The second aim of my dissertation is to identify reproductive costs of individual variation in timing of breeding. To address the two aims of my dissertation, we tested the following hypotheses in free-living female songbirds:

1.1: *Variation in daily rhythms is an underlying mechanism driving individual variation in seasonal onset of timing of breeding*

1.2: *Variation in maximum E_2 levels are related with female activity onset during the breeding season*

2.1: *Energetic costs of upregulation of the humoral immune response will reduce offspring survival and have a disproportionately higher impact on reproductive success earlier in the breeding season*

2.2: *Individuals that breed earlier have higher rates of telomere loss*

Aim 1: Identify Potential Interactions Between Endogenous Rhythms and Reproductive Timing Behavior and Physiology in Free-Living Organisms

The Role of Endogenous Circadian Rhythms in Reproduction

Seasonal activation of the reproductive system displays a strong interaction with endogenous daily (i.e., circadian) rhythms (Helm and Visser 2010). Many organisms display internally driven physiological and behavioral rhythms that are shorter or longer than 24 hours in length when external cues (e.g. photoperiod, temperature changes) are absent (Aschoff 1988). Laboratory studies in multiple species using 1 hr light-pulses and varying the length of the dark phase (i.e., nighttime) have found that exposure to light during a specific time of the circadian phase activates the long day reproductive response (Hamner 1963; Follett and Sharp 1969; Milette and Turek 1986; Paul et al. 2008a). This seasonal photoperiodic response across taxa occurs during what is referred to as the photo-inducible phase of the circadian system, which was first proposed in 1936 by Erwin Bünning (Paul et al. 2008a). The photo-inducible phase is the timing of the rhythm predicted to occur during darkness under short photoperiods, but coincide with light under a long photoperiod (Chapter 2, Figure 2) (Hazlerigg and Wagner 2006). Laboratory studies have also shown that photoperiodic control of endogenous daily rhythms is linked with seasonal reproductive responses. For example, shortening the length of circadian rhythms via mutation of the *tau* gene in reproductive Syrian hamsters (*Mesocricetus auratus*) causes testicular regression to occur more quickly (under 10 h of darkness compared to 12 h of darkness) than in controls (Stirland et al. 1996). In *Arabidopsis*, knocking out genes associated with circadian regulation delays flowering (Wang and Tobin 1998)*.*

Significant individual variation in length of endogenous circadian rhythms (Helm and Visser 2010) may vary the timing of the photo-inducible phase on an individual basis. In Japanese quail (*Cortunix japonica*), the photo-inducible phase occurs between 10 and 16 hours after dawn (Nicholls et al. 1983). We would predict that the photo-inducible phase of an individual with a shorter endogenous circadian

rhythm may occur earlier in the day compared to an individual with a longer rhythm. However, social interactions and other environmental cues entrain the circadian system to the earth's 24 hr cycle (Crowley and Bovet 1980) which does not allow us to measure the length of an individual's circadian rhythm in the wild. As natural selection acts upon phenotypes, one way we can measure phenotypic output of circadian rhythms is by measuring chronotype, or the timing of an individual relative to a cue (e.g. onset of activity relative to sunrise) (Lehmann et al. 2012). Prior studies have found that individuals with a shorter circadian period also become active earlier than individuals with longer rhythms (Aschoff and Wever 1966; Duffy et al. 2001)). Circadian rhythms are also heritable, allowing selection upon these rhythms to occur (McClung 2006; Helm and Visser 2010). We would expect natural selection to act upon chronotype, the timing phenotype, in free-living organisms. Yet this pathway remains largely unexplored in the wild, where selective pressures are acting upon phenotypes. Thus, variation in circadian rhythms may underlie the individual variation in seasonal timing of breeding. Chapter 2 of my dissertation tests the hypothesis that variation in daily rhythms is an underlying mechanism influencing individual variation in seasonal onset of timing of breeding by measuring daily onset of activity in females and correlating it with first egg of the season.

Hormonal Influences on Behavior and Reproduction

Behavioral output of daily rhythms are plastic and influenced by many environmental and physiological factors. For example, prolonged increases in levels of hormones like melatonin have the capacity to delay awakening time and timing of reproduction in songbirds (Greives et al. 2012; Greives et al. 2015). Alternatively, women with higher levels of the sex-steroid, estradiol (E_2), wake up earlier than females with lower levels of E_2 (Bracci et al. 2014). In female hamsters, E_2 implants lead to shortened free-running circadian period and earlier chronotypes when compared to females with empty implants (Morin et al. 1977; Takahashi and Menaker 1980). There appears to be a lack of information on the relationships between E_2 and circadian rhythms in non-mammalian vertebrates; however, higher levels of plasma testosterone (T), another sex-steroid hormone, has been found to be correlated with higher levels of plasma E_2 in female birds (Rosvall et al. 2013). Experimentally induced and naturally increasing levels (i.e., seasonal increases) of T have been found to lengthen the active period of male birds and shorten circadian period length during the breeding season (Gwinner 1974; Gwinner 1975). This suggests a

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similar mechanistic relationship between sex-steroids and daily rhythms in birds that has been observed in mammalian vertebrates.

Studies measuring baseline hormone levels do not often find a relationship between the hormone and the behavioral or morphological variable of interest (Damassa et al. 1977; Chastel et al. 2003; Williams et al. 2004; Mcglothlin et al. 2008; Huyghe et al. 2009; Eikenaar et al. 2011; Caro et al. 2013; Burtka et al. 2016). Many samples in these studies were collected during the day or after individuals had been in captivity for several hours. Recently it was found that nighttime levels of T in both male and female songbirds are, on average, twice as high as daytime levels (Laucht et al. 2011). While capturing wild individuals at night may not always be feasible in the wild, recent evidence suggests that gonadotropin-releasing hormone (GnRH) induced T levels are reflective of nighttime T levels (Needham et al. 2017). If, like T, nighttime levels of E_2 are more informative of maximum levels experienced during the breeding season, we may observe stronger relationships between levels of GnRH-induced sex-steroid hormones and reproductive timing behaviors compared to baseline levels. Chapter 3 of my dissertation tests the hypothesis that variation in maximum E_2 levels are related with female activity onset during the breeding season by exposing females to a GnRH challenge and correlating maximal levels of E_2 and T with daily onset of activity.

Aim 2: Identify Reproductive Costs of Individual Variation in Timing of Breeding

Additive Energetic costs and Timing of Reproduction

In addition to understanding the mechanisms that determine timing of breeding in individuals, it is important to understand why most of the population does not breed early, when higher reproductive success is observed (Perrins 1970; Bourdon and Brinks 1982; Festa-Bianchet 1988; Landa 1992; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004). Early breeders appear able to produce more and larger offspring than late breeders (Perrins 1970; Festa-Bianchet 1988; Ribble 1992; Rieger 1996; Holand et al. 2006; Williams 2012b) increasing overwinter survival and recruitment rates compared to smaller young of the year (Krementz et al. 1989; Linden et al. 1992; Sedinger et al. 1995; Naef-Daenzer et al. 2001; Monrós et al. 2002; Low et al. 2015). The observed delay in reproduction from what seems to be optimal reproductive timing (Verhulst and Nilsson 2008) suggests there are likely costs to early breeding that delay reproduction in most of the population.

Offspring rearing is energetically demanding (Williams 1966; Trivers 1972); therefore, additional energetic challenges during this life-history stage may allocate energy away from offspring care, decreasing reproductive success. Cool temperatures and reduced food availability early in the season may reduce energy available for other energetically expensive processes, like immune system activation, compared to later in the season (Ardia 2005). Activation of the immune system is energetically expensive (Demas et al. 1997; Martin et al. 2003) and incompatible costs associated with reproduction and immune function have been found to result in a trade-off (Deerenberg et al. 1997; Hanssen et al. 2004; Hanssen et al. 2005; French et al. 2007; Martin et al. 2008). Immune system activation generally requires an individual to conserve energy, but offspring care (e.g. feeding, predator defense) requires energy use that could be allocated toward immune function and adult self-maintenance (Adelman and Martin 2009). A number of studies have looked at the effects of immune system activation during the breeding season on adult survival (Hanssen et al. 2004), changes in offspring feeding rate (Råberg et al. 2000; Ardia 2005), and offspring growth rates (Lozano and Ydenberg 2002), yet seasonal variation in ultimate effects on offspring survival are not as well studied. Therefore, we would hypothesize this trade-off may be amplified for early breeding individuals, resulting in reduced ability to handle an energetically demanding immune challenge. As a result, early breeding individuals would have significantly reduced offspring survival compared to later in the season. Chapter 4 of my dissertation tests the hypothesis that energetic costs of upregulation of the humoral immune response will reduce offspring survival and have a disproportionately higher impact on reproductive success earlier in the breeding season. To test this hypothesis, I exposed incubating females to an immune challenge (or control injection) and compared nest success of early and late breeding females.

Biological Aging as a Cost of Reproduction

While the trade-off between reproduction and survival is well studied (Smith 1958; Blomquist 2009), little is known about how seasonal variation in reproductive timing may play a role in rates of aging. Exposure to more stressful conditions early in the breeding season may alter homeostasis, leading to increased levels of oxidative stress and thus an increased rate of aging (Finkel and Holbrook 2000; Costantini 2008). Recently, telomere dynamics have become a potential biomarker to study aging (Hornsby 2006; Mather et al. 2011). Telomeres, the repetitive DNA sequences on linear eukaryotic

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chromosomes, protect the chromosome ends from losing coding sequences during the replication process, however, they limit the number of replications a cell can undergo without losing coding DNA (Monaghan 2010). Higher rates of telomere loss also correlate with reduced survival (Haussmann and Marchetto 2010), thus if early breeding individuals have higher rates of telomere loss, it would suggest increased aging rate is a cost of early reproduction. Chapter 5 of my dissertation tests the hypothesis that individuals that breed earlier have higher rates of telomere loss using a longitudinal dataset to measure change in telomere length in the year following a breeding attempt and correlating it with timing of breeding.

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CHAPTER 2: EARLY TO RISE, EARLY TO BREED: A ROLE FOR ENDOGENOUS RHYTHMS IN SEASONAL REPRODUCTION[1]

Abstract

Vertebrates use environmental cues to time reproduction to optimal breeding conditions. Numerous laboratory studies have revealed that light experienced during a critical window of the circadian (daily) rhythm can influence reproductive physiology. However, whether these relationships observed in captivity hold true under natural conditions and how they relate to observed variation in timing of reproductive output remains largely unexplored. Here we test the hypothesis that individual variation in daily timing recorded in nature (i.e., chronotype) is linked with variation in timing of breeding. To address this hypothesis and its generality across species, we recorded incubation behavior data to identify individual patterns in daily onset of activity for two temperate-breeding songbird species, the dark-eyed junco (*Junco hyemalis aikeni*) and the great tit (*Parus major*). We found that females who first departed from their nest earlier in the morning (earlier chronotype) also initiated nests earlier in the year. Date of data collection and ambient temperature had no effect, but stage of incubation influenced daily onset of activity in great tits. Our findings suggest a role for daily rhythms as one mechanism underlying the observed variation in seasonal timing of breeding.

Keywords: chronotype, seasonal timing, daily rhythms

Background

Seasonal bouts of reproduction have been observed in many vertebrate species and have long thought to have been shaped by selection (Baker 1938; Hau 2001; Bronson 2009). The physiological and behavioral changes that animals must undergo to prepare for seasonal breeding are predominantly timed by photoperiod (Dawson et al. 2001; Dawson 2003). Photic control of endogenous daily (i.e. circadian) rhythms has been linked to seasonal photoperiodic responses through a variety of laboratory-based

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studies. Specifically, animals possess a photo-inducible phase of the circadian rhythm, whereby light experienced during this phase induces reproductive responses appropriate for long days, whereas the absence of light during this critical phase of the circadian rhythm leads to short-day reproductive responses (Hamner 1963; Follett and Sharp 1969; Milette and Turek 1986; Hazlerigg and Wagner 2006; Paul et al. 2008a). In vertebrates breeding during long days, exposure to light during this photo-inducible phase of the circadian rhythm will be encoded as a reproductively stimulatory day length (Hazlerigg and Wagner 2006). In Japanese quail (*Cortunix japonica*), this photo-inducible phase ranges between 10 and 16 hours after the lights come on, the laboratory equivalent of dawn (Nicholls et al. 1983), suggesting that timing of the photo-inducible phase is variable between individuals of the same species.

Variation among individuals in behavioral and physiological outputs of circadian rhythms is commonly observed in animals held under constant laboratory conditions (Aschoff and Wever 1966; Horne and Östberg 1977; Duffy et al. 2001) and recent work in wild caught individuals has suggested there is a heritable component to these rhythms (Helm and Visser 2010). Whether individual variation in endogenous daily rhythms may influence variation in the time of day an individual is sensitive to photo-induction, and thus responses to light exposure at the end of an early spring day, remains unknown. For example, an individual with a shorter free-running circadian rhythm may have a slightly advanced photo-inducible phase compared to an individual with a longer free-running rhythm, leading to earlier reproductive responses under the same photoperiod (Figure 2). Thus, individual variation in circadian rhythms may underlie individual variation in seasonal timing of breeding as suggested by Helm and Visser (Helm and Visser 2010) by shifting the timing of the photo-inducible phase. This hypothesis has not yet been tested in the wild, where natural selection pressures are acting and these traits are being expressed.

Measuring the period length of an individual's circadian rhythm in the wild, however, presents many challenges. The daily light-dark cycle, social interactions and other environmental cues entrain the circadian system to a period of 24hrs (Crowley and Bovet 1980; Johnson et al. 2003; Dunlap et al. 2004), thus making it impossible to measure the endogenous free-running circadian period of an individual in the wild. Alternatively, we can measure the phenotypic output of circadian rhythms by measuring the chronotype, or the timing exhibited by an individual relative to a salient environmental cue (e.g. sunrise)

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and other population members (Lehmann et al. 2012; Dominoni, Helm, et al. 2013). Evidence in humans and birds has uncovered clear links between endogenous rhythms and chronotype; individuals with a shorter free-running circadian period exhibit an earlier chronotype and become active earlier in the day (Aschoff and Wever 1966; Duffy et al. 2001; Dominoni, Helm, et al. 2013); c.f. Majoy and Heideman 2000; Helm and Visser 2010). As selection acts upon phenotypes expressed in nature, we would expect it to act upon chronotype in free-living organisms.
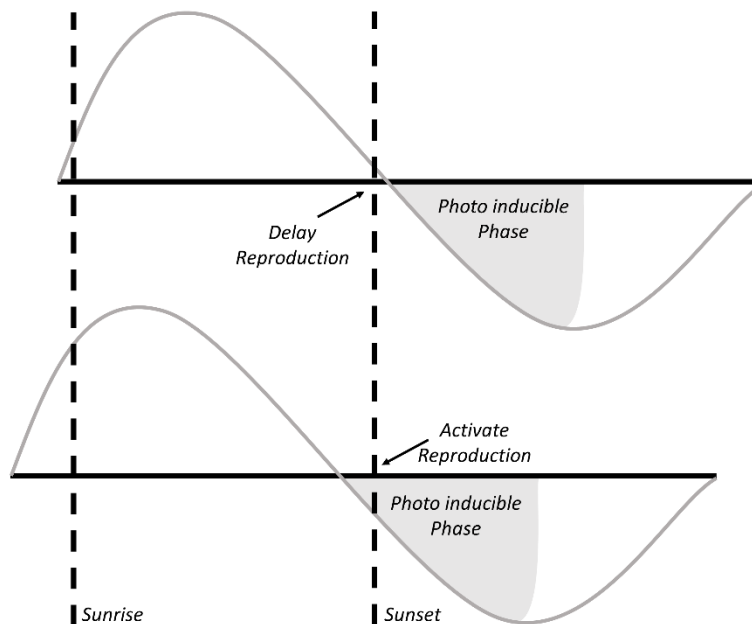
Figure 2. Two individuals entrained to a 24-hour day; however, differing chronotypes (i.e., earlier onset of activity with respect to sunrise in the bottom individual) change the phase of the endogenous rhythm at which light is experienced. Hence, timing of the photo inducible phase varies the reproductive response within an individual.

Here we test the hypothesis that variation in daily rhythms, expressed as chronotype, is an underlying mechanism driving individual variation in seasonal timing of breeding. Specifically, we predicted that female songbirds who show an earlier seasonal activation of reproductive function and thus initiate egg laying earlier, would also be active earlier in the morning (i.e., a chronotype related to a shorter circadian period) compared to females with later chronotypes that departed from their nest later in the morning. We assessed the chronotype of females repeatedly during incubation to quantify the repeatability of this trait and collected data from females of two free-living species of songbird on two

continents, dark-eyed juncos (*Junco hyemalis*) and great tits (*Parus major*) to determine the generality of this pattern across species.

Methods

Ethics

All animal use was conducted with approval from the NDSU Institutional Animal Care and Use Committee (Protocol #A13063) and the animal ethics committee of the state of Baden-Württemberg, Germany.

Study System

We studied a population of dark-eyed juncos (*Junco hyemalis aikeni*) near Lead, SD, USA (44°14'38"N, 103°51'55"W), from May 28 to July 5, 2015 and May 15 to June 26, 2016. This subspecies of dark-eyed junco is a small, ground nesting passerine that resides primarily in pine forests in the central US and typically begins laying eggs around mid to late May with an incubation period of approximately 12 days (Nolan et al. 2002).

In addition, data collected from a free-living population of great tits (*Parus major*) near Radolfzell, Germany (47°44'24"N, 8°58'48"E) from April 20 to 28, 2010 were utilized. Great tits are small, secondary cavity nesting songbirds that readily breed in nest boxes (Drent 1987; Cramp and Perrins 1993). Egg-laying at this study site begins in early April and incubation lasts approximately 12 days (Cramp and Perrins 1993).

Daily Timing

Thermochron iButtons (Model DS1921G-F5# with iButton Connectivity Kit Model SK-IB-R) were placed in the nest of incubating great tits and dark-eyed juncos. The iButtons were programmed to record nest temperature to the nearest 0.5^{O}C every 2-3 minutes. At least 2-8 mornings worth of data were collected for every individual. iButtons were also placed near the nest of dark-eyed juncos to collect ambient temperature every 30 min in 2015, while Onset HOBO data loggers (Model UA-002-08) were used to collect ambient temperature and light intensity every 5 min in 2016. Ambient temperature and light intensity was not collected for great tits.

Determining Onset of Activity

Observer (JLG) was kept blind to nest initiation date while determining activity onset times for all individuals. Daily onset of activity was calculated by subtracting onset of activity time from sunrise times derived for each study site from the United States Naval Observatory data base (www.usno.navy.mil/USNO) and determined by graphing incubation bouts from 03:00 to 08:00 and finding the first major dip in temperature indicating nest departure (Figure S1). This method has previously been shown to correlate strongly with video recorded nest departure times (Joyce et al. 2001). The dip was generally greater than 2°C below the average nest temperature from 03:00 to 08:00, however, this was not true in all cases. Eight dark-eyed juncos (10 mornings total) and 2 great tits (4 mornings total) had 1 or 3 mornings of data where nest temperature did not drop more than 2°C. Removing those mornings did not change the results, so all measures were included in analysis.

Statistical Analyses

All statistical analyses were performed using the free software R 3.2.2 (R Core Team 2015) with the package "lme4" (Bates et al. 2015) for mixed-effect modeling. To calculate repeatability of activity onset, a linear mixed-effects model was run with day of incubation included as a fixed effect and nest ID as a random effect. The resulting variance of the intercept was then divided by the sum of the variance of the intercept and variance of error (Dingemanse and Dochtermann 2013). To calculate 95% confidence intervals, "confint" was used. Lower and upper values were squared to get standard deviations for the variance of intercept and variance of error. Standard deviation for variance of the intercept was divided by the sum of standard deviations for variance of the intercept and variance of error for upper and lower values. This provided confidence intervals of repeatability.

Separate linear mixed-effects models were used to determine if activity onset was related to first egg of the season for individual dark-eyed juncos and great tits (hereafter referred to as egg 1 date), the day of incubation squared (Cooper and Voss 2013), and date of data collection (included as fixed effects). Year (for dark-eyed juncos) and nest ID were included as random effects. Ambient temperature data were not collected for great tits and ambient temperature did not influence onset of activity for the dark-eyed junco; thus temperature was not included in the final models. In year 2 for the dark-eyed juncos, we

additionally collected light intensity at the nest. A third linear mixed-effects model was used to determine if light intensity at sunrise was related to onset of activity in this reduced data set. Significance level was set at $\alpha = 0.05$.

Results

Dark-eyed junco activity onset ranged from 04:25 to 07:13 MDT. When calculated with respect to sunrise, times varied from 41 min pre-sunrise to 117 min post-sunrise. Repeatability of activity onset was 0.73 (*CI*: 0.67, 0.76) when controlling for day of incubation ($n = 36$). Onset of activity in female great tits ranged from 06:15 to 07:04 CEST. When calculated with respect to sunrise activity onset varied from 8 min pre-sunrise to 43 min post-sunrise. Repeatability of onset of activity was 0.27 (*CI*: 0.02, 0.42) when controlling for day of incubation ($n = 13$).

We found that the date a female laid her first egg of the season was positively related to daily onset of activity in both dark-eyed juncos ($F = 5.300$, $df = 1$, 19.45, $p = 0.03$, Figure 3A) and great tits ($F = 8.621$, $df = 1$, 9.64, $p = 0.01$, Figure 3B): individuals that laid their first egg earlier in the year had earlier daily activity onset. We also found the closer a female's eggs were to hatching, the onset of activity became later in great tits ($F = 6.996$, $df = 1$, 11.47, $p = 0.02$), but not in dark-eyed juncos ($F = 2.295$, $df = 1$, 85.80, $p = 0.13$). There was no relationship of activity onset with calendar date of data collection in either species (dark-eyed juncos: $F = 0.454$, $df = 1$, 33.35, $p = 0.50$; great tits: $F = 0.003$, $df = 1$, 16.54, $p = 0.95$).

When we restricted our data to only include dark-eyed juncos with data for light intensity at sunrise ($n = 24$), light intensity was significantly related to onset of activity ($F = 7.838$, $df = 1$, 84.80, $p < 0.01$). We still see a trend for individuals that laid eggs earlier in the year to also have earlier onset of activity ($F = 3.349$, $df = 1$, 21.97, $p = 0.08$). Calendar date of data collection and day of incubation showed no relationship with onset of activity (date of data collection: $F = 0.003$, $df = 1$, 23.65, $p = 0.96$; day of incubation: $F = 1.483$, $df = 1$, 75.15, $p = 0.23$).
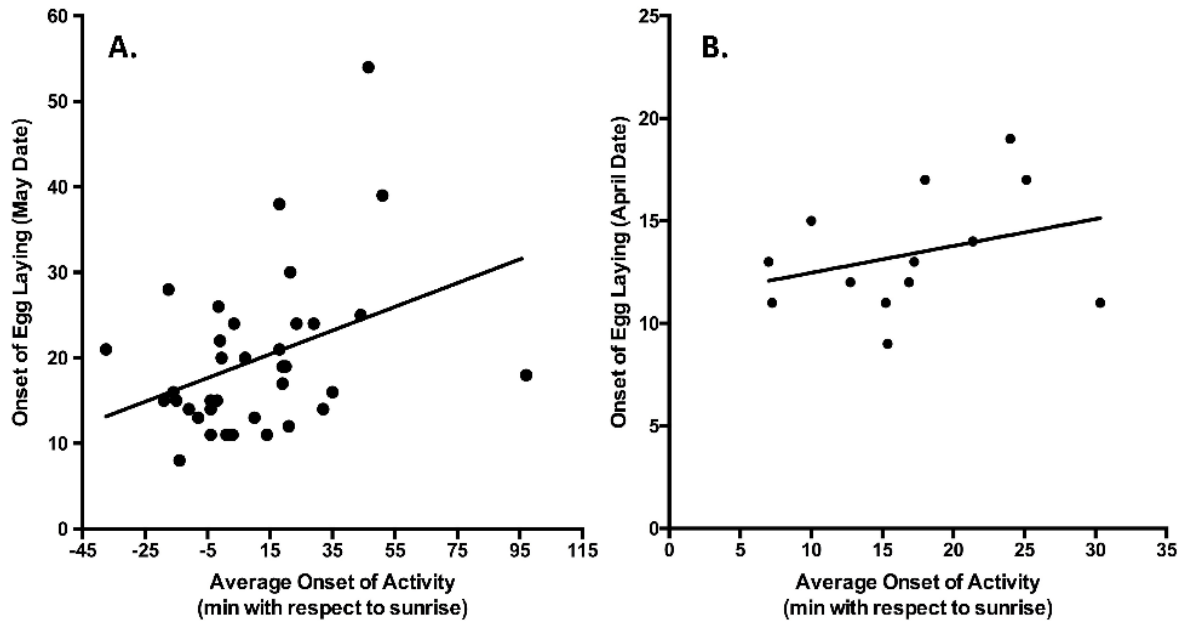
Figure 3. Individual averages of raw data for onset of activity graphed against first egg of the season shows as nest initiation dates shifts later in the season, females become active later in the day. Both dark-eyed juncos (A) and great tits (B) show this pattern

Discussion

Our data support the hypothesis that individual variation in daily rhythm phenotype influences individual variation in seasonal onset of breeding. Females of both dark-eyed juncos and great tits that became active earlier in the morning (i.e., earlier chronotype) had earlier nest initiation dates than females that became active later in the morning. As previous findings have demonstrated that earlier chronotypes are manifestations of shorter circadian period lengths (Aschoff and Wever 1966; Duffy et al. 2001; Dominoni, Helm, et al. 2013), our findings of a relationship between early onset of activity and clutch initiation provide evidence that variation in endogenous circadian rhythms may act as a mechanism influencing individual variation in seasonal timing of breeding in wild populations.

We observed consistent individual differences in daily onset of activity during the breeding season. While our study necessarily collected timing data after the decision to lay eggs had been made, we believe that our findings are indicative of an individual's chronotype prior to nest initiation. Other studies, including in our study species, have also observed within year consistency in individual female awakening and nest departure times both during and outside the breeding season (Steinmeyer et al. 2010; Schlicht et al. 2014; Stuber et al. 2015). In addition, onset of activity for six dark-eyed junco females

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in our study was collected during a known second nesting attempt. Nest initiation date for the first nesting attempt of these individuals was known and was included in the model with activity onset recorded during the second attempt. Females that laid eggs early during the first nesting attempt still fit the observed pattern of becoming active earlier, even on a second nesting attempt, compared to late breeding females. This provides some support that females with early chronotypes consistently exhibit early chronotypes compared to those with late chronotypes, regardless of time of season. Interestingly, timing of reproductive characteristics like egg laying date in females and onset of dawn song in males, are repeatable (Noordwijk et al. 1980; Sydeman and Eddy 1995; Nussey et al. 2005; Murphy et al. 2008; Naguib et al. 2010). Males that sing earlier also pair with early breeding females (Murphy et al. 2008), suggesting males who awaken earlier (i.e. have shorter endogenous rhythms) are pairing with females that breed earlier and also have shorter endogenous rhythms. These combined observations suggest that an individual's onset of daily activity is consistent with respect to other individuals in the population, and likely reflects individual variation in endogenous rhythms, though future work is needed to confirm this result.

A recent study found no relationship between chronotype and timing of breeding in captive-housed great tits (Helm and Visser 2010). One reason for this deviation may have been due to using artificial lighting in aviaries while organisms are better able to respond more precisely to natural external cues (Fleissner and Fleissner 2002; Helm and Visser 2010). Captive great tits do show altered timing of sleep behaviors, including earlier awakening and nest departure times and shortened sleep duration, even when kept under naturalistic conditions compared to their free-living counterparts (Stuber et al. 2015). A study in European blackbirds (*Turdus merula*) did find that chronotype measured during the breeding season under free-living conditions was related to the length of an individual's free-running rhythm measured in captivity later on in the same breeding season (Dominoni, Helm, et al. 2013). While the study by Helm and Visser (2010) contained important findings, our study utilized measurements of rhythmic daily and seasonal behaviors in a natural environment. This may indicate the importance of measuring these traits and their relationship with seasonal timing decisions in the wild.

It has been generally observed that early breeding individuals in temperate zone habitats are few in number compared to the rest of the population, but have higher reproductive success compared to

individuals that breed later (Perrins 1970; Bourdon and Brinks 1982; Olsson and Shine 1997; Dawson and Clark 2000). This observed pattern is consistent with patterns observed in the length of endogenous rhythms; that shorter rhythms are rare compared to longer rhythms while an intermediate rhythm length is most common (Helm and Visser 2010). Flexibility in timing of breeding is important for adapting to year to year variation in the environment, but variation in the length of endogenous rhythms may be one mechanism regulating individual reproductive timing relative to other individuals within a given year. The relationship between individual variation in behavioral rhythms and clutch initiation suggests the possibility that individual variation in the timing of the photo-inducible phase in the early spring may directly influence seasonal reproductive onset.

Light intensity at the nest at sunrise appears to play an important role in onset of activity as well. Though we only collected a single year of data for dark-eyed juncos, our findings agree with other studies showing that higher light intensity leads to an advanced onset of daily activity (Aschoff 1979; Newberry et al. 1988; Dominoni et al. 2014; Stuber et al. 2015). Urban dwelling birds are exposed to higher light intensities at night and will also advance their timing of reproduction compared to forest dwelling birds (Dominoni, Quetting, et al. 2013). With nighttime and sunrise light intensity affecting onset of activity (Dominoni et al. 2014), roosting behavior prior to the breeding season could be an important factor in determining onset of reproduction by influencing intensity of light experienced by an individual during this crucial time.

Additional non-photic cues that interact with endogenous daily rhythms may also play a role in onset of activity and observed timing of reproduction. For example, a high fat diet is capable of lengthening activity rhythms in mice (Kohsaka et al. 2007). The ability to obtain a high-quality diet under pre-breeding conditions could then advance the onset of reproduction. A common phenomenon observed in songbirds is the tendency for older females to initiate reproduction earlier in the season than younger females (Perrins 1970; Mills 1973; Nol and Smith 1987). The age of the individuals in the current investigation was unknown, but it may be likely that age-related changes in endogenous rhythms influence age-related changes in clutch initiation in birds. Age related shifts in the endogenous rhythms and chronotypes of humans have been extensively studied, showing a significant increase in early rising behavior in advanced age (Tankova et al. 1994; Hur and Bouchard 1997; Roenneberg et al. 2004;

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Roenneberg et al. 2007). Studies in *Drosophila* and birds have obtained similar results, with older individuals spending less time sleeping and awakening earlier in the day, though many of these studies are cross-sectional (Shaw et al. 2000; Steinmeyer et al. 2010; Stuber et al. 2015). To our knowledge, connections between age-related shifts in chronotype and reproductive timing have not been tested. The findings from our study suggest that changes in chronotype with age may at least in part underlie the observed pattern of older individuals initiating seasonal reproduction earlier than first year individuals in temperate breeding species (Mills 1973; Nol and Smith 1987). However, future work is needed to address the potential relationship between advances in chronotype and timing of breeding from year to year.

Conclusion

We found female songbirds that became active earlier, exhibiting an early chronotype, initiated clutches earlier in the season compared to females with a late chronotype that became active later. This supports our hypothesis that variation in daily rhythms are a likely underlying mechanism driving individual variation in seasonal onset of timing of breeding, though further experiments directly measuring length of free-running endogenous rhythms would strengthen these findings. Endogenous daily rhythms may also underlie other commonly observed patterns related with variation in timing of breeding (e.g. age), as well as other seasonal phenomena like migration. The fact that similar results were obtained in two unrelated species from two continents attests to the generality of our findings. Further research should aim at addressing the detailed mechanistic underpinnings of the observed relationships, test the hypothesis that similar patterns are also found in males, and examine possible seasonal and ecological variations in the connections between daily and seasonal rhythms.

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CHAPTER 3: FREE-LIVING FEMALE DARK-EYED JUNCOS (*JUNCO HYEMALIS*) WITH HIGHER MAXIMAL ESTRADIOL LEVELS SHOW EARLIER ONSET OF ACTIVITY

Abstract

Many studies have tried to relate baseline levels of sex steroid hormones (i.e., estradiol, testosterone) to reproductive timing, behavior, and morphological traits, yet few studies have found significant relationships. Interestingly, experimental increases in these hormones significantly alters daily rhythms and we recently found a correlation between onset of activity and timing of breeding in two species of songbirds. This suggests the circadian system may play a role in seasonal timing of wild animals and maximal hormone levels may regulate this relationship. Natural, maximal levels of sex-steroid hormones have been found to occur at night. Additionally, they correlate highly with plasma levels after an injection of gonadotropin releasing hormone during daytime. Thus, we hypothesized maximal levels of estradiol correlate with onset of daily activity in free-living species. We measured onset of activity in incubating female dark-eyed juncos (*Junco hyemalis*), then injected them with gonadotropin releasing hormone to measure maximal levels of estradiol. Our findings suggest an interaction between estradiol and the circadian system to regulate daily onset of activity in free-living females. We suggest future studies examine the relationship between maximal sex-steroid hormones and other reproductive behaviors potentially associated with age.

KEYWORDS: Estradiol, Circadian behavior, Activity onset, Reproductive timing

Background

Biological rhythms are highly conserved across taxa (Dunlap et al. 2004) and daily timing behaviors in the laboratory have been shown to be correlated with internally driven circadian (i.e., daily) rhythms (Aschoff and Wever 1966; Duffy et al. 2001). In free-living populations, the timing of peak function is likely important to survival strategies (Pittendrigh 1954). For example, predators may be more abundant at certain times of day and low alertness or vigilance could decrease survival (DeCoursey et al. 2000). Similarly, timing daily and seasonal expression of reproductive behavior and morphology likely have a strong influence on mating success (Hau et al. 2017).

Photoperiodic control of the reproductive system is found in many taxa (Tauber and Tauber 1976; Thomas and Vince-Prue 1996; Dawson et al. 2001; Hau 2001; Dawson 2003). Specifically, light

experienced during specific timing of the circadian rhythm, termed the 'photo-inducible phase', induces the appropriate reproductive response to long or short day lengths (Hamner 1963; Follett and Sharp 1969; Milette and Turek 1986; Hazlerigg and Wagner 2006; Paul et al. 2008b). Understanding mechanisms of how physiology regulates daily timing behaviors may be important for understanding differences in fitness related traits.

The influence of sex-steroid hormones on circadian rhythms in captivity are well studied. In captive female hamsters, estradiol (E_2) implants lead to earlier awakening times when compared to females with empty implants (Morin et al. 1977; Takahashi and Menaker 1980). In males, experimentally induced and naturally increasing (i.e., seasonal) levels of testosterone (T), have been found to lengthen the active period of captive birds during the breeding season (Gwinner 1974; Gwinner 1975). Even seasonal peaks in E_2 levels correlate with earlier female emergence times relative to sunrise in birds (Williams et al. 2004; Schlicht et al. 2014). Awakening time and levels of estradiol (E_2) are correlated in women; females with higher levels of E_2 have an earlier awakening time than females with lower levels of E_2 (Bracci et al. 2014). Yet there appears to be a lack of information on the relationships between E_2 and circadian rhythms in non-human, free-living female vertebrates. However, we lack information on how maximal levels of hormones like T and E_2 relate to activity driven by endogenous daily rhythms in the wild.

Circulating levels of baseline sex-steroid hormones are typically measured from samples collected during the day or after individuals had been in captivity for several hours. Previous research attempting to relate baseline levels of plasma T and E_2 to reproductive timing (Chastel et al. 2003; Williams et al. 2004), behavior (Damassa et al. 1977; Wells 1984; Eikenaar et al. 2011; Burtka et al. 2016) and phenotypic traits (Mcglothlin et al. 2008; Huyghe et al. 2009) often do not observe consistent relationships. Evidence suggests that an injection of gonadotropin-releasing hormone (GnRH) induces a short term increase in plasma T to maximal levels (Jawor et al. 2006; Jawor et al. 2007) that are more correlated with reproductive traits in males than baseline levels (McGlothlin et al. 2007). Recently it was found that in a captive songbird, the house sparrow (*Passer domesticus*) nighttime levels of T in both males and females are significantly higher than daytime levels (Laucht et al. 2011). While capturing wild individuals at night may not always be feasible, recent evidence suggests that GnRH induced T levels are reflective of nighttime T levels (Needham et al. 2017). In a different songbird, the dark-eyed junco (*Junco*

hyemalis), higher levels of pooled plasma T have been found to be correlated with higher levels of plasma E_2 (Rosvall et al. 2013). GnRH challenges provide an alternative method to measure maximal, nighttime-like levels of T and E_2 in free-living organisms and may reveal stronger relationships with circadian timing behaviors compared to daytime, baseline levels.

We tested the hypothesis that variation in maximum E_2 levels are related with female activity onset during the breeding season by assessing the first daily off-bout during incubation in female dark-eyed junco's (*Junco hyemalis*) and measuring GnRH-induced levels of E_2 just prior to laying and during incubation. Specifically, we predicted that females departing from the nest earlier in the morning would have higher GnRH-induced E_2 levels compared to females that depart from the nest later in the morning. We additionally measured GnRH-induced plasma T to test for a correlation between T and E_2, which has previously been shown using pooled plasma samples (Rosvall et al. 2013). A correlation between these values would allow future researchers to measure T as a proxy for E_2, which requires less than one third the amount of plasma to assay using commercially available enzyme immuno-assays. Lack of collinearity between these values would allow us to address whether T or E_2 is more closely related to onset of activity in free-living females.

Methods

Ethics

All methods used in the study were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC Protocol #A13063).

Study Species and Location

We monitored and studied a breeding population of dark-eyed juncos (*Junco hyemalis aikeni*) near Lead, SD, USA ($44^O14'38"$N, $103^O51'55"$W). Dark-eyed juncos are a small songbird that nests on the ground, making nests easy to find and monitor (Nolan et al. 2002). Females in this population have a relatively short breeding season (May – July) and some early breeders produce two successful clutches in a season (J. Graham, pers. obs.). On average, females lay four eggs per nest with an incubation period of approximately 12 days (Nolan et al. 2002).

Females were passively trapped prior to the breeding season using baited potter traps from April 17, 2016 – May 9, 2017, a time when the reproductive system should be primed for breeding and responsive to GnRH. Immediately upon capture, females were weighed and given a single mass-dependent intramuscular injection of 62.5 µg/kg chicken GnRH-I (American Peptide product #54-8-23, Sunnyvale, CA, USA) dissolved in PBS into the pectoralis muscle (Needham et al. *In prep*; Jawor et al. 2007). Females were held in cloth bags until 30 min post-injection when a blood sample was collected. Due to the large amount of plasma required by our assay to measure E_2 and regulation limits on volume of blood that can be collected, only post-GnRH samples were collected. A pilot study conducted in this species shows that females increase E_2 in response to a GnRH challenge after 30 minutes compared to control injected females (Needham et al. *In prep*). Blood samples were stored on ice until centrifugation to collect plasma. Plasma samples were stored at -20 until assayed for plasma estradiol. Testosterone was not assayed in these samples because plasma was also used to measure very low-density lipoprotein (Needham et al. *In prep*). All samples were collected prior to the first egg of the season being laid.

Determining Onset of Activity

Exhaustive nest searching was conducted from May through June 2016. To determine onset of activity for individual females, Thermocron iButtons (Model DS1921G-F# with iButton Connectivity Kit Model SK-IB-R) were placed in the nest of incubating females to record nest temperature to the nearest $0.5^{\circ}C$ every 3 minutes for 4 consecutive mornings (Graham, Cook, et al. 2017). All times are calculated with respect to sunrise. We have previously shown onset of activity to be a highly repeatable behavior within this population (Graham, Cook, et al. 2017). A subset of females in this study were part of the previous study ($n = 24$ of 30).

After the 4th morning of iButton data collection, females were captured from the nest using either a butterfly net or strategically placed mist nets (Graham, Mady, et al. 2017). GnRH injections followed the same protocol as used with pre-breeding females. A total of 30 females had complete activity data and a blood sample collected during incubation for measurement of T and E_2. Of the 30 females, 13 females also had pre-breeding GnRH-induced E_2 samples. This allowed us to test whether variation in maximum E_2 levels are related with female activity onset at a physiologically relevant time when females are

preparing for reproduction (pre-breeding) and a temporally similar time to the behavior measured (incubation). Blood samples were stored on ice until centrifugation to collect plasma. Plasma samples from incubation were assayed for plasma estradiol and testosterone.

Hormone Assays

Plasma E_2 levels were measured following the manufacturer's guidelines using a commercially available enzyme immunoassay kit (Enzo Life Sciences, Cat # ADI-900-174)(Needham et al. *In prep*; Gall et al. 2013; Wilcoxen et al. 2015). Briefly, hormones were extracted (3x) from 100μL of plasma using diethyl ether, dried in nitrogen gas at $25^{\circ}C$, and reconstituted in assay buffer overnight (1:4.3 dilution). When 100 μL of plasma was not available, the concentration was adjusted ($n = 1$). Reconstituted samples were plated in duplicate (100 μL per well) and concentrations were calculated using a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc). A 7-point standard curve with known concentrations ranging from 1000 pg/mL – 15.6 pg/mL was run on the plate to determine unknown concentrations. The sensitivity of the assay is 14.0 pg/mL and this value was conservatively assigned to any individuals measuring below the detection limit ($n = 4$ of 13 pre-breeding, $n = 22$ of 30 incubating). Pre-breeding females were run as part of a larger set of samples ($n = 13$ of 94) across three plates. Inter-plate variation was 5.4 % and intra-plate variation ranged from 3.0 % – 8.8 % (plates 1: 4.2 %, 2: 3.0%, 3: 8.8 %) (Needham et al. *In prep*). All incubating female samples fit on a single plate and intra-plate variation was calculated at 3.71 %.

Plasma T was also measured following the manufacturer's guidelines of a commercially available enzyme immunoassay kit on a single plate (Enzo Life Sciences, ADI-900-065). Hormones were extracted (2x) from 30 μL of plasma using diethyl ether, dried under nitrogen gas at $25^{\circ}C$, and reconstituted with assay buffer overnight (1:10 dilution). Concentration was adjusted for samples that did not have 30 μL of plasma available after running the E_2 assay ($n = 7$ of 30). Reconstituted samples were plated in duplicate (100 μl per well) and concentrations determined using a five-parameter logistic curve-fitting program (Microplate Manager, Bio-Rad Laboratories, Inc.). A 6-point standard curve with known concentrations ranging from 2000 pg/mL – 1.95 pg/mL was run on the plate to determine unknown concentrations. Samples that were below detection limit ($n = 3$) were set at sensitivity of the assay (5.67 pg/μL). Intra-plate variation was calculated at 4.12 %.

Statistical Analyses

All statistical analyses were conducted in R version 3.2.2 (R Core Team 2015) using package lme4 (Bates et al. 2015). Hormones were natural log transformed for all models to achieve a normal distribution. To confirm there was no collinearity between E_2 and T, we ran a linear model to determine if E_2 and T were correlated in incubating females with detectable levels of both T and E_2. Lack of a relationship allowed us to include E_2 and T values as independent variables in a single model.

We used a linear mixed-effects model with onset of activity recorded over four days as the dependent variable and pre-breeding sex-steroid levels during incubation as the independent variable. A second linear mixed-effects model was run comparing onset of activity with T and E_2 values in all 30 incubating females and additionally included the day of the year final behavioral samples were measured as timing of behavior may change across the reproductive season (Graham, Cook, et al. 2017). In both models, female ID was included as a random effect to control for repeated measures in nest departure time. Effect sizes (r) for the mixed-effects model was calculated using the formula provided by Nakagawa and Cuthill (2007).

Results

Pre-breeding levels of post-GnRH E_2 ranged from undetectable (set to 14.0 pg/mL) to 112.11 pg/mL in the 13 females that also had later recorded incubation behavior. During incubation, circulating levels of post-GnRH E_2 ranged from undetectable (set to 14.0 pg/mL) to 118.69 pg/mL, while circulating levels of T post-GnRH injection ranged from undetectable (set to 5.67 pg/mL) to 1503.11 pg/mL. There was not a significant correlation between GnRH-induced T and E_2 in incubating females ($F_{1, 6} = 0.35$, $p = 0.57$).

Higher levels of GnRH-induced E_2 pre-breeding ($F_{1, 11} = 5.27$, $p = 0.04$, r = –0.27, Figure 4) and during incubation ($F_{1, 26} = 6.26$, $p = 0.02$, r = –0.18, Figure 5) were both correlated with earlier nest departure times recorded from incubating females. GnRH-induced T showed no relationship with onset of daily activity ($F_{1, 26} = 0.65$, $p = 0.43$, r = 0.06, Figure 6). We additionally observed a trend for females sampled later in the season rising earlier relative to sunrise compared with females sampled earlier in the season ($F_{1, 26} = 3.66$, $p = 0.06$, r = –0.14).
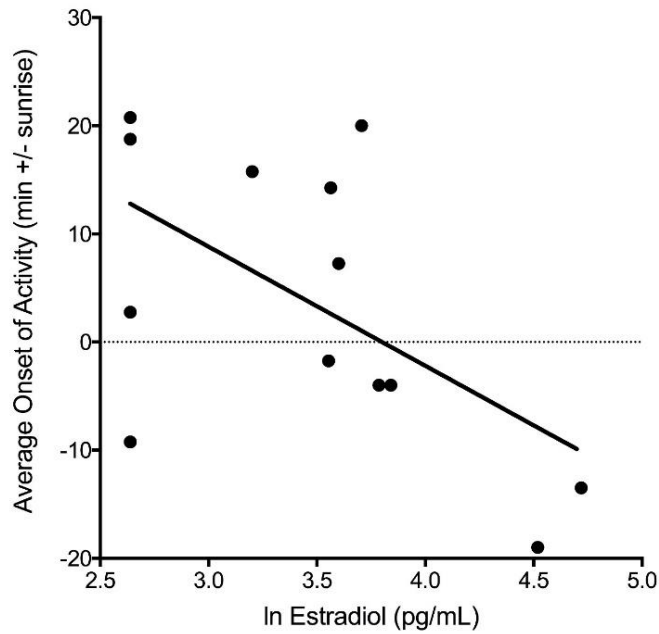
Figure 4. The 13 of females with pre-breeding levels of GnRH-induced estradiol showed a significant correlation between early onset of activity and higher maximal estradiol levels (r = -0.27, *p* = 0.04).
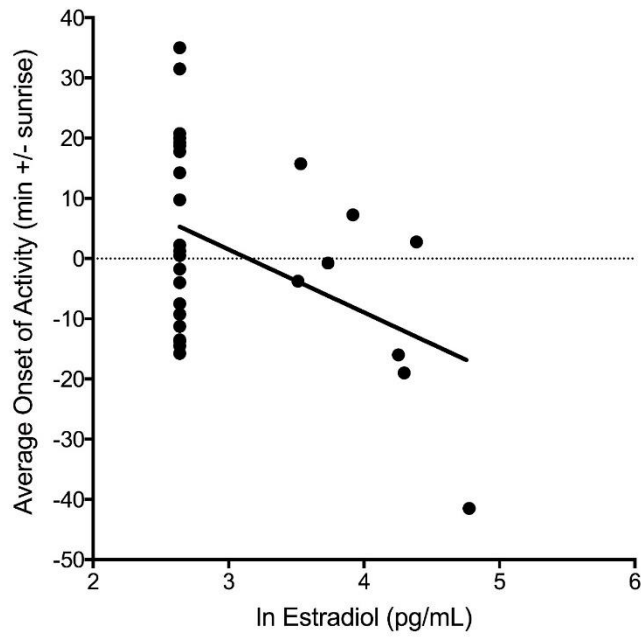
Figure 5. GnRH-induced levels of estradiol are correlated with onset of activity in incubating female dark-eyed juncos (r = -0.18, *p* = 0.02).
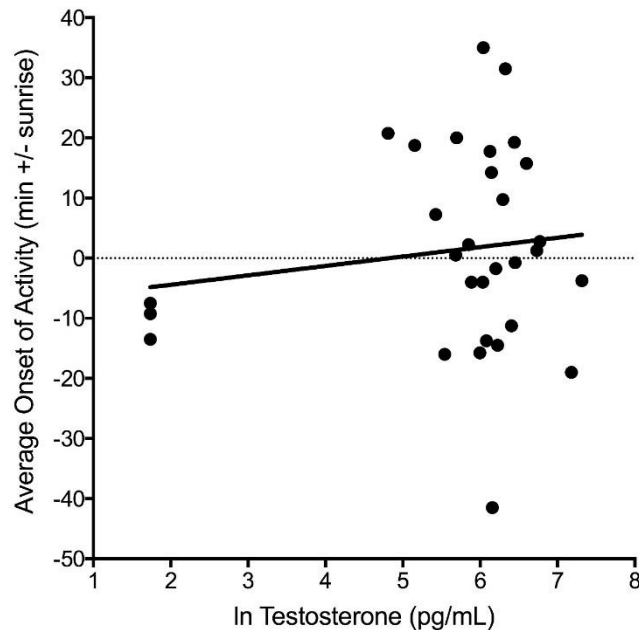
Figure 6. While most females had detectable levels of GnRH-induced testosterone, there was no relationship with onset of activity (r = 0.06, *p* = 0.43).

Discussion

Our study provides the first correlative evidence that individual levels of GnRH-induced estradiol (E$_2$) are related to daily (i.e. circadian) behavior patterns in free-living female songbirds. In incubating females, maximal levels of T were not correlated with onset of activity. There was a trend for incubating females recorded later in the season to awaken earlier relative to sunrise compared with females recorded earlier in the season. We additionally did not find a relationship between GnRH-induced plasma testosterone (T) and estradiol (E$_2$).

We found that GnRH-induced E$_2$ levels pre-breeding and during incubation are correlated with onset of activity (i.e., first nest departure), with earlier circadian behavior correlated with higher levels of E$_2$. We chose to collect our data both from pre-breeding and incubating females. Pre-breeding females were thus sampled at a time when females are preparing for reproduction and likely to have an active HPG axis, though it was temporally distinct from behavioral sampling. Incubating female dark-eyed juncos were sampled because we have previously shown high repeatability of onset of activity in this population during incubation (Graham, Cook, et al. 2017) and we additionally wanted to measure hormone levels at the same time as the behavioral observations. Our findings are in agreement with previous studies that

observed that experimentally increasing circulating sex-steroid hormones leads to earlier awakening times (Gwinner 1974; Gwinner 1975; Morin et al. 1977; Takahashi and Menaker 1980; Albers 1981) and the correlation between early awakening and higher levels of E_2 in women (Bracci et al. 2014).

While a clear relationship was observed between post-GnRH E2 and onset of daily activity during incubation, we observed many undetectable hormone levels while females were incubating. Regulatory mechanisms during this time period may dampen the responsiveness of the pituitary and/or ovary to GnRH and explain why so many E_2 samples collected during incubation were below the detection limit of an assay capable of detecting GnRH induced levels of E_2 in this species pre-breeding (Needham et al. *In prep*). E_2 levels in mammals fluctuate drastically from the beginning of the estrous cycle through parturition and can drop to non-breeding levels during this time period (Elias et al. 1984). In birds, E_2 peaks during rapid yolk development, declining throughout the laying cycle (Williams et al. 2004). In support of this hypothesis that regulatory mechanisms alter responsiveness to GnRH during incubation, we observed higher proportion of detectable E_2 samples pre-breeding (69.2 %) compared to post egg-laying (26.7 %). Additionally, increasing levels of E_2 prior to egg-laying and a decline to lower levels post egg-laying correlates with changes in female emergence times, which is earliest relative to sunrise just prior to the egg laying phase (Schlicht et al. 2014). Our finding that GnRH-induced E_2 levels measured pre-breeding are still correlated with onset of activity during incubation suggests that between individual relationships between estradiol and circadian behavior persists across different reproductive phases; those individuals with the highest maximal levels of E_2 are likely always awakening earliest. Studies measuring GnRH-induced E_2 levels and activity onset just prior to egg laying may produce an even stronger relationship than the one we found, particularly as 10-fold variation in plasma E_2 has already been shown in starlings with yolky follicles (Williams et al. 2004).

The trend of a negative relationship between day of year and onset of activity in this population was unexpected. We have previously shown in this population that early rising females breed earlier in the season (Graham, Cook, et al. 2017). However, the relationship observed in this study may likely be caused by including data collected during the second nesting attempts of early breeding females. We did not know the nest initiation date for all females in this data set; however, at least 5 of the last 7 females to be recorded during the season had known early first nest initiation dates, but due to inability to record

during their first attempt (e.g., nests depredated prior to sampling) these females were sampled on their second nesting attempt of the season. Early rising females sampled late in the season are likely continuing to rise early with respect to sunrise on their second nesting attempt and may be driving this pattern.

The lack of a correlation between T and E_2 was also unexpected. Rosvall et al. (2013) reported a positive correlation between pooled E_2 and T samples after a GnRH-challenge in photo-stimulated, captive female dark-eyed juncos. Lack of a correlation in our study may be due to greater variation in physiology and responsiveness of females sampled in captivity versus in the wild (Calisi and Bentley 2009). For example, there may be higher individual variation in aromatase levels (serving as a limiting enzyme in the conversion of T to E_2 (Simpson et al. 1994)) in the wild compared to captivity. Individual females in the wild may differ in how they respond physiologically to supplementary environmental cues (Chastel et al. 2003; Williams 2012a; Caro et al. 2013; Greives et al. 2016) that are lacking when housed indoors (Rosvall et al. 2013). In vitro output of T from ovarian follicles collected from free-living camels (*Camelus dromedarius*) does not change with follicle size or across the breeding season, but in vitro E_2 output increases with follicle size and from non-breeding to peak breeding season, suggesting individual and seasonal variation in aromatase activity (Sghiri and Driancourt 1999). Importantly, aromatase levels have been found to vary between free-living individuals during the breeding season (Silverin et al. 2004). In birds with the opportunity to re-nest later in the season, aromatase levels may be important for maintaining the ability to re-nest quickly after a nest fails while maintaining optimal hormone levels if the nest is successful. Maximal T levels have been found to be related to female parental care behaviors, like increased provisioning rate and decreased brooding time (O'Neal et al. 2008; Cain and Ketterson 2013), but very little is known about how maximal E_2 levels affect incubation and parental behavior (Hunt and Wingfield 2004). While females in our study were incubating when T was measured, variation in aromatase could have been due to how long a female had been incubating the current clutch or even time of season. Future work in larger individuals would allow us to collect both a baseline and maximal sample to measure responsiveness of the HPG axis during the incubation phase.

Recent evidence points to a relationship between daily rhythms and reproductive timing in the wild where those who awaken earliest also breed earliest (Graham, Cook, et al. 2017). Some hormones,

like melatonin, when provided as continuous release implants, have been shown to significantly delay onset of activity in males and timing of breeding in females (Greives et al. 2012; Greives et al. 2015), further suggesting a relationship between daily rhythms and seasonal timing. There is a pattern seen in many vertebrates for the earliest seasonal breeders to obtain highest reproductive success compared to later breeding individuals (Perrins 1970; Bourdon and Brinks 1982; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004; Low et al. 2015). However, few individuals breed during this optimal time and the mechanisms enabling certain individuals to breed early is under continued exploration (Verhulst and Nilsson 2008; Wilson and Nussey 2010; Low et al. 2015). Our finding that higher levels of GnRH-induced E_2 correlates with earlier activity onset suggests variation in hormonal regulation of daily rhythms may additionally fine-tune yearly reproductive timing decisions at the individual level, but requires further research in free-living individuals.

While the age of the females in our study was unknown and not part of the current study, females with detectable E_2 levels may have been older and have had prior reproductive experience. Evidence in the literature shows that reproductively experienced females elevate reproductive hormones, like GnRH content in GnRH neurons and circulating vitellogenin levels, and have rapid follicular growth compared to photo-naïve individuals (Sockman et al. 2004; Salvante et al. 2013a). This is particularly important with respect to our previous finding of a relationship between onset of activity and timing of breeding (Graham, Cook, et al. 2017). Cross-sectional studies have found a tendency for older individuals to awaken and depart from the nest box earlier than younger individuals (Steinmeyer et al. 2010; Stuber et al. 2015). If older or more experienced females are upregulating their reproductive hormones earlier than first time breeders, sex-steroid levels may be acting as a mechanism to regulate onset of activity as well as timing of breeding, though we lack a sufficient sample size to test this hypothesis.

Conclusion

Our study provides evidence for a relationship between maximum-induced levels of the sex-steroid hormone estradiol and circadian behavior in the wild. This is an important first step in understanding how hormones regulate daily timing behaviors, yet future work is needed to determine if these findings hold in other species and are related to timing during other life history traits. We suggest future studies with access to radio-telemetry or RFID readers address similar relationships between E_2

and daily behavioral patterns during the pre-breeding and egg laying stages of reproduction in free-living females. Our findings lay the foundation for understanding how estradiol influences reproductive behavior and reproductive timing in seasonally breeding species. However, our current understanding of how maximal production of E_2 from the hypothalamic-pituitary-gonadal (HPG) axis relates to individual variation in behavior is limited and future experimental work is needed to better understand the regulatory effects of estradiol on timing of behavior in free-living organisms.

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CHAPTER 4: EXPERIMENTAL IMMUNE ACTIVATION USING A MILD ANTIGEN DECREASES

REPRODUCTIVE SUCCESS IN FREE-LIVING FEMALE DARK-EYED JUNCOS[2]

Abstract

Seasonal animals time breeding so offspring rearing coincides with favorable conditions. Offspring rearing is energetically demanding; therefore, additional energetic challenges during this life-history stage may allocate energy away from offspring care, decreasing reproductive success. Activation of the immune system may be one such energetic challenge, and may have a disproportionately higher impact on reproductive success earlier in the breeding season when resources are less abundant and thermoregulatory demands are greater. We monitored nestling growth and survival in incubating female Dark-eyed Juncos (*Junco hyemalis* Linnaeus, 1758) injected with a mild antigen to stimulate antibody production and induce an energetic challenge. We found nests of treated females were more likely than controls to fail prior to six days post-hatch, coinciding with timing of peak antibody production. No effect of season was detected. Offspring mass did not differ between treatments prior to failure, suggesting failure was potentially due to differences in behaviour other than nestling feeding. Our findings indicate a trade-off between immunity and nest survival that is not affected by time of season. Based on the results of our study, we suggest future research be directed toward how immune activation influences behaviours, including nest guarding and predator aggression, and mediates this trade-off.

Keywords: Dark-eyed Junco, *Junco hyemalis,* reproductive trade-offs, seasonal effects, keyhole limpet hemocyanin, eco-immune

Introduction

Life history theory predicts a trade-off between current reproductive effort and survival and future reproduction (Harshman and Zera 2007). Indeed, experimentally increased reproductive effort has been shown to support this hypothesis (Smith 1958; Gustafsson and Pärt 1990; Hanssen et al. 2005).

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[2]The material in this chapter was co-authored by Jessica Graham, Rachael Mady, and Timothy Greives. Jessica Graham has primary responsibility for experimental design, sample collection, running experiments, data collection, data analysis, and developing the first draft of this chapter. Rachael Mady assisted with sample collection and revisions of this chapter. Timothy Greives was the primary provider of funding for materials and assisted in sample collection, forming conclusions, and revisions of this chapter. This publication can be found under, "Graham, J.L., Mady, R.P., and Greives, T.J. 2017. Experimental immune activation using a mild antigen decreases reproductive success in free-living female Dark-eyed Juncos. Canadian Journal of Zoology, 95(4): 263-269."

Reproduction is temporally separated from other energetically demanding life-history events, like molting and migration in birds, which increases the probability of adult and offspring survival (Hemborg and Lundberg 1998; Stutchbury et al. 2011). However, while there is separation of costly life-history events from each other, trade-offs with other energetically expensive processes still may occur within the breeding season.

Activation of the immune system is energetically expensive (Demas et al. 1997; Martin et al. 2003) and competing costs associated with reproduction and immune function has been found to result in a trade-off (Deerenberg et al. 1997; Hanssen et al. 2004; Hanssen et al. 2005; French et al. 2007; Martin et al. 2008). Immune system activation generally requires an individual to conserve energy for self-maintenance (Adelman and Martin 2009), but offspring care requires energy for incubation, nestling feeding and defense from predators. A number of studies have measured the effects of immune system activation during the breeding season on adult survival (Moret and Schmid-Hempel 2000; Hanssen et al. 2004), changes in offspring feeding rate (Råberg et al. 2000; Ardia 2005), and offspring quality and growth rates (Lozano and Ydenberg 2002; Stahlschmidt et al. 2013), yet ultimate effects on nest survival, particularly under activation of the humoral immune response, are not as well studied (Ilmonen et al. 2000; Råberg et al. 2000; Bonneaud et al. 2003)

In seasonally breeding species early breeders often have greater reproductive success (Perrins 1970; Bourdon and Brinks 1982; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004). However, cool temperatures increasing thermoregulatory demands combined with reduced food availability early in the season may reduce energy available for other energetically expensive processes, like immune system activation(Kelley 1980; Maniero and Carey 1997; Cichoń et al. 2002; Ardia 2005). In generalist species, like the Dark-eyed Junco (*Junco hyemalis* Linnaeus, 1758), parents feed their offspring primarily arthropods (Nolan et al. 2002) which have a peak in abundance during the breeding season (Pérez et al. 2016). Adult Dark-eyed Juncos consume both insects and vegetable matter during the summer (Nolan et al. 2002). High numbers of insects and the increase in vegetable matter consumption later in the season (Nolan et al. 2002; Ardia 2005) may allow females to compensate for increased energetic demands. Therefore, we hypothesize this trade-off may be amplified for early breeding individuals, resulting in reduced ability to handle an energetically demanding immune

challenge. As a result, early breeding individuals would have significantly reduced offspring survival compared to later in the season.

Here, we experimentally induced activation of the humoral immune response and recorded effects on offspring growth and nest survival in a ground nesting songbird, the Dark-eyed Junco (*Junco hyemalis*). Specifically, we injected incubating females in a free-living population with keyhole limpet hemocyanin (KLH), a relatively mild antigen that activates the humoral immune response (Lee 2006; O'Neal et al. 2011) and has not been found to elicit sickness behaviours, though evidence of this across taxa is limited (Demas et al. 1997). By injecting females during the incubation stage, we ensured that antibody production would occur during the energetically expensive offspring rearing phase, as this dose produces a measureable immune response by 11 days post-injection in the Dark-eyed Junco and other avian species (Hasselquist et al. 1999; O'Neal et al. 2011). We hypothesize that energetic costs of upregulation of the humoral immune response will reduce offspring survival. Specifically, we predict that females exposed to a mild antigen would have smaller nestlings and successfully rear fewer offspring than females that were not exposed to an immune challenge. We further predicted that this trade-off would be more exaggerated early in the breeding season, with greater reduction in nest survival, compared to nests initiated later in the season when temperatures are milder and resources more abundant (Ardia 2005).

Materials and Methods

All procedures used in this study were approved by the NDSU Institutional Animal Care and Use Committee (Protocol # A13063). Capture of birds was conducted under permit # 47553 from Virginia Department of Game and Fish.

Study Organism

The Dark-eyed Junco (*Junco hyemalis carolinensis*) is a medium sized sparrow that nests on the ground, making natural nests easy to find and monitor (Nolan et al. 2002). The population at and around Mountain Lake Biological Station (MLBS) in Giles County, Virginia, USA has been studied since 1983 and nest failure (i.e. no young fledge) on average is 60.3 ± 4.2% (mean ± SEM), though significant year to year variation occurs, with annual failure rates prior to fledging ranging from 20% – 85% (Nolan et al.

2002; Clotfelter et al. 2007). This population is also multi-brooded, with females capable of having up to 4 successful nests in one breeding season (Nolan et al. 2002). The nesting period of the junco includes 1 day for each egg laid (modal clutch size = 4) followed by 12 days of incubation typically beginning the same day the last egg is laid. Hatch day (nestling age 0) occurs on day 12 of incubation followed by 11–12 days in the nest. Fledging occurs at nestling age 11-12 (Nolan et al. 2002).

Field Methods

We searched for nests at and around the Mountain Lake Biological Station over the course of two years (April 21 to June 30, 2014 and April 24 to July 15, 2015). Nests found during the building or egg laying stage (n = 34) were checked regularly for the onset of incubation. Incubating females were captured and injected on day 6, the midpoint, of the incubation period. For nests found with unknown incubation initiation dates (n = 14), females were captured and injected the morning following finding the nests; the date of injection relative to incubation initiation was calculated after eggs hatched based on the known incubation period of this species (Nolan et al. 2002). Injection date in these females ranged from day 2 – 12 of incubation. Combined (known age nests and unknown age nests), the average day of incubation that injections were performed was day 6.18 ± 0.23 (mean ± SEM) of incubation. Average day of incubation for injections was 6.62 ± 0.23 for saline injected females (range: 3 – 12 days) and 5.78 ± 0.24 for immune challenged females (range 2 – 10 days).

To investigate seasonal effects, nests were assigned as early (first nesting attempt of the season) or late (second or later attempt of the season). Intensive nest searching was conducted by members of the field crew to strengthen ability to correctly assign early vs. late nesting attempts for all females in the population. In some cases a female's actual first nest attempt may have been missed if it was not found prior to depredation or abandonment. Thus, nests of all females found after the first known re-nest observed in the population (first egg date of earliest re-nest: May 20, 2014; May 11, 2015) were considered to be late nests, and nests that were initiated prior to the first known re-nest in the population were considered early nests.

On the date of injection, females were captured off the nest (0538–1011h) using a 6 m mist net or directly off the nest with a butterfly net. Upon capture, females were weighed to the nearest 0.1 *g* (Pesola spring scale, 30 *g*, Pesola AG), tarsus length to the nearest 0.1 *mm* (SPI dial plastic caliper, 150*mm*,

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AVINET, Inc), wing length to the nearest 0.5 *mm* (Economy wing ruler, 15*cm*, AVINET, Inc.), and clutch size was recorded. Each female was given a single intramuscular injection of 100 µl of 1 *mg ml*[1] Keyhole Limpet Hemocyanin (KLH, Enzo Life Sciences, Lot # 01091429, *n* = 26) in 1 *mL* of Freund's incomplete adjuvant (Thermo-Scientific 77145, Lot # OJ190038) or a saline control (physiological saline: 0.9%, *n* = 22)(O'Neal et al. 2011). Freund's incomplete adjuvant can magnify antibody production in response to an antigen (Dixon et al. 1966; French et al. 1970); thus saline was chosen as a control so comparisons could be made between immune challenged and non-immune challenged birds. Previous work in songbirds, including juncos, indicates a significant increase in KLH-specific antibodies in response to KLH in Freund's incomplete adjuvant approximately 6 days post-injection that peaks approximately 12 days post-injection (Hasselquist et al. 1999; O'Neal et al. 2011). Thus, this manipulation enabled us to address our hypothesis by increasing energy investment into immunity over the span of several days, rather than a single point in time (i.e. acute phase response). KLH was prepared for immunization following the rapid vortex method (Flies and Chen 2003). Females were assigned a treatment the day before injections. If only 1 female was to be injected, treatment was alternated to try and equalize sample size over the course of the season. On several occasions there were multiple injections (up to four) scheduled for a single morning. Females were randomly assigned to a treatment (equal in number if number of females was even), but nest failure sometimes occurred prior to the injection. We adjusted on a case by case basis to try and keep sample sizes as equal as possible without injecting all females with the same treatment in a single day. However, when this occurred multiple mornings in a row, and the nests assigned a saline injection failed both mornings, we ultimately ended up with a slightly uneven sample size. Due to the small number of females available for re-capture during the nestling phase (see results), we were unable to quantify antibody production or changes in adult mass; however, this dose has previously been shown to induce a robust response in Dark-eyed Juncos (O'Neal et al. 2011).

Following injection, nests were monitored daily. Days nestlings were not weighed; nests were checked from a distance with binoculars to reduce nest visitation rates that may adversely influence nest failure. A nest was considered to have failed if all eggs or nestlings were missing. Hatch day was recorded and nestlings were weighed and measured on hatch day, 3 days post-hatch, 6 days post-hatch, and 11 days post-hatch, following the standardized protocol for this population. Nestlings that survived to

11 days post-hatch and subsequently not found in the nests were considered to have fledged successfully.

Temperature data was supplied by Mountain Lake Biological Station's long term data set. Temperature at the station is recorded every 30 min throughout the year, providing 48 data points per calendar day. For the purpose of our study, we restricted the temperatures included in the model to the breeding season (April 1 – June 30).

Statistical Analyses

To ensure groups did not differ prior to treatment, pre-treatment comparisons of female mass, wing length, tarsus length, clutch size, and clutch initiation date were analyzed using univariate ANOVA with treatment, nest attempt (early vs. late), and treatment by nest attempt interaction included as covariates. In nests that were successful through hatching, nestling mass was compared using a linear mixed-effects model performed using the free software R 3.2.2 (R Core Team 2015) with the package "lme4" (Bates et al. 2015) on day 3 and 6 post-hatch. Treatment, nest initiation day, and nest attempt were included as fixed effects and female ID was included as a random effect to control for multiple nestlings within each nest. Nest survival to hatching (i.e. 6 days post injection, when antibody levels begin to rise), 12 days post-injection (i.e. 6 days post-hatch, during peak antibody production) (Hasselquist et al. 1999; O'Neal et al. 2011) and fledging success (nest failed vs nest fledged successfully) was analyzed using binomial logistic regressions. Treatment, year, and nest attempt (early vs late) were included as covariates in the model. These days were chosen in advance of data analysis and no other days were statistically analyzed to reduce the potential for type II error. Additionally, we analyzed temperature over the course of the breeding season using a linear mixed-effects model in R 3.2.2. Day of the year was included as a fixed effect and time of day was included as a random effect. Results were considered significant at $\alpha \leq 0.05$. Unless otherwise stated, statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA).

Results

Prior to treatment, no significant differences were observed in wing length, tarsus length, or clutch size (all $p > 0.05$, Table 1). Female mass did not differ between treatment groups ($F_{1,44} = 2.27$ $p = 0.14$)

and no treatment by nest attempt interaction on mass was observed ($F_{1,44}$ = 0.33, p = 0.57). However, females on their first nest attempt were, on average, 0.91 g heavier than females on later nesting attempts ($F_{1,44}$ = 7.82, p = 0.01, Table 1). Temperature increased significantly over the course of the breeding season in 2014 (F_{4367} = 3106.2, p < 0.001, Figure 7A) and 2015 (F_{4367} = 3714.9, p < 0.001, Figure 7B).

Table 1. Morphometric and clutch size measurements for female Dark-eyed Juncos (*Junco hyemalis* Linnaeus, 1758) prior to injection grouped by treatment and season.

		Mass (g)	Wing (mm)	Tarsus (mm)	Clutch Size	Clutch Initiation Day
Early	Saline	23.2 ± 0.3	77.9 ± 0.8	22.0 ± 0.2	3.8 ± 0.2	124.9 ± 4.1
	KLH	22.5 ± 0.3	77.7 ± 0.7	21.6 ± 0.2	3.9 ± 0.2	127.2 ± 3.9
Late	Saline	22.1 ± 0.3	78.4 ± 0.8	21.8 ± 0.2	3.7 ± 0.2	154.6 ± 4.1
	KLH	21.8 ± 0.3	77.7 ± 0.7	21.6 ± 0.2	3.8 ± 0.2	151.4 ± 3.6

Note: Treatment average ± 1 SEM shown in table. Combined, early season females were significantly heavier than late season females, but there was no difference in mass between treatments.

Thirteen of 22 saline injected females (59%) and 14 of 26 immune challenged females (54%) had nests survive to hatching (~6 days post-injection). No effect of treatment was observed on the proportion of nests that survived to hatching (x^2_1 = 0.06, p = 0.80). Further, no effect of year (x^2_1 = 0.48, p = 0.49), or season (early vs late nest) (x^2_1 = 3.39, p = 0.07) was observed on likelihood of nest survival to hatching. The proportion of nests that survived to 6 days post-hatch significantly differed between treatments (x^2_1 = 4.21, p = 0.04, Figure 8), with nests of immune challenged females more likely to have failed; 8 of 22 saline injected females (36%) and 3 of 26 immune challenged females (12%) still had active nests 6 days post-hatch (~12 days post-injection). Additionally, we observed a higher proportion of surviving nests for saline injected females compared to immune challenged females on days 5, 7, and 8 compared to day 6 post-hatch, though no statistical analyses were performed on these days (Table 2). However, neither year (x^2_1 = 0.59, p = 0.53) nor season (early vs late nest) (x^2_1 = 1.45, p = 0.23) significantly affected nest success to 6 days post-hatch.
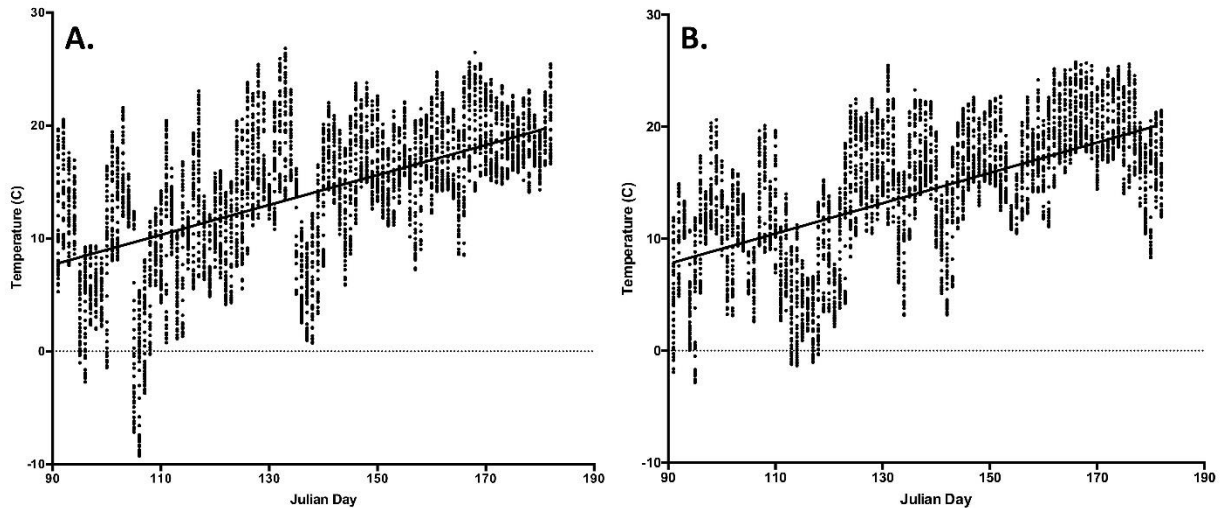
Figure 7. Temperature data from MLBS show an increase in temperature from April 1 to June 30 in 2014 (A) and 2015 (B).

The effect of treatment seen 6 days post-hatch was no longer observed at fledging (treatment: χ^2_1 = 2.32, $p = 0.13$, Figure 9; year: χ^2_1 = 1.27, $p = 0.26$; nest attempt: χ^2_1 = 0.67, $p = 0.41$); 5 of 22 saline injected females (23%) and 2 of 26 immune challenged females (8%) had at least 1 nestling still alive at fledgling (~17 days post-injection).

Nestling mass 3 days post-hatch (~9 days post-injection) did not significantly differ between treatments (F_{14} = 0.35, p =0.56, Table 3) or early and late nest attempts (F_{14} = 0.01, $p = 0.92$). There was also no effect of nest initiation date (F_{14} = 0.004, $p = 0.95$). Nestling mass 6 days post-hatch (~12 days post-injection) also did not differ, but only 3 nests for immune challenged females were still active at this time point (F_7 < 0.001, $p = 0.99$, Table 3) or early and late nest attempts (F_7 = 0.05, $p = 0.83$). There was also no effect of nest initiation date (F_7 = 0.62, $p = 0.45$).

Discussion

Our findings support our hypothesis that activation of the humoral immune response with a mild antigen decreases offspring survival, demonstrating a trade-off between reproduction and immune function. We observed a significant reduction in nest survival to 6 days post-hatch for females treated with KLH in Freund's incomplete adjuvant compared with saline treated females. Interestingly, the greatest extent of nest failure in immune challenged individuals occurred between hatch and 6 days post-hatch, a

time period coinciding with increasing and peak antibody production (i.e. ~12 days following the challenge) (Hasselquist et al. 1999; O'Neal et al. 2011). Contrary to our predictions no effect of season (early versus late nest attempt) was observed.
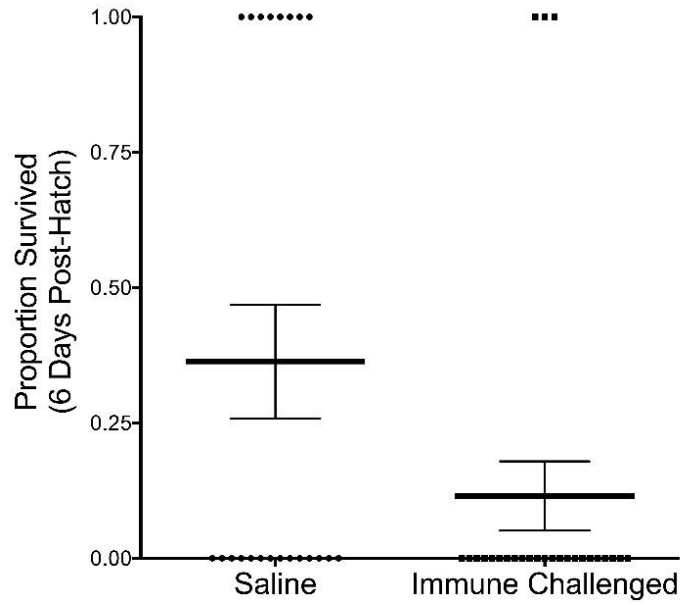
Figure 8. Proportion of nestling survival to 6 days post-hatch (i.e., coinciding with peak antibody production) was significantly lower for immune challenged females (n = 26) compared to saline injected females (n = 22).
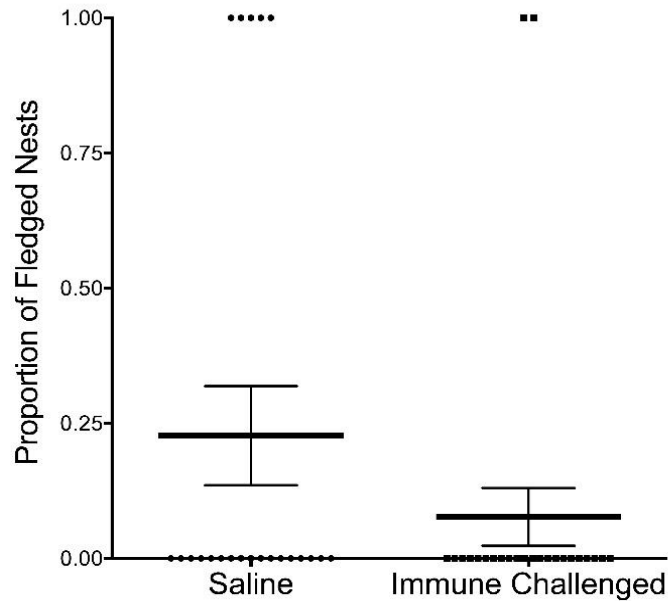
Figure 9. Saline injected females (n = 22) did not have significantly higher fledging success compared to immune challenged females (n = 26).

Table 2. Differences in proportion of surviving nests was highest from when nestlings were 5 days old to 8 days old, though the significant difference between immune challenged and control females was statistically analyzed when nestlings were 6 days old.

Nestling Age (Days post hatch)	Saline Treated	Immune Challenged	Difference
0	0.59	0.54	0.05
1	0.55	0.42	0.13
2	0.55	0.42	0.13
3	0.50	0.31	0.19
4	0.50	0.31	0.19
5	**0.50**	**0.23**	**0.27**
6	**0.36**	**0.12**	**0.24**
7	**0.36**	**0.08**	**0.28**
8	**0.36**	**0.08**	**0.28**
9	0.27	0.08	0.19
10	0.23	0.08	0.15
11	0.23	0.08	0.15

Note: Bolded days indicate when saline injected females had a higher proportion of surviving nests compared to immune challenged females that was equal to or greater than the significant difference shown on day 6.

Table 3. Average individual offspring mass 3 days (KLH: $n = 36$ nestlings, Saline: $n = 35$ nestlings) and 6 days (KLH: $n = 13$ nestlings, Saline: $n = 27$ nestlings) post-hatch did not significantly differ between treatment and control female Dark-eyed Juncos.

	Mass 3 days post-hatch (g)	Mass 6 days post-hatch (g)
Saline	6.72 ± 0.21	13.45 ± 0.67
KLH	6.99 ± 0.28	13.17 ± 0.31

Note: Mass reported as the average of all nestlings (mean ± 1 SEM).

The significant reduction in nest survival seen at 6 days post-hatch corresponds with the peak in antibody production and the related energetic costs associated with activation of the humoral immune response (Demas et al. 1997; Hasselquist et al. 1999; O'Neal et al. 2011). Prior to hatching (< 6 days

post-injection) antibody titers would be undetectable, so we would predict no increased energetic demands due to immune system activation during the egg laying and incubation stage. As expected, there was no difference in survival to hatching observed between treatment and control females with 54% and 59% of nests surviving to hatch, respectively. For females whose nest successfully hatched, 62% of control nests were active for an additional 6 days post-hatch (8 of 13) compared to only 21% of the nests for immune challenged females that were still active to six days post-hatch (3 of 14), a time frame corresponding to increasing and peak antibody production (Hasselquist et al. 1999; O'Neal et al. 2011). Additionally, the highest difference in nest survival between saline injected and immune challenged females was from 5 to 8 days post-hatch, also corresponding with previously reported antibody production. Thus, our findings support that the difference seen at 6 days post-hatch was due to an effect of treatment.

The reduction in body mass observed from early season to late season females is likely a result of later nesting females having already invested energy into early nest attempts (Kennamer and Hepp 1987; Barzen and Serie 1990). We may not have observed a seasonal effect of nest survival because early breeding females were heavier than late breeding females, suggesting greater stored energy in the early season was equivalent to external resource availability later (Nagy et al. 2007). This would allow early breeders to utilize internal stores to support immune system activation earlier in the season (Ots et al. 2001; Eraud et al. 2005) while during later nests external energy resources are abundant (Ardia 2005). However, regardless of internal or external energy resources, our findings suggest that in this species activation of the immune system and investment in self-maintenance comes at the costs of investment in reproduction and reproductive success. This strategy may also be adaptive in other multi-brooded or ground nesting species.

While we found clear differences in the number of active nests between immune challenged and saline treated females up to 6 days post-hatch, no difference in nest success was observed at fledging. This may be a result of a reduced sample size in both treatment groups due to high nest predation. Out of all females in the study, only 3 nests of immune challenged and 8 nests of saline treated females remained at 6 days post-hatch, thus we may lack the statistical power to detect a difference between treatment groups. Average yearly nest failure in this population of Dark-eyed Juncos is 60.3 ± 4.2%, but

ranges from 20% to 85% with up to 95% of nest failures being caused by predation (Nolan et al. 2002; Clotfelter et al. 2007). Failure rates in 2014 and 2015 were well above average, even in control females who had a 77% failure rate.

One additional reason for a lack of observed treatment effect could be due to increased activity at the nest, attracting more predators as nestlings age. It has been shown that increases in parental activity at the nest after hatching cause a corresponding increase in daily predation rate in open nesting species (Martin et al. 2000) and feeding rate increases significantly with nestling age (Freed 1981; Ketterson et al. 1992). Nest success in ground nesting birds also declines as small mammal population density increases (Ketterson et al. 1996; Schmidt 2003; Clotfelter et al. 2007). Therefore, the longer nests remained active the higher the likelihood of both control and treatment nest being discovered by predators, particularly in a high predation year.

While this investigation found a significant effect of treatment on nest failure prior to the mid-point of the nestling rearing phase, the cause of nest failure in immune challenged females was not part of the current investigation; future work will be needed to address this. One possibility is that immune challenged females are reducing their feeding efficiency in response to an increase in energy expenditure. For example, if they are reducing feeding behavior to allocate more time to thermoregulation, nestlings may suffer as a consequence (Todd et al. 2016). If females allocate more energy to self-maintenance in response to an immune challenge, we would predict correlated reductions in mass and growth of offspring of treated females compared to controls (Ilmonen et al. 2000). In our study, nestling mass at days 3 and 6 post-hatch did not differ between treatments; however, both male and female Dark-eyed Juncos care for and feed nestlings (Nolan et al. 2002), thus an increase in male parental care may be capable of compensating for a decline in female parental care early in the nestling rearing stage. This effect has been seen in other species with bi-parental care where experimental reductions in female feeding efficiency induced reciprocal increases in male provisioning rates and nestling care (e.g. removal of fecal sacs, and time spent at nest)(Wright and Cuthill 1989; Markman et al. 1995; Bonneaud et al. 2003; Paredes et al. 2005); c.f. (Ilmonen et al. 2000; Råberg et al. 2000; Sanz et al. 2000). Indeed, in one year of nestling care observations following female injection, we observed a trend toward males paired with immune challenged females increasing nest visitation rate, even though

females did not appear to be reducing nest visitation; however, due to high failure prior to recording, observed sample size was too small for analysis (J. Graham and R. Mady unpublished data). More work should be conducted to understand the effects of an immune challenge on female feeding and potential male compensation in non-cavity nesting species.

Another possibility for the observed reduction in nest success in immune challenged females is reduced female aggression toward predators as a cost of activating the immune response. Female aggression is important for nest success in this species (Cain and Ketterson 2012) and, compared to males, females are more aggressive toward nest predators (Ketterson et al. 1996; Nolan et al. 2002). If an immune challenge alters aggressive behaviour in ground nesting females, then this may significantly increase nest predation risk. We would predict that if Dark-eyed Junco males are expending more energy to feed nestlings in response to decreased female parental effort (Wright and Cuthill 1989; Markman et al. 1995; Bonneaud et al. 2003; Paredes et al. 2005), nest defense may decline, increasing likelihood of failure due to predation. However, to our knowledge, no work has investigated whether female aggression or response to predators is altered during activation of the humoral immune system. Future work is needed to clarify whether impacts of the humoral immune response on nest success is the direct result of an energetic trade-off leading to decreased feeding efficiency, or through indirect effects on non-energetic related behaviours in males and/or females.

Activation of the immune response influences reproductive success and the findings of our study reinforce the importance of understanding the fitness consequences of immune system activation during energetically challenging life history stages. The few studies examining effects of adult humoral immune challenges on nest survival after reproduction has been initiated have found mixed results. This variation was likely due to differences in whether the humoral immune or acute phase response (e.g. fast-acting response to pathogens including sickness behavior, cytokine activation and hyper or hypothermia) was targeted and level of environmental pollution at the nest site (Ilmonen et al. 2000; Råberg et al. 2000; Bonneaud et al. 2003), suggesting further investigation in free-living populations is needed.

In conclusion, for females that had already initiated reproduction, exposure to even a mild immune challenge, known to induce a humoral immune response, negatively impacted nest survival. Our data indicate that the cause of nest failure is not likely a result of offspring starvation. Thus it will be

important for future work to uncover the mechanisms, including changes in behaviour, driving the observed trade-off between activation of the immune system and care of offspring.

Acknowledgements

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CHAPTER 5: EARLY BREEDING FEMALES EXHIBIT ACCELERATED TELOMERE SHORTENING

Abstract

Reproductive success is often highest in early seasonal breeders, yet relatively few individuals are observed breeding during this apparently optimal time. This suggests females likely incur costs by breeding early. We hypothesized that accelerated senescence may be one cost of breeding early. Telomeres, the repetitive DNA sequences on the ends of chromosomes, may be good proxies of biological aging as they shorten with age and in response to oxidative stress. Using historical data from a long-term study population of dark-eyed juncos (*Junco hyemalis*), we found earlier breeding females exhibited greater telomere loss compared with later breeding females, regardless of chronological age. High telomere attrition rates were correlated with cooler temperatures during the egg laying and incubation period, suggesting that challenging environmental conditions may be the primary cause of accelerated telomere attrition rates in early breeders.

Keywords: Aging, Reproductive Timing, Telomeres

Introduction

In seasonally breeding species, individuals that breed earlier tend to enjoy greater reproductive success than those that breed later (Perrins 1970; Bourdon and Brinks 1982; Festa-Bianchet 1988; Landa 1992; Olsson and Shine 1997; Dawson and Clark 2000; Lepage et al. 2000; Doody et al. 2004). Early breeders may have higher reproductive success for several reasons, including the ability to fit more clutches or litters within a season, replace failed reproductive attempts before the season ends, and/or appropriately time offspring growth with peaks in food abundance (Ribble 1992; Rieger 1996; Williams 2012b). In addition to these factors, early breeders also tend to produce larger offspring (Perrins 1970; Festa-Bianchet et al. 1997; Holand et al. 2006), which typically have higher overwinter survival and recruitment rates compared to smaller individuals (Krementz et al. 1989; Linden et al. 1992; Sedinger et al. 1995; Naef-Daenzer et al. 2001; Monrós et al. 2002; Low et al. 2015). However, despite these observed benefits of breeding early, most individuals breed later than the earliest breeders (Verhulst and Nilsson 2008). This suggests that while there may be significant reproductive benefits to breeding early, there are likely costs that delay seasonal reproduction in most of the population.

Costs associated with early breeding may be due to several factors, including reduced food availability (Perrins 1970; Bradbury and Vehrencamp 1977; Nilsson 1994), a reduction in future fecundity and survival (Visser and Lessells 2001; Hanssen et al. 2005), and additional energy expenditure as a result of exposure to colder spring temperatures (Speakman 2008; Visser et al. 2015). One potential cost of early reproduction that remains largely unexplored is accelerated senescence. Recent studies suggest that telomere dynamics may serve as useful biomarkers of senescence (Hau et al. 2015) as telomere length and loss rate have been shown to be predictive of longevity across a diverse array of taxa (Haussmann et al. 2003; Haussmann et al. 2005; Bize et al. 2009; Heidinger et al. 2012). Telomeres are repetitive, non-coding DNA sequences that form protective caps at the ends of linear eukaryotic chromosomes. While telomeres enhance genome integrity, they shorten during normal cell division and limit the lifespan of the cell (Blackburn 2005). There is also evidence that stress exposure can shorten telomeres (Epel et al. 2004; Kotrschal et al. 2007; Haussmann and Marchetto 2010; Hau et al. 2015; Herborn et al. 2016). For example, harsh environmental conditions can lead to significant telomere loss while moderate conditions may reduce or even reverse telomere attrition (Angelier et al. 2013; Mizutani et al. 2013).

Early breeding may increase telomere erosion through many routes. During the early stages of reproduction, females may have to expend additional energy to cope with cooler temperatures and reduced food availability (Ardia 2005; Pretzlaff et al. 2010; Graham, Mady, et al. 2017). Cooler temperatures can even lead to reduced food availability (Anthony et al. 1981; Schekkerman et al. 2003). In birds, females have been shown to lose body mass and significantly increase energy expenditure during colder weather (Conway and Martin 2000; Tulp and Schekkerman 2006; Tulp et al. 2009; Nord et al. 2010). Additionally, as early breeders have the potential to produce more offspring within a season (Perrins 1970; Ribble 1992), accelerated telomere attrition could occur due to increased reproductive output over the breeding season (Bauch et al. 2013).

Observed variation in reproductive timing has been partially attributed to older or more experienced individuals tending to breed earliest (Perrins 1970; Mills 1973; Nol and Smith 1987; Perdeck and Cavé 1992; Sockman et al. 2004; Salvante et al. 2013b). Older individuals may be better able to pay costs associated with early reproduction because they are more experienced and better able to

accumulate the necessary resources to initiate reproduction (Verhulst and Nilsson 2008; Froy et al. 2013; Salvante et al. 2013b). Alternatively, older individuals may be more willing to pay higher costs of reproduction because they are likely to have fewer future reproductive opportunities (Bauer et al. *In review*; Pianka and Parker 1975; Bonneaud et al. 2004; Fischer et al. 2008). However, these studies are often cross-sectional, rather than following individuals across years. This makes it difficult to discern whether the relationship between age and timing of breeding is due to within-individual advancement of timing or higher quality individuals breeding earlier and living longer (Forslund and Pärt 1995; Wilson and Nussey 2010). Long-term studies with multiple observations from the same individuals across multiple years are necessary to better understand why older individuals tend to breed earlier (McCleery et al. 2008; Froy et al. 2013).

The goal of this study was to use longitudinal data to test hypotheses addressing chronological age related with molecular senescence in relation to variation in initiation of seasonal reproduction. Our study organism was the dark-eyed junco (*Junco hyemalis*), a medium sized, ground-nesting sparrow (Nolan et al. 2002). Data from this population (near Mountain Lake Biological Station in Pembroke, VA, USA) spans 34 years, making it possible to measure telomere lengths from the same individuals over multiple years (Nolan et al. 2002). First, we confirmed that breeding earlier increases reproductive success within a season by comparing timing of first clutch initiation with the total number of eggs laid and offspring fledged over the breeding season. Second, we asked whether chronological age was related to nest initiation date, as would be expected if birds breed progressively earlier as they age or if early breeding individuals have a longer lifespan. Third we tested whether telomere attrition is a cost of early breeding by examining changes in telomere length from one breeding season to the next in females with known first clutch initiation dates. Specifically, we predicted that females with earlier nest initiation dates would display greater annual telomere loss than females with later nest initiation dates. Finally, we used temperature data to determine whether average daily temperatures experienced during the egg laying and incubation stage of reproduction were correlated with telomere loss. We predicted that females experiencing cooler conditions would experience more telomere loss, particularly if they laid more eggs.

Materials and Methods

Study Species

This study took place at and around the University of Virginia Mountain Lake Biological Station (MLBS), Salt Pond Mountain, Giles County, Virginia, USA (37°22'32"N, 80°32'20"W, elevation 1,160 meters). Dark-eyed juncos are the most common ground-nesting bird in the study area and their population has been monitored since 1983 (Nolan et al. 2002). Early season trapping and nest searching begins in mid – late April each year. Trapping occurs in the same locations on the field site every year using mist nets and walk-in potter traps. Upon capture, individuals are banded with a numbered aluminum federal ID band. A unique color band combination is also used so individuals can be identified through binoculars.

Determining Lay Date

In most years, a team of researchers searched the field site for nests from late April through mid-July. If a nest was found after the female had started incubating, the day the female laid her first egg was determined by backdating from day of hatching (Nolan et al. 2002). Dark-eyed juncos are capable of having up to four successful nests within a season (Nolan et al. 2002). To be certain we were measuring the first egg of the season for each female, a conservative cut-off date for each year was defined as the day before the first known re-nest of the year (Graham, Mady, et al. 2017). To be able to compare first egg dates across multiple years, an individual's first egg date is represented by the number of days before (-) or after (+) the population average for that year that she laid her first egg. The population average for first egg was calculated as the mean first egg date for all nests prior to the established cut-off date. We used 143 known age females (with first nests in two or more consecutive years) to address whether individual females breed progressively earlier as they age chronologically. Nests were included from 1983–2016. We additionally included the total number of eggs laid and number of successful fledglings in a single season as a measure of seasonal reproductive investment and success, respectively.

To be included in the telomere portion of the study, a banded female was required to have (1) blood samples collected in two or more consecutive years, and (2) a nest with a first egg of the season occurring before the annual cut-off in Year 1. Long term monitoring of the population allows for a large

proportion of the population to be of known age. Known age females were banded either as nestlings or juveniles. Additionally, one-year-old individuals have identifiable plumage characteristics and iris color which allows them to be reliably identified (Nolan et al. 2002). While most females were known age ($n =$ 83), we expanded our sample size to include females of unknown age ($n = 23$, for a total sample size of n $= 106$) when we were comparing nesting records from one year to the next within an individual.

Telomere Measurements

Starting in 1990, blood samples were collected from the alar wing vein using heparinized microhematocrit capillary tubes. Longmire's lysis buffer solution (1 mL) was added to the erythrocytes for long term storage at 2^oC. Blood samples are included only for females from 1990 and later, as no blood samples were collected prior to that year. Avian erythrocytes are an ideal tissue for longitudinal telomere measurements as they are a nucleated, highly mitotic tissue that can be sampled with no harm to the bird (Nussey et al. 2014).

Genomic DNA was extracted from red blood cells using Macherey-Nagel Nucleospin Blood Kits (Macherey-Nagel, Bethlehem, PA, USA) (Bauer et al. *In review*; Heidinger et al. 2012; Bauer et al. 2016 Aug 3). We added 100 μL of erythrocytes in Longmire's solution to 100 μL of phosphate buffer solution and then followed the manufacturer's instructions. DNA concentration and purity (260/280 ratios above 1.7 and 260/230 ratios above 1.8) were verified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Quantitative PCR (qPCR) was used to measure relative telomere length in extracted samples with respect to a single copy control gene (Glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*) (Criscuolo et al. 2009) following methods adapted for the Dark-eyed Junco (Bauer et al. *In review*; Bauer et al. 2016 Aug 3). *GAPDH* primer sequences were: *forward GAPDH* (5'-AACCAGCCAAGTACGATGACAT-3′) and *reverse GAPDH* (5′-CCATCAGCAGCAGCCTTCA-3′). Telomere primers were: *forward tel1b* (5'- CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and *reverse tel2b* (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). Reaction volumes were 25 μL and contained 6 uL DNA (20 ng per well), 12.5 μL perfecta SYBR green supermix Low ROX (stratagene), and 200 nM/200 nM *forward GAPDH/reverse GAPDH or* 200 nM/200 nM *forward tel1b/reverse tel2b*. A standard curve (40, 20, 10, 5, and 2.5 ng) from a single reference sample was run

in triplicate on every plate to control for inter-plate variation. Females were randomized across plates to more reliably detect any potential differences between years, but all samples for a single female were run on the same plate in duplicate. *GAPDH* and telomere reactions were run separately. Reaction conditions for telomere plates were as follows: 10 min at 95°C; 27 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C; and 1 min at 95°C, 30 s at 58°C, and 30 s 95°C. *GAPDH* plates were run under the following conditions: 10 min at 95°C; 40 cycles of 30 s at 95°C and 30 s at 60°C; and 1 min at 95°C, 30 s at 58°C, and 30 s 95°C.

For each sample, the number of PCR cycles (C_t) to reach a threshold was measured. A standard curve was included on every plate to control for differences within and between plates. The 20 ng dilution reference sample was used to calculate intra- and inter-assay variation. Intra-assay coefficient of variation for C_t of telomere plates was 0.72 % and inter-assay coefficient of variation was 3.22 %. For *GAPDH* plates intra and inter-assay coefficient of variation for C_t was 0.23 % and 1.55 %, respectively. Average standard curve efficiency for telomere plates was 89.4 % (range: 85.5 – 93.5 %) and 93.8 % (range: 91.2 – 98.0 %) for *GAPDH* plates. The ratio of telomere repeats (TTAGGG) to the number of copies of *GAPDH*, or T/S ratio, was used to calculate relative telomere length. The formula $2^{-\Delta\Delta Ct}$ was used to calculate T/S ratio where $\Delta\Delta C_t = (C_t\ ^{telomere} - C_t\ ^{GAPDH})$ reference $- (C_t\ ^{telomere} - C_t\ ^{GAPDH})$ focal (Cawthon 2002).

Temperature Data

MLBS has its own weather station with historical data (from 1994–2015) freely available online (http://mlbs.virginia.edu/meteorological-data). Temperature ($^{\circ}$C) is measured every 30 min year round. We analyzed average daily temperature during the egg laying and incubation stage (~15 days long) because this is a metabolically demanding time for females (Tulp et al. 2009). Egg production is energetically expensive (Williams 2012b) and incubation in this species is performed only by females with no partner feeding by the males (Nolan et al. 2002).

Statistical Analysis

All statistical analyses were conducted in R version 3.2.2 (R Core Team 2015). To control for females having repeated measures, linear mixed effects models were run using package lme4 (Bates et

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al. 2015). All models included female ID and year as random effects. For models involving change in telomere length, the plate a female's blood samples were run on was included as a random effect. Significance was set at $\alpha = 0.05$.

Analysis 1: Does Reproductive Investment and Success Decline Across the Season?

        To confirm that our population shows a seasonal decline in reproductive output and success, we ran two models using 147 females of known age. Our first model compared an individual's reproductive output (measured as total number of eggs laid within a season) to her date of first nest initiation. The second model compared an individual's reproductive success (measured as number of fledglings surviving to 11 d post-hatch) to her first nest initiation day. Female age was included as a covariate in both models.

Analysis 2: Do Individual Females Breed Earlier as they Age?

        To determine the relationship between age and nest initiation date, we ran a model to compare chronological age and nest initiation date (measured as number of days before (-) or after (+) the population average for that year). To determine if the decline in nest initiation date with age was due to birds breeding progressively earlier as they age (i.e., within-individual variation) or because early breeding individuals tend to live longer and thus predominantly make up older age classes (among-individual variation), we ran a second model replacing age with two calculations of variation (Van de Pol and Wright 2009; Herborn et al. 2016). The among-individual value was calculated as the average age of a female across all sampling points. By calculating average age of an individual, this form of variation tests whether the pattern is driven by high quality individuals breeding earlier and living longer. Calculating within-individual variation as (age – average age) controls for among-individual variation by scaling individuals around 0 and focuses on whether birds breed earlier as they get older. We accounted for individual random slopes by including the within-individual component of age correlated with female ID as a random effect (Dingemanse and Dochtermann 2013). To be included in this analysis, females with nests in subsequent years were needed and not blood samples; thus, more females are included in this analysis compared to the telomere analyses.

Analysis 3: Do Early Breeding Females Exhibit Accelerated Telomere Loss?

We ran a model comparing when a female laid her first egg of the season to subsequent annual telomere loss. It is important to include starting telomere length when measuring telomere loss as longer telomeres have been found to show greater shortening rates compared to shorter telomeres (Nordfjäll et al. 2009). In order to include starting telomere length as a covariate, we calculated an individual's change in telomere loss by correcting for the regression to the mean (Berry et al. 1984; Verhulst et al. 2013). Chronological age was originally included in the model, but was not significant ($F_{1,120} = 0.002$, $p = 0.97$), thus it was removed from subsequent analyses. Chronological age additionally reduced sample size ($n = 83$ known age females and $n = 23$ unknown age females) without changing the significance of the other variables; thus, we removed it from the final model. The final model included 106 individual females.

Analysis 4: Is Accelerated Telomere Loss Related to Environmental Conditions and/or Reproductive Output?

To test the hypothesis that change in telomere length may vary with total reproductive output (i.e., number of eggs laid in a season), we ran a model to compare number of eggs laid with change in telomere length. Alternatively, adverse environmental conditions experienced during the early breeding season may contribute to telomere loss, thus to test the hypothesis that thermoregulatory demand influence change in telomere length we additionally included average daily temperature during egg laying and incubation in the model. Temperature data were available from 1995–2016, which allowed us to analyze 89 separate females with at least one year of weather data and a blood sample from the following year. We also included the number of days between collection of samples in year 1 and samples in year 2 as a fixed effect in this and the previous model because not all samples were collected exactly 365 days apart.

Results

Reproductive Investment and Success Decline Across the Season

As predicted, earlier laying females laid more eggs within a season ($F_{1, 329} = 10.81$, $p = 0.001$, table 4) and successfully fledged more offspring ($F_{1, 321} = 12.65$, $p < 0.001$, table 4). Chronological age did not influence number of eggs laid or number of offspring fledged (both $p > 0.95$, table 4). This result

confirmed our population is suitable for addressing questions measuring potential costs of early reproduction.

Individuals Breed Earlier as they Age

We analyzed nesting records from 147 known age females who nested in the population for at least 2 consecutive years (max: 5 consecutive years). Our analysis found that females progressively laid their first clutch earlier each year they bred in the population ($F_{1, 302} = 5.40$, $p = 0.02$, table 4). Within-individual variation was significant ($F_{1, 196} = 11.66$, $p < 0.001$, table 4), but among-individual variation had no effect ($F_{1, 124} < 0.001$, $p = 0.98$, table 4). This suggests the negative relationship between egg lay date and chronological age is driven by individuals breeding progressively earlier as they age.

Table 4. Slope and standard error of slope for each model run. Bolded and "*" slope values are significant. Repeated measure from each model is listed under dependent variable and all random effects included in each model are listed.

	Dependent Variable	*Independent Variable*	*B*	*SE B*	*Random Effects*
Analysis 1	*Model 1*: Eggs	First Egg Date	**-0.09***	0.03	Female ID Year
		Chronological Age	0.00	0.17	
	Model 2: Fledglings	First Egg Date	**-0.07***	0.02	Female ID Year
		Chronological Age	-0.01	0.12	
Analysis 2	*Model 1*: First Egg Date	Chronological Age	**-0.75***	0.32	Female ID Year
	Model 2: First Egg Date	Among Individual	0.01	0.44	Female ID Year
		Within Individual	**-1.60***	0.47	
Analysis 3	*Model 1*: Change in Telomere Length	First Egg Date	**0.01***	0.00	Female ID Year Plate
		Days Between Samples	0.00	0.00	
Analysis 4	*Model 1*: Change in Telomere Length	Number of Eggs Laid	-0.01	0.01	Female ID Year Plate
		Average Temperature (^{O}F)	**0.03***	0.01	
		Days Between Samples	0.00	0.00	

Early Breeding Females Experience Greater Telomere Attrition

With 267 blood samples from 106 females, we found that females breeding earlier in the season incurred greater telomere loss between subsequent breeding seasons ($F_{1,146}$ = 8.43, *p* = 0.004, Fig. 10, table 4). The average number of days between collection of two samples was 371 (range: 263 – 472) and did not have a significant effect on change in telomere length ($F_{1,99}$ = 1.41, *p* = 0.24, table 4).

Greater Telomere Attrition is Related to Cooler Temperatures, but not Reproductive Investment

Average daily temperature during the egg laying and incubation stage (range: 6.08 – 18.19°C) could be calculated for 89 females. Females that experienced lower daily temperatures during this period had greater annual telomere loss compared with females that nested during warmer periods ($F_{1,79}$ = 5.89, *p* = 0.02, Fig. 11, table 4). As in the previous model, the number of days between samples did not influence change in telomere length ($F_{1,65}$ = 2.13, *p* = 0.15, table 4). Interestingly, although earlier laying females laid more eggs within a season and successfully fledged more offspring, change in telomere length was not related to the number of eggs laid by the female that year ($F_{1,117}$ = 1.82, *p* = 0.18).
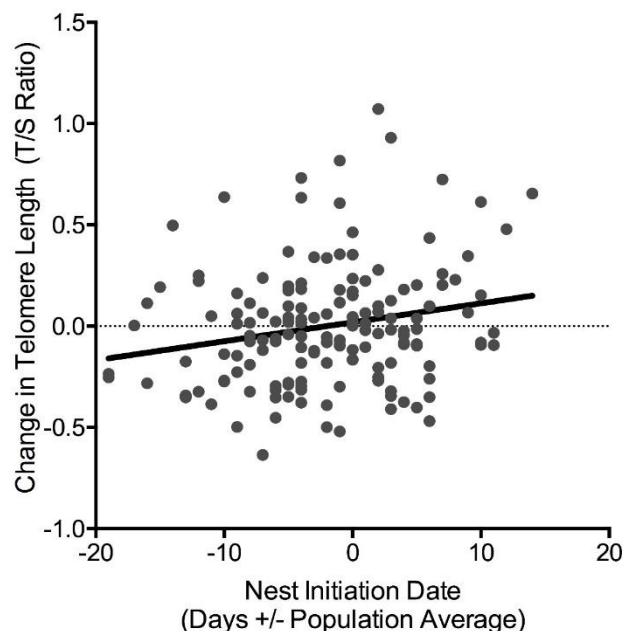

Figure 10. Earlier breeding females experienced higher rates of telomere loss from one breeding season to the next with respect to the rest of the population. Change in telomere length was corrected for the regression to the mean and was scaled to zero so loss is negative and gain is positive. The data in this figure is not corrected for the other variables included in the analysis.
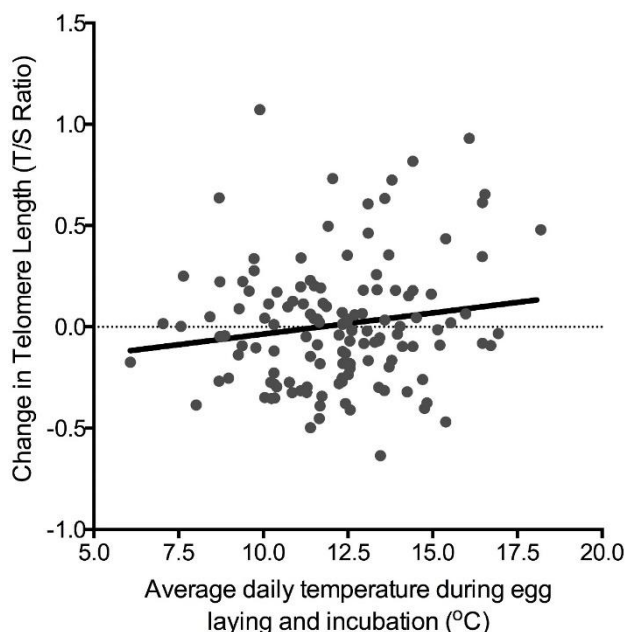
Figure 11. Colder temperatures during the egg laying and incubation stages positively relate with attrition rates. The data in this figure is not corrected for the other variables included in the analysis.

Discussion

The results of this study support our hypothesis that reproductive success declines across the season and the observation that early breeding females are older is due to individuals breeding earlier as they age rather than early breeding individuals making up the majority of older age classes because they're of higher quality and, thus, live longer. We additionally found that early breeding females experience accelerated telomere loss compared to later breeding females, therefore suggesting females experience a molecular cost by breeding early in the season. We found additional support that this cost is related to cooler temperatures experienced during the first nesting attempt of the season, which is a time when reproductive investment is primarily controlled by the female. However, telomere dynamics (i.e., annual loss) do not appear to be influenced by reproductive investment or chronological age.

We found that individual females in this population breed earlier as they age. This is important in helping distinguish age related changes in reproductive timing from higher quality individuals breeding earlier and living longer than low quality individuals (McCleery et al. 2008). Birds may breed earlier as they age because they increase their reproductive experience, which can lead to higher levels of reproductive hormones and earlier activation of the reproductive system (Wooller et al. 1990; Sockman et al. 2004; Angelier et al. 2007; Salvante et al. 2013b). This could additionally be related to the ability of

older females to accumulate necessary resources more quickly through efficient foraging or pairing with familiar males (Weimerskirch 1992; Fowler 1995).

Accelerated telomere loss in early breeding individuals suggests that a female's reproductive timing decision includes trade-offs between telomere loss and reproductive success. In addition to increased telomere attrition in early breeding individuals, we also observed telomere lengthening in later breeding females. Telomere lengthening can occur via upregulated production of the enzyme telomerase, and has been documented in other studies (Ilmonen et al. 2008; Bize et al. 2009; Ujvari and Madsen 2009; Turbill et al. 2012; Angelier et al. 2013; Mizutani et al. 2013). Telomere lengthening in later breeding females may be due to the ability to upregulate telomerase activity, perhaps via access to better resources, though future work on this topic is needed (Lin et al. 2012). Furthermore, while we found that females breed progressively earlier as they age, this trend didn't hold true for all individuals. Delaying reproduction in one year could reduce the overall cost of breeding early the following year, but further work is needed to understand this relationship.

We saw higher telomere loss in females experiencing cooler temperatures, which are more likely to occur early in the breeding season. Cold temperatures may increase oxidative stress via an increase in reactive oxygen species and a decrease in antioxidants. Females may be increasing reactive oxygen species (ROS) via shivering and increased food intake because of an increase in metabolic heat production (Selman et al. 2002; Stier et al. 2014). Increasing anti-oxidants neutralizes ROS and helps preserve telomere length (Liu et al. 2003; Tarry-Adkins et al. 2008), even when reproductive workload is experimentally increased (Beaulieu et al. 2011). However, under acute cold stress, non-hibernating mammals do not upregulate ROS detoxifying enzymes, which are important determinants of aging rate (Buzadžić et al. 1997; Teramoto et al. 1998). Because we were using historical samples, we were unable to measure anti-oxidant capacity, but anti-oxidant rich arthropods become more available later in the breeding season, when the first clutches begin hatching (Arnold et al. 2010) and may be unavailable to early breeding females during egg laying and incubation.

Interestingly, accelerated aging was not related to reproductive investment. This is counter to the finding that females with 10d old nestlings had higher telomere attrition compared to females with failed nests (Bauch et al. 2013). The difference between species may be due to Bauch et al. (2013) studying a

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single brooded sea bird. Nest failure in a single-brooded species would result in reduced reproductive investment, as the next reproductive attempt will be the following year. Contrary to this, the dark-eyed junco is multi-brooded and capable of having up to 4 successful nests within a single season (Nolan et al. 2002); thus nearly all females will re-nest after a failed first attempt and continue to breed through the end of the season. Our finding instead suggests that the conditions experienced during reproduction are more important than the total number of offspring produced by female passerines within a season.

Telomere loss and length are of increasing importance to understanding lifespan and longevity under varying conditions. Telomere loss can have significant effects on individuals, including shortened lifespan (Haussmann et al. 2005; Haussmann and Marchetto 2010). Furthermore, DNA repair mechanisms are not as effective in telomeric regions compared to other parts of the chromosome; thus, increased oxidative stress may result in senescence (Barja and Herrero 2000; von Zglinicki 2002). While we were unable to assess oxidative stress in our samples, the relationship between lower temperatures and increased telomere loss suggests greater oxidative stress in earlier laying females exposed to cooler temperatures. Thus, early breeders likely had shorter telomeres, which may cause them to die earlier (Fairbairn 1977; Brown and Brown 1999; Sheldon et al. 2003; Angelier et al. 2013; Mizutani et al. 2013).

Our unique, longitudinal data set in a short-lived songbird demonstrates that regardless of chronological age, breeding early in the season comes with a significant molecular cost via telomere loss that may be a result of breeding when temperatures are cooler. Telomere dynamics may be a better predictor of reproductive timing and performance than chronological age (Bauer et al. *In review*; Bauch et al. 2013). There may additionally be molecular benefits to delayed reproduction, though this avenue requires further exploration.

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CHAPTER 6: CONCLUSION

Seasonal bouts of reproduction are timed so that offspring are born when conditions are optimal for raising young. Photoperiod is the initial cue for priming the reproductive system to breed at the appropriate time each season, but the variation in precise reproductive timing is less well understood. This lack of understanding of the mechanisms underlying variation in reproductive timing is particularly true in females, who ultimately make the decision of when offspring are born. In addition to the variation seen in reproductive timing, the few, earliest breeding individuals tend to produce the most and the largest offspring. However, our understanding of the costs that prevent most individuals from breeding at this time are not well understood. The work described here aimed to 1) identify potential interactions between endogenous rhythms and reproductive timing behavior and physiology in free-living organisms and 2) identify reproductive costs of individual variation in timing of breeding.

In Chapter 2, I examined the relationship between circadian behavior (i.e., daily onset of activity) and reproductive timing in two free-living species of songbirds. I found support for my hypothesis that variation in daily rhythms is an underlying mechanism driving individual variation in seasonal onset of timing of breeding. In both the North American dark-eyed junco (*Junco hyemalis*) and the European great tit (*Parus major*), females who departed from the nest earliest in the morning also laid their first egg earliest in the season. To continue to understand how circadian behavior, like daily activity onset, is influenced by an individual's physiology, I performed GnRH-challenges in pre-breeding and incubating female dark-eyed juncos. I found that females departing from the nest earlier had higher maximal levels of the sex-steroid hormone estradiol, supporting my hypothesis that variation in maximum E_2 levels are related with female activity onset during the breeding season (Chapter 3). These results suggest that individual variation in mechanisms regulating circadian rhythms are likely to play an important role in determining which females breed earliest. Circadian behaviors, however, are influenced by many variables, including maximal hormone levels. These findings, combined with prior research showing earlier activation of reproductive hormones in reproductively experienced individuals and shifts toward earlier activity onset with age could additionally explain why older females tend to breed earliest.

In addition to understanding the mechanisms that lead to precise decision making in reproductive timing, I also aimed to address costs associated with early reproduction. To test the hypothesis that

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energetic costs of upregulation of the humoral immune response will reduce offspring survival and have a disproportionately higher impact on reproductive success earlier in the breeding season (Chapter 4), I gave an immune challenge or control injection to incubating female dark-eyed juncos. I found that a mild immune challenge led to significant nest failure in both early and late breeding females compared to controls. While increasing energetic demands did not affect offspring survival of early breeders more so than late breeders, I decided to examine how biological aging of the female could be a cost of early reproduction. I used a long-term study population of dark-eyed juncos to test the hypothesis that individuals that breed earlier have higher rates of telomere loss (Chapter 5). While females breed earlier as they age chronologically, the earliest breeders experience greater annual telomere attrition compared to late breeders. This could not be attributed to the number of eggs laid within a season, but was correlated with cooler average daily temperatures during egg laying and incubation; a period of time when females provide most of offspring care and experiencing high energetic demands. These findings suggest early breeding females are experiencing costs of early reproduction through telomere loss, which may lead to reductions in overwinter survival under harsh conditions.

There is an abundance of research on the adaptation of organisms to climate change (Parmesan 2006; Moore and Huntington 2008; Moritz et al. 2008; Visser 2008; Bickford et al. 2010). In particular with respect to reproduction, which ultimately determines survival of a species [Amphibians, (Beebee 1995; Blaustein et al. 2001); Birds, (Crick et al. 1997); Insects, (Visser and Holleman 2001; Bale et al. 2002); Mammals,(Réale et al. 2003; Post and Forchhammer 2008; Bronson 2009; Stirling and Derocher 2012); Plants, (Myneni et al. 1997)]. Reproduction occurs in periodic bouts for many tropical, temperate, and arctic species and it is important for those species to use environmental cues to time reproduction with optimal conditions for offspring rearing (Baker 1938; Hau 2001; Bronson 2009). While organisms should time reproduction to occur during this optimal time, few individuals take advantage of highest reproductive success by breeding when conditions produce highest fitness (Perrins 1970). Our findings suggest that circadian timing of behavior and physiology are important mechanisms underlying observed individual variation in reproductive timing. As circadian rhythms are highly conserved across taxa (Dunlap et al. 2004), these results are broadly applicable and are worthy of further study in many organisms.

Furthermore, this research increases our understanding of how selection has shaped individual variation in timing of breeding. My findings under Aim 2 suggest that early breeding females suffer from molecular costs of early reproduction. The inability of early breeding females to prevent telomere loss warrants future research on seasonal changes in antioxidant availability (Arnold et al. 2010) and how levels of reactive oxygen species change under cold stress when females are allocating resources toward reproduction. A better understanding of the proximate and ultimate mechanisms regulating these decisions, and the potential costs and benefits of early or late breeding, are necessary to enable understanding of how species' adapt to their environment. At a time when animals are being exposed to rapidly changing environments via climate change and other human-induced factors, this research will aid in understanding the ability of animals to adaptively alter timing of breeding.

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