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Early-breeding females experience greater telomere loss

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Abstract

Annual reproductive success is often highest in individuals that initiate breeding early, yet relatively few individuals start breeding during this apparently optimal time. This suggests that individuals, particularly females who ultimately dictate when offspring are born, incur costs by initiating reproduction early in the season. We hypothesized that increases in the ageing rate of somatic cells may be one such cost. Telomeres, the repetitive DNA sequences on the ends of chromosomes, may be good proxies of biological wear and tear as they shorten with age and in response to stress. Using historical data from a long-term study population of dark-eyed juncos (*Junco hyemalis*), we found that telomere loss between years was greater in earlier breeding females, regardless of chronological age. There was no relationship between telomere loss and the annual number of eggs laid or chicks that reached independence. However, telomere loss was greater when temperatures were cooler, and cooler temperatures generally occur early in the season. This suggests that environmental conditions could be the primary cause of accelerated telomere loss in early breeders.

KEYWORDS
biological ageing, life history evolution, reproductive timing, telomeres

1 | INTRODUCTION

In seasonally breeding species, individuals that breed earlier often have greater annual reproductive success than those that breed later (mammals: Bourdon & Brinks, 1982; Festa-Bianchet, 1988; birds: Dawson & Clark, 2000; Price, Kirkpatrick, & Arnold, 1988; reptiles: Bauwens & Verheyen, 1985; Doody, Georges, & Young, 2004: amphibians; Morin, Lawler, & Johnson, 1990; Tejedo, 1992; invertebrates: Landa, 1992). Early breeders may have higher annual reproductive success than later breeders for several reasons, including the ability to fit more clutches or litters within a season, replace failed reproductive attempts before the season ends, appropriately time offspring growth with peaks in food abundance or ephemeral habitat availability, and/or reduce predation risk of offspring (Cox, Thompson III, & Faaborg, 2012; Morin et al., 1990; Ribble, 1992; Rieger, 1996; Williams, 2012). In addition to these factors, early breeders also tend to produce larger offspring (Holand et al., 2006; Perrins, 1970), which typically have higher overwinter survival and recruitment rates compared to smaller individuals (Low, Arlt, Pärt, & Öberg, 2015; Monrós, Belda, & Barba, 2002; Naef-Daenzer, Widmer, & Nuber, 2001; Sedinger, Flint, & Lindberg, 1995). However, despite these observed benefits of initiating reproduction early in the season, there is a lack of evidence for response to directional selection on breeding date (Verhulst & Nilsson, 2008). This suggests that while there may be significant reproductive benefits to breeding early, there are likely costs that delay onset of seasonal reproduction in most of the population and these costs may only be sustainable by high-quality individuals.

There may be many potential costs of initiating breeding early, including less food early in the year for adults (Bradbury &

Vehrencamp, 1977; Perrins, 1970), a reduction in future fecundity and survival resulting from increased current reproductive output (Dijkstra et al., 1990; Hanssen, Hasselquist, Folstad, & Erikstad, 2005), and additional energy expenditure as a result of exposure to colder spring temperatures during the first breeding attempt (Speakman, 2008; Tattersall et al., 2016). Cold temperatures may increase shivering and food intake, leading to increased metabolic heat production, and resulting in an increase in reactive oxygen species (ROS) and oxidative stress (Selman et al., 2002; Stier, Massemin, & Criscuolo, 2014). Under acute cold stress, nonhibernating mammals do not upregulate ROS-detoxifying enzymes, which may play a significant role in senescence (Buzadžić et al., 1997; Teramoto, Uejima, Kitahara, Ito, & Ouchi, 1998). Further, an imbalance between ROS and antioxidant defences is thought to contribute significantly to DNA damage (Barnes, Fouquerel, & Opresko, 2018). Early-breeding individuals may face higher energetic costs than late-breeding individuals during their first breeding attempt of the season, as early breeders must allocate energy to both reproductive functions (e.g., incubation, pregnancy) and increased thermoregulatory demands. The combined effects of reproduction (Heidinger et al., 2012) and cold stress may increase DNA damage compared to individuals that delay the onset of reproduction.

It has been observed that older or more experienced individuals tend to start breeding earliest in the season (Perdeck & Cavé, 1992; Perrins, 1970; Salvante, Dawson, Aldredge, Sharp, & Sockman, 2013; Sockman, Williams, Dawson, & Ball, 2004). Thus, 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 (Piper, 2011; Salvante et al., 2013; Verhulst & Nilsson, 2008). 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., 2018; Fischer, Perlick, & Galetz, 2008; Heidinger, Nisbet, & Ketterson, 2006). However, these studies are often cross-sectional samplings of a population, 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 (Heidinger et al., 2006; Wilson & 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 (Froy, Phillips, Wood, Nussey, & Lewis, 2013; McCleery, Perrins, Sheldon, & Charmantier, 2008).

While other studies have measured senescence via reduced fecundity and survival (Boonekamp, Salomons, Bouwhuis, Dijkstra, & Verhulst, 2014; Bouwhuis, Choquet, Sheldon, & Verhulst, 2012; Gustafsson & Pärt, 1990), one potential cost of early seasonal reproduction that remains largely unexplored is age-related declines in somatic integrity (Young, 2018). Investment in reproduction and increased reproductive effort have the potential to reduce adult survival (Cox et al., 2010; Dijkstra et al., 1990; Smith, 1958), and this reduction in survival may be mediated by telomere dynamics (Bauch, Becker, & Verhulst, 2013; Monaghan & Haussmann, 2006). Telomeres are repetitive, noncoding DNA sequences that form protective caps at the ends of linear eukaryotic chromosomes, being conserved in many eukaryotic organisms from coral to mammals (Gomes, Shay, & Wright, 2010). Telomeres enhance genome integrity, but shorten during normal cell division and can limit the lifespan of the cell (Blackburn, 2005). Reproduction has been found to shorten telomeres in birds and mammals (Heidinger et al., 2012; Kotrschal, Ilmonen, & Penn, 2007). There is also evidence that stress exposure can shorten telomeres (Epel et al., 2004; Haussmann & Marchetto, 2010; Kotrschal et al., 2007; Reichert & Stier, 2017). For example, environmental conditions and telomere dynamics have been linked (Debes, Visse, Panda, Ilmonen, & Vasemägi, 2016; Mizutani, Tomita, Niizuma, & Yoda, 2013; Wilbourn et al., 2017). Recent studies suggest that telomere dynamics may serve as useful biomarkers of biological ageing (Hau et al., 2015; Young, 2018) and telomere length and loss rate have been shown to be predictive of longevity in many mammals, birds and even some reptiles (Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Fairlie et al., 2016; Haussmann et al., 2003; Heidinger et al., 2012; Tricola et al., 2018; c.f., Olsson, Pauliny, Wapstra, & Blomqvist, 2010; Ujvari & Madsen, 2009). Experimental increases in telomere length increase lifespan in nematodes (*Caenorhabditis elegans*) (Joeng, Song, Lee, & Lee, 2004), and high telomerase activity is also thought to explain longevity in species with indeterminate growth (Reviewed by Gomes et al., 2010).

Initiating reproduction early in the season may increase telomere loss through many routes. While these factors have the capacity to influence both males and females, females ultimately dictate when offspring are born and fitness consequences of mistimed reproduction may be more serious for females while territory establishment and courtship behaviour may have a more significant impact on males (Ball & Ketterson, 2008; Kunz & Orrell, 2004). During the early stages of reproduction, females may have to expend additional energy to cope with cooler temperatures and reduced food availability in addition to the added costs of egg laying or pregnancy (Ardia, 2005; Graham, Mady, & Greives, 2017; Miles, Sinervo, & Frankino, 2000; Nicol, Mare, & Stolp, 1995; Pretzlaff, Kerth, & Dausmann, 2010). In birds, incubating females have been shown to lose body mass and significantly increase energy expenditure during colder weather (Conway & Martin, 2000; Nord, Sandell, & Nilsson, 2010; Tulp et al., 2009), suggesting energetic costs associated with reproduction during cold weather could lead to trade-offs.

Early breeders have the potential to produce more offspring within a season (Perrins, 1970; Ribble, 1992). Experimental increases in brood size can reduce adult survival (Dijkstra et al., 1990), and reproducing more frequently has been correlated with shorter telomeres (Plot, Criscuolo, Zahn, & Georges, 2012). Thus, accelerated telomere loss could occur due to increased annual reproductive output (Bauch et al., 2013). In birds, increased parental effort has been found to increase oxidative stress (Christe, Glaizot, Strepparava, Devevey, & Fumagalli, 2012), while reproductive effort reduces resistance to reactive oxygen species (ROS) (Reviewed

by Metcalfe & Alonso-Alvarez, 2010). Production of more and larger offspring may therefore lead to increases in oxidative stress. Such variation in oxidative status may also mediate the effects of stressful conditions or higher reproductive effort on telomere loss (Reichert & Stier, 2017).

The goal of this study was to understand relationships between chronological age, telomere loss and variation in initiation of seasonal reproduction using longitudinal data. Our study organism was the dark-eyed junco (*Junco hyemalis*), a medium-sized, ground-nesting passerine (Nolan et al., 2002). Data from this sedentary population (near Mountain Lake Biological Station in Pembroke, VA, USA) span 34 years, making it possible to measure telomere lengths from the same individuals over multiple years. The modal clutch size is 4 eggs (making clutch size consistent across nesting attempts) and females are multibrooded, capable of having as many as 5 nesting attempts within a season (though >3 successful nests is unlikely) (Nolan et al., 2002). First, we confirmed that initiating reproduction earlier in the season increases annual reproductive success by investigating the relationship between timing of first clutch initiation and (i) the number of eggs laid and (ii) offspring fledged annually. 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 loss is a correlate of early breeding by examining changes in telomere length from one breeding season to the next in females with known first clutch initiation dates. Finally, we used nesting and temperature data to determine whether (i) number of eggs laid annually, (ii) number of offspring fledged annually and/or (iii) temperatures experienced during the egg laying and incubation stages of the first reproductive attempt were correlated with annual telomere loss. Specifically, we predicted that females with earlier first nest initiation dates would display greater annual telomere loss than females with later first nest initiation dates. Early-breeding females are likely expending additional energy by breeding when temperatures are cooler and by producing more offspring annually than late-breeding females.

2 | MATERIALS AND METHODS

2.1 | Study species

This study took place at 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 metres). Dark-eyed juncos are a common ground-nesting bird in the study area, and their population has been monitored since 1983 (Nolan et al., 2002). Early-season captures and nest searching begin in mid-April through late April each year. Captures occur at 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 aluminium federal ID band. A unique colour band combination is also used, so individuals can be identified without being recaptured.

2.2 | 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 the day of hatching (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 renest of the year by any female occurred (Graham et al., 2017). In our population, the first egg date of the season (range: April 5–May 4) and the average first egg date for the population (range: April 21–May 19) vary considerably. This variation means that two females breeding in two separate years, but on the same absolute day of the year, could be an early and a late breeder. Thus, to normalize and compare first egg dates across years (1983–2016), we represented an individual's first egg date as the number of days before (–) or after (+) the population average for that year in which 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. To address whether individual females breed progressively earlier as they age chronologically, we examined 147 known-age females with first nest attempts found in two or more consecutive years. In addition, we included the total number of eggs laid and number of successful fledglings in a single season as a measure of seasonal reproductive output and breeding success, respectively.

Females included in the telomere analysis were those that had blood samples collected in two or more consecutive years and produced a nest with a first egg of the season occurring before the annual cut-off in their first year. As a result of ongoing studies, many of the birds that breed at the field site are individually marked and are of known age. Known-age females were banded either as nestlings, juveniles or first-year breeders. Not all nestlings and juveniles are caught every year, and young females may disperse into the population. However, first-year breeders (i.e., one-year-old individuals) have identifiable plumage characteristics and variation in iris colour that allows them to be reliably aged (Nolan et al., 2002). Although most females included in the study were of known age (n = 83), when we were comparing nesting records from one year to the next within an individual, we expanded our sample size to include females of unknown age (i.e., first captured and banded when they were older than one year; n = 23), for a total sample size of 106. Blood sampling did not begin until 1990, reducing the number of females with samples for telomere analyses. Additionally, not all known-age females from the age analysis had blood samples collected in consecutive years (i.e., they were not captured during the annual census, but had been banded in previous years). This resulted in a lower sample size of known-age females for telomere analyses compared to the reproductive performance and age analyses. Final sample sizes for each analysis are indicated in the corresponding Statistical Analysis section.

TABLE 1 Average C_t values ± SEM for samples across all 10 assays

	GAPDH	Telomere
Plate #	Average Sample C_t	Average Sample C_t
1	26.32 ± 0.12	15.35 ± 0.09
2	24.92 ± 0.07	16.55 ± 0.08
3	25.16 ± 0.05	15.38 ± 0.09
4	24.92 ± 0.06	15.67 ± 0.09
5	25.49 ± 0.06	16.37 ± 0.15
6	25.02 ± 0.13	16.24 ± 0.06
7	24.97 ± 0.06	15.26 ± 0.10
8	25.10 ± 0.05	16.21 ± 0.08
9	24.94 ± 0.05	15.56 ± 0.07
10	25.17 ± 0.05	15.84 ± 0.18

Note. All sample values were within the range of the standard curve.

2.3 | 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°C. Avian erythrocytes are an ideal tissue for longitudinal telomere measurements as they are a nucleated, highly mitotic tissue that can be nondestructively sampled (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., 2018; Bauer, Heidinger, Ketterson, & Greives, 2016). 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 8,000 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., 2018, 2016). *GAPDH* primer sequences were as follows: *forward GAPDH* (5'-AACCAGCCAAGTACGATGACAT-3′) and *reverse GAPDH* (5′-CCATCAGCAGCAGCCTTCA-3′). Telomere primers were as follows: *forward tel1b* (5'- CGGTTTGTTTGG GTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and *reverse tel2b* (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′). Reaction volumes were 25 µl and contained 6 uL DNA (20 ng per well) (Schmidt et al., 2016), 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 interplate variation (Schmidt et al., 2016). These values were chosen because they consistently produce reactions with optimal efficiencies (Bauer et al., 2018, 2016; Schmidt et al., 2016). The curve was made by mixing red blood cell samples from six one-year-old male dark-eyed juncos and extracting DNA from the pooled sample four times. Males were chosen to reduce the possibility that a female used in the study was part of the standard. The four extractions were then pooled and mixed prior to dilution for the standard curve. 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 at 95°C.

For each sample, the number of PCR cycles (C_t) to reach a threshold set by the 20 ng dilution of the reference sample in the standard curve was measured (Schmidt et al., 2016). The 20 ng reference was chosen because a preliminary study randomizing plate location of 29 individuals across 2 plates showed repeatability of T/S ratio was 0.89 (repeatability calculated following methods of Dingemanse & Dochtermann, 2013). The average C_t value for samples across all GAPDH and telomere plates was 25.20 ± 0.03 (range: 24.92 (plate 4) – 26.32 (plate 1), Table 1) and 15.85 ± 0.04 (range: 15.26 (plate 7) – 16.55 (plate 2), Table 1), respectively. All C_t values fell within the range of the standard curve. The ratio of number of telomere repeats (TTAGGG) to the number of copies of *GAPDH* (or T/S ratio) was used to quantify relative telomere length using the formula: T/S ratio = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})$ reference – $(C_t^{\text{telomere}} - C_t^{\text{GAPDH}})$ focal (Bauer et al., 2018; Cawthon, 2002). The 20 ng dilution reference sample from the standard curve was used to calculate the T/S ratio (as the reference C_t value in the calculation). Intra-assay variation was calculated by averaging the coefficient of variation across all 10 plates for each point on the standard curve (Table 2). The interassay variation was calculated by measuring the C_t coefficient of variation for all 5 points on the standard curve. The average intra- and interassay C_t variation of the telomere plates was 0.68% and 2.93%, respectively. For *GAPDH* plates, the intra- and interassay C_t variation was 0.32% and 1.61%, respectively. A standard curve was included on every plate to calculate the efficiencies. 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.

As blood samples were collected across 26 years, we used a linear mixed-effects model to check for any effects of storage time on degradation of telomeres. Plate number and female ID were included as random effects to control for repeated samples. Number of years the sample was stored had no influence on telomere length ($F_{1,119} = 0.40$, $p = 0.53$). Thus, we proceeded normally with the rest of our analyses.

TABLE 2 Coefficient of variation (%CV) calculated for each point on the standard curves

	GAPDH			Telomere		
	Average C_t	Interassay CV (%)	Intra-assay CV (%)	Average C_t	Interassay CV (%)	Intra-assay CV (%)
40 ng	24.01 ± 0.09	2.02	0.51	14.51 ± 0.10	3.30	0.67
20 ng	25.01 ± 0.07	1.50	0.22	15.64 ± 0.11	3.32	0.70
10 ng	26.00 ± 0.07	1.47	0.17	16.65 ± 0.11	3.14	1.10
5 ng	27.18 ± 0.09	1.62	0.44	17.88 ± 0.10	2.34	0.38
2.5 ng	28.25 ± 0.08	1.44	0.27	18.87 ± 0.11	2.46	0.48

Note. Average C_t values reported ± standard error of the mean (SEM).

2.4 | Temperature data

MLBS has its own weather station with historical data (from 1994 to 2016) freely available online (http://mlbs.virginia.edu/meteorological-data). Temperature (°C) is measured every 30 min year-round. We analysed average daily temperature during the egg laying and incubation stages (~15 days long) because this is a metabolically demanding time for females (Tulp et al., 2009). Additionally, females are almost exclusively responsible for offspring care during this time (Nolan et al., 2002). Egg production is energetically expensive (Williams, 2012), and incubation in this species is performed only by females with no partner feeding by the males (Nolan et al., 2002).

2.5 | 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, Maechler, Bolker, & Walker, 2015). Table 3 summarizes the correlation value (r) between explanatory variables used in models to show where collinearity occurs using package rmcorr (Bakdash & Marusich, 2017). All reported correlation values use rmcorr to control for repeated measures. All models included female ID and year as random effects. For models involving change in telomere length, the plate number corresponding with analysis of each female's samples was included as a random effect. Significance was set at $\alpha = 0.05$.

2.6 | Analysis 1: does annual reproductive output and success decline with later initiation of seasonal reproduction?

To ask whether nest initiation date in our population shows a seasonal decline in annual number of eggs laid and number of successful fledglings, we ran two models using 147 females of known age. Our first model investigated the relationship between the total number of eggs laid within a season for each individual and her date of first nest initiation. Clutch size in dark-eyed juncos is very consistent (Nolan et al., 2002) and annual number of eggs laid is highly reflective of the number of nesting attempts ($r = 0.91$). Thus, annual number of eggs laid is also a good proxy for total number of nesting attempts. The second model investigated the relationship between the total number of fledglings surviving to 11 d posthatch and her first nest initiation day. Female age was included as a covariate in both models.

TABLE 3 Correlation values (*r*) between explanatory variables used in analyses

Variable 1	Variable 2	Correlation (*r*)
First egg date	Chronological age	−0.24
First egg date	Days between samples	0.09
First egg date	Temperature	0.75
Number of eggs laid	Number of fledglings	0.23
Number of eggs laid	Days between samples	−0.06
Number of eggs laid	Temperature	0.05
Number of fledglings	Days between samples	−0.01
Number of fledglings	Temperature	−0.10
Days between samples	Temperature	0.15

Note. Only the relationship between first egg date and temperature provides concern for collinearity between explanatory variables.

2.7 | 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 investigate the relationship between chronological age and nest initiation date (measured as number of days before (-) or after (+) the population average for that year). To determine whether any decline in nest initiation date with age was due to birds breeding earlier as they age (i.e., within-individual variation) as opposed to longer lived individuals being earlier breeders (among-individual variation), we ran a second model replacing age with two calculations of variation (Herborn et al., 2016; Van de Pol & Wright, 2009). We calculated within-individual variation as (age – average age). By centring an individual's age around 0, we can identify whether there are longitudinal patterns of individual birds breeding earlier as they age. The among-individual value was calculated as the average age of a female across all sampling points. This calculation helps identify cross-sectional patterns of relationships between age and timing of

TABLE 4 Slope and standard error of slope for each model run

	Dependent Variable	Independent Variable	B	SE B	Random Effects
Analysis 1	*Model 1*: No. eggs	First egg date	**−0.09***	0.03	1. Female ID
		Chronological age	0.00	0.17	2. Year
	Model 2: No. fledglings	First egg date	**−0.07***	0.02	1. Female ID
		Chronological age	−0.01	0.12	2. Year
Analysis 2	*Model 1*: First egg date	Chronological age	**−0.75***	0.32	1. Female ID
					2. Year
	Model 2: First egg date	Among individual	0.01	0.44	1. Individual random slopes (correlation of female ID and within-individual variation)
		Within individual	**−1.60***	0.47	
					2. Year
Analysis 3	*Model 1*: Change in telomere length	First egg date	**0.01***	0.00	1. Female ID
					2. Year
		Days between samples	0.00	0.00	3. Plate
Analysis 4	*Model 1*: Change in telomere length	Number of eggs laid	−0.01	0.01	1. Female ID
		Number of fledglings	0.01	0.01	2. Year
		Average temperature	**0.03***	0.01	3. Plate
		Days between samples	0.00	0.00	

Note. Bolded and "*" slope values are significant ($p < 0.05$). Repeated measures from each model are listed in the dependent variable column, and all random effects included in each model are significant.

breeding. Longer lived individuals will have a higher average age than shorter lived individuals, and a significant correlation between average age and nest initiation date would indicate that the pattern was driven by high-quality individuals consistently breeding earlier and living longer. We accounted for individual random slopes by including the within-individual component of age correlated with female ID as a random effect (Dingemanse & Dochtermann, 2013). To be included in this analysis, females with nests in subsequent years were needed and not blood samples; thus, more females ($n = 147$) are included in this analysis compared to the telomere analyses.

2.8 | Analysis 3: do early-breeding females exhibit accelerated telomere loss?

We ran a model investigating the relationship between when a female laid her first egg of the season and subsequent annual telomere loss. It is important to include starting telomere length when measuring telomere loss as some studies suggest that longer telomeres show greater shortening rates compared to shorter telomeres (Nordfjäll et al., 2009). In order to include starting telomere length, we calculated an individual's change in telomere loss by correcting for the regression to the mean (Berry, Eaton, Ekholm, & Fox, 1984; Verhulst, Aviv, Benetos, Berenson, & Kark, 2013). To correct for regression to the mean, we first calculated $\rho = (2r\sigma_1\sigma_2)/(\sigma_1^2 + \sigma_2^2)$. In this equation, r is the correlation between starting telomere length (TL1) and ending telomere length (TL2); σ_1 is the standard deviation of all TL1 samples; σ_2 is the standard deviation of all TL2 samples; σ_1^2 is the sample variance for all TL1 samples; and σ_2^2 is the sample variance for all TL2 samples. We then calculated $D = -1(\rho(x_1 + \bar{x}_1)-(x_2 + \bar{x}_2))$, where x_1 is the focal TL1 sample; x_2 is the focal TL2 sample; $\bar{x}_1$ is the average of all TL1 samples; and $\bar{x}_2$ is the average of all TL2 samples. D is used in the model as change in telomere length, and because it accounts for starting telomere length, we did not include initial telomere length as a covariate in our statistical models. Chronological age was originally included in the model, but was not significantly correlated with loss ($F_{1,120} = 0.002$, $p = 0.97$); thus, it was removed from subsequent analyses. Including chronological age in the analysis 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.

2.9 | Analysis 4: is accelerated telomere loss related to temperature or annual reproductive output and success?

To test the hypothesis that annual change in telomere length may vary with annual reproductive output or reproductive success, we ran a model to investigate (i) the correlation between change in telomere length and (ii) number of eggs laid and (iii) number of successful fledglings. As the correlation value between number of eggs laid and number of successful fledglings was relatively low, they were included in the model together ($r = 0.23$, Table 3). Alternatively, adverse environmental conditions experienced during the early breeding season may contribute to telomere loss. Therefore, to test the hypothesis that thermoregulatory demands influenced change in telomere length, we additionally included average daily temperature during egg laying and incubation for the first nest of the season in the model. We found a highly significant

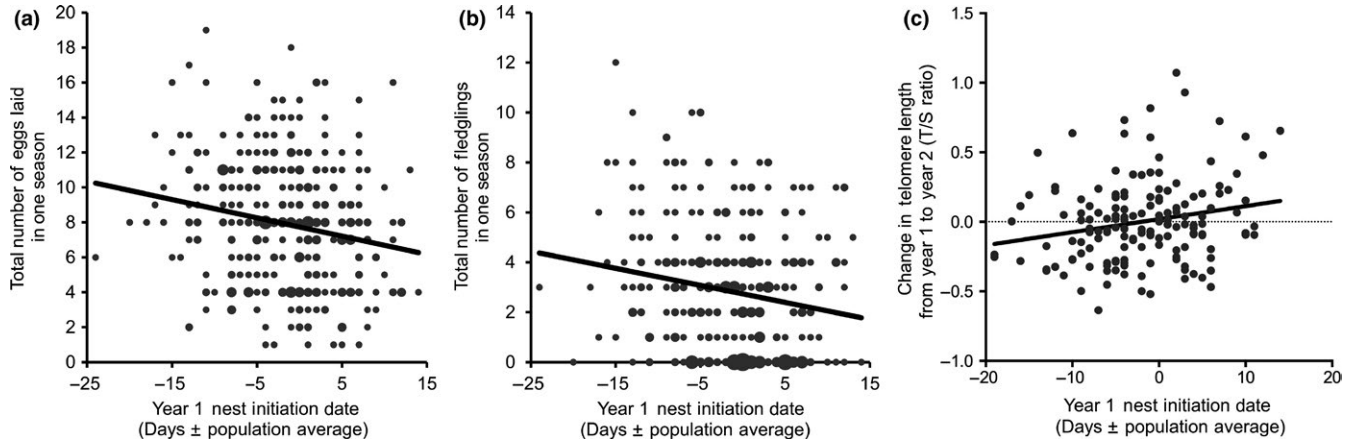

FIGURE 1 Decline in annual number of eggs laid (a) and fledglings produced (b) with initiation of seasonal reproduction. To indicate where overlapping points occur, larger point sizes indicate more individuals. Females that started breeding earlier in the season experienced telomere loss from one breeding season to the next as compared to the rest of the population (c). Change in telomere length was corrected for regression to the mean and was scaled to zero, so loss is negative and gain is positive. During analysis, the number of days between collection of blood samples was taken into account; however, the data in this figure are not corrected for this effect

correlation between day of year and temperature ($F_{1, 125} = 90.05$, $p < 0.001$, $r = 0.75$, $\beta = 0.21$) (Graham et al., 2017); thus, collinearity between temperature and day of year does not allow us to include day of year in the model. Therefore, any effect of temperature must be interpreted with caution, as we are unable to separate the effects of day and temperature. Temperature data were available from 1994–2016. The full model (including reproductive output, reproductive success and temperature) included 89 separate females with blood samples from two consecutive years. 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.

3 | RESULTS

A summary of the statistical models run within each of the four analyses reported below appears in Table 4. Also included in the table are the (i) slope of each independent variable, (ii) standard error of the slope and (iii) random effects included in each model. Values reported are ±standard error of the mean (*SEM*).

3.1 | Annual reproductive output and success decline with nest initiation date

The average bird laid 7.67 ± 0.45 eggs and fledged 2.62 ± 0.34 nestlings per year. As predicted, females that initiated reproduction earlier in the season laid more eggs ($F_{1, 329} = 10.81$, $p = 0.001$, $r = -0.55$, Figure 1a) and successfully fledged more offspring ($F_{1, 321} = 12.65$, $p < 0.001$, $r = -0.26$, Figure 1b) that year. For every day a female delayed reproduction, the annual number of eggs laid and fledglings produced declined by 0.09 ± 0.03 and 0.07 ± 0.02, respectively (Table 4). Chronological age did not influence the number of eggs laid or the number of offspring fledged (both $p > 0.95$).

3.2 | Individuals breed earlier as they age

We analysed nesting records from 147 known-age females who nested for at least 2 consecutive years (max: 5 consecutive years). Our analysis found that females laid their first clutch of the season 0.75 ± 0.32 days earlier each year they bred in the population ($F_{1, 302} = 5.40$, $p = 0.02$, $r = -0.30$, Figure 2a, Table 4). Within-individual variation had a significant effect on lay date ($F_{1, 186} = 11.42$, $p < 0.001$, $r = -0.45$, Figure 2b), with females laying their first egg 1.60 ± 0.47 days earlier each year (Table 4). Among-individual variation had no effect on lay date ($F_{1, 125} < 0.001$, $p = 0.95$). This suggests the negative relationship between egg lay date and chronological age is driven by individuals breeding progressively earlier as they age.

3.3 | Females that start breeding earlier experience greater annual telomere loss

With 267 blood samples from 106 females, we found that females initiating reproduction earlier in the season incurred greater telomere loss between breeding seasons ($F_{1,146} = 8.43$, $p = 0.004$, $r = 0.30$, Figure 1c). Telomere loss was reduced by 0.01 ± 0.003 for each day a female delayed nest initiation (Table 4). The average number of days between collection of two samples was 371 (range: 263–472). The number of days between the collection of a female's samples did not have a significant effect on change in telomere length ($F_{1,99} = 1.41$, $p = 0.24$).

3.4 | Greater telomere loss is related to cooler temperatures, but not reproductive performance

Average daily temperature during the egg laying and incubation stages of the first nesting attempt (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 ($F_{1,88} = 6.75$,

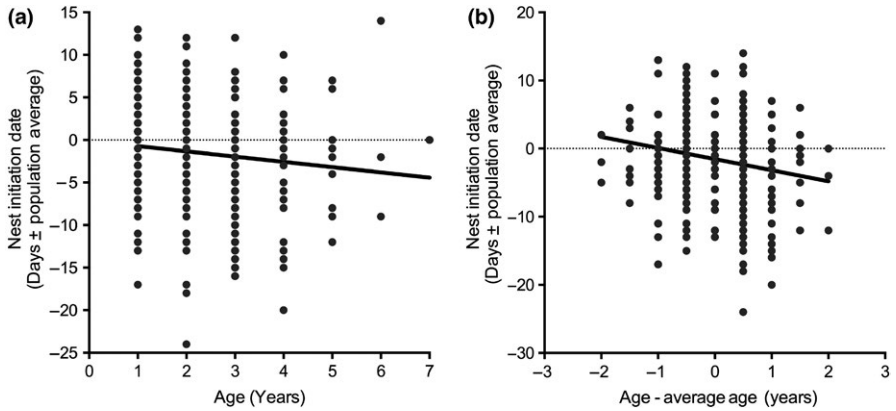

FIGURE 2 As females aged, we saw earlier nest initiation dates (a). This was due to within-individual variation (b) rather than among-individual variation. The data presented in these figures show the trend at the population level, though statistical analyses included individual identity as a random effect

$p = 0.01$, $r = 0.16$, Figure 3). For every degree Celsius increase in temperature, telomere loss was reduced by 0.03 ± 0.01 (Table 4). As in the previous model, the number of days between samples did not influence change in telomere length ($F_{1,67} = 1.87$, $p = 0.18$). Interestingly, although earlier laying females laid more eggs within a season and successfully fledged more offspring and initiation date was positively related to change in telomere length, change in telomere length was not significantly related to the number of eggs laid ($F_{1,117} = 1.94$, $p = 0.16$) or fledglings produced ($F_{1,121} = 0.97$, $p = 0.33$).

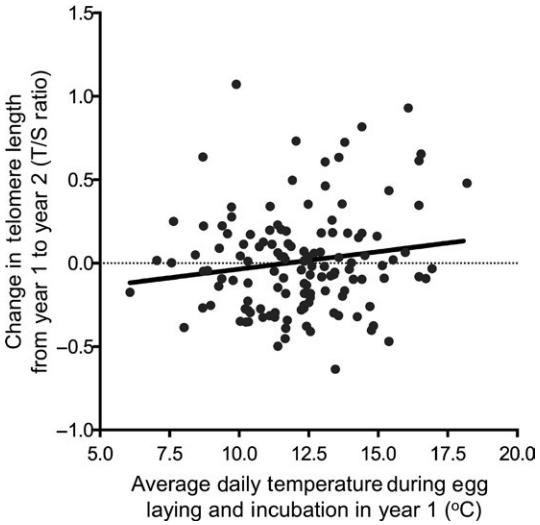

FIGURE 3 Temperatures during the egg laying and incubation stages of the first nest of the season positively correlate with annual change in telomere length. The data in this figure are not corrected for other variables included in the statistical analysis, which were (1) number of eggs laid over the season and (2) number of days between collection of blood samples

4 | DISCUSSION

The results of this study support the hypothesis that early-breeding females have greater annual reproductive success than females that breed later in the season because they lay more eggs and successfully fledge more offspring in a season. We also found that females breed earlier as they age. Thus, the relationship between timing of breeding and age is not simply an outcome of older age classes consisting of higher quality individuals that have always bred earlier and live longer than lower quality individuals (McCleery et al., 2008). Females breeding earlier as they age may be due to prior reproductive experience, which can lead to higher levels of reproductive hormones and earlier activation of the reproductive system (Angelier, Weimerskirch, Dano, & Chastel, 2007; Salvante et al., 2013; Sockman et al., 2004). Older females may also accumulate necessary resources more quickly through efficient foraging, have better knowledge of their environment or reduce time allocated to prelaying activities by pairing with familiar males (Brown, Brown, & Brazeal, 2008; Fowler, 1995; Piper, 2011; Weimerskirch, 1992).

We also found that early-breeding females exhibit more telomere loss as compared to females breeding later, suggesting that increased annual telomere loss may be a cost of initiating reproduction early in the season. We additionally found that annual change in telomere length is related to cooler temperatures experienced during the first nesting attempt of the season, which is a time when offspring care is primarily controlled by the female. However, annual change in telomere length does not appear to be influenced by annual reproductive output as there was no relationship with number of eggs laid or number of chicks that successfully reached independence.

Observed annual telomere loss in early-breeding individuals suggests that a female's reproductive timing influences trade-offs between telomere maintenance and annual reproductive success. In addition to increased telomere loss in early-breeding individuals, we also observed telomere lengthening in later breeding females. Observations of telomere preservation, and even elongation, observed in females that initiated reproduction later in the season could be a result of measurement error (Martin-Ruiz et al., 2014), but it is also possible that true lengthening occurs (Bateson & Nettle, 2017). Telomere lengthening can occur via upregulated production

of the enzyme telomerase and has been documented in other studies (Bize et al., 2009; Ilmonen, Kotrschal, & Penn, 2008; Turbill, Smith, Deimel, & Ruf, 2012; Ujvari & Madsen, 2009). Telomere lengthening in later breeding females may be due to increased food availability. For example, when supplemental food is provided to free-living edible dormice (*Glis glis*), telomeres are elongated when compared to both starting telomere length and nonsupplemented controls (Hoelzl, Cornils, Smith, Moodley, & Ruf, 2016). Furthermore, a study in black-tailed gulls (*Larus crassirostris*) found telomere elongation during an El Niño year, which produced mild weather and low sea surface temperatures (Mizutani et al., 2013). Differences in habitat quality early and late in the breeding season may also influence telomere loss (Angelier, Vleck, Holberton, & Marra, 2013), though we are unable to measure this given our available data set. Further work is needed to understand the relationship between environmental conditions, food availability and telomere dynamics.

Telomere loss in individuals that initiate reproduction earlier in the season suggests that a female's reproductive timing influences trade-offs between telomere maintenance and annual reproductive success. We also saw decreasing telomere length in females experiencing cooler temperatures during the first nesting attempt of the season. While we are unable to separate the effects of timing of breeding and temperature, cooler temperatures are more likely to occur early in the breeding season (Graham et al., 2017) and may be a mechanism increasing telomere loss. Cold temperatures may increase oxidative stress via an increase in reactive oxygen species (ROS) and reduced antioxidant availability. For example, females may be exposed to acute cold stress during egg laying or pregnancy, resulting in an increase in ROS (Selman et al., 2002; Stier et al., 2014) and inhibition of ROS-detoxifying enzymes (Buzadžić et al., 1997; Teramoto et al., 1998). Whether oxidative damage to telomeres occurs directly or indirectly (e.g., through processing of oxidized purines (Barnes et al., 2018)) via ROS, there is accumulating evidence from both correlative and manipulative experiments that oxidative stress accelerates telomere loss (Barnes et al., 2018), though in vivo studies suggest the effects may be tissue- or species-specific and more work is needed (Reichert & Stier, 2017).

Increasing antioxidants can neutralize ROS and help preserve telomere length (Liu, Trimarchi, Navarro, Blasco, & Keefe, 2003; Tarry-Adkins, Martin-Gronert, Chen, Cripps, & Ozanne, 2008). Because we were using historical samples, we were unable to measure antioxidant capacity. A diet rich in arthropods (known to be a source of antioxidants (Catoni, Peters, & Schaefer, 2008)) could counteract oxidative stress and telomere loss (Badás et al., 2015). In addition to seasonal increases in overall food availability (Ardia, 2005), antioxidants can reduce oxidative damage to tissues (Liu et al., 2003; Tarry-Adkins et al., 2008). Increasing antioxidants helps reduce DNA damage (Liu et al., 2003; Tarry-Adkins et al., 2008), even when reproductive workload is increased experimentally (Beaulieu, Reichert, Le Maho, Ancel, & Criscuolo, 2011). However, antioxidant-rich arthropods do not become available until later in the breeding season, when the first clutches begin hatching (Arnold, Ramsay, Henderson, & Larcombe, 2010) and may be unavailable to early-breeding females during egg laying and incubation of their first clutch.

Interestingly, change in telomere length was not related to the annual number of eggs laid or the number of chicks that survived to leave the nest. This is in contrast to the finding that female common terns (*Sterna hirundo*) with more chicks that survived to 10 days of age had higher telomere loss compared to females with failed nests (Bauch et al., 2013). This difference between species may be due to differences in how reproductive performance was measured. In the common terns, females either succeeded or failed in a single breeding attempt (Bauch et al., 2013), while dark-eyed juncos are multibrooded and capable of attempting to raise as many as 5 clutches within a season (though >3 successful attempts are unlikely) (Nolan et al., 2002). Thus, the common terns did not have the opportunity to renest after a failed attempt, unlike dark-eyed juncos. Telomere length may be more influenced by whether an individual breeds at all, rather than the number of reproductive bouts. For example, breeding zebra finches (*Taeniopygia guttata*) exhibited higher telomere loss compared to nonreproductive individuals, but the number of reproductive bouts in breeding birds was not correlated with telomere length (Heidinger et al., 2012). The lack of a relationship between annual change in telomere length and reproductive performance is additionally supported by a recent study in Magellanic penguins (*Spheniscus magellanicus*) (Cerchiara et al., 2017). Our finding that cooler temperatures are correlated with telomere shortening could instead suggest that the conditions experienced during reproduction affect telomere dynamics more strongly than the total number of offspring produced by female passerines within a season. It is also possible that our sample size did not enable us to detect a relationship between change in telomere length and reproductive performance; no blood samples were collected from birds prior to 1990, reducing the sample size for this analysis.

Our unique, longitudinal data set in a short-lived songbird demonstrates that regardless of chronological age, breeding early in the season results in significant annual telomere loss that may be a result of initiating reproduction when temperatures are cooler. Telomere length and loss are of increasing importance for understanding the influence of varying conditions on longevity (Haussmann & Marchetto, 2010; Salomons et al., 2009), and telomere dynamics may be a better predictor of reproductive timing than chronological age (Bauch et al., 2013; Bauer et al., 2018). Our study provides the first evidence of telomere loss as a potential cost to early reproductive timing.

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AUTHOR CONTRIBUTIONS

JLG, CMB, EDK, BJH and TJG conceived the idea. JLG, CMB, BJH and TJG designed methodology. EDK contributed all samples and database information. BJH and CMB developed qPCR protocol for dark-eyed juncos. JLG did DNA extractions and qPCR. JLG, CMB, BJH and TJG performed data analysis. JLG wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

DATA ACCESSIBILITY

All R-code and data files used for analyses presented in this manuscript are available on the Dryad Digital Repository (https://doi.org/10.5061/dryad.sd8dp40).

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