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Experimentally elevated testosterone shortens telomeres across years in a free-living songbird

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Abstract

Reproductive investment often comes at a cost to longevity, but the mechanisms that underlie these long-term effects are not well understood. In male vertebrates, elevated testosterone has been shown to increase reproductive success, but simultaneously to decrease survival. One factor that may contribute to or serve as a biomarker of these long-term effects of testosterone on longevity is telomeres, which are often positively related to lifespan and have been shown to shorten in response to reproduction. In this longitudinal study, we measured the effects of experimentally elevated testosterone on telomere shortening in free-living, male dark-eyed juncos (*Junco hyemalis carolinensis*), a system in which the experimental elevation of testosterone has previously been shown to increase reproductive success and reduce survival. We found a small, significant effect of testosterone treatment on telomeres, with testosterone-treated males exhibiting significantly greater telomere shortening with age than controls. These results are consistent with the hypothesis that increased telomere shortening may be a long-term cost of elevated testosterone exposure. As both testosterone and telomeres are conserved physiological mechanisms, our results suggest that their interaction may apply broadly to the long-term costs of reproduction in male vertebrates.

KEYWORDS
ageing, life-history evolution, reproduction, senescence, telomeres, testosterone

1 | INTRODUCTION

Life-history theory predicts that because organisms must balance investment in reproduction against self-maintenance, greater reproductive investment will accelerate the rate of senescence (Harshman & Zera, 2007; Roff, 2002; Stearns, 1992). Although the "cost of reproduction" is considered a cornerstone of life-history theory, empirical studies have produced mixed results (Santos & Nakagawa, 2012), and several factors could contribute to discrepancies among studies. For example, individuals often vary in their ability to acquire resources to allocate to reproduction and self-maintenance, which can mask life-history trade-offs in nonexperimental studies (Reznick et al., 2000; Van Noordwijk & Dejong, 1986). Furthermore, studies are often conducted over a single year, and when the costs are paid over longer timescales, they may go undetected (Boonekamp et al., 2014). Importantly, the mechanisms involved in mediating these long-term costs remain poorly understood (Boonekamp, Salomons, et al., 2014; Harshman & Zera, 2007), particularly in free-living populations (Monaghan et al.,

2008; Nussey et al., 2013). Addressing this knowledge gap is important for predicting the evolution of life-history trade-offs, but requires longitudinal experimental studies (Boonekamp, Salomons, et al., 2014; Monaghan et al., 2008; Nussey et al., 2013).

Hormones often have pleiotropic effects on suites of traits and underlie life-history trade-offs (Cohen et al., 2012; Ketterson & Nolan, 1999; Ricklefs & Wikelski, 2002; Zera & Harshman, 2001). In male vertebrates, the sex steroid testosterone influences reproductive function by stimulating the development of secondary sexual traits, sperm production and mating behaviours (Nelson, 2011). Variation in the seasonal pattern of testosterone secretion is associated with broad-scale differences in life-history strategies and has been implicated in underlying the trade-off between reproduction and survival (Hau, 2007; Ketterson & Nolan, 1992; Mills et al., 2009; Wingfield et al., 2001). For example, across bird species, peak testosterone levels during the breeding season tend to be lower in tropical species, which are also often characterized by lower annual reproductive output and longer lifespans than birds that breed at temperate latitudes (Goymann et al., 2004). In some species, experimentally elevated testosterone also increases reproductive success, but simultaneously decreases survival (Mills et al., 2009; Reed et al., 2006), but this pattern is not universal (Khaw et al., 2007; Taff & Freeman-Gallant, 2014). Regardless, the mechanisms that mediate associations between testosterone and reduced longevity are not well understood.

Elevated testosterone could decrease survival by increasing annual mortality or the pace of senescence via several nonexclusive routes including increasing activity and exposure to predation risk, metabolic rate, stress exposure (Buchanan et al., 2001; Ketterson et al., 1991; Schoech et al., 1999), and oxidative damage (Alonso-Alvarez et al., 2007, 2009), but see (Carlos Noguera et al., 2011; Taff & Freeman-Gallant, 2014) or reducing immune function (Grindstaff et al., 2001), but see (Roberts et al., 2004). One previously unexplored factor that could be part of this suite of mechanisms linking elevated testosterone exposure to longevity and/or serve as a biomarker of the long-term costs of testosterone exposure is telomeres. Telomeres are highly conserved, repetitive, noncoding DNA sequences that form protective caps at chromosome ends that enhance genome integrity (Blackburn, 2005), but shorten during cell division and in response to stress (Boonekamp et al., 2014; Hau et al., 2015; Herborn et al., 2014; Nettle et al., 2013; Reichert & Stier, 2017; von Zglinicki, 2002). Telomeres limit cellular lifespan because once they become critically shortened, cells stop dividing and often have altered secretory profiles that can increase inflammation (Blackburn, 2005). Telomeres are also often predictive of longevity, with individuals with shorter telomeres and faster telomere loss having reduced lifespans (Asghar et al., 2015; Barrett et al., 2013; Cawthon et al., 2003; Fairlie et al., 2016; Heidinger et al., 2012; Wilbourn et al., 2018). There is also increasing evidence that metabolically demanding activities that increase oxidative stress such as reproduction increase the rate of telomere loss (Bauch et al., 2013; Graham et al., 2019; Heidinger et al., 2012; Sudyka, 2019; Sudyka et al., 2014, 2019).

In this study, we tested the hypothesis that exposure to experimentally elevated testosterone increases telomere loss in free-living, male dark-eyed juncos (*Junco hyemalis carolinensis*). Previous research in this system has demonstrated that males exposed to experimentally elevated testosterone have higher reproductive success (Raouf et al., 1998), but lower survival (Reed et al., 2006), compared to controls with normal levels of testosterone. In this historical, longitudinal study, males were given subcutaneous implants packed with crystalline testosterone that experimentally elevated testosterone to physiologically relevant levels or empty, control implants. Implants remained in place throughout the breeding season and were removed at the end of the summer. If males were recaptured in subsequent years, they were blood sampled and implanted again with the same treatment. To test whether an experimental elevation of testosterone was associated with increased telomere shortening, we examined a subset of these historical samples collected from known-age males that were resampled at least once. This allowed us to examine whether experimentally elevated testosterone was associated with greater testosterone shortening with age within individuals across years.

2 | METHODS

2.1 | Study system, experimental treatment and sample collection

Research was conducted on a free-living population of the Carolina subspecies of the dark-eyed junco (*Junco hyemalis carolinensis*), a socially monogamous songbird that breeds at the Mountain Lake Biological Station in Giles County, Virginia, USA (37°22′N, 80°32′W); for a detailed description of the study site see (Ketterson & Nolan, 1992). As part of the long-term, ongoing research in this system, chicks in all of the nests that are found, and all of the recently fledged juveniles that are caught each year are individually marked with U.S. Fish and Wildlife Service bands and hence are of known age.

Between 1993 and 2000, Ketterson and her colleagues conducted a unique, longitudinal experimental manipulation of testosterone in breeding male juncos (Ketterson & Nolan, 1992). At the beginning of each breeding season, males were captured in mist-nets and walk-in Potter traps. Upon capture, small blood samples were collected by venipuncture from the alar vein into heparinized capillary tubes and stored in Longmire's solution at 4°C until frozen at −20°C until analyses. During this initial capture, males were grouped by age and capture site and randomly assigned to an experimentally elevated testosterone or control treatment. Males in the experimentally elevated testosterone group received two subcutaneous, 10-mm-long silastic implants (Dow Corning; 1.47 mm i.d., 1.96 mm o.d.) containing crystalline testosterone (Sigma Chemical); males in the control group received empty implants (Ketterson & Nolan, 1992; Ketterson et al., ,,,,1991, 1992; Klukowski et al., 1997). Numerous studies in this system have established that this

experimental treatment effectively elevates testosterone and maintains it at peak, physiologically relevant levels throughout the breeding season (Ketterson & Nolan, 1992; Ketterson et al., 1991, 1992; Klukowski et al., 1997). To examine the longitudinal influence of the testosterone treatment on telomeres, this study necessarily focused on a subset of birds from this larger experimental study that were of known age and, if treated in more than one year, received the same experimental treatment. Telomeres were measured in blood samples that were collected at the time of the first implantation and again when the birds were recaptured during subsequent breeding seasons (see statistical methods for details).

2.2 | Telomere analyses

Telomeres were measured in red blood cells (RBCs), which are nucleated in birds and well suited for longitudinal telomere analyses (Nussey et al., 2014). We extracted DNA from RBCs suspended in Longmire's buffer solution using Macherey-Nagel Whole Blood Kits (Macherey-Nagel) as per the manufacturer's instructions (Bauer et al., 2016). DNA quantity was measured using a Nanodrop 8000 spectrophotometer (Thermo Scientific). DNA quality was verified by electrophoresis on a 2% agarose gel. There was no indication that storage time impacted the quality of these samples and telomere length was not significantly correlated with time spent in storage (i.e., the year of collection; $r = .074$, $p = .240$).

Relative telomere length was measured using quantitative polymerase chain reaction (qPCR) on an Mx3000P (Stratagene), following the methods of (Cawthon, 2002) adapted for dark-eyed juncos (Bauer et al., 2016). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the single copy control gene. We verified the suitability of GAPDH using a melt curve analysis, which indicated that the dissociation curve had a single peak at the expected melting temperature (T_m) of 82.0°C. In addition, the PCR product was run on a 2% agarose gel to confirm the amplification of a single product at the expected 98 bp.

Samples were randomized with respect to treatment across plates and all of the samples for an individual were run on the same plate. GAPDH and telomere reactions were run in triplicate on separate plates and the number of PCR cycles necessary to accumulate sufficient fluorescent signal to cross a threshold (C_t) was measured. Each 25-µl reaction contained 20 ng of DNA and either telomere or GAPDH primers at a 200/200 nM concentration mixed in 12.5 µl of perfeCTa SYBR green supermix Low ROX (Stratagene). We used the following primers to amplify the reactions: telomeres—forward tel1b (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and reverse tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′) and zebra finch GAPDH—forward (5′-AACCAGCCAAGTACGATGACAT-3′) and reverse GAPDH (5′-CCATCAGCAGCAGCCTTCA-3′). The qPCR conditions were as follows: telomeres—10 min at 95°C, followed by 27 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C, finishing with 1 min at 95°C, 30 s at 58°C and 30 s at 95°C and GAPDH—10 min at 95°C, followed by 40 cycles of 30 s at 95°C and 30 s at 60°C, finishing with 1 min at 95°C, 30 s at 55°C and 30 s at 95°C.

Each plate also contained a dark-eyed junco reference sample that was used to create a five-point standard curve (40, 20, 10, 5, 2.5 ng) to ensure that all samples fell within the bounds of the standard curve and to measure reaction efficiencies. Average C_t values were used to calculate the relative telomere length (T/S ratio) according to the following formula: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})$ reference sample $- (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})$ focal sample (Stratagene, 2007). All samples fell within the range of the standard curve. Reaction efficiencies were similar: GAPDH (mean ±1 *SEM*: 91.9 ± 0.81, range 89.6%–94.9%) and telomere (mean ±1 *SEM*: 89.3 ± 0.58, range 85%–94.2%). At the time of assay optimization, the repeatability of the T/S ratio (0.89, $p < .001$) was calculated by running 29 juncos from this long-term study in random well locations across two plates. The single Intraclass correlation coefficient (ICC) was 0.89, $p < .001$, 95% confidence interval lower bound 0.77 and upper bound 0.95, as previously reported (Graham et al., 2019). The repeatability of the telomere C_t (0.93, $p < .001$) and GAPDH C_t (0.98, $p < .001$) triplicates was calculated using all of the samples following the methods of (Dingemanse & Dochtermann, 2013).

2.3 | Statistics

In total, we analysed telomeres (mean ± *SEM* T/S ratio 1.00 ± 0.0213) in 254 blood samples collected from $n = 114$ males ($n = 50$ with experimentally elevated testosterone and $n = 64$ controls). All individuals were resampled at least once, at least 1 year after implantation, and telomere length was significantly repeatable within individuals across years (0.257, $p < .01$), calculated following the methods of (Dingemanse & Dochtermann, 2013).

There were no significant differences between treatment groups in the age at first implantation (first breeding season: $n = 45$ testosterone males and $n = 54$ control males; later ages: $n = 5$ testosterone and $n = 10$ control; $F_{1,112} = 0.037$, $p = .847$); the number of times individuals were resampled and received implants (once: $n = 42$ testosterone and $n = 55$ control males; more than once: $n = 9$ control and $n = 8$ testosterone males; $F_{1,112} = 0.614$, $p = .435$); how much time elapsed (number of years) between sample collections (mean ±1 *SEM*: 1.56 ± 0.105 years; range = 1–8 years; $F_{1,112} = 0.032$, $p = .858$); or telomere length at the start of the experiment (i.e., at the time of first implantation; $F_{1,102.28} = 0.590$, $p = .444$). There was also no significant effect of year on telomere length ($F_{1,161.504} = 0.769$, $p = .382$), and the number of individuals sampled from each treatment group did not significantly differ among years (Wald chi-square = 0.921, $p = .921$). Thus, these variables were not considered in subsequent analyses.

To examine the potential influence of treatment on the change in telomere length with age, we used linear mixed models that included all of the telomere samples collected for each individual.

Importantly, any change in telomere length with age could be due to both within- and among-individual effects and in this study, we were specifically interested in within-individual changes. Therefore, we separated the within- and among-individual effects, by using within-subject centring and partitioning age into two new variables, an average age variable and a delta age variable (van de Pol & Wright, 2009). Average age is calculated as the average of all of the actual ages at which an individual's samples were collected and represents the among-individual effect of age. Whereas delta age is calculated as the actual age at the time each sample is collected minus the average age and represents only the within-individual effect of age (i.e., telomere shortening with age within individuals). For example, if an individual's actual ages at three sampling events were 1, 2 and 3 years old, the average ages that would be recorded for each of these samples would be 2, and the delta ages that would be recorded for each of these samples would be −1, 0 and 1 respectively.

To test whether telomeres declined more across years within males that received testosterone than control implants, we ran a linear mixed model that included telomere length (all of the measurements for each individual) as the dependent variable and treatment, delta age (the within-individual component of age), and an interaction between treatment and delta age as fixed effects. Individual was included as a random effect to account for the fact that individuals were sampled multiple times and plate was included as a fixed effect to control for any potential variation among plates (Table S1).

Variance structures were estimated using restricted maximum likelihood (REML) and all models had normal error structures. All statistical analyses were performed in IBM SPSS Statistics 23 (IBM Corp.).

3 | RESULTS

There were no significant main effects of treatment ($F_{1,97.089}$ = 1.930, p = .168; effect size −0.069 ± 0.0497 T/S ratio units per year) or delta age ($F_{1,141.842}$ = 0.970, p = .326; effect size 0.0259 ± 0.0257 T/S ratio units per year) on telomeres. However, males exposed to experimentally elevated testosterone experienced significantly more telomere shortening with age than males that received control implants (indicated by a significant treatment × delta age interaction effect on telomeres: $F_{1,139.26}$ = 5.681, p = .018; effect size −0.089 ± 0.0373 T/S ratio units per year; r = −.21 for testosterone males and r = .049 for control males; Figure 1).

One male was sampled over a relatively longer time span (i.e., he was first implanted and sampled when he was 1 year old and last sampled when he was 9 years old), but if this individual is removed from the analysis, the treatment × delta age interaction effect on telomeres remains significant and quantitively similar ($F_{1,139.97}$ = 5.917, p = .016; effect size −0.103 ± 0.0426 T/S ratio units per year). There was also a significant effect of plate on telomere length ($F_{13,124.053}$ = 2.395, p = .007), but if plate is also removed from the analysis, the treatment × delta age interaction effect on telomeres remains significant and quantitatively similar ($F_{1,137.469}$ = 6.158, p = .014; effect size −0.095 ± 0.0383 T/S ratio units per year).

4 | DISCUSSION

Here we demonstrate that telomeres shorten more with age in males exposed to experimentally elevated testosterone than controls.

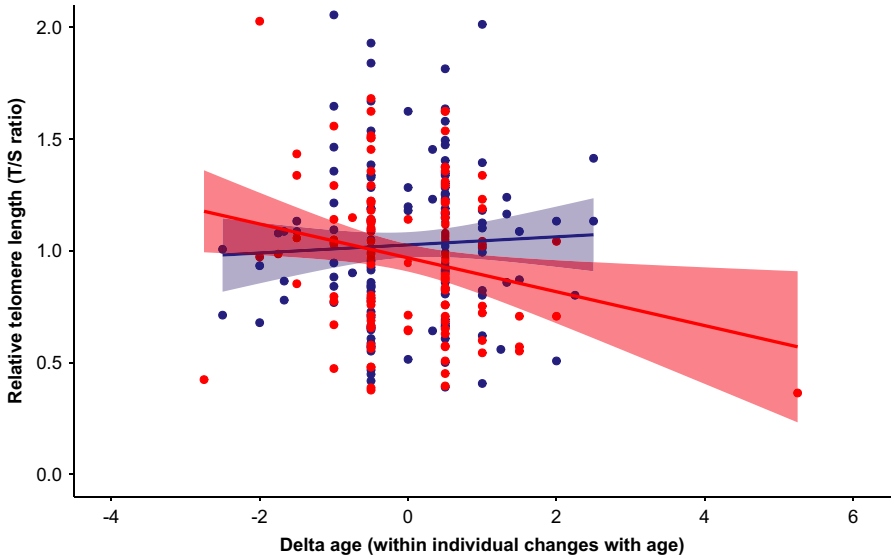

FIGURE 1 The relationship between delta age (longitudinal change in telomere length with age within individuals) and relative telomere length (T/S ratio) in male dark-eyed juncos (*Junco hyemalis carolinensis*) that received testosterone (red dots, line, and 95% confidence interval) or control (blue dots, line, and 95% confidence interval) implants. Males with testosterone implants experienced significantly more telomere loss across years than controls (indicated by a significant treatment × delta age interaction effect on telomeres: $F_{1,139.26}$ = 5.681, p = .018; effect size: −0.089 ± 0.0373 T/S ratio units per year; r = −.21 for testosterone males and r = .049 for control males)

Previous research in this system has demonstrated that males with experimentally elevated testosterone benefit from higher reproductive success (Raouf et al., 1998), but also suffer from increased mortality (Reed et al., 2006). Furthermore, males with endogenously higher peak testosterone levels also have lower return rates (McGlothlin et al., 2010). In humans, other mammals and birds, longer-lived individuals often have longer telomeres (Asghar et al., 2015; Barrett et al., 2013; Bize et al., 2009; Cawthon et al., 2003; Fairlie et al., 2016; Heidinger et al., 2012). Thus, our findings are consistent with the idea that increased telomere shortening is one potential long-term cost of elevated testosterone that may be associated with reduced longevity.

The relationship between testosterone and telomeres may indicate direct functional impacts of testosterone on lifespan, may be a biomarker of accumulated stress exposure incurred during reproduction (i.e., defending territories, displaying for females, etc.), or both and we are unable to distinguish between these two nonexclusive possibilities. The effect of testosterone on telomeres is somewhat small, but our study design may have caused us to underestimate it. To assess the influence of testosterone on telomere loss within individuals, we necessarily focused on a subset of birds that survived for at least 1 year after treatment. Annual survival of males in this population varies, but on average is around 50% (Nolan et al., 2020; Reed et al., 2006) and these "survivors" may have been of higher quality or in better condition, which could have made it more difficult to detect an effect of testosterone on telomeres. Regardless, an important implication of these results is that the effects of testosterone on telomeres accrue across years and such physiological costs would be missed in shorter term experimental manipulations.

There are many nonexclusive pathways through which elevated testosterone could accelerate telomere shortening. Testosterone may increase telomere loss by elevating stress exposure. Previous research in this system has found that males with experimentally elevated testosterone have increased glucocorticoid stress hormones (Ketterson et al., 1991) and a stronger stress response (Schoech et al., 1999). In other organisms, there is evidence that elevated testosterone increases oxidative stress (Alonso-Alvarez et al., 2007, 2009) and that oxidative stress can accelerate telomere loss (Geiger et al., 2012; Reichert & Stier, 2017; Stier et al., 2015; Taff & Freeman-Gallant, 2017), but this is not always the case (Boonekamp et al., 2017).

Elevated testosterone may also affect telomerase, an enzyme that can extend telomere length (Blackburn, 2005). In humans, telomerase is often down-regulated in somatic tissues soon after birth as an anticancer protection mechanism (Blackburn, 2005), but in some bird species it continues to be expressed in some tissues even into old age (Haussmann et al., 2007). Testosterone has also been shown to decrease telomerase expression both in vitro and in vivo (Culig et al., 2002; Meeker et al., 1996; Moehren et al., 2008; Teske et al., 2002), but see (Bar et al., 2015). Interestingly, recent studies in cancer cells suggest that androgens may have direct effects on telomere stability, but whether this is also true in healthy cells is currently unknown (Zhou et al., 2013).

Although the effect of testosterone on telomeres has been little studied, our results are consistent with a correlational study in humans, in which male children and adolescents with higher peak testosterone and a slower testosterone recovery following social stress had shorter telomeres (Drury et al., 2014). However, recent research in yellow-legged gull chicks suggests that these effects may vary across tissues, as exogenous testosterone negatively affected telomeres in the liver, but not in the brain, heart (Parolini et al., 2018) or blood (Parolini et al., 2019). Given that both testosterone and telomeres are conserved mechanisms, a negative relationship between testosterone and telomeres may be widespread and could be indicative of increased biological ageing or form a part of the mechanistic pathway underlying the long-term costs of reproduction in male vertebrates. Future experimental studies will be necessary to determine the mechanisms through which testosterone impacts telomeres, whether these effects vary across life-history stages and tissues, and the degree to which they impact longevity.

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AUTHORS' CONTRIBUTIONS

B.J.H. conceived of the current study, conducted the statistical analysis and drafted the manuscript; S.P.S. located the blood samples; A.E.S. and J.K. conducted DNA extraction and telomere measurements; N.M.G. compiled the long-term database used to locate the blood samples; E.D.K. conceived of the long-term study, oversaw the field research and contributed the blood samples. All authors provided comments that improved the final manuscript and gave approval for publication.

DATA AVAILABILITY STATEMENT

The data file used for the analyses presented in this paper are available on the Dryad Digital Repository (https://doi.org/10.5061/dryad.k3j9kd564).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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