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Developmental stress and telomere dynamics in a genetically polymorphic species

Running title: Telomere dynamics in a polymorphic species

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Abstract

A central objective of evolutionary biology is understanding variation in life-history trajectories and aging rate, or senescence. Senescence can be affected by tradeoffs and behavioral strategies in adults, but may also be affected by developmental stress. Developmental stress can accelerate telomere

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degradation, with long-term longevity and fitness consequences. Little is known regarding whether variation in developmental stress and telomere dynamics contribute to patterns of senescence during adulthood. We investigated this question in the dimorphic white-throated sparrow (*Zonotrichia albicollis*), a species in which adults of the two morphs exhibit established differences in behavioral strategy and patterns of senescence, and also evaluated the relationship between oxidative stress and telomere length. Tan morph females, which exhibit high levels of unassisted parental care, display faster reproductive senescence than white females, and faster actuarial senescence than all of the other morph-sex classes. We hypothesized that high oxidative stress and telomere attrition in tan female nestlings could contribute to this pattern, since tan females are small and potentially at a competitive disadvantage even as nestlings. Nestlings that were smaller than nest mates had higher oxidative stress, and nestlings with high oxidative stress and fast growth rates displayed shorter telomeres. However, we found no consistent morph-sex differences in oxidative stress or telomere length. Results suggest that oxidative stress and fast growth contribute to developmental telomere attrition, with potential ramifications for adults, but that developmental oxidative stress and telomere dynamics do not account for morph-sex differences in senescence during adulthood.

Keywords: Telomeres, developmental stress, genetic polymorphism, disassortative mating, white-throated sparrow

Introduction

Understanding factors that affect individual-level differences in life-history trajectories is a central objective of evolutionary biology. Of particular interest are factors affecting rates of senescence, or the decline in physical and reproductive performance with age (Monaghan, 2008; Ricklefs, 2010; Bouwhuis *et al.*, 2012). Much of life-history theory focuses on tradeoffs affecting adult organisms, most notably the tradeoff between self-maintenance versus reproductive effort (Fisher, 1930; Roff, 1992; Nussey *et al.*, 2009). How different forms of reproductive effort affect life-history trajectories

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and senescence has also generated debate. For instance, does intense competition over mates, as observed under sexual selection, lead to earlier or faster senescence than does investment in parental care (Bonduriansky *et al.*, 2008)?

In addition to dynamics in adults, developmental tradeoffs and environments can also play a role in determining individual differences in life-history trajectories and patterns of senescence. Exposure to developmental stress can have wide-ranging phenotypic effects that persist into adulthood (Metcalfe & Monaghan, 2001; Monaghan, 2008; Spencer & MacDougall-Shackleton, 2011). For instance, developmental stress can reduce growth rates and body size (Searcy *et al.*, 2004; Verhulst *et al.*, 2006), affect cognitive ability (Peters *et al.*, 2014; Bateson *et al.*, 2015a) and metabolic rate (Criscuolo *et al.*, 2008), and alter stress physiology and expression of sexually selected ornaments in adults (Spencer *et al.*, 2003; Spencer *et al*., 2009; Spencer & MacDougall-Shackleton, 2011; Banerjee *et al.*, 2012; Marasco *et al*., 2012).

Stress-related changes in telomere dynamics during development may have particularly strong effects on life history trajectories (Haussmann *et al.*, 2012; Monaghan & Haussmann, 2006; Boonekamp *et al.*, 2014; Monaghan, 2014). Telomeres are conserved terminal repeats of the sequence TTAGGG, in vertebrates, which protect DNA from degradation and distinguish healthy from damaged chromosomes (Blackburn, 1991). Telomere attrition regulates senescence by triggering apoptosis and can lead to genomic instability, disease, and decreases in reproductive performance and survivorship (Haussmann *et al.*, 2005; Monaghan & Haussmann, 2006). Fast growth and cellular division during development can result in rapid telomere attrition (Heidinger *et al.*, 2012), and developing organisms may be sensitive to stress-related increases in telomere degradation due to poorly developed physiological coping mechanisms (Boonekamp *et al.*, 2014; Herborn *et al.*, 2014; Monaghan, 2014). A longitudinal study in zebra finches (*Taeniopygia guttata*) found that post-fledging telomere length more strongly predicted longevity than telomere length later in life, suggesting that developmental telomere dynamics can fundamentally alter life history trajectories and lifetime fitness (Heidinger *et al.*, 2012).

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The mechanisms that influence telomere degradation rates during development remain contentious. Research suggests that stressful environmental conditions during development, such as intense sibling competition (Nettle *et al.*, 2013, 2015), can accelerate telomere degradation, with fitness and longevity consequences (Heidinger *et al.*, 2012). Elevated stress hormone (corticosterone) levels (Haussmann *et al.*, 2012; Quirici *et al.*, 2016) and rapid growth (Geiger *et al.*, 2012; Stier *et al.*, 2015) during development have been linked to increased telomere loss, and might both influence telomere dynamics by increasing oxidative stress (OS) (Geiger *et al.*, 2012). Understanding how OS influences rates of developmental telomere attrition has received particular attention. Telomeres are sensitive to OS in cell culture (von Zglinicki, 2002). However, studies that assess the relationship between OS and telomere length in vivo have yielded mixed results. Some report a positive relationship between OS and telomere degradation rate (Geiger *et al.*, 2012; Stier *et al.*, 2015). For instance, king penguin (*Aptenodytes patagonicus*) chicks that engaged in catch up growth did so at the expense of oxidative status and telomere length (Geiger *et al.*, 2012). On other hand, other studies find no relationship between OS and telomere degradation rate (Boonekamp *et al.*, 2017), calling the relationship between OS and telomere dynamics into question.

Despite interest in the mechanisms and implications of developmental telomere dynamics, little is known regarding whether variation in developmental telomere dynamics is related to differences in life history strategies and senescence during adulthood. We employed a unique model species, the dimorphic white-throated sparrow (*Zonotrichia albicollis*) (Fig. 1), to address this question and evaluate the contended relationship between OS, growth rate and telomere length. White-throated sparrows display a genetically-determined polymorphism in both sexes (Fig. 1). White morph birds are heterozygous for an inversion-based supergene on chromosome 2 (ZAL2^m/2), whereas tan morph birds are homozygous, lacking the rearrangement (ZAL2/2) (Thorneycroft, 1966; Tuttle *et al.*, 2016). White birds are more aggressive, less parental and sing more than tan counterparts, and white males (WMs) are promiscuous (Knapton & Falls, 1983; Kopachena & Falls, 1993; Tuttle, 2003; Zinzow-Kramer *et al.*, 2015). Furthermore, white-throated sparrows pair disassortatively by morph, producing a pair type with biparental care (tan males (TMs) × white females (WFs); T×W), and a pair type with

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female-biased care (WMs × tan females (TFs); W×T) (Knapton & Falls, 1983; Tuttle, 2003). We previously found that TFs, which engage in relatively unassisted parental care, display faster reproductive senescence than WFs (Grunst et al., 2018), and faster actuarial senescence (decreased survivorship with age) than all other morph-sex classes (Grunst et al., *in review*). These results suggest that parental care, rather than intense competition for mates as observed in WMs, is particularly likely to promote senescence. Here we address the contingency that variation in developmental stress and telomere attrition also contributes to morph-sex differences in senescence.

Most published work on morph differences in the white-throated sparrow focuses on adults. Thus, whether nestlings differ in physiology is largely unknown. However, white birds are larger than tan birds even as nestlings (Tuttle *et al.*, 2017). We predicted that the genetic and behavioral divergence between the morphs could also affect OS and telomere dynamics in nestlings. Compared to white counterparts, tan nestlings might suffer more developmental stress via sibling competition, since tan birds are generally smaller and less aggressive (Kopachena & Falls, 1993; Tuttle, 2003; Tuttle *et al.*, 2016). On the other hand, since they attain higher body mass, WM nestlings might suffer accelerated telomere degradation rates during development (Tuttle *et al.*, 2017). Indeed, past studies report that male nestlings display faster telomere degradation than females (Stier *et al.*, 2015). Our study is the first to investigate morph differences in developmental OS and telomere dynamics in a species with genetically-determined morphs that display differences in reproductive strategy and life history. Moreover, the unique disassortative pairing and parental system of the white-throated sparrow provided an opportunity to explore whether parental strategies exert distinct physiological effects on offspring of different morphs.

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Methods

Field methods

We studied white-throated sparrows breeding at Cranberry Lake Biological Station (44°15'N, 74°48'W; Adirondack Mountains, New York). The study site consists of ~32 hectares, with the sparrows favoring forest edges, bogs, and glades. White-throated sparrows at Cranberry Lake have been monitored for over 27 years, and territory boundaries are relatively stable and well delineated. We banded adults with Fish and Wildlife aluminum bands and a combination of three color bands. We morphed adults using visual criteria (Lowther, 1961, Piper & Wiley, 1989, Tuttle, 1993, 2003), and sexed adults based on presence of a brood patch (females) or cloacal protuberance (males).

From early May until early August, 2014 and 2015, we located nests through behavioral observations and systematic search. White-throated sparrows sometimes rear two broods per season and repeatedly re-nest after clutch loss. Thus, for some pairs we collected data from multiple nests per season. We located most nests during the building or laying stages and were thus able to predict hatching dates. We checked nests found during incubation at least every two days so that hatching date was known. We marked the tarsi of nestlings using non-toxic markers, to enable individual identification. From day zero (hatch day) until ~day 6, we measured mass (± 0.25 g) daily. We calculated growth rates using the slope of a linear regression of mass versus nestling age (in days; no evidence of non-linearity). We determined nestling size rank by ordering nestlings within broods from largest to smallest. The largest nestling received a rank of 1 and the smallest a rank equal to the brood size. Size rank may influence the competitive stress experienced by nestlings. To minimize disruption and nestling stress levels, we never removed all nestlings from the nest simultaneously, and took measurements ~10 meters from the nest, and returned nestlings to the nest as quickly as possible. Daily handling could nonetheless have elevated nestling stress levels. However, this is unlikely to have biased our results, since nestlings of both morphs and sexes were treated equivalently.

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We aimed to banded and bleed nestlings on day 6. Some nestlings were banded on day 5, because some nestlings hatched one day later than nest mates. Only data from 5- and 6-day old nestlings were included in models involving telomere length, which is known to erode during development (Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Bateson *et al.*, 2015a, b). We occasionally located nests when nestlings were older than 6 days, in which case nestlings were immediately banded and bled, and also occasionally banded and bled 4-day old nestlings due to time constraints. We included these nestlings in models involving OS, because age had no effect on OS in statistic models (age range 4-10 days, mean ± SE = 5.9 ± 0.04). We collected ~100 µL blood samples immediately before banding using 26-gage needles and two heparinized microcapillary tubes. Blood samples were stored on ice in the field and processed within 5 hours.

Processing samples

Following collection of blood samples, we separated cell fraction from plasma via centrifugation. We expelled erythrocytes from the first microcapillary into Longmire's buffer (Longmire *et al.*, 1992), and stored these samples at 4°C for use in genetic sexing and morphing. We expelled erythrocytes from the second microcapillary into glycerol buffer (50 mM Tris-Cl, 5 mM MgCl, 0.1 mM EDTA, 40% glycerol), and plasma from both microcapillaries into a microvial. We stored erythrocytes in glycerol and plasma samples in liquid nitrogen pending transport to Indiana State University (ISU). At ISU, we stored samples at -20°C until performing telomere length and OS assays, 6-8 months after sample collection.

Genetic sexing and morphing

We extracted DNA from samples in Longmire's buffer using the DNA IQ® magnetic extraction system (Promega Corp; Madison, WI). We determined nestling sex using the P2 and P8 primers to amplify a region of the CHD gene on the W and Z sex chromosomes (Griffiths *et al.*, 1998), and

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nestling morph using a process modified from Michopoulos *et al.* (2007). We then determined the sex and morph composition of broods. Adult sex and morph was also confirmed using genetic techniques.

Telomere length

We extracted DNA from erythrocytes stored in glycerol buffer using the DNeasy Blood and Tissue Kit (Qiagen). We determined telomere length using a relative real-time qPCR assay modified from Criscuolo et al. (2009), which measures telomere length relative to a single copy reference gene. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as our reference gene. We amplified GAPDH using the primers GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3') and GAPDH-R (5'-CCATCAGCAGCAGCCT TCA-3'). These primers are specific to the zebra finch (*Taeniopygia guttata*) (Criscuolo *et al.*, 2009), but have been used in other passerine species (Bize *et al.*, 2009; Barrett *et al.*, 2013; Bádas *et al.*, 2015; Nettle *et al.*, 2013, 2015; Soler *et al.*, 2017).

We amplified telomere sequences using the primers Tel1b (5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'), which amplify telomere sequences across avian species. For both telomeres and GAPDH, we ran 15 µL qPCR reactions containing 7.5 µL of FastStart Essential DNA Green Master (Roche Diagnostic Corporation, Indianapolis, IN), 0.3 µL each of forward and reverse primers (concentration: 10 µM), 3.9 µL of water, and 3.0 µL of DNA (concentration: 1 ng/µL).

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We performed qPCR using a LightCycler®96 System (Roche). We ran telomere and GAPDH qPCR reactions on separate 96-well plates. Telomere thermocycling conditions were: 10 min preincubation at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C. GAPDH conditions were: 10 min preincubation at 95°C, followed by 40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 20 sec at 72°C. We used a ramp speed of 4.4°C/sec, and followed both amplification programs with high resolution melting curve analysis.

Each 96-well plate contained a serial dilution (12 ng, 6 ng, 3 ng, 1.5 ng, 0.75 ng, and 0.375 ng) of DNA from the same reference bird, run in duplicate, which was used to determine and control for the qPCR's amplification efficiency. Amplification efficiency was 79-97% for GAPDH reactions and 71-81% for telomere reactions. Each plate also contained a "golden standard" reference sample, derived from a single individual. We ran all samples in duplicate and in the same position on the GAPDH and telomere plates. Negative controls were included on every plate, and melting curve analysis confirmed amplification of a single product of appropriate length.

In qPCR, the C_T (crossing threshold) is the number of amplification cycles needed for products to exceed a threshold florescent signal. We used the following formula to calculate calibrator-normalized relative telomere length (RTL; amount of telomere sequence relative to GAPDH; T/S ratio):

$$RTL = E_T^{CtT(C)-CtT(S)} * E_R^{CtR(S)-CtR(C)}.$$

E_T is the efficiency of the telomere qPCR reaction (e.g. 88% efficiency = 1.88), CtT(S) is the C_T of each sample, and CtT(C) is the C_T of the calibrator. E_R is the efficiency of the GAPDH qPCR reaction, CtR(S) is the C_T of each sample, and CtR(C) is the C_T of the calibrator (Pfaffl, 2001).

The inter-plate coefficient of variation for telomere length, calculated by running the calibrator sample on each plate, was 4.66%. Intra-assay coefficients of variation for RTL, calculated from duplicate samples run on each plate, averaged 7.17%.

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OS assays

We measured total antioxidant capacity (TAC) in plasma using the OXY-adsorbent kit (Diacron International, distributed by Cedar Creek Laboratories in the United States). We measured reactive oxygen metabolites (ROMs) in plasma using Diacron International's d-ROMs kit (Costantini & Dell'Omo, 2006; Costantini *et al.*, 2007). We performed assays using an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA), capable of temperature control.

For both the OXY-adsorbent and d-ROMs assays, we followed the manufacturer's protocol, but reduced plasma volume from 5 to 2 μL. For the OXY-absorbent assay, we diluted plasma 1:100 with distilled water. We then generated a standard curve of solutions capable of neutralizing 0, 175, 350, and 700 mM of hypochloric acid (HOCl), a generic antioxidant. We added 200 μL of HOCl and 5 μL of plasma or standard to a 96-well microplate, and performed a pre-read to control for variation in sample absorbance. After a 5-min incubation at 37 °C, we added 2 μL of chromogenic solution and read absorbance at 490 nm. We report results in terms of mM HOCl neutralized.

For the d-ROMs assay, we generated a standard curve consisting of 0, 0.94, 1.88, 3.76 and 7.52 mM H_2O_2 (peroxide, the most common reactive oxygen metabolite). We then added 200 μL of buffer solution and 2 μL of plasma to a microplate, and performed a pre-read to control for variation in plasma absorbance. Following the pre-read, we added 2 μL of chromogenic solution. We incubated the plate for 45 min at 37 °C, after which absorbance was read at 490 nm. We report results in mM H_2O_2 equivalents.

For both assays, the standard curve and all samples were run in duplicate. Intra-assay coefficients of variation averaged 11.7% for the OXY-adsorbent assay and 13.1% for the d-ROMs assay. We calculated OS as: mM ROMs/mM HOCl neutralized (TAC) x 1,000 (Costantini *et al*., 2007).

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Statistical analyses

We performed statistics in R 3.1.2 (R Core Team, 2014). We used linear mixed effect models (R packages lme4; Bates *et al*., 2012) to analyze differences in OS and RTL, with nest, father, and mother identity as random effects. As fixed effects, we entered nestling morph-sex (e.g. TM vs. WF), the morph composition of the parental pair, brood size, clutch initiation date, time of blood sampling (excluded in the telomere model), year, growth rate, size rank and age. OS level was additionally included as a fixed effect in the model predicting RTL.

To assess whether rearing conditions affected the morph-sex classes differently, we tested two-way interactions, with nestling type interacting with parental pair composition, brood size, clutch initiation date, year, and size rank. Testing overly complex global models can lead to problems with overfitting, inaccurate parameter estimates, model convergence, and interpretability (Harrison et al. 2018). Moreover, robustly testing interactions involving four-level (nestling type) or two-level (sex, morph) factors is especially difficult, and requires a relatively large sample size, even for one such interaction (Harrison et al. 2018). Thus, to avoid the problems discussed above, we tested each interaction separately, by including all main effects in the global model but only one interaction at a time.

We performed an additional model to assess the effect of brood composition on both OS and RTL. In this model, we used the morph and sex composition of the brood (proportion male and proportion white morph). We included an interaction with nestling morph-sex class in the initial model and year and time of sampling as covariates.

We sequentially reduced models by eliminating the predictor variable with the highest p-value first. When morph-sex type was non-significant, this factor was simplified to morph and sex, entered separately, to further assess the potential effects of these variables. In final models, all predictor

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variables were significant at $\alpha = 0.05$. *P*-values were derived by using Sattherwaite approximations to estimate degrees of freedom (R package lmerTest; Kuznetsova *et al.*, 2013). To normalize residuals for the model involving OS, we natural-log transformed OS and eliminated four outlying data points.

Results:

Oxidative stress

Nestling OS (mM ROMs/mM TAC ×1000) averaged 3.91 ± 0.13 (range: 0.57-15.77; $N = 244$). TAC averaged 288.47 ± 4.44 mM (range: 111.80-499.3 mM; $N = 248$), and ROMs averaged 1.09 ± 0.03 mM (range: 0.18-3.20 mM; $N = 248$).

Nestlings had lower OS in 2015 than in 2014, and higher OS later in the day (Table 1). In addition, OS was higher in nestlings that were smaller than others relative to nest mates (in nestlings with higher size ranks) (Table 1, Fig. 2). Nestling size rank tends to vary with morph-sex class, with WMs less likely to place low in the hierarchy than TFs (Poisson GLMM: $\beta = -0.31 \pm 0.13$, $z = -2.32$, $P = 0.020$). Thus, nestling morph-sex class could affect OS via an effect on size rank. However, we found no consistent evidence that nestling morph-sex affects OS. The relationship between nestling morph and OS differed between years (significant morph × year interaction; Table 1). In 2014 white nestlings tended to have lower OS than tan nestlings (LMM: $\beta = -0.12 \pm 0.07$, $t_{114} = -1.76$, $P = 0.087$). In 2015 this relationship reversed, with white nestlings tending to display higher OS ($\beta = 0.16 \pm 0.09$, $t_{117} = 1.76$, $P = 0.080$).

Across years, OS did not vary with nestling morph-sex type ($F_{3, 211} = 0.07$, $P = 0.973$), being 3.94 ± 0.25 in WMs, 4.04 ± 0.27 in TMs, 3.89 ± 0.27 in WFs, and 4.00± 0.27 in TFs (Fig. 3a). Moreover, when data were combined across years the morphs had virtually identical OS ($\beta = 0.01 \pm 0.05$, t_{230} =

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0.246, P = 0.805), with white nestlings having levels of 3.91 ± 0.18, and tan nestlings displaying levels of 3.91 ± 0.17. The two sexes also displayed similar OS (β = 0.03 ± 0.05, t_{213} = 0.51, P = 0.608), with males displaying levels of 3.98 ± 0.18, and females displaying levels of 3.92 ± 0.19.

The other predictors examined were also unrelated to nestling OS. Nestling OS was unrelated to the morph composition of the parental pair (β = -0.04 ± 0.06, t_{76} = -0.69, P = 0.487), growth rate (β = -0.11 ± 0.15, t_{161} = -0.73, P = 0.461), brood size (LMM: β = -0.02 ± 0.03, t_{131} = -0.57, P = 0.566), nest date (β = 0.002 ± 0.001, t_{85} = 1.42, P = 0.157), or nestling age (β = -0.02 ± 0.03, t_{86} = -0.76, P = 0.452). Furthermore, these variables did not interact with nestling sex or morph to predict OS (P > 0.06 for interaction terms). Brood composition, quantified by the proportions of male and white nestlings, was also unrelated to OS (P > 0.10 for main effects and interactions). P-values for non-significant predictors are reported from models containing only variables that were retained in the final model and the non-significant predictor of interest.

Telomere length

RTL in nestlings ranged from 0.334 to 2.37 with a mean ± SE of 1.137 ± 0.028. Nestlings had shorter telomeres in 2015 (mean ± SE = 0.974 ± 0.034, N = 113) than in 2014 (mean ± SE = 1.311 ± 0.039, N = 105). When accounting for this year effect on RTL, nestlings with higher growth rates and higher OS had shorter telomeres (Table 2; Fig. 4a, b). Removing one outlining data point (OS = 15.77; Fig. 4a) did not qualitatively change results of this model (β = -0.02 ± 0.01, t_{156} = -2.44, P = 0.036; effect of OS with this data point removed).

We found no evidence for an effect of nestling ($F_{3, 160}$ = 0.11, P = 0.951) or parental (β = -0.12 ± 0.08, t_{56} = -1.46, P = 0.150) morph-sex type on nestling RTL. RTL of nestlings averaged 1.154 ± 0.058 for WMs, 1.108 ± 0.049 for TMs, 1.169 ± 0.058 for WFs, and 1.169 ± 0.070 for TFs (Fig. 3b). RTL was similar between nestlings from broods produced by the two pair types, being 1.153 ± 0.040 for nestlings of W×T pairs, and 1.124 ± 0.040 for nestlings of T×W pairs.

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RTL was unrelated to brood size (β = -0.03 ± 0.03, t_{66} = -0.978, P = 0.331) and nest date (β = 0.001 ± 0.001, t_{43} = 0.546, P = 0.587), which could both affect environmental stress. Size rank was not related to RTL (β = 0.03 ± 0.02, t_{178} = 1.27, P = 0.204), despite the association between size rank and OS. However, nestlings that were smaller than brood mates also exhibited slow growth rates (β = -0.10 ± 0.01, t_{244} = -10.76, P < 0.001), potentially explaining absence of high telomere attrition in these individuals. Finally, the morph-sex composition of broods was also unrelated to RTL (P > 0.05 for all main effects and interaction terms).

Discussion

A primary objective of our study was to assess whether morph-sex differences in OS or telomere length occur in nestling white-throated sparrows, and have the potential to explain different patterns of senescence during adulthood. Apart from our investigation of morph differences, our study also contributes to the current debate regarding the relationship between OS and telomere dynamics (Monaghan, 2014; Boonekamp *et al.*, 2017). Results provide no evidence that nestlings of different morphs or sexes differ in developmental stress levels or telomere length. Lack of morph-sex differences in nestling OS and telomere lengths suggest that the morph-sex differences in reproductive and actuarial senescence observed in white-throated sparrows are not linked to developmental telomere dynamics.

On the other hand, results support a role for both OS and growth rate in determining rates of developmental telomere degradation. Individuals with higher OS and faster growth rates had shorter telomeres at day 5 to 6 of the nestling stage. OS clearly leads to telomere attrition in cell cultures, but whether OS leads to telomere degradation in vivo is the subject of more debate (Boonekamp *et al.*, 2017). A few past studies report a relationship between accelerated growth, OS and telomere length, in accordance with our results (Geiger *et al.*, 2012; Bádas *et al.*, 2015; Stier *et al.*, 2015). However, others find no relationship between OS and telomere length or attrition (Reichert *et al.*, 2014; Nettle *et al.*, 2015, 2017; Boonekamp *et al.*, 2017; Young *et al.*, 2017). Boonekamp *et al.* (2017) failed to find

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a relationship between multiple markers of OS and telomere attrition in jackdaw (*Corvus monedula*) nestlings, and suggested that cell proliferation rates during development may be more important to determining rates of telomere loss than OS. However, our results suggest a role for both variables in determining nestling telomere lengths. Studies that do not concurrently measure both growth rate and OS could potentially fail to find an effect of either variable. However, in our dataset, the relationship between OS and telomere length remained significant even when excluding growth rate from the model ($P = 0.025$). Of course, our results are correlational, and cannot prove a causal relationship between OS and telomere length, which would require experimental manipulation.

Results also suggest that the competitive environment within broods can contribute to levels of developmental stress, and particularly to OS. Specifically, we found that nestlings that were smaller than nest mates had higher OS. The finding that relatively small nestlings had higher OS is consistent with past research reporting higher stress in nestlings at the bottom of brood hierarchies (Martínez-Padilla *et al.*, 2004; Merkling *et al.*, 2014; Stier *et al.*, 2014, 2015). On the other hand, unlike some studies (Nettle *et al.*, 2013, 2015; Stier *et al.*, 2015), we did not find that nestlings that were smaller than brood-mates suffered higher telomere attrition. Given our finding that fast growth was associated with shorter telomeres, one explanation for this null result is that higher OS in smaller nestlings was accompanied by slower growth, depressing overall rates of telomere loss. Indeed, nestlings low in the competitive hierarchy displayed slow growth rates. The sex and morph composition of broods did not affect OS, suggesting that the morph-sex identity of nestlings does not strongly influence competitive dynamics.

Although TF nestlings tend to be smaller than nest mates, whereas WM nestlings tend to be larger, we also found no evidence for morph-sex differences in nestling OS or telomere length. Some studies have found sex differences in telomere dynamics during development. For instance, in male great tit (*Parus major*) nestlings suffer faster telomere degradation than females, potentially because males attain higher mass and are under more OS (Stier *et al.*, 2015). Higher testosterone in males could contribute to faster telomere degradation, although the above study found no sex difference in testosterone levels (Stier *et al.*, 2015). However, consistent with our results, many studies also fail to

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find sex differences in telomere dynamics (Barrett & Richardson, 2011; Herborn *et al.*, 2014; Reichert *et al.*, 2014, 2015).

These results suggest that neither developmental telomere dynamics nor OS during development can explain morph-sex differences in patterns of reproductive and actuarial senescence in adult white-throated sparrows. Rather, as we propose elsewhere, differences in the reproductive strategies and pairing dynamics may explain morph-sex differences in senescence (Grunst *et al.*, 2018). Specifically, unassisted parental care might elevate stress and induce faster reproductive and actuarial senescence in TFs. As a caveat, we only measured telomere length near the end of the developmental period, not telomere degradation rate. Some research suggests that the rate of telomere attrition is more reflective of developmental stress and more predictive of longevity than nestling telomere length alone (Boonekamp *et al.*, 2014). However, others have found that the rate of telomere attrition and telomere length in nestlings are strongly correlated, and can both predict fitness and behavior in adults (Heidinger *et al.*, 2012; Bateson *et al.*, 2015b).

We also found no evidence that the morph-sex composition of the parental pair affects nestling OS or telomere length. The pair types tend to exhibit unique parental systems, with T×W pairs exhibiting biparental care and W×T pairs exhibiting female-biased care (Tuttle, 2003; Tuttle *et al.*, 2016). However, results suggest that nestlings from the two different pair types do not experience different developmental stress levels, perhaps because overall provisioning rates do not consistently differ between pair types.

Finally, we found year effects on both OS and telomere length, suggesting cohort effects. Other studies have also found significant cohort effects on these variables (Watson *et al.*, 2015). Thus, cohort effects could play a substantial role in determining levels of developmental stress and telomere length, and shaping future life history trajectories.

In conclusion, our study supports the hypothesis that OS and growth rates (levels of cell proliferation) concurrently affect telomere attrition during development. On the other hand, results fail to support our prediction that genetically determined morphs differ in developmental OS levels

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and telomere dynamics. Thus, results suggest no role for developmental telomere dynamics in explaining morph-sex differences in life histories, and particularly aging rates. However, absence of morph-sex effects does not preclude a role for developmental telomere dynamics in influencing individual life histories, independent of morph-sex identity. OS and telomere loss in developing nestlings may contribute to the fitness and longevity of adults within each morph-sex class. Establishing a link between telomere attrition during development and adult fitness and longevity will require longitudinal studies that track individuals into adulthood.

Acknowledgements

TBD

Data accessibility

Data available on the *Dryad Digital Repository*.

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Tables

Table 1. LMM predicting nestling OS from size rank, morph and year.

	$\beta \pm$ SE	Df	t	$P > \mid t \mid$
Intercept	1.09 ± 0.16	111.76	6.81	< 0.001
Size rank	0.05 ± 0.02	217.52	2.08	0.038
Time	0.02 ± 0.01	73.52	2.02	0.047
Nestling morph*	-0.11 ± 0.08	229.49	-1.42	0.156
Year (factor)†	-0.54 ± 0.08	128.09	-6.73	< 0.001
Morph × year	0.26 ± 0.11	233.97	2.26	0.024
Random effects	Variance	SD	N	
Nest ID	0.0006	0.02	84	
Father ID	< 0.0001	< 0.0001	51	
Mother ID	< 0.0001	< 0.0001	53	
Residual	0.201	0.449	240	

*White contrasted to tan morph.

†2015 contrasted to 2014.

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Table 2. LMM predicting RTL from OS and growth rate.

	$\beta \pm$ SE	Df	T	$P > \lvert t \rvert$
Intercept	1.96 ± 0.20	170.20	9.63	< 0.001
OS	-0.02 ± 0.01	156.50	-2.44	0.015
Growth rate (g/day)	-0.20 ± 0.08	177.17	-2.50	0.013
Year (factor)*	-0.35 ± 0.06	60.69	-5.27	< 0.001
Random effects	Variance	SD	N	
Nest ID	0.02	0.14	76	
Father ID	0.05	0.23	46	
Mother ID	<0.001	<0.001	46	
Residual	0.06	0.25	196	

*2015 contrasted to 2014.

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Figures

Figure 1.

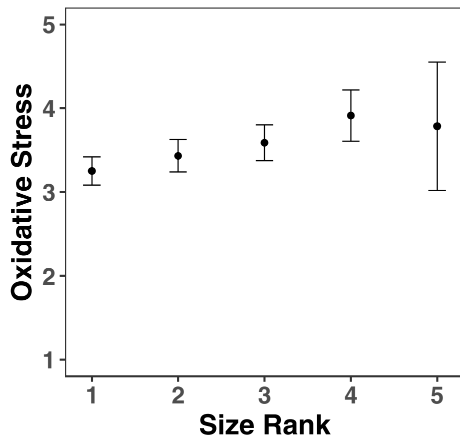

Figure 2.

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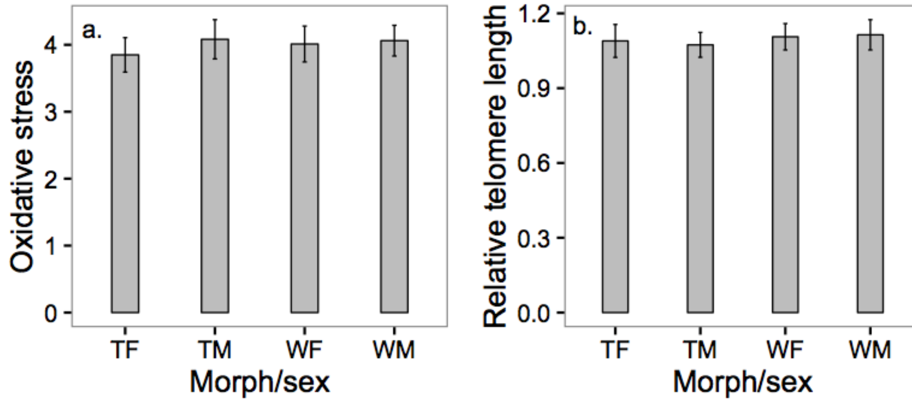

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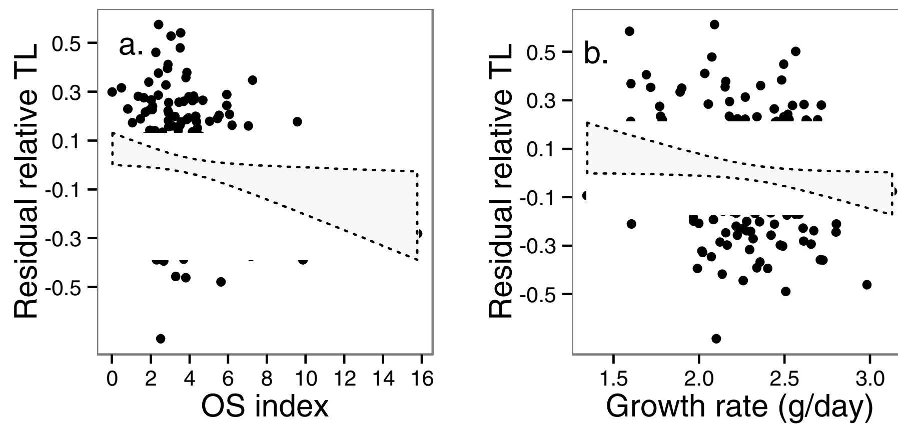

Figure 4.

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Figure legends

Fig. **1**. The white morph of the white-throated sparrow (left) is visually distinguished from the tan morph (right) by brighter crown plumage coloration, and genetically distinguished by an inversion-based supergene on chromosome 2.

Fig. 2. Relationship between size rank and oxidative stress (OS) in nestling white-throated sparrows. Nestlings with larger size ranks were smaller than nest mates, and had higher OS levels. Points are back-transformed least square mean values extracted using the model in Table 1, and error bars represent standard error.

Fig. **3**. Bar graphs showing no relationship between OS (a) or telomere length (b) and morph-sex class in nestling white-throated sparrows. Bars represent means and error bars represent standard error.

Fig. **4**. The relationship between OS (a), growth rate (b) and RTL. RTL is represented as residuals from the mixed model shown in Table 2, excluding the focal variable of interest (i.e. OS for plot a. and growth rate for plot b.). Shaded regions represent 95% confidence intervals.

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