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HIGHLIGHTED STUDENT RESEARCH

Brood size is associated with apparent telomere lengthening in nestling barn swallows

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Abstract

Early life for animals is often a time of rapid growth and development. In a resource-limited environment, life history theory predicts that there must be trade-offs between resource sinks in ways that optimize future survival and reproductive success. Telomeres have emerged as putative indicators of these early life trade-offs, but there are conflicting accounts as to how developmental traits and conditions impact telomere length and dynamics. For 2 years, we studied the nestlings of a breeding population of barn swallows from day 6 to day 12 of life, measuring various ontogenetic factors to understand to what extent they explain variation in telomere length and dynamics. We unexpectedly found that telomeres lengthened between the two sampling points. Nestlings in large broods had shorter telomeres, but surprisingly, individuals that grew faster from day 6 to day 12 had longer telomeres and more telomere lengthening. Nestlings with higher mass relative to their nestmates on d6 had shorter telomeres, suggesting that the relatively fast growth barn swallows experience early in development is more costly than the relatively slower growth later in development. These effects were only found in the first year of study. Telomere lengthening may be due to the initiation of new hematopoietic cell lines during development or the expression of telomerase early in life. Favorable early life conditions and high parental investment could allow for more growth with little to no cost to telomere length or dynamics.

Keywords Early life · Hematopoiesis · Relative size · Sibling competition · Telomere lengthening

Introduction

Life history theory dictates that an organism's allocation of finite resources is optimized to maximize reproductive success and survival. Optimization toward one strategy must result in siphoning resources from other strategies, resulting in a trade-off (Stearns 1976). Trade-offs that occur early in an individual's life can have far reaching consequences on fitness and lifespan (e.g., Felitti et al. 1998; Monaghan 2010; Eastwood et al. 2019), and there is evidence that telomeres may indicate the long-term costs of developmental trade-offs. Telomeres are protective structures composed of repeating TTAGGG nucleotide sequences on the ends of eukaryotic chromosomes that prevent damage to coding regions of DNA. Telomere length changes over the life of an organism, and this change is a balance of shortening and lengthening processes (Monaghan et al. 2010). Furthermore, telomere length can change substantially early in life (e.g., Hall et al. 2004; Haussmann and Mauck 2008; Ballen et al. 2012; Cram et al. 2017) and seems to have long-term consequences for survival (Eastwood et al. 2019), reproductive

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This undergraduate-led study is a comprehensive, 2-year investigation of relative telomere length and dynamics in nestling barn swallows. The strengths of this work include the large number of nestlings sampled almost entirely from one breeding population, which allows us to fully leverage a substantial number of predictor variables using a linear mixed modeling approach. The most novel finding is the identification of substantial telomere lengthening during this period of nestling development, and we suggest a potential explanation of new hematopoietic cell line initiation during development that, to our knowledge, has not been previously explored in this context.

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success (Parolini et al. 2017; Eastwood et al. 2019), and lifespan (Heidinger et al. 2012). Telomere length has been linked to developmental conditions such as brood size (Nettle et al. 2016), parasite load (Asghar et al. 2015), body size (Ringsby et al. 2015), weather (e.g., Pérez et al. 2016; Sauve et al. 2021), parental care (Brown et al. 2021), and growth rate (Salmón et al. 2021), making telomere measures potentially useful as a proxy for individual state during and after early life development (Monaghan 2010). While many factors have been found to be related to telomere length, the relative importance of each factor in determining early life telomere length and dynamics requires further clarification.

Telomere length changes over the life of an organism, and this change is a balance of shortening and lengthening processes (Monaghan et al. 2010). Shortening can result from two processes: a gradual reduction in telomere length from cellular replication, and oxidative damage caused by an abundance of free radicals in the cellular environment (von Zglinicki 2002; Reichert and Stier 2017; Armstrong and Boonekamp 2023). To prevent oxidative telomere shortening, organisms produce or consume antioxidants that can neutralize oxidative free radicals (e.g., Badás et al. 2015; Pérez-Rodríguez et al. 2019). Furthermore, the protein telomerase can actively lengthen telomeres, serving as a mechanism to maintain and restore telomere length; however, telomerase expression is typically restricted to germline and stem cells (Jafri et al. 2016). Telomerase expression in the bone marrow (the site of blood cell production) of passerine birds is relatively high in the nestling phase, but typically drops off by fledging and remains low for the rest of the organism's life (Haussmann et al. 2007). The limits on telomerase expression combined with the damage that telomeres consistently accrue typically result in the shortening of telomeres over an organism's lifetime.

For most organisms, early life development is characterized by the energetically demanding processes of extensive growth and tissue maturation. Oxidative damage appears to be the cause of the majority of telomere shortening (Haussmann and Marchetto 2010), and damage may be further exacerbated by the increased metabolic demands of early life growth. Therefore, both increased size (e.g., Ringsby et al. 2015) and growth rate (e.g., Grunst et al. 2019; Salmón et al. 2021) are associated with greater telomere shortening in nestling birds. However, there is also evidence that larger body size (Nettle et al. 2016) and increased growth rates (e.g., Voillemot et al. 2012; Costanzo et al. 2017; Vedder et al. 2017; Wolf and Rosvall 2021) are unrelated to—or are positively associated with—less telomere shortening in certain conditions. These differences suggest that growing in favorable conditions can be less costly in terms of telomere shortening, but there can also be conditions where energy dedicated toward growth comes at the cost of telomere maintenance (Nettle et al. 2016; Vedder et al. 2017).

Brood size may be an important factor predicting early life telomere length and dynamics, as larger broods can have increased sibling competition, which can result in reduced per-individual resources and increased social stress. Larger brood sizes can lead to reduced telomere length by the end of development because there might be less energy available to allocate toward telomere maintenance processes such as telomerase expression or endogenous antioxidant synthesis (e.g., Boonekamp et al. 2014; Young et al. 2017; Gil et al. 2019). Sibling competition can also increase baseline circulating glucocorticoid levels (Quirici et al. 2016; Gil et al. 2019), which can increase the generation of reactive oxygen species and decrease the production and efficacy of antioxidant defenses, resulting in increased oxidative damage to telomeres (Haussmann and Marchetto 2010). Experimental brood size reductions and enlargements have provided causational evidence for the negative consequences of larger broods on telomere length (e.g., Voillemot et al. 2012; Boonekamp et al. 2014; Young et al. 2017; Gil et al. 2019), but the relative importance of resource availability or social stress in determining telomere length and dynamics remains less clear.

Telomere length may also be determined by an interaction between brood size and body size, where the relative mass of an individual with respect to its nestmates has implications for telomere length. Nettle et al. (2013, 2015) suggest that position within the brood size hierarchy can affect telomeres differently than just brood size alone. Having more relatively heavy nestmates can lead to shorter telomeres in the relatively lighter individuals, potentially due to increased social stress and competition for resources (Nettle et al. 2013, 2015, 2016). However, there is also evidence that being a relatively heavy individual in a small brood results in shortened telomeres (Nettle et al. 2016), suggesting that there may be a cost to growing larger than nestmates that may not be ameliorated by a competitive advantage.

While some aspects of telomere dynamics and their molecular causes are well documented in the literature, the relative contribution of the ontogenetic factors that modulate telomere length and dynamics is less clearly understood. We collected data on several ontogenetic variables (brood size, nestling size, growth rate, developing plumage color, ectoparasite intensity, corticosterone (CORT) level) that may affect telomere length and dynamics and then assessed the relative influence of each in explaining variation in barn swallow (*Hirundo rustica erythrogaster*) nestling telomere length and dynamics. This 2-year study consisted of an initial year of data collection with no manipulation followed by a year with a brood-size reduction in a subset of nests aimed at increasing the total number of small broods in the dataset. In the second year, we measured CORT levels to determine the relationship between telomere measures, brood size, and CORT.

We predicted that brood size would explain at least some of the variation in telomere length, with individuals in smaller broods having longer telomeres and experiencing less telomere shortening due to increased resource availability and decreased sibling competition. We predicted that growth rate would be negatively related to telomere length in small broods where being relatively large does not necessarily confer a competitive advantage, but positively related to telomere length in large broods where being a larger competitor might lead to better resource acquisition (Caro et al. 2016). Additionally, we expected that individuals in larger broods would have higher circulating CORT levels, due to the potential for sibling competition to increase CORT. We included measures of nestling plumage color and intensity of ectoparasitic fowl mites *Ornithonyssus sylviarum,* which parasitize nestlings, because both measures provide potentially important information about nestling and parental phenotype and the developmental environment. High-quality (e.g., dark ventral plumage) adult males had fewer nest mites in their nests and were more likely to settle in a territory containing a nest that had mites removed via heat than paler males (Hund et al. 2021). Additionally, parental care behaviors differed depending on whether the nest contained mites (Hund et al. 2015). Finally, Hubbard et al. (2015) demonstrated that nestling plumage color is heritable, and that nestling ventral plumage color is predictive of their adult color. Given this, we would predict that nestlings raised in nests with fewer nest mites and nestlings with darker developing plumage would have longer telomeres and/or less attrition.

Methods

Study site and species

We studied Barn Swallows (*Hirundo rustica erythrogaster*) in Knox County, Ohio (40.3756° N, 82.3971° W) during the breeding season (May to August) in 2020 and 2021. Barn swallows typically breed in colonies, building their nests in the rafters of barns or other structures. Approximately 50 pairs breeding in one large colony were studied each year, with a few small broods (16 individuals from 7 broods with ≤ 3 nestlings) included in 2021 from other sites within 5 miles of the focal colony. Individual nests contained anywhere from one to seven nestlings, and we focused our study on first and replacement (laid following predation) broods from May to July in both years.

Field methods

Nests were checked for eggs every 2 to 3 days, and 14 days after the penultimate egg was laid, nests were checked daily to confirm the hatch date. We returned to each nest 6 (d6) and 12 (d12) days after the hatch date and briefly removed the nestlings from the nest, counting the number of nestlings in each nest to determine brood size. On d6, we uniquely clipped a toenail on each nestling, allowing us to differentiate between individuals on d12. We measured wing length, tarsus length, mass (precision 0.1 g), and counted ectoparasitic fowl mites on each nestling. We collected approximately 20 µL of blood from the brachial vein. We returned on d12 and placed a USGS aluminum band on the leg of all nestlings. In addition to repeating all previous measurements, we plucked a cluster of developing ventral feathers from each nestling. These feathers were taped on a white notecard in the field and stored in a dark container until analyzed. In 2021, we took approximately 50 µL of blood from d12 nestlings to have a sufficient amount to quantify CORT levels. Because blood samples were taken > 5 min from the time nestlings were removed from the nest, they are more likely to represent stress-induced measures. Blood was suspended in Longmire's lysis buffer and refrigerated at 4 ℃ until DNA extraction took place no more than 2 months after sampling. DNA extracted from Longmire's lysis buffer performs well in tests of telomere measure repeatability (e.g., Eastwood et al. 2017). In 2021, a separate aliquot of whole blood was stored on ice until plasma separation. Plasma was stored at – 80 ℃ until analysis.

In 2021, we reduced 12 clutches of 4 ($n = 5$) or 5 ($n = 7$) eggs to 3 eggs midway through incubation to balance the sample sizes of broods and increase statistical power to detect brood size effects on telomere length in these smaller broods. In addition to these 12 reduced nests, we had 9 unmanipulated nests with 3 eggs. Eggs were removed randomly with no knowledge of lay order, and used for an unrelated study. Eggs from these nests and others were removed briefly to photograph for yet another study, and we have no indication that handling eggs disrupts incubation behavior in these birds. No females abandoned their nests after egg collection or handling. All other nests remained unmanipulated, and all manipulations occurred at the same site used in both years. There were 178 individuals sampled in 2020 from 44 nests and 207 individuals were sampled in 2021 from 54 nests (sample sizes by brood size used in final analyses in Table S1). Seventeen individuals in 2020 and eleven individuals in 2021 died or were depredated before d12, for mortality rates of 9.55% in 2020 and 5.05% in 2021. Natural brood reduction was rare, occurring only in three nests each year, where 1–2 nestlings in broods of 4–5 died between d6 and d12. Brood size used in our analysis was determined by the number of nestlings on d6.

Plumage color quantification

Ventral feather color (brightness, hue, and chroma) of breast feathers of d12 nestlings was quantified using an

Ocean Insight Flame spectrometer (Dunedin, FL, USA) and a pulsed xenon light (PX-2, Ocean Insight) or a deuterium–tungsten-halogen light (DH-MINI, Ocean Optics) (Jenkins et al. 2013; Levin et al. 2018). Reflectance measurements were standardized against both a white and a dark background and were recorded using the OceanView software (v2.0 Ocean Insight). Three measurements were recorded per feather cluster, and average brightness, hue, and chroma values were generated using the R package *Pavo* (Maia et al. 2013). Hue values from 2020 were not reliable because of deuterium interference in the spectra, and therefore not included in the 2020 analyses.

DNA extraction

DNA was extracted from 200 µL of lysis buffer containing blood using a Qiagen DNEasy Blood and Tissue kit (Qiagen, MD USA). DNA was eluted twice in 50 µL of elution buffer each time for a total of 100 µL of concentrated DNA stored at – 20 ℃ until analyses. The purity and concentration of DNA were measured using a Take3 Micro-Volume Plate in an Epoch 2 microplate reader (BioTek, VT, USA), with 260/280 > 1.75 as a cut-off for inclusion in analyses.

Nestling sex determination

Previously verified primers targeting the sex chromosomes of male and female birds P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') were used to determine nestling sex (Griffiths et al. 1998). PCR was performed in a BioRad T100 thermal cycler (Hercules, CA) using One*Taq* Quick-Load 2 × Master Mix (New England BioLabs, MA, USA). The PCR cycle times and temperatures were as follows: initial denaturation at 94 °C for 1 min; followed by 35 cycles of 94 °C for 45 s, 48 °C for 45 s, and 68 °C for 45 s; followed by a final extension at 68 °C for 5 min. PCR products were run on a 1.5% agarose gel and females, being the heterogametic sex, were identified by the presence of two bands.

Quantification of relative telomere length

We quantified relative telomere length as a ratio of telomeric DNA to the single copy, non-telomeric gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using qPCR. While there are interstitial telomeres located in interior regions of chromosomes, previous research found no evidence for long interstitial sequences in barn swallows (Parolini et al. 2015). Short sequences should not affect relative telomere quantification, as they are likely to be ubiquitous but relatively constant in the species. Furthermore, previous studies have found high correlations between absolute measures (e.g., telomere restriction fragment method) of telomere length and estimates obtained in those same individuals by qPCR (Cawthon et al. 2009; Criscuolo et al. 2009; Parolini et al 2015). Telomere primers were telg (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTAGTGT-3') and telc (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3') and GAPDH primers were GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3') and GAPDH-R (5'-CCATCAGCAGCAGCCTTCA-192 3') diluted to final concentrations of 0.25 µM for telc/telg and 0.05 µM for GAPDH. Individuals (duplicate) as well as standards (triplicate) were run in the same positions in 96-well plates for telomere and GAPDH quantification (USA Scientific, FL, USA) and an individual's d6 and d12 samples were always run on the same plate. Individuals from the same nest were almost always run on the same plate. We analyzed a total of 23 plates (plus 23 for GAPDH), and samples were analyzed at similar intervals from sampling in both years (within 2–6 months). Standards consisted of the same set of pooled d12 nestlings ($n = 10$) that were diluted once and split into separate aliquots, sealed and frozen. We used an ABI 7500 Real-Time qPCR System (ThermoFisher, MA, USA) and SsoAdvanced Universal SYBR Green Supermix reagents (BioRad, CA, USA). Total reaction volume was 20µL, and the final concentration of DNA per reaction was 0.125 ng/µL. PCR conditions for telc/telg were: 95 °C for 15 min, followed by two cycles of 94 °C for 15 s and 49 °C for 15 s, and 30 cycles of 94 °C for 15 s, 62 °C for 10 s, and 74 °C for 15 s with signal acquisition. The program ended with a melt curve run from 62 to 95 °C with 0.3 °C increments each for 5 s to check for non-specific amplification and primer-dimer artifacts. GAPDH PCR conditions were as follows: 95 °C for 15 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s with signal acquisition. The program ended with the same melt curve described above. In both reactions, the number of PCR cycles (C_t) necessary to accumulate a sufficient fluorescent signal to cross a threshold was measured; individuals with relatively short telomeres are characterized by more cycles to cross that threshold. In addition to the 40 samples on each plate, we also ran 5 standards at concentrations 10 ng, 5 ng, 2.5 ng, 1.25 ng, 0.625 ng, each run in triplicate. Any sample that had a C_t standard deviation of > 0.5 for the duplicate set was reanalyzed.

Relative telomere length was expressed as the ratio of telomere to single copy (T/S) amplicon. The T/S ratio was calculated using this formula: $2^{\Delta\Delta Ct}$, $\Delta\Delta C_t = (C_t^{\text{Telomere}} — C_t^{\text{GAPDH}})$ reference—$(C_t^{\text{Telomere}} — C_t^{\text{GAPDH}})$ focal sample (Cawthon 2002). The reference was the 2.5 ng standard, or the "golden sample", which was a dilution of ten pooled barn swallow nestlings run on all plates. The average qPCR efficiencies were 99.76% for GAPDH and 91.21% for telc/telg. The intra-assay repeatability for T/S was $R = 0.996$ (± 0.0003). Our golden sample (the pooled set of individuals

we set to T/S = 1) was our only individual run on all plates, which prevents the calculation of an inter-assay repeatability for T/S; however, using the C_t values instead, we find an $R = 0.943$ (± 0.024) and $R = 0.948$ (± 0.0232) for telc/telg and GAPDH, respectively.

Corticosterone quantification

We used a corticosterone ELISA (Enzo Life Sciences, NY, USA) to quantify whether CORT on d12 co-varied with brood size, telomere length, and change in length, or both in 2021 nestlings. Because a larger blood sample is required to quantify CORT and telomere length, we only included this measure from d12 nestlings. Whole blood was centrifuged at 2000 × g for 10 min to separate plasma from red blood cells (RBCs). The plasma was removed from the packed RBCs using a Hamilton syringe (Hamilton Company, NV, USA) and frozen at -80℃ until the assay was performed. The ELISA protocol followed the manufacturer guidelines (ADI-900–097, Enzo Life Sciences, NY, USA) with modifications for barn swallows used by Jenkins et al. (2013). In brief, plasma was diluted to a 1:40 concentration, a standard curve of six duplicate concentrations (20,000, 4,000, 800, 160, 32, 6.4 pg/mL) was run on each plate alongside samples, which were run in duplicate. Individuals from the same nest were run on the same plate. The average inter-assay CV was 8.73% and the intra-assay CV was 7.35%. CORT concentrations were related to the order in which individuals were sampled (LMM: $\beta = 2.01 \pm 0.64$, $p = 0.002$), with chicks sampled later having higher CORT.

Statistical analysis

We assessed which fixed effects predicted telomere length and dynamics using linear mixed models (LMMs) in R (R Core Team 2021). We used the formula (d12 measure-d6 measure)/6 to assess average growth rate and average daily mass gain, which were based on structural tarsus length ad body mass, respectively. In addition to assessing the absolute mass of each individual, we calculated z-scores of nestling mass, standardizing the weight of individual nestlings to the average weight of their nestmates as done in Nettle et al. (2016), which allows assessment of the location of individuals within the nest size hierarchy. We found that this relative mass measure was correlated ($r > 0.70$) with other measures of mass and size (Tables S2; S3). Therefore, in models that included relative mass, we did not include other measures of nestling morphology. Nest mite intensity, feather color (brightness and hue), sex, and hatch date were included as covariates.

We used LMMs for predicting telomere length and dynamics between d6 (early) and d12 (late) in development. Change in telomere length was not correlated with d6 measures (Fig. S1C, D). We constructed a set of biologically relevant global models (Table S4) for each response variable and used the *lme4* (Bates et al. 2015) and *MuMIn* (Barton 2020) R packages to generate model sets using every combination of the predictors which we evaluated using an information theoretic approach. Late-state measures such as mass, mite intensity, and feather color were never included in models of early relative telomere length. Models contained a maximum of 11 predictors, below the conservative n/k estimate proposed by Harrison et al. (2018) to avoid overfitting. Models were run separately by year due to a detectable year effect (LMM; $\beta = -0.06 \pm 0.03$, $p = 0.05$), and CORT and feather hue were only available in 2021. Julian hatch date was included in all global models as a covariate and nest ID was included as a random effect to account for the lack of independence between nestmates. Interaction terms were included for the relationship between mass and brood size, as well as relative mass and brood size, as the size of brood may change the effect that mass has on telomere length (e.g., Nettle et al. 2016). Brood sizes of one individual ($n = 5$) were omitted from analyses due to low sample size. After the omission of a small number of individuals with telomere length measures identified as outliers by a Grubb's test ($n = 4$) or individuals missing a measurement in at least one category ($n = 47$), sample sizes were 152 nestlings from 40 nests in 2020 and 177 nestlings from 47 nests in 2021 (Table S1).

Models were assessed via AICc and models with a delta AICc < 2 were included in the top model sets. Statistical significance of each fixed effect was evaluated using the R package *lmerTest* (Kuznetsova et al. 2017). We used conditional model averaging when we had multiple top models, where coefficients are averaged only across models where they appear. Residuals were checked for adherence to normality and homoscedasticity.

Results

Descriptive statistics and ontogenetic trends

Nestmates typically had similar relative telomere lengths; the random effect of nest identity accounted for approximately 53.49% and 39.05% of the variation in telomere length and 6.62% and 0% of the variation in telomere dynamics in 2020 and 2021, respectively. The average nestling mass on d12 was equivalent in 2020 (20.17 ± 1.39 g) and 2021 (20.18 ± 1.5 g), and body mass at d12 declined with increasing brood size (Tables S5 Fig. S2A, B). Average daily mass gain only marginally declined with increasing brood size in 2020 ($\beta = -0.04 \pm 0.02$, $p = 0.07$) and was unrelated to brood size in 2021 ($\beta = -0.02 \pm 0.03$, $p = 0.51$; Figure S2C, D). Relative mass did not decline with increasing brood size,

indicating that the size hierarchy of individuals was similar across brood sizes despite average mass varying substantially (Table S5). Corticosterone levels were unrelated to brood size ($\beta = -0.02 \pm 1.02$, $p = 0.98$). Nest mites were highly variable between nests in both years (2020: range = 0–330. 2021: range = 0–533). There was no difference in telomere length ($\beta_{2020} = -0.05 \pm 0.08$, $p = 0.50$; $\beta_{2021} = -0.04 \pm 0.05$, $p = 0.41$) or dynamics ($\beta_{2020} = -0.02 \pm 0.05$, $p = 0.74$; $\beta_{2021} = 0.006 \pm 0.04$, $p = 0.874$) between the sexes.

Relative telomere length and dynamics

Relative telomere length measures on d6 and d12 were correlated within individuals in 2020 ($r = 0.86$) and 2021 ($r = 0.78$) (Fig. S2), and relative telomere length on d12 was more variable in 2020 (T/S = 1.03 ± 0.64) than in 2021 (T/S = 0.75 ± 0.42) (Fig. 1; Levene's test, $p = 0.002$). On average, relative telomere length increased from d6 to d12 in both years of study (one sample t test, $t_{2020} = 4.62$, $df_{2020} = 152$, $p_{2020} < 0.0001$, $n = 153$; $t_{2021} = 3.03$, $df_{2021} = 181$, $p_{2021} = 0.003$, $n = 182$; Fig. 1). The increase was more substantial in 2020 (an average 13.7% increase) than in 2021 (8.1% increase) (two samples t test, $t = 2.23$, $df = 217.34$, $p = 0.03$, $n = 364$; Fig. 1).

Predictors of telomere length and dynamics: 2020

Brood size, average daily mass gain, growth rate, and d6 relative mass were common predictors in the top models explaining telomere length and dynamics in 2020 (Table 1, S6). Individuals in smaller broods had longer telomeres on both d6 and d12 while also experiencing more telomere lengthening (Fig. 2A, C, E). Nestlings that had a larger average daily mass gain between the two sampling periods had more telomere lengthening (Table 1). While absolute mass was not a predictor of telomere length or dynamics, individuals that were heavy relative to their siblings (i.e., grew faster than their nest mates prior to d6) had increased telomere shortening (Table 1). Growth rate was retained in some top model sets, but did not provide additional explanation for variation in d12 relative telomere length and dynamics. Hatch date, nest mite intensity, feather brightness and chroma, and sex did not predict telomere length or dynamics in 2020. No interaction terms were retained in the top set of models. Full top model sets can be found in Table S7. Note that in some cases (d6 length, change in length), the null model appears in the top model sets.

Predictors of telomere length and dynamics: 2021

Models with telomere dynamics as a response variable were run with and without the reduced broods, and we found no change in the top model set. Contrary to findings from 2020, 6-day old nestlings in small broods had shorter telomeres in 2021; however, the null model was included within the top model set (Table 2, Tables S6, S7, Fig. 2B). Brood size was not related to relative telomere length on d12 or telomere dynamics (Table 2, Fig. 2D, E). For d12 relative

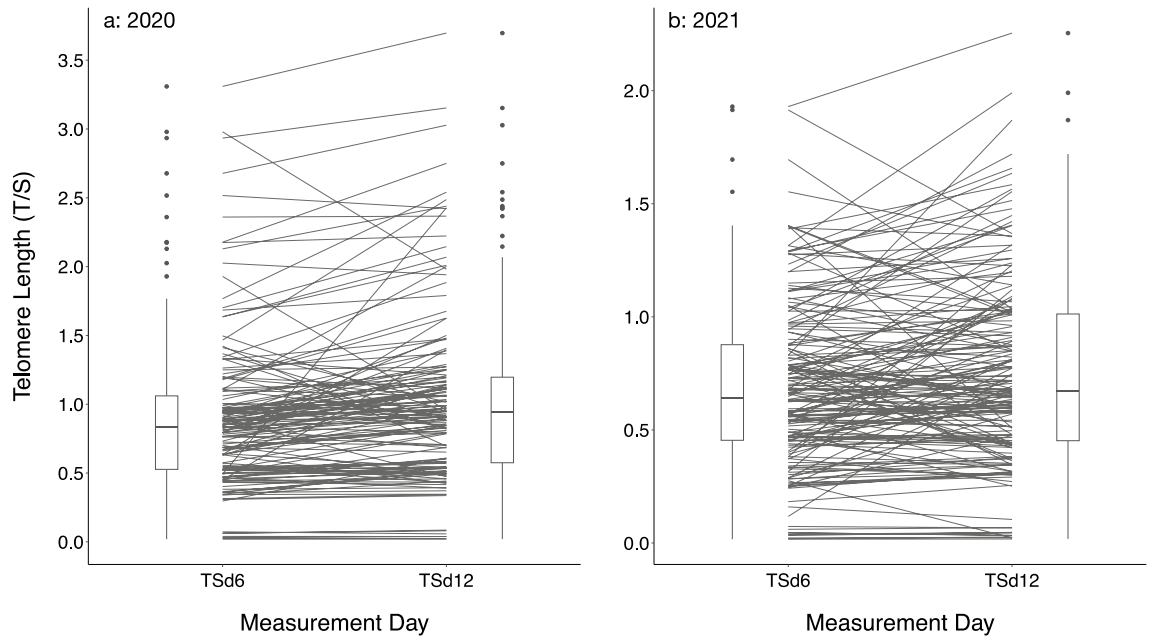

Fig. 1 Changes in nestling barn swallow telomere length from day 6 to day 12 post-hatching in 2020 (**a**) and 2021 (**b**). Telomere lengthening occurred in both years, but was more pronounced in 2020 than in 2021 (t test, $t = 2.23$, $df = 217.34$, $p = 0.03$, $n = 364$). Boxplots show median values, with the boundaries of the boxes indicating quartiles and the points illustrating outliers (1.5 × IQR)

Table 1 Model-averaged predictors of relative telomere length and dynamics of nestling barn swallows in 2020

Response variable	Model-averaged predictors	β	SE	*p*
Day 6 telomere length	Intercept	1.41	0.49	0.001
	Brood size	**− 0.18**	**0.07**	**0.009**
Day 12 telomere length	Intercept	2.16	0.32	< 0.001
	Brood size	**− 0.25**	**0.072**	**0.001**
	Growth rate	0.0006	0.55	0.99
Change in telomere length	Intercept	0.03	0.31	0.92
	Average daily mass gain	**0.26**	**0.11**	**0.018**
	Brood size	**− 0.08**	**0.03**	**0.006**
	Growth rate	− 0.24	0.35	0.51
Change in telomere length*	Intercept	0.14	0.3	0.65
	Daily mass gain	**0.26**	**0.11**	**0.02**
	Brood size	**− 0.08**	**0.03**	**0.004**
	Relative mass d6	**− 0.08**	**0.03**	**0.005**
	Growth rate	− 0.24	0.35	0.51

Coefficients were calculated using conditional averaging of linear mixed models that had a delta AICc < 2. The fixed effects shown are the only predictors that were retained in top model set for each response variable, Table S4 lists all the predictors included in the models. Growth rate is based on the change in tarsus length, while average daily mass gain is used as an alternative way to look at change in size between sampling points. Bolding indicates significant model-averaged predictors. Sample size is 153 nestlings

*Model utilized relative mass measurements rather than absolute mass measurements

telomere length and dynamics, no combination of predictors was an improvement over the null model containing the random effect of nest ID. Hatch date, growth rate, average daily mass gain, nest mite intensity, sex, feather brightness, hue, and chroma were not predictors of telomere length or dynamics in 2021. Additionally, CORT did not predict telomere length or dynamics regardless of whether sampling order, which did explain some variation in CORT measures, was accounted for in the model. No interaction terms were retained in the top models. Full top model sets can be found in Table S7.

Discussion

We measured a range of ontogenetic traits of barn swallow nestlings during development to determine the relative impact of these measures on telomere length and dynamics. We discovered that nestling relative telomere length increased on average from d6 to d12 in both years. We also found that brood size predicted telomere length differently in each year and that average daily mass gain and relative mass were predictors of telomere length in 2020, when greater variation in telomere length was found. This inter-annual variation suggests that the factors determining telomere length may be more dependent on environmental conditions (e.g., van Lieshout et al. 2021).

We found an unexpected pattern of lengthening in our measures of nestling telomeres sampled from erythrocytes. Cases of telomere lengthening within individuals have been reported (e.g., Spurgin et al. 2017; Wolf et al. 2021; van Lieshout et al. 2021); however, to our knowledge, only one other study found widespread lengthening, also in nestling barn swallows (Parolini et al. 2015). Instances of lengthening occur in studies using both telomere restriction fragment analysis (Pauliny et al. 2012) and qPCR (Parolini et al. 2015; Spurgin et al. 2017; Wolf et al. 2021; van Lieshout et al. 2021) measurement techniques. Lengthening could simply be due to measurement error; however, we have evidence of high precision of telomere length measurements in this study. One potential explanation for the observed telomere lengthening involves the hematopoietic source of RBCs in embryonic and nestling development which changes throughout the developmental timeline. Red blood cells in chicken (*Gallus gallus domesticus*) first originate from blood islands located extra-embryonically, but quickly transition to the spleen, bursa, then finally the bone marrow (Elsaid et al. 2020). As new hematopoietic cell lines initiate production of RBCs throughout early development, telomeres could appear to lengthen if new cell lines have longer telomeres. Telomerase may also be partly responsible for the lengthening; telomerase expression is relatively high early in development to compensate for the damage done by reactive oxygen species generated by metabolism (Haussmann et al. 2007; Noguera and Velando 2021). Slowing growth rates (like those seen in barn swallows approaching d12 (Stoner 1935; Morales Fernaz et al. 2012)) coupled with continued telomerase expression would then cause telomeres to be lengthened past the length observed in the initial measurement. Blood volume increases associated with mass gains

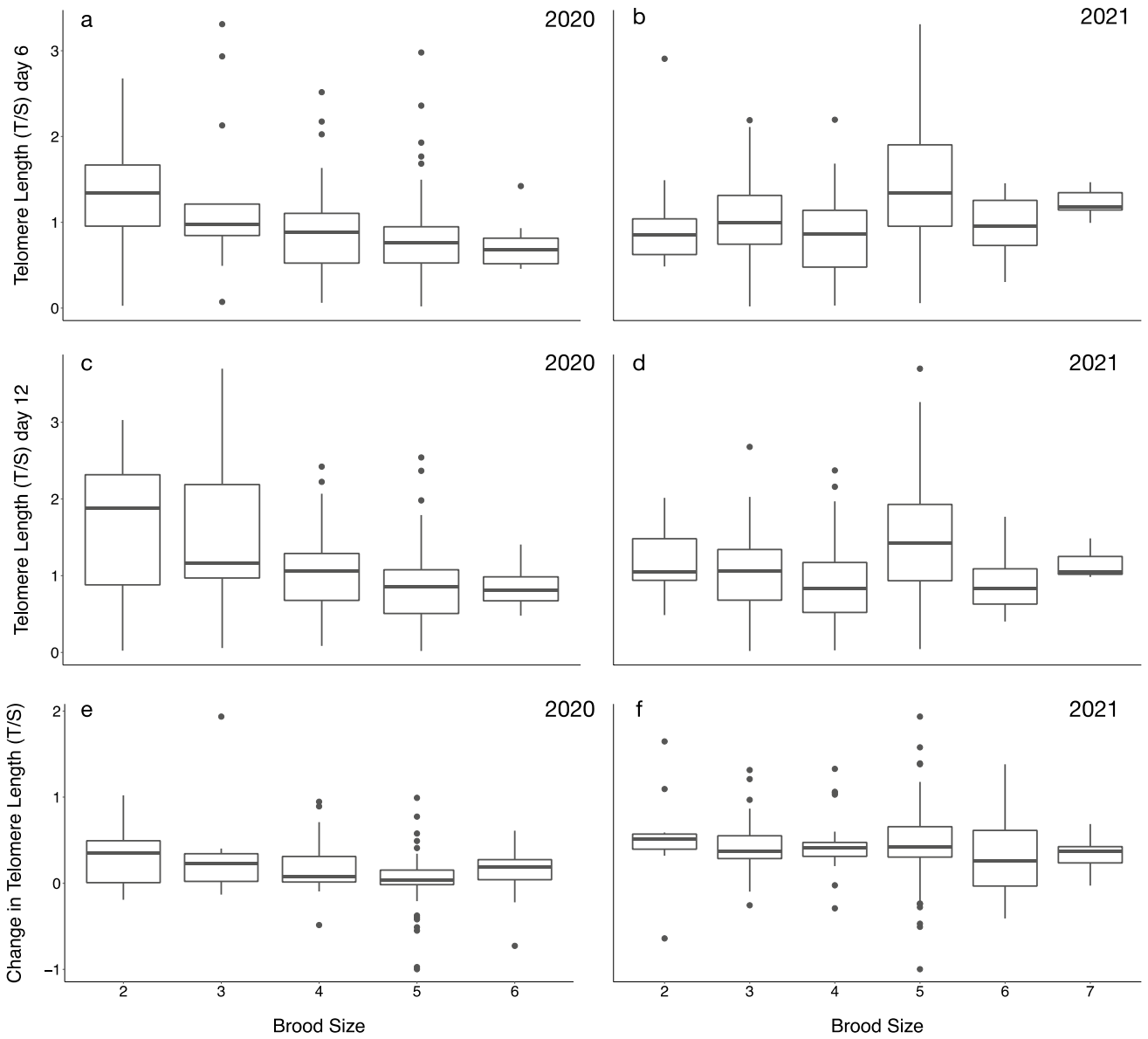

Fig. 2 The effect of brood size on day 6 telomere length (**a**, **c**), day 12 telomere length (**b**, **d**), and dynamics (**e**, **f**) of nestling barn swallows in 2020 (**a**, **c**, **e**) and 2021 (**b**, **d**, **f**). Brood size was negatively related to telomere dynamics in 2020, but positively related in 2021. Boxplots show median values, with the boundaries of the boxes indicating quartiles and the points illustrating outliers (1.5×IQR). Sample sizes by brood in 2020 were 2 ($n=11$), 3 ($n=14$), 4 ($n=36$), 5 ($n=80$), 6 ($n=12$) and in 2021 were 2 ($n=12$), 3 ($n=41$), 4 ($n=30$), 5 ($n=81$), 6 ($n=11$), 7 ($n=7$)

(Newell and Schaffner 1950) might also contribute to the apparent telomere lengthening. Populations of RBCs expand substantially during the periods of rapid growth in chickens (Prinzinger et al. 2015), pigeons (Gayathri et al. 2004), and storm petrels (Kostelecka-Myrcha and Myrcha 1989). Passerine RBCs have an average lifespan of approximately 17–21 days (Bauchinger and McWilliams 2009), and new RBCs—from potentially different hematopoietic sources—could increase the average telomere length in the population of erythrocytes. Because we sampled all individuals twice, we cannot quantify whether blood sampling on d6 affected our results due to an increase in blood volume beyond what is typical during development.

Shorter telomeres in individuals reared in larger broods are consistent with most previous research (e.g., Boonekamp et al. 2014; Cram et al. 2017; Costanzo et al. 2017). Individuals with shorter telomeres may live shorter lives and have reduced fitness (Heidinger et al. 2012; Eastwood et al. 2019). This relationship may partly explain why the most common brood size in our populations is five, but the largest broods we observe are six to seven individuals. The cliff-edge hypothesis suggests that the average brood size will

Table 2 Model-averaged predictors of relative telomere length and dynamics of nestling barn swallows in 2021

Response variable	Model-averaged predictors	β	SE	*p*
Day 6 telomere length	Intercept	0.59	0.13	< 0.001
	Brood size	**0.07**	**0.03**	**0.02**
Day 12 telomere length	Intercept	0.72	0.08	< 0.001
Change in telomere length	Intercept	0.06	0.02	0.003

Coefficients were calculated using conditional averaging of linear mixed models that had a delta AICc < 2. The fixed effects shown are the only predictors that were retained in top model set for each response variable, Table S4 lists all the predictors included in the models. Growth rate is based on the change in tarsus length, while average daily mass gain is used as an alternative way to look at change in size between sampling points. Bolding indicates significant model-averaged predictors. Sample size is 182 nestlings

be less than the maximum due to asymmetrically poor survivorship in large broods (Mountford et al. 1968; Morris et al. 1992). However, we found no negative relationship between telomere length and brood size in 2021, driven by the longest telomeres in broods of five individuals. It may be that the impact of brood size on nestling telomere length and dynamics depends on the quality and physiological state of the parents and how much energy they can dedicate to nestling provisioning (Morris et al. 1996; Bauch et al. 2013; Heidinger et al. 2016; Brown et al. 2021). Under favorable environmental conditions, parents can invest in larger brood sizes such that there is little to no cost to long-term somatic maintenance, whereas trade-offs may occur in more environmentally challenging years. Warmer days (with maximum temperatures above 18℃) and lower precipitation has consistently been related to increased nestling survival in aerial insectivores like barn swallows (Weegman et al. 2017; Cox et al. 2020; Garrett et al. 2022), and favorable environmental conditions in 2021 could have eliminated the stratification in telomere length that occurs between brood sizes when breeding conditions are more challenging. We observed a 50% reduction in mortality from 2020 to 2021, and favorable environmental conditions in 2021 could explain why broods of five nestlings included individuals with the longest telomeres in 2021.

CORT levels were unrelated to brood size or relative telomere length, which is in contrast to our prediction that larger broods would have higher CORT levels and relatively shorter telomeres, a pattern found in other systems (Pegan et al. 2019; Powolny et al. 2020). However, other studies have concluded that CORT is unrelated to telomere length (e.g., Ouyang et al. 2016; Gil et al. 2019), suggesting that the relationship between CORT and telomere length is not straightforward. It is possible that the favorable environmental conditions in 2021 mitigated any negative effects of higher CORT on nestling telomere length. Our CORT measures must be cautiously interpreted, as we did detect an effect of sampling order on CORT levels; however, accounting for sampling order did not qualitatively change model outputs. Nestlings who had a longer latency until sampling (based on sampling order) tended to have higher CORT, as expected due to stress of handling and removal from the nest.

In 2020, individuals that were relatively large compared to their nestmates on d6 had more telomere shortening from d6 to d12, but nestlings that grew more from d6 to d12 experienced more telomere lengthening. Barn swallows grow two and a half times faster from hatching to d6 than from d6 to d12 (Stoner 1935), and from this we might infer that the relatively fast growth barn swallows experience early in development (pre-d6) is more costly than the relatively slower growth later in development (between d6 and d12). Relatively fast growth rates are often associated with more telomere shortening (e.g., Hall et al. 2004; Herborn et al. 2014; Noguera et al. 2015; Stier et al. 2015; Salmón et al. 2021) but there is also evidence for longer telomeres in individuals that grow faster (Monaghan and Ozanne 2018; Wolf and Rosvall 2021). It may be that the possible competitive advantage associated with rapidly growing to a relatively large size early in development requires a trade-off with telomere maintenance mechanisms. With this interpretation, telomeres would shorten because telomere maintenance cannot keep pace with the extensive damage of peak growth. When growth slows down, telomere length increases because maintenance would outpace damage, at least in high-quality individuals that have sufficient resources to grow well in the first place. Alternatively, it is feasible that as new hematopoietic tissue is activated throughout development (Orkin and Zon 2008), these new cell lines begin to produce RBCs that have long telomeres, increasing the average telomere length of the sample. Depending on the time point that telomeres are measured, rapid growth may either shorten the telomeres of an older hematopoietic line (Friedrich et al. 2001) or stimulate the initiation of a new line sooner, resulting in shorter or longer observed telomeres depending on when sampling occurs. Measures of telomere length at multiple, different time points would help clarify how hematopoietic patterns during development might explain changes in telomere length.

Generally, the lack of consistent relationships between brood size, growth, and telomere length indicates that telomere length and dynamics may be at least partially contingent on the developmental context of the individual. Large sample size studies in natural populations are imperative to determine the generalizability of studies that have focused on captive organisms or smaller scale experiments. Many studies where brood size is manipulated lack large numbers of control nests that span the natural range of brood sizes,

which can obscure the complex nature of these relationships or overestimate the effect of brood size on relative telomere length. Furthermore, we need more longitudinal work to determine the impacts of environmental variability on telomere length and dynamics. Our finding of consistent telomere lengthening suggests that when studying even short-term telomere change, different populations of erythrocytes within an individual organism are potentially being studied at each time point. Further research into the timing of RBC expansion and the impact of telomerase expression on early life telomere dynamics will aid in understanding how the timing of telomere measurement(s) may impact findings and interpretations. We hope researchers continue to report telomere lengthening when it is found and avoid terms such as "less shortening" where instances of lengthening occur.

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Author contributions IL, TT, and CV conceived and designed the study. IL, TT, YL, and CV collected the samples. CV, IL, YL, and KA conducted the associated lab work, and CV analyzed the data. CV and IL wrote the manuscript with input from other authors.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Analyses were done in R, and code is available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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