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SHORT COMMUNICATION

Embryonic growth rate affects telomere attrition: an experiment in a wild bird

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ABSTRACT

High growth rate is associated with a short lifespan, but the physiological basis for this trade-off is not well known. Telomere length predicts individual lifespan and in this study we investigated whether embryonic growth rate, manipulated using incubation temperature, affects erythrocyte telomere length in a wild bird species, the common tern (*Sterna hirundo*). A 1°C lower incubation temperature decreased growth rate by 5%, without affecting size at hatching. The slower growth was associated with an average telomere length that was 147 base pairs longer at hatching. If carried through to adulthood, this effect would correspond with an approximately 3 year longer lifespan. Our results thus suggest that an effect of growth rate on lifespan may be mediated by telomere dynamics or a physiological process reflected by telomere length.

KEY WORDS: Ageing, Embryonic development, Cell division, Life-history trade-off, Lifespan, Senescence

INTRODUCTION

A trade-off between individual growth rate and lifespan is a fundamental prediction from life-history theory, and found across taxa (Metcalfe and Monaghan, 2003; Ricklefs, 2006), but the underlying mechanism of this trade-off is poorly known. Telomeres are DNA–protein structures at chromosome ends that shorten with age. At the cellular level, telomeres maintain chromosome stability (Blackburn, 1991), and, at the organismal level, telomere length predicts individual variation in lifespan across taxa (Heidinger et al., 2012; Boonekamp et al., 2013; Bauch et al., 2014; Wilbourn et al., 2018). Telomeres shorten with cell division, and generally shorten fastest during organismal growth (Rufer et al., 1999; Baerlocher et al., 2007; Boonekamp et al., 2014; Vedder et al., 2017a). Whether the effect of growth on telomere attrition only reflects the number of cell divisions or whether faster growth is additionally associated with a higher rate of telomere loss per cell division is currently unknown (Monaghan and Ozanne, 2018). This, however, is of interest because, in the latter case, telomere dynamics may explain the observation that high growth rates are associated with a short lifespan.

Oviparous animals are ideal to test the effect of growth rate on telomere dynamics, because eggs allow embryonic growth to be manipulated independent of the mother and nutrient availability. In this study, we manipulated embryonic growth rate by varying the incubation temperature of common tern eggs, and hypothesized that slower growth would lead to an increased telomere length at hatching.

MATERIALS AND METHODS

Study species and experimental protocol

The common tern, *Sterna hirundo* Linnaeus 1758, is a relatively long-lived bird species, whose natural telomere dynamics are well documented (Bauch et al., 2013, 2014; Vedder et al., 2017a). Common terns have a clutch of 1–3 eggs per breeding attempt; eggs are laid with 1–2 day intervals and hatch in the order of laying, and chicks are semi-precocial (Becker and Ludwigs, 2004). In 2015, we studied a natural common tern breeding colony at a raft of about 4×8 m within the city of Wilhelmshaven at the German North Sea coast (53°36′N, 08°06′E). The colony was visited on a daily basis, and all newly laid eggs of all 53 clutches produced in the colony were collected and numbered according to clutch identity and laying order within the clutch. Collected eggs were replaced with model eggs, roughly similar in size and colour, which were readily accepted by the parents. All collected eggs were weighed (to the nearest 0.1 g with a digital balance) and placed in an incubator within 2 h of collection. We used fresh egg mass as an indicator of egg size, which is highly correlated with egg volume in common terns (r^2=0.94; Vedder et al., 2017b).

For all clutches, the first egg was randomly assigned to incubation at 36.5 or 37.5°C. If present, the second egg of the clutch was always incubated at the alternative temperature (see details below). Detailed knowledge of the natural or optimal incubation temperature in common terns is not available, but Bosveld et al. (1995) report a hatching success of 95% (n=115) of common tern eggs with artificial incubation at 37.5°C, and we therefore adopted this temperature for the baseline treatment. The 1°C lower incubation temperature for the alternative treatment was chosen with the aim of delaying growth while trying to avoid an effect on hatching success. If present, the third egg of the clutch was incubated at 37.5°C, but as these were not assigned randomly to an incubation temperature, they were not included in further analyses.

Detailed incubation procedure

We used three digital forced-air incubators (Rcom max 50, Autoelex Co. Ltd, Gimhae-si, South Korea). These incubators can be programmed with a resolution of 0.1°C and 1% relative humidity, and maintain temperature and humidity within a range of ±0.1°C and ±1%, respectively. One incubator was programmed with a temperature of 37.5°C and 50% relative humidity. A second, completely identical, incubator was programmed with a temperature of 36.5°C and 50% relative humidity. The 1.0°C lower incubation temperature and the accuracy of the incubators were confirmed with a ‘check-up’ incubator thermometer (Brinsea Products Inc., Titusville, FL, USA) prior to the experiment, and the two

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incubators were positioned adjacent to each other in the same room such that there was no difference in atmospheric pressure. In both incubators, eggs were automatically turned approximately 90 deg around their axis every hour and we manually and randomly shuffled the position of the eggs inside each incubator every day to prevent bias due to potential temperature gradients within incubators. After 18 days of incubation, all eggs were transferred to a third incubator, set at the intermediate temperature of 37.0°C and 60% relative humidity, without egg turning. In this incubator, eggs were placed in individual compartments, constructed using wire mesh, to ensure that a hatched chick could be linked to its egg. A digital camera was programmed to take a picture of the content of the hatching incubator every half hour, allowing the determination of total incubation time with a 0.5 h resolution. At hatching, chicks were weighed (to the nearest 0.1 g with a digital balance), blood sampled (approximately 25 µl) and kept within the incubator until they were returned, within a day, to a nest in the colony (see Vedder et al., 2017b, for more details).

Telomere length measurements

All blood samples were stored in 2% EDTA buffer at 3–7°C until transferred into a 40% glycerol buffer within 2 weeks from sampling and snap-frozen for storage at −80°C. From the blood sample, average erythrocyte telomere length was measured by in-gel terminal restriction fragment (TRF) analysis (following Vedder et al., 2017a), which excludes interstitial telomeric sequences. The glycerol buffer was removed and the blood cells were washed with 2% EDTA. DNA was isolated from erythrocytes (7 µl cells) using a CHEF Genomic DNA plug kit (Bio-Rad, Hercules, CA, USA) and subsequently digested simultaneously with *Hin*dIII (60 U), *Msp*I (60 U) and *Hin*fI (30 U) in NEB2 buffer (New England Biolabs, Inc., Beverly, MA, USA) at 37°C for ca. 18 h. One-third of the DNA from every sample was added in a 0.8% non-denaturing agarose gel (Pulsed Field Certified Agarose, Bio-Rad) and separated by pulsed field electrophoresis for 22 h at 14°C (3 V cm^{-1}, initial switch time 0.5 s, final switch time 7.0 s). ^{32}P-labelled size ladders [one lane per gel: 1 kb DNA ladder (range 1–10 kb), New England Biolabs, Inc.; two lanes per gel: DNA Molecular Weight Marker XV (range 2–48 kb), Roche Diagnostics, Basel, Switzerland] were added for size calibration on every gel, as was a standard sample (blood of a single chicken) as a control. Gels were dried with a gel dryer (model 538, Bio-Rad) and hybridized overnight at 37°C with a ^{32}P-labelled oligonucleotide ($5'$-C_3TA_2-$3'$)$_4$ that binds to the telomeric single-strand overhang. Subsequent washing of the gel with 0.25× SSC buffer at 37°C removed unbound oligonucleotides. The gel was then exposed to a phosphor screen for 24 h (MS, PerkinElmer, Inc., Waltham, MA, USA) to detect the radioactive signal, which was visualized by a phosphor imager (Cyclone™ Storage Phosphor System, PerkinElmer, Inc.). Telomere length distributions were quantified using Image J (version 1.38x, open source) and the average was calculated for every sample (in base pairs). The lower limit of the telomere length distribution was lane-specifically set at the point with the lowest signal. At the upper end of the distribution, we set a fixed limit at 30 kb because the background noise in the region of the longest telomeres was more variable. This selected range was previously shown to represent the telomere distribution with the highest intra-individual repeatability for average telomere length in common terns (Bauch et al., 2013). The background value was subtracted lane specifically from the optical density measurements.

To assess the intra-blood sample repeatability of average erythrocyte telomere length, we extracted DNA from 17 samples twice, one sample three times, and one sample four times. These separate DNA extractions were randomly distributed over four gels, and repeatability (intra-class correlation coefficient) of telomere length was tested with blood sample identity (all from different hatchlings) as the single random effect in a mixed model with average telomere length as the dependent variable. Repeatability was 78%, but when correcting for between-gel variation, by adding the average telomere length of the standard chicken sample run on all gels as a covariate (coefficient±s.e.=0.59±0.14, χ^2_1=18.04, P<0.001), the repeatability increased to 86%. Hence, although the coefficient of variation (CV) of the standard sample based on all gels used for this study was low (CV=2.8%, n=8), we opted to further minimize the effect of between-gel variation by always running DNA extractions from siblings from the first two eggs (always of different incubation treatment) in adjacent lanes on the same gel. In case clutch size was one, or one of the first two eggs of a clutch did not hatch, samples of different treatment were randomly distributed over gels, and we therefore added the value of the standard sample as a covariate to the model to correct for between-gel differences.

Statistical analyses

For testing effects of incubation treatment on the normally distributed response variables (fresh egg mass, incubation time, hatchling mass and telomere length), we used linear mixed models with a normal error distribution that included ‘clutch identity’ as a random effect. For telomere length, we added the value of a standard sample, run on all gels, as a covariate to the model, to correct for between-gel differences (see above). In a follow-up analysis, we verified that our results were not confounded by potential effects of laying order and egg size occurring despite the randomization of treatments. Differences in hatching success between incubation treatments were tested using a generalized linear mixed model with a binomial error distribution and a logit link function, and clutch identity as a random effect. All models were run in MLwiN 2.02 (Rasbash et al., 2004) and significance (P<0.05, two-tailed) was assessed using the Wald statistic.

RESULTS AND DISCUSSION

The assignment of incubation treatment was unbiased with respect to fresh egg mass (χ^2_1=0.14, P=0.71). In total, we incubated 96 first and second eggs of 53 clutches, of which 83 (86.5%) hatched. Hatching success was not significantly influenced by incubation temperature (36.5°C: 83.0%, n=47; 37.5°C: 89.8%, n=49; χ^2_1=0.94, P=0.33), nor was mass at hatching (36.5°C: mean±s.e.m.=14.3±0.17 g; 37.5°C: 14.2±0.19 g; χ^2_1=0.08, P=0.77).

The average incubation periods for both treatments were well within the natural range of the species (21.1–23.4 days; Murk et al., 1996). However, embryos developing at the lower incubation temperature hatched 1.1±0.1 days (mean±s.e.m.) later than those developing at the baseline incubation temperature (Fig. 1A; χ^2_1=119.5, P<0.001). In accordance with the hypothesized negative effect of growth rate on telomere length, the telomeres of embryos developing at the lower temperature were on average 147±63 base pairs (bp) longer at hatching (Fig. 1B, Table 1). Clutch identity explained 76% of variance in telomere length (χ^2_1=16.6, P<0.001), indicating that telomere length of siblings was strongly positively correlated. The significant effect of incubation treatment on telomere length persisted when the analysis was restricted to clutches where both siblings with alternative treatments hatched (Fig. 2; χ^2_1=3.98, P=0.046; n=32 clutches).

Although the assignment to experimental treatment was unbiased with respect to laying order and fresh egg mass, these variables may

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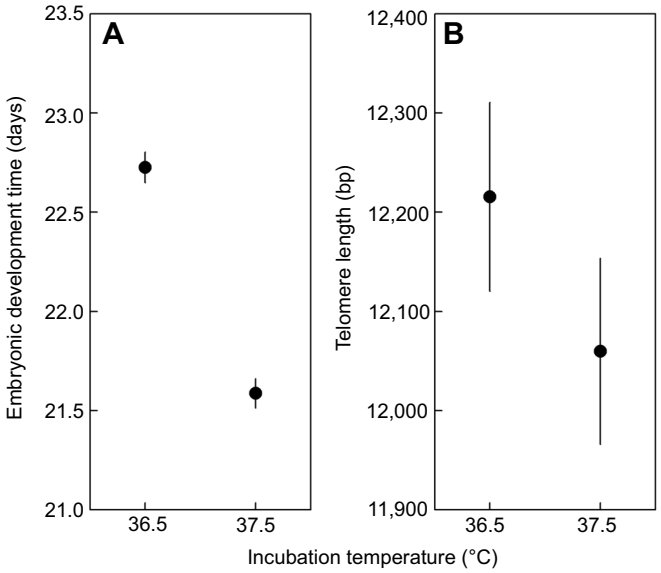

Fig. 1. Total duration of embryonic development and erythrocyte telomere length for common tern hatchlings incubated at 36.5 and 37.5°C. (A) Embryonic development time; (B) telomere length (bp, base pairs). Raw data are plotted (means±s.e.m.), but note that in the statistical analyses we include clutch identity as a random effect, thereby reducing the standard error of the treatment effect due to the high similarity between siblings, particularly in telomere length, as illustrated in Fig. 2.

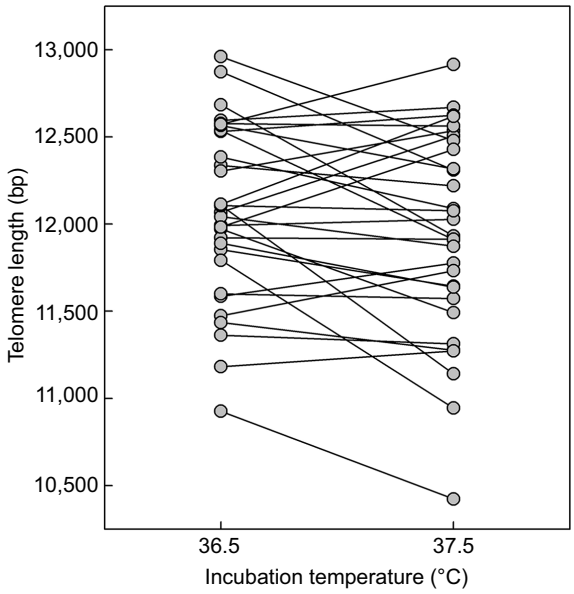

Fig. 2. Average individual erythrocyte telomere length at hatching for common tern hatchlings incubated at 36.5 and 37.5°C. Lines connect siblings from the same clutch incubated at different temperatures.

nevertheless affect telomere length (Noguera et al., 2016). We tested whether this was the case by separately adding them to the model in Table 1. These analyses showed that neither laying order (second versus first: estimate±s.e.=15.4±62.4 bp, χ^2_1=0.06, P=0.81) nor fresh egg mass (estimate±s.e.=−31.2±41.3 bp g^{-1}, χ^2_1=0.57, P=0.45) was associated with telomere length at hatching. Moreover, the effect of incubation treatment on telomere length remained significant with these additional variables included in the model (χ^2_1=5.04, P=0.025).

Embryos incubated at 36.5°C hatched with a similar body mass to embryos incubated at 37.5°C, but they required more than a day extra (5%) for total development. This suggests that the lower incubation temperature decelerated the rate of cell division without decreasing the total number of cell divisions. As incubation at 36.5°C not only decelerated development but also resulted in longer telomeres at hatching, our finding provides experimental evidence that growth rate is causally linked to telomere attrition. However, this will only be independent of the number of cell divisions if cell mass or cell turnover during embryogenesis is independent of incubation temperature.

We manipulated growth rate by varying incubation temperature after eggs were laid. Faster development with increasing temperature is a universal phenomenon across life, presumably because of the underlying physical law of higher temperature increasing the rate of biochemical reactions (Gillooly et al., 2002). Our result may thus be explained by reactions that determine the rate of embryonic growth being more responsive to temperature than those involved in telomere maintenance. Alternatively, a trade-off between growth rate and telomere length may be caused by constraints on the availability of nutrients, such as antioxidants (Monaghan and Ozanne, 2018). Oxidative stress can increase telomere attrition (Reichert and Stier, 2017) and faster growth can increase oxidative stress (Smith et al., 2016). Hence, temperature-induced faster growth, without a concomitant increased availability of nutrients required for oxidative defence within the egg, may have caused the increased telomere attrition we observed. However, other mechanisms that are independent of nutrient availability, like a speed–accuracy trade-off in tissue formation (Ricklefs, 2006), are equally likely.

Individual differences in telomere length early in life tend to be carried through to adulthood (Heidinger et al., 2012; Boonekamp et al., 2014), and remaining lifespan among adults in this population increased by approximately 1 year per 50 bp telomere length (Bauch et al., 2014). The loss of an extra 147 bp during embryonic development can therefore potentially shorten lifespan by 3 years, or approximately 30%, given that the average adult lifespan of common terns is 10 years (Becker and Ludwigs, 2004). Yet, such extrapolation must be treated with caution, as the few studies that tested for effects of incubation temperature on adult performance in birds suggest low incubation temperature to increase adult mortality rate (Berntsen and Bech, 2016; Nord and Nilsson, 2016). Ultimately, long-term follow-up of individuals with manipulated growth rate is required to resolve whether a trade-off between growth rate and lifespan is mediated by telomere dynamics or a physiological process reflected by telomere length.

Table 1. Summary of the linear mixed model testing for the effect of incubation treatment (independent variable) on average telomere length at hatching (dependent variable)

Independent variable	Coefficient (s.e.)	χ^2	Δd.f.	P
Clutch ID (random)		16.6	1	<0.001
Standard sample per gel	1.16 (0.25)	21.6	1	<0.001
Incubation treatment (37.5°C=ref.)	147.0 (63.1)	5.4	1	0.020

n=83 hatchlings, 50 clutches.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: O.V., S.V., S.B.; Methodology: O.V., S.V., E.Z.; Formal analysis: O.V., E.Z.; Investigation: O.V., E.Z., S.B.; Data curation: O.V., E.Z.; Writing - original draft: O.V.; Writing - review & editing: O.V., S.V., E.Z., S.B.; Visualization: O.V.; Supervision: O.V., S.B.; Project administration: O.V.; Funding acquisition: O.V., S.B.

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