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Experimentally increased reproductive effort alters telomere length in the blue tit (*Cyanistes caeruleus*)

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Abstract

Telomeres have recently been suggested to play important role in ageing and are considered to be a reliable ageing biomarkers. The life history theory predicts that costs of reproduction should be expressed in terms of accelerated senescence, and some empirical studies do confirm such presumption. Thus, a link between reproductive effort and telomere dynamics should be anticipated. Recent studies have indeed demonstrated that reproduction may trigger telomere loss, but actual impact of reproductive effort has not received adequate attention in experimental studies. Here, we experimentally manipulated reproductive effort by increasing the brood size in the wild blue tit (*Cyanistes caeruleus*). We show that parents attending enlarged broods experienced larger yearly telomere decay in comparison to control birds attending unaltered broods. In addition, we demonstrate that the change in telomere length differs between sexes, but this effect was independent from our treatment. To our knowledge, this is the first experimental study in the wild revealing that telomere dynamics may be linked to reproductive effort. Thus, telomere shortening may constitute one of the potential proximate mechanisms mediating the costs of reproduction.

Introduction

Telomere biology keeps on receiving substantial interest among researchers, as the understanding of their role and patterns of erosion may provide important insight into ageing process. Telomeres are short tandem repeats of DNA $(TTAGGG)_n$ found at the ends of eukaryotic chromosomes. With each cell division, they become shorter due to incomplete replication at the lagging DNA strand (so-called end replication problem). However, the largest portion of telomere loss is attributed to damaging action of reactive oxygen species that are inevitably generated as a by-product of aerobic metabolism (von Zglinicki *et al.*, 2001). Telomeric sequences can be restored due to the activity of telomerase, a ribonucleoprotein enzyme (Blackburn, 2005), protective shelterin proteins or chromosome recombination (Dunham *et al.*, 2000; De Lange, 2005). There is a growing evidence that shortening of telomeres may reflect intrinsic biological process of ageing (reviewed in Monaghan, 2010 and Sahin & Depinho, 2010), and the dynamics of telomere decay and restoration may potentially explain some of the observed variation in longevity (Haussmann & Mauck, 2008). For this reason, telomeres may constitute a reliable biomarker of ageing (Boonekamp *et al.*, 2013).

The rate of ageing is influenced by numerous factors and reproduction could be one of its major contributors. Elevated reproductive effort has been shown to accelerate ageing and reduce lifespan in a number of taxa (e.g. Partridge & Farquhar, 1981; Wiersma *et al.*, 2004; Hamel *et al.*, 2010; Flatt, 2011; Shi & Murphy, 2013). Various mechanisms have been proposed to explain how these negative effects of elevated reproduction may arise. Immunity suppression (Knowles *et al.*, 2009), accumulation of oxidative damage (Metcalfe & Monaghan, 2013) or reduction of insulin/IGF signalling through activation of transcription factors (Kenyon, 2010; Antebi, 2013) have been proposed as

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potential mechanisms mediating costs of reproduction. Telomere shortening has also been considered as a potential mechanistic link between reproductive rate and future survival (Monaghan & Haussmann, 2006). It can be argued that increased reproductive demands may elevate cellular turnover rate and increase the oxidative stress leading to faster telomere deterioration. However, the link between telomere shortening and the level of reproductive effort has received surprisingly little attention in experimental studies to date.

Telomere loss has been reported in breeding laboratory mice (*Mus musculus;* Kotrschal *et al.*, 2007) and captive zebra finches (*Taeniopygia guttata;* Heidinger *et al.*, 2012). In these studies, reproducing animals experienced telomere attrition, whereas the ones prevented from breeding showed slight telomere elongation. To our knowledge, only one study, performed in a laboratory population of zebra finches, manipulated the level of reproductive effort to investigate telomere dynamics. This study reported that elevated reproductive effort causes telomere reduction (Reichert *et al.*, 2014). However, the studies performed in the wild do not provide such clear results. Plot *et al.* (2012) showed that the leatherback turtles (*Dermochelys coriacea*) reproducing more frequently had shorter telomeres than individuals breeding at lower rates. Similarly, in free-ranging common terns (*Sterna hirundo*), shorter telomeres were found among birds exhibiting high breeding success in comparison to the individuals with low breeding success (Bauch *et al.*, 2012). However, the two latter studies did not manipulate the reproductive rates, so interpretation of their findings is not straightforward: the results may alternatively be explained as reflecting individual differences in quality. The only study to date that experimentally altered the level of parental effort in the context of telomere dynamics was performed on the wild Adélie penguins (*Pygoscelis adeliae*), but the authors did not detect any effects of the manipulation of breeding effort on telomere length (Beaulieu *et al.*, 2011). Consequently, the potential link between the level of reproductive effort and telomere dynamics remains unclear.

Here, we examined telomere dynamics in response to experimental manipulation of reproductive effort in the wild population of blue tits (*Cyanistes caeruleus*). We experimentally forced some parents to elevate their reproductive effort by increasing brood size, whereas some breeding pairs were left nonmanipulated constituting a control group. The change in telomere length from a year of manipulation to the subsequent year was quantified in the blood cells. We expected that shortening of telomeres between the two subsequent years should be larger among parents experiencing experimentally elevated reproductive effort in comparison to control nonmanipulated birds.

Materials and methods

Study system and sampling

The study was carried out in the wild population of nest-box breeding blue tits inhabiting the southern part of the Baltic island of Gotland (57°01′N 18°16′E). The study area consists of deciduous woodland patches separated by agricultural grounds. The forest is rich in oak (*Quercus robur*) and ash (*Fraxinus excelsior*), but there are some coniferous plots dominated by pine (*Pinus sylvestris*) with the presence of birch (*Betula pubescens*). The majority of the local blue tit population uses nest-boxes for breeding. Females usually lay one clutch per season of median size of 11 eggs. Young hatch after two weeks of incubation and fledge after another 17–20 days.

Nest-boxes were regularly inspected from the end of April to record all breeding attempts. Within each experimental year, nests with similar hatching date and nestlings number (±1) were matched in pairs. One randomly chosen nest in each pair was enlarged (experimental nest) by three nestlings (ca. 30% increase in brood size). Extra nestlings originated from donor nests (which provided various number of nestlings, from 3 to 9, and were not included in further analyses) and had identical hatching date as nestlings from recipient broods. In addition, half of the nestlings was exchanged between a pair of experimental nests. Brood enlargement and cross-fostering took place 2 days after hatching. Nestlings were uniquely marked by nail clipping at the time of manipulation. Our experimental design did not include broods with reduced number of nestlings. We decided to do so, first due to logistic reasons (matching nests by hatching date and brood size was facilitated by having to match two instead of three nests), and second, because in most studies concerning reproductive costs reduced broods do not differ from nonmanipulated controls (see Results of recent meta-analysis by Santos & Nakagawa, 2012). Adults were caught although feeding nestlings either with mist nets set in the vicinity of the nest-box or traps installed inside the box, on the 14th day after hatching, invariably for both experimental groups. Blood sample was taken via brachial vein puncture. Adult sex was determined by the presence of a brood patch, whereas age was assessed on the basis of ringing data or according to a colour difference between the great and primary wing coverts. The latter method allows distinguishing one year old from older birds (Svensson, 1992). In the subsequent years, survivors were caught at the nest and blood sampled.

The brood size manipulation experiment was performed over 11 years (from 2003 to 2013). Overall, 203 nest pairs were established during study years. However, only 50 parents from control and 45 parents from experimental nests were recaptured a year after the treatment. Blood samples from both captures were

available for parents attending 26 experimental and 33 control nests. We excluded one control nest showing exceptionally high nestling mortality rate (64%), as there is a risk that only one parent was attending this nest (in fact, we failed to capture the other parent). In all nests but two, only one parent was caught in the subsequent year. To be conservative, in these two cases where both parents were recaptured (one enlarged and one control nest), we excluded the parent that may potentially generate larger difference between groups (in the enlarged group, we excluded the one with larger telomere loss and in control the one that had smaller telomere loss). We also ran an analysis in which these two nests were excluded and confirmed that the outcome remains unaffected. As a result, our final data set comprises of 25 parents attending enlarged broods and 31 attending nonmanipulated, control broods. This sample included 20 females and 36 males. Due to various recapture rates and sample losses, the samples were not equally distributed across years. Number of fledglings significantly differed between enlarged and control nests in this subset of data (median brood size at fledging: 13 vs. 10; Mann–Whitney $U = 85.5$, $N = 56$, $P < 0.001$), whereas initial brood size (number of hatchlings) before the treatment did not differ between enlarged and control nests (median brood size at hatching: 11 vs. 10; Mann–Whitney $U = 336.5$, $N = 56$, $P = 0.389$).

Telomere measurements

Blood is an appropriate tissue for measuring telomere length in birds as erythrocytes are nucleated and experience relatively fast turnover rate. Moreover, sampling is not invasive, and blood telomere length is correlated with telomere length in other tissues (Reichert *et al.*, 2013). Blood was stored in 96% ethanol immediately after sampling. Then, DNA was extracted with Blood Mini kit (A&A Biotechnology, Gdynia, Poland). Manufacturer's protocol was followed, but the protein lysis was modified by adding overnight incubation in 37 °C. DNA concentration and purity was measured by means of a NanoDrop 1000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). Poor DNA quality can lead to biologically irrelevant variance in telomere lengths (Barrett *et al.*, 2012), so integrity of each sample was confirmed by electrophoresis on a 1% agarose gel.

Telomere length was assessed using real-time quantitative PCR (qPCR) assay adapted for birds (Criscuolo *et al.*, 2009). It is based on the amplification of telomeric $(TTAGGG)_n$ sequences. Relative telomere length is expressed as the ratio (T/S) of telomere copy number (T) and control single gene copy (S) (Cawthon, 2002). We used primer sets designed by Criscuolo *et al.* (2009). Primers for the control single copy gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were GAPDH-F (5′-AACCAGCCAAGTACGATGACAT-3′) and GAPDH-R (5′-CCATCAGCAGCAGCCTTCA-3′). These primers have been developed for the zebra finch, but can be successfully employed as a single control gene for blue tit as shown by our study. The melt curves showed a single peak, indicating that one specific product was amplified, and there was no amplification in the negative control. Even though those primers seemed to be not invariant in blue tit samples in preliminary study of Atema *et al.* (2013), they worked well in our assay, judging first by the gel electrophoresis product size verification and then by the melt curve (Fig. S1). Finally, low values of standard deviation of the Ct for the control gene (mean CV = 1.1%) show that the variation in amplification between individuals was negligible. This specific amplification in the GAPDH region of blue tit DNA is also independent of DNA concentration as demonstrated by the mean determination coefficient of a serial dilution standard curve $r^2 = 0.988$. Telomere primers were Tel1b (5′-CGGTTTG TTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCC TTACCCT-3′). MicroAmp Fast Optical 96-Well Reaction Plates were manually loaded and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA). 5 ng of DNA per reaction was used in qPCR for both sets of primers. GAPDH and telomere primers concentrations were 80 nM and 40 nM, respectively, for final volume of 10 μL per reaction well containing 5 μL of Brilliant III Ultra-Fast SYBR QPCR MM (Agilent Technologies, Santa Clara, CA, USA). The qPCR assays were performed using an Applied BioSystems 7500 Fast instrument (15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 25 s annealing at 58 °C and 30 s extending at 72 °C; data were collected during the extension phase). Both amplicons (GAPDH and telomeres) were placed on one plate along with a negative control for each and run in duplicate. Both samples from one individual were assayed on the same plate, whereas experimental and control individuals were evenly and alternately distributed on a plate. If the variation between technical replicates (Ct SD) exceeded 0.5, both samples for individual were measured again. In total, we ran 12 plates.

To generate standard curve for amplification efficiency, each plate included serial two-fold dilutions of a reference DNA (mixed DNA of three birds not included in the study) run in duplicate form 10 to 0.16 ng for telomere and from 10 to 0.62 ng for GAPDH. The same DNA was used as a GOLDEN sample to account for interplate variation (run in triplicate). Mean amplification efficiency and the determination coefficient (r^2) of the standard curve were 89% (range 86–98%) and 0.988 (range 0.964–0.997) for GAPDH and 103% (range 96–112%) and 0.954 (range 0.923–0.978) for telomeres, respectively, which lies within qPCR standards (Bustin *et al.*, 2009). To adjust for the differences in efficiencies

between telomere and GAPDH amplifications, we calculated relative telomere length using Gallup & Ackermann (2008) method. To calculate the amount of relative template in the sample for the telomere (X_{OTEL}) and GAPDH (X_{OGAP}), we used the equation $X_O = 10^{[(Ct-b)/m]}$; where *Ct* is the cycle at which the amplification plot of the focal sample crosses the threshold (CT), whereas *b* is intercept and *m* is slope of the log of the standard curve. The amount of telomere per sample (X_{OS}) normalized by the GAPDH amplification was expressed as $X_{OS} = X_{OTEL}/X_{OGAP}$. To account for between plate variability, we divided X_{OS} by X_{OGOLD} (where $X_{OGOLD} = X_{OTEL}/X_{OGAP}$) and this is our final T/S ratio for a sample.

Mean intraplate coefficient of variation (100xSD/mean value) was 2.3% for the *Ct* values of the telomere and 0.6% for the *Ct* values of the GAPDH, and interplate coefficient of variation calculated on GOLDEN sample *Ct* was 12.7% for telomeres and 4.6% for GAPDH. Telomere length of the same individual was strongly correlated between the year of treatment and the subsequent year ($r = 0.494$, $P < 0.001$ across individuals). This can be seen as lower bound estimate of repeatability of telomere measurements, as variation among years may also be affected by year, age and treatment effects.

Statistical analyses

To test whether telomere dynamics differs between birds experiencing elevated reproductive effort and birds attending nonmanipulated broods, we employed linear mixed models based on REML. The difference between relative telomere length (T/S ratio) in a year of treatment and a year after (telomere length $_{\text{year after manipulation(x+1)}}$ − telomere length $_{\text{year of manipulation(x)}}$) was defined as the dependent variable. To account for potential effects of individual sex and age, those variables were introduced as explanatory independent variables, whereas year of experiment was introduced as a random effect. Age was defined as a continuous variable. In all analyses, we tested for nonlinearity of age effects by introducing a quadratic term, but the quadratic term was retained in the model and reported only if significant. In further analyses, we also checked interactions between factors, but they all appeared nonsignificant ($P > 0.05$). It is important to note that it was not possible to consider paired design in our analyses as only single parents from nest couples we recaptured in the subsequent year. To compare telomere length (log transformed for normality) between parents attending experimental and control nests in the year of manipulation (x) and in the following year (x+1), we performed linear mixed models with brood size treatment, sex and age as explanatory variables and year of manipulation as a random effect. The same factors were considered in the logistic regression testing whether telomere change (treated as independent continuous variable) affects survival to the second year after manipulation (x+2). Of the 56 considered birds, we knew the exact age (fitted as years) of 45 individuals, whereas for 11 birds, we could only assign their minimal age. Yearly survival rate in this population is relatively low (ca. 30%, own unpublished data); therefore, we assumed that those 11 individuals are two years old at their first capture. The median age of the parents did not differ between birds attending enlarged and control nests (1 year for both treatments; Mann–Whitney $U = 326.5$, $N = 56$, $P = 0.214$) and between sexes (1 year for both sexes; Mann–Whitney $U = 354$, $N = 56$, $P = 0.899$). All analyses were performed in IBM SPSS 21 and R, version ASREML-R v 3.0.1 (Butler, 2009; R Core Team, 2014).

Results

Telomere length in the year of manipulation (x) did not differ between experimental groups ($F_{1,47.5} = 0.028$, $P = 0.868$) and sexes ($F_{1,49.4} = 2.282$, $P = 0.137$), but significantly decreased with individual age in a nonlinear manner (age: $F_{1,50.2} = 4.455$, $P = 0.040$; age^2: $F_{1,50.8} = 6.700$, $P = 0.013$). Telomere length observed a year after treatment (x+1) was also not significantly affected by experiment ($F_{1,52} = 2.592$, $P = 0.113$), sex ($F_{1,52} = 2.273$, $P = 0.138$), but was not linked to individual age ($F_{1,52} = 0.004$, $P = 0.952$).

The experimental treatment significantly predicted the change in telomere length observed between the year of manipulation and the subsequent year (Table 1, Fig. 1a). In comparison to parents attending nonmanipulated control broods, parents raising experimentally enlarged broods showed a significant negative change in telomere length. The change in telomere length also differed significantly between sexes (Table 1). It was negative among males, whereas it appeared positive among females (Fig. 1b). However, the difference in telomere change between sexes was independent of experimental treatment (nonsignificant interaction experiment by sex; $F_{1,48.2} = 2.018$, $P = 0.162$). Interestingly, the changes in telomere length between years

Table 1 Results of a linear mixed model analysing variation in telomere change in response to brood size manipulation (enlarged vs. control nonmanipulated). The model accounted for potential effect of study year introduced as a random effect and fixed effects of sex and individual age.

Source of variation	Estimates ± SE	T	d.f.	*F*	*P*
Experiment	0.344 ± 0.16	2.197	1, 47.808	4.827	**0.033**
Sex	0.514 ± 0.16	3.131	1, 48.344	9.802	**0.003**
Age	0.111 ± 0.09	1.227	1, 51.147	1.506	0.225

Variance estimate for random effect year = 0.056 ± 0.067.
Significant values ($P < 0.05$) shown in bold.

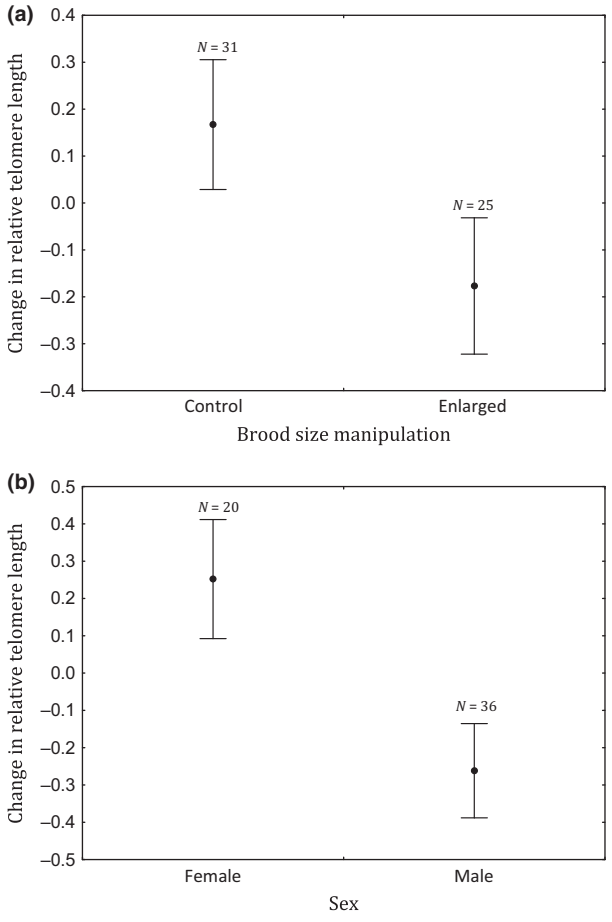

Fig. 1 Change in the relative telomere length [the ratio (T/S) of telomere copy number (T) and control single gene copy (S)] in adult blue tits between a year of experimental treatment and the subsequent year (least squares means ± 1SE) according to the treatment (a) and sex (b). The numbers above bars indicate sample size.

were not significantly different from zero in neither of experimental groups (sexes combined) nor among females. Only males experienced significant telomere shortening (when tested without accounting for other factors as in the global model above, Table S1).

Our brood size manipulation and the change in telomere length had no significant effect on the probability of survival between year x+1 and x+2 (Table 2).

Table 2 Results of logistic regression analysing probability of survival between a year after manipulation (x+1) and the subsequent year (x+2) in response to telomere change between year of treatment (x) and year after treatment (x+1). Model accounted for effects of experimental treatment, sex, individual age and year as a random effect.

Term	Estimates ± SE	*Z*	*F*.con	d.f.	*P*
Telomere change	0.651 ± 0.57	1.138	1.332	1, 51	0.254
Experiment	0.114 ± 0.74	0.154	0.025	1, 51	0.876
Sex	1.271 ± 0.88	1.449	2.179	1, 51	0.146
Age	−1.669 ± 1.05	−1.596	2.652	1, 51	0.110

Variance estimate for random effect year = 0.146 ± 0.55.

Discussion

Here, we show that experimentally elevated reproductive effort triggers telomere shortening. As the blue tit is a relatively short-living species, the observed loss of telomere length over one year should be considered as a long-term effect spanning throughout the reproductive lifespan of an individual. To our knowledge this is the first experimental study performed in a wild population to support the hypothesis that elevated reproductive effort may incur costs in terms of telomere attrition. Previous studies suggested the existence of a link between breeding effort and telomere dynamics, but as the reproductive effort was not experimentally manipulated, such outcomes are open for alternative explanations (Bauch *et al.*, 2012; Plot *et al.*, 2012). The only previous experimental work in the wild did not show any effect of modified parental effort on telomere length (Beaulieu *et al.*, 2011). However, in accordance with our result, manipulated reproductive status (reproducing vs. nonreproducing) in mice and zebra finches significantly affected telomere dynamics (Kotrschal *et al.*, 2007; Heidinger *et al.*, 2012). Recently, experimental laboratory study of zebra finch (Reichert *et al.*, 2014) reported that increased reproduction led to a reduction in telomere length, which persisted up to 1 year.

Our data do not allow to infer on physiological mechanism behind the observed effect of brood manipulation on telomere dynamics. We can presume that observed telomere decay could stem from faster cellular turnover directly related to end replication problem or from higher levels of oxidative stress resulting from elevated reproductive effort. Brood size manipulation affects parental workload (Nur, 1984) and subsequent metabolic rates (Skibiel *et al.*, 2013). Enhanced aerobic metabolism inevitably generates highly reactive oxygen species that, unless neutralized, damage various cellular structures. Damaged cells require extra renewal, so this may increase their turnover rate and telomere loss occurring during each cellular division (Monaghan & Haussmann, 2006). Free oxygen species may also directly damage structure of telomeres resulting in their attrition as telomeric sequences seem to be particularly susceptible to the action of free radicals (Monaghan & Haussmann, 2006; Metcalfe & Monaghan, 2013). Reactive oxygen species are neutralized by antioxidative defences, but any investments to such protection may be limited in favour of allocation to reproduction (Metcalfe & Monaghan, 2013). Another hypothetical mechanism by which enhanced breeding performance

may cause telomere decay is suppression of the activity of telomerase or protective proteins. Failure in telomeric restoration may be derived from prioritizing resource allocation to meet elevated reproductive demands at the costs of telomere protection.

We found that the change in telomere length differed significantly between sexes, but this effect was independent from the experimental treatment. The change was negative only among males, whereas it appeared positive among females. This is quite unexpected and difficult to explain. We can speculate that the positive change in telomere length between years among females may invoke sex-specific telomerase activity, which may be accounted to sex hormones, as transcription and activation of telomerase is promoted by oestrogen (Kyo *et al.*, 1999; Grasselli *et al.*, 2008). Oestrogen has also been found to affect DNA repairs through p53 pathway (Sengupta & Wasylyk, 2004). Sex-specific differences in telomere dynamics have been rarely reported in birds (reviewed in Barrett & Richardson, 2011), whereas abundant human studies show that females display longer telomeres than males, but only if the Southern blot method is used to assess telomere length (see recent meta-analyses by Gardner *et al.*, 2014).

Telomere length has been shown to predict longevity (Pauliny *et al.*, 2006; Bize *et al.*, 2009; Salomons *et al.*, 2009; Heidinger *et al.*, 2012; Barrett *et al.*, 2013), hence the negative changes in telomere length observed by us may potentially compromise future survival. Interestingly, previous studies on the costs of reproduction in blue tit reported survival costs (eg. Nur, 1988). However, our own data (Table S2) do not show any significant negative effects of brood size manipulation on survival probability from the year of manipulation to the subsequent year. More importantly, here we were not able to demonstrate any effects of change in telomere length between years and brood size manipulation on the probability of survival from year x+1 to x+2. So, we are not able to directly show that the observed change in telomere length might mediate cost of reproduction in terms of survival. However, our sample size is far too small to reliably assess survival probability, as measured under field condition when mortality estimates are strongly influenced by recapture probability. Several studies have reported that individuals with the greatest telomere shortening are less likely to survive (e.g. Bize *et al.*, 2009; Salomons *et al.*, 2009). Therefore, it is plausible that nonsurvivors experienced the largest telomere shortening due to our brood manipulation, and the small subsample of survivors may in fact represent superior individuals paying negligible costs of elevated reproductive effort.

To conclude, our study provides the first experimental evidence on the suggested link between the level of reproductive effort and the dynamics of telomere length in a wild population. Our results imply that variation in reproductive investments may add up to the huge variation in telomere length observed within single age classes (Monaghan & Haussmann, 2006). Future studies should explore direct physiological mechanisms that link telomere attrition with elevated reproductive effort. The studies performed in larger scale are also needed to confirm whether the telomere dynamics may explain the expected relationship between reproductive effort and future survival and longevity.

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References

Antebi, A. 2013. Regulation of longevity by the reproductive system. *Exp. Gerontol.* **48**: 596–602.

Atema, E., Oers, K.V. & Verhulst, S. 2013. GAPDH as a control gene to estimate genome copy number in Great Tits, with cross-amplification in Blue Tits. *Ardea* **101**: 49–54.

Barrett, E.L.B. & Richardson, D.S. 2011. Sex differences in telomeres and lifespan. *Aging Cell* **10**: 913–921.

Barrett, E.L.B., Boner, W., Mulder, E., Monaghan, P., Verhulst, S. & Richardson, D.S. 2012. Absolute standards as a useful addition to the avian quantitative PCR telomere assay. *J. Avian Biol.* **43**: 001–006.

Barrett, E.L.B., Burke, T.A., Hammers, M., Komdeur, J. & Richardson, D.S. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. *Mol. Ecol.* **22**: 249–259.

Bauch, C., Becker, P.H. & Verhulst, S. 2012. Telomere length reflects phenotypic quality and costs of reproduction in a longlived seabird. *Proc. R. Soc. B* **280**: 20122540.

Beaulieu, M., Reichert, S., Maho, Y.L., Ancel, A. & Criscuolo, F. 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. *Funct. Ecol.* **25**: 577–585.

Bize, P., Criscuolo, F., Metcalfe, N.B., Nasir, L. & Monaghan, P. 2009. Telomere dynamics rather than age predict life expectancy in the wild. *Proc. R. Soc. B* **276**: 1679–1683.

Blackburn, E.H. 2005. Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Lett.* **579**: 859–862.

Boonekamp, J.J., Simons, M.J.P., Hemerik, L. & Verhulst, S. 2013. Telomere length behaves as biomarker of somatic redundancy rather than biological age. *Aging Cell* **12**: 330–332.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M. *et al.* 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**: 611–622.

Butler, D. 2009. asreml: asreml() fits the linear mixed model. R package version 3.0-1.

Cawthon, R.M. 2002. Telomere measurement by quantitative PCR. *Nucleic Acids Res.* **30**: e47.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N.B., Foote, C.G., Griffiths, K. *et al.* 2009. Real-time PCR quantification assay for measurements of bird telomeres. *J. Avian Biol.* **40**: 342–347.

De Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19**: 2100–2110.

Dunham, M.A., Neumann, A.A., Fasching, C.L. & Reddel, R.R. 2000. Telomere maintenance by recombination in human cells. *Nat. Genet.* **26**: 447–450.

Flatt, T. 2011. Survival costs of reproduction in *Drosophila*. *Exp. Gerontol.* **46**: 369–375.

Gallup, J.M. & Ackermann, M.R. 2008. The 'PREXCEL-Q method' for qPCR. *Int. J. Biomed. Sci.* **4**: 273–293.

Gardner, M., Bann, D., Wiley, L., Cooper, R., Hardy, R., Nitsch, D. *et al.* 2014. Gender and telomere length: systematic review and meta-analysis. *Exp. Gerontol.* **51**: 15–27.

Grasselli, A., Nanni, S., Colussi, C., Aiello, A., Benvenuti, V., Ragone, G. *et al.* 2008. Estrogen receptor-α and endothelial nitric oxide synthase nuclear complex regulates transcription of human telomerase. *Circ. Res.* **103**: 34.

Hamel, S., Gaillard, J.-M., Yoccoz, N.G., Loison, A., Bonenfant, C. & Descamps, S. 2010. Fitness costs of reproduction depend on life speed: empirical evidence from mammalian populations. *Ecol. Lett.* **13**: 915–935.

Haussmann, M.F. & Mauck, R.A. 2008. Telomeres and longevity: testing an evolutionary hypothesis. *Mol. Biol. Evol.* **25**: 220–228.

Heidinger, B.J., Blount, J.D., Boner, W., Griffiths, K., Metcalfe, N.B. & Monaghan, P. 2012. Telomere length in early life predicts lifespan. *PNAS* **109**: 1743–1748.

Kenyon, C. 2010. A pathway that links reproductive status to lifespan in *Caenorhabditis elegans*. *Ann. N.Y. Acad. Sci.* **1204**: 156–162.

Knowles, S.C.L., Nakagawa, S. & Sheldon, B.C. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. *Funct. Ecol.* **23**: 405–415.

Kotrschal, A., Ilmonen, P. & Penn, D.J. 2007. Stress impacts telomere dynamics. *Biol. Lett.* **3**: 128–130.

Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y. *et al.* 1999. Estrogen activates telomerase. *Cancer Res.* **59**: 5917–5921.

Metcalfe, N.B. & Monaghan, P. 2013. Does reproduction cause oxidative stress? An open question. *Trends Ecol. Evol.* **28**: 347–350.

Monaghan, P. 2010. Telomeres and life histories: the long and the short of it. *Ann. N.Y. Acad. Sci.* **1206**: 130–142.

Monaghan, P. & Haussmann, M.F. 2006. Do telomere dynamics link lifestyle and lifespan? *Trends Ecol. Evol.* **21**: 47–53.

Nur, N. 1984. Feeding frequencies of nestling blue tits (*Parus caeruleus*): costs, benefits and a model of optimal feeding frequency. *Oecologia* **65**: 125–137.

Nur, N. 1988. The consequences of brood size for breeding blue tits. III. Measuring the cost of reproduction: survival, future fecundity, and differential dispersal. *Evolution* **42**: 351–362.

Partridge, L. & Farquhar, M. 1981. Sexual activity reduces lifespan of male fruitflies. *Nature* **294**: 580–582.

Pauliny, A., Wagner, R., Augustin, J., Szep, T. & Blomqvist, D. 2006. Age-independent telomere length predicts fitness in two bird species. *Mol. Ecol.* **15**: 1681–1687.

Plot, V., Criscuolo, F., Zahn, S. & Georges, J.Y. 2012. Telomeres, age and reproduction in a long-lived reptile. *PLoS ONE* **7**: 1–6.

R Core Team 2014. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Reichert, S., Criscuolo, F., Verinaud, E., Zahn, S. & Massemin, S. 2013. Telomere length correlations among somatic tissues in adult zebra finches. *PLoS ONE* **8**: e81496.

Reichert, S., Stier, A., Zahn, S., Arrivé, M., Bize, P., Massemin, S. *et al.* 2014. Increased brood size leads to persistent eroded telomeres. *Front Ecol. Evol.* **2**: 1–11.

Sahin, E. & Depinho, R.A. 2010. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature* **464**: 520–528.

Salomons, H.M., Mulder, G.A., Zande, L., Haussmann, M.F., Linskens, M.H.K. & Verhulst, S. 2009. Telomere shortening and survival in free-living corvids. *Proc. R. Soc. B* **276**: 3157–3165.

Santos, E.S.A. & Nakagawa, S. 2012. The costs of parental care: a meta-analysis of the trade-off between parental effort and survival in birds. *J. Evol. Biol.* **25**: 1911–1917.

Sengupta, S. & Wasylyk, B. 2004. Physiological and pathological consequences of the interactions of the p53 tumor suppressor with the glucocorticoid, androgen, and estrogen receptors. *Ann. N. Y. Acad. Sci.* **1024**: 54–71.

Shi, C. & Murphy, C.T. 2013. Mating induces shrinking and death in *Caenorhabditis* mothers. *Science* **343**: 536–540.

Skibiel, A.L., Speakman, J.R. & Hood, W.R. 2013. Testing the predictions of energy allocation decisions in the evolution of life-history trade-offs. *Funct. Ecol.* **27**: 1382–1391.

Svensson, L. 1992. *Identification Guide to European Passerines*. Naturhistoriska Riksmuseet, Stockholm.

Wiersma, P., Selman, C., Speakman, J.R. & Verhulst, S. 2004. Birds sacrifice oxidative protection for reproduction. *Proc. R. Soc. B (Suppl.)* **271**: S360–S363.

von Zglinicki, T., Bürkle, A. & Kirkwood, T.B.L. 2001. Stress, DNA damage and ageing – an integrative approach. *Exp. Gerontol.* **36**: 1049–1062.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 GAPDH melt curve for two different individuals.

Table S1 Annual telomere length change (T/S ratio) in groups.

Table S2 Results of logistic regression analysing probability of survival between a year of manipulation (x) and the subsequent year (x+1) in response to brood size manipulation (enlarged vs. control nests).

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