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RESEARCH ARTICLE

Telomere dynamics from hatching to sexual maturity and maternal effects in the 'multivariate egg'

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ABSTRACT

Avian eggs contain a large number of molecules deposited by the mother that provide the embryo with energy but also potentially influence its development via the effects of maternally derived hormones and antibodies: the avian egg is thus 'multivariate'. Multivariate effects on offspring phenotype were evaluated in a study on captive zebra finches, by simultaneously manipulating maternally derived antibodies (MAb) by lipopolysaccharide (LPS) treatment of mothers and injection of testosterone into the egg yolk. LPS treatment had a positive effect on body mass growth at 30 days after hatching and immune response at sexual maturity, while egg testosterone treatment positively influenced immune response at fledging and courtship behaviour in sexually mature male offspring. Maternal effects are known to modulate offspring telomere length (TL). However, the multivariate effects of egg-derived maternal components on offspring telomere dynamics from hatching to sexual maturity are undefined. Here, we tested: (1) the effects of LPS and testosterone treatments on TL from hatching to sexual maturity (day 82); (2) how LPS treatment modulated TL over reproduction in adult females; and (3) the relationship between maternal and offspring TL. We predicted that TL would be shorter in LPS fledglings (as a cost of faster growth) and that TL would be longer in sexually mature adults after yolk testosterone treatment (as a proxy of individual quality). In adult females, there was an overall negative relationship between laying and rearing investments and TL, this relationship was weaker in LPS-treated females. In chicks, there was an overall negative effect of LPS treatment on TL measured at fledging and sexual maturity (day 25–82). In addition, at fledging, there was a Sex×LPS×Testosterone interaction, suggesting the existence of antagonistic effects of our treatments. Our data partially support the hypothesis that telomeres are proxies of individual quality and that individual differences in TL are established very early in life.

KEY WORDS: Maternal effects, Egg, Growth, Maternal antibodies, Testosterone, Telomere, Bird

INTRODUCTION

Telomeres are non-coding DNA sequences that control DNA-end recognition and fusion in linear chromosomes (Blackburn, 1991). However, because of the non-replication of the 3′-end of DNA during cell division, telomeres shorten over the lifetime of a cell (Blackburn, 2000). In addition, telomere ends seem to be preferred targets of oxidative stress, an observation albeit currently better documented at the cellular (von Zglinicki, 2002) than at the whole organism level (Boonekamp et al., 2017; Reichert and Stier, 2017). Telomere maintenance mechanisms also exist, which may counterbalance telomere erosion (Beery et al., 2012; Blackburn and Epel, 2012), and studies across a range of taxa have shown that telomere dynamics are more complex than a simple, regular rate of shortening over age (Fairlie et al., 2016). In fact, several recent studies have highlighted evidence for telomere lengthening in different ecological or experimental conditions (Hoelzl et al., 2016a,b; Lieshout et al., 2019; Spurgin et al., 2018). This idea of negative/positive telomere dynamics has led to the suggestion that telomeres represent one of the cell mechanisms underpinning life-history trade-offs, and that they are a key molecular tool to explore inter-individual differences in ageing trajectories and fitness (Young, 2018). The growth versus ageing trade-off and its concomitant changes in telomere length (TL) have attracted extended interest (reviewed in Monaghan and Ozanne, 2018) because damaged or shortened telomeres are good candidates to explain future consequences of variable offspring investment in the soma (Monaghan and Haussmann, 2006; Reichert et al., 2015a). Indeed, telomere erosion during development has the potential to effectively mediate the effect of early-life stress as a predictor of future individual fitness (Heidinger et al., 2012; Marasco et al., 2019). Accordingly, TL at the end of growth correlates with remaining lifespan in birds and mammals (e.g. Heidinger et al., 2012; Lieshout et al., 2019), even though causality between TL and fitness traits remains an open question (Simons, 2015).

Growth is a period of extensive cell division and of high energy expenditure (2–6 times basal metabolic rate; Dunn, 1980; Kirkwood, 1991; Vleck and Vleck, 1980), both of which potentially influence telomere erosion in early life (albeit putatively at different degrees, see Boonekamp et al., 2017). As such, fast growth is predicted to be costly in terms of TL (Jennings et al., 1999; Metcalfe and Monaghan, 2001). Numerous previous studies conducted on birds have addressed how growth trajectories and telomere dynamics are intertwined but have produced equivocal results (reviewed in Vedder et al., 2017). Most studies report negative effects of stressful growth environments on chick telomeres but rarely confirm a causal relationship with (a high) growth rate (e.g. Boonekamp et al., 2014; Reichert et al., 2015a; Voillemot et al., 2012). Growth rate has evolved as a compromise between the benefit and the costs of rapid growth for newborn organisms in any given environment (Dmitriew, 2011). The environment in which early-life growth takes place is thus widely recognized to affect the phenotype through ontogeny. In this early-life environment, the non-genetic effects derived from the mother's physiological or behavioural traits – so-called 'maternal' effects – are of prime importance in determining the offspring phenotype (Mousseau and

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Fox, 1998). For instance, pre-natal adjustments in yolk components like hormones should have additive effects on the genetic programme of development of the embryo (Groothuis et al., 2005). Thus, maternal effects may drive the adaptation of offspring phenotype to current environmental conditions (i.e. maternally derived adaptive phenotypic plasticity), including the modulation of trade-offs with body maintenance and their ageing consequences. A large panel of egg components, including macronutrients, hormones and antibodies, might act as developmental regulators. This diversity of egg components that can putatively modulate the phenotype of the developing organism (in addition to their interactions) has led to the concept of the 'multivariate egg' (Postma et al., 2014; Williams and Groothuis, 2015). Because virtually all components of the egg may have their own effects, and potentially act in synergistic or antagonistic ways, this requires integration of multiple interactive factors when studying maternal effects on offspring phenotype (Possenti et al., 2018; Torres et al., 2019). For example, manipulation of yolk-deposited carotenoids and testosterone showed that their independent deleterious effects on chick oxidative stress while triggering a higher growth rate were buffered when both factors were increased simultaneously (Giraudeau et al., 2017). Interestingly, deleterious effects of steroid hormones on TL have been previously shown (e.g. testosterone; Drury et al., 2014), providing a potential mechanism by which maternal effects may modulate telomere shortening in offspring (Marchetto et al., 2016). In birds, pre-natal as well as post-natal maternal investment in reproduction have been studied in the context of TL of avian chicks: TL decreases with laying order (Noguera et al., 2016) and with increased egg content in corticosterone (Haussmann et al., 2012; Tissier et al., 2014); instability in incubation temperature shortened TL at hatching (Stier et al., 2019 preprint) whereas lower incubation temperature decreased growth rate and telomere erosion (Vedder et al., 2018). In addition, telomere shortening is associated with immune system activation in chicks and adult birds (Asghar et al., 2015; Lardy et al., 2018). In this case, telomere erosion may be due to higher rate of cell division or to side deleterious effects of the production of oxidative molecules by immune cells (Criscuolo et al., 2018). Using lipopolysaccharide (LPS) injection in mothers before egg-laying (Boulinier and Staszewski, 2008; Torres et al., 2019), the impact of yolk-deposited maternal antibodies on offspring telomeres can be experimentally tested.

Maternal effects on TL are part of the broader question of how TL is passed on to the next generation, i.e. how Gene×Environment interactions determine telomere inheritance (Dugdale and Richardson, 2018). Early studies suggested TL to be mainly genetically determined (Graakjaer et al., 2004) and with a more pronounced mother-offspring resemblance in birds (Becker et al., 2015; but see Atema et al., 2015), but contrasting data are accumulating suggesting a key influence of early-life environmental conditions in parent–offspring telomere resemblance, as seen for parental care-derived effects (Viblanc et al., 2020). Among those, a role for epigenetic-like effects from paternal gametes has been recently reported in birds (Bauch et al., 2019), but the potential role of maternally derived egg effects on offspring telomeres remains relatively unknown.

Until recently, most studies on telomere dynamics have focused on the effects of early-life conditions and development rather than changes in early adulthood. This is likely to have important consequences for our understanding of how adult telomere dynamics might play a role in trade-offs between current and future reproduction or adult survival (Bauch et al., 2013; Bichet et al., 2020; Reichert et al., 2014). This is based on the hypothesis that telomere maintenance is energy demanding and thus TL will reflect trade-offs (Bauch et al., 2016; Young, 2018). However, another possibility is that individual differences are mainly established during development, at birth or very early in adulthood (i.e. before adult maturity), which may indicate that individual variation in TL is only weakly affected by environmental stress or trade-offs over adult life (Bichet et al., 2020). As TL has been related to adult foraging efficiency (Young et al., 2015) or breeding success (Bauch et al., 2014), it was suggested to be a biomarker of individual quality (Angelier et al., 2019; Le Vaillant et al., 2015), individual quality being mostly interpreted as a multivariate characteristic inherited from parents and then further modulated very early in life.

The present study follows up on the study by Torres et al. (2019), which focused on multivariate maternal effects on offspring phenotype. This previous study showed that LPS treatment did not change the reproductive output of females and had only a small effect on growth trajectories of chicks: male offspring of LPS-challenged females were heavier at 30 days old (Torres et al., 2019). However, the immune response of chicks to a phytohaemagglutinin test (PHA) was higher in chicks from testosterone-treated eggs at day 26 after hatching (end of growth), and in chicks raised by LPS-treated mothers at day 82 after hatching (sexual maturity). In addition, courtship behaviour of males was significantly and positively affected by the testosterone treatment, although there were no interaction effects of LPS and testosterone on the chick phenotype. Thus, male chicks could have benefited from (i) the mother's LPS challenge (i.e. being larger at day 30) and (ii) egg testosterone injections resulting in males of higher quality than control males at sexual maturity. Similarly, testosterone- and LPS-treated chicks had a better immune response at the end of growth and at sexual maturity. In the present study, we considered telomere dynamics in adult female zebra finches [*Taeniopygia guttata* (Vieillot 1817)] during reproduction and in their chicks (offspring) from hatching to sexual maturity. Our predictions rely on the adult and immature phenotypes previously observed (Table 1). We present data on: (1) changes in TL in adult female zebra finches in relation to reproductive investment, including females that were LPS immune challenged prior to egg production: we predicted that telomeres of mothers should shorten in relation to reproductive effort (i.e. with increasing clutch/brood size) and this effect would be enhanced in immune-challenged females; (2) the relationship of TL between parents (mothers) and offspring (chicks): we expect a positive relationship for mother-female offspring TL at least in the control groups, where inheritance has not been modulated by experimental treatments; (3) effects of chick growth and age on TL in chicks from eggs where we manipulated both yolk testosterone or maternal antibodies to determine how these two factors are interacting in the set-up of growth trajectories and immune response in offspring, and how these in turn were related to telomere dynamics: based on the cost of growth hypothesis, we expected telomeres of chicks at the end of growth to be shorter in males from the maternal LPS treatment group. Based on the trade-off between growth and immune system maturation, chick and fledgling TLs should be shorter in experimental groups, except for males of the testosterone group, which should have longer telomeres when reaching sexual maturity.

MATERIALS AND METHODS

Full details of our experimental protocol and sampling design are given in Torres et al. (2019). Zebra finches were housed under

Table 1. Predictions for effects of experimentally induced maternal effects (LPS) and egg-yolk content (testosterone) on chick telomere length after embryonic development and post-hatching growth (day 26) and young adult final development (day 82) in zebra finch

		Immature phenotype (day 26)			Young adult phenotype (day 82)		
	Sex	Growth	Immune function	TL	Immune function	Adult quality	TL (days 26-82)
Yolk testosterone (T)	M	0	+	–	0	+	+
	F	0	+	–	0	0	0
LPS treatment (LPS)	M	+	0	–	+	0	–
	F	0	0	0	+	0	–
Interaction T×LPS	M	0	0	0	0	0	0
	F	0	0	0	0	0	0

Those predictions are based on our previous sister paper (Torres et al., 2019) main findings (indicated in grey) that LPS-induced maternal effect accelerated growth rate in male chicks at the end of post-hatching growth, and that immune response at the end of post-hatching growth (egg-yolk treated chicks) and at the end of young adult maturation period (chicks produced by LPS-treated mothers) was enhanced. Males of the testosterone group that were found of better pairing quality should have longer telomeres when reaching sexual maturity. Predictions either followed the trade-off/telomere hypothesis. Table modified from Torres et al. (2019).

controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant 14 h light:10 h dark, lights on at 07.00 h) and fed with a mixed seed (millet) diet, water, grit and cuttlefish bone (calcium) provided *ad libitum*, and a multivitamin supplement in the drinking water once per week. Experienced adult male and female birds >90 days old (i.e. birds that had been paired or laid eggs previously) were randomly paired and housed in individual breeding cages (51×39×43 cm), each with an external nest box (14×14.5×20 cm). Breeding pairs had access to 6 g egg food supplement (20.3% protein: 6.6% lipid) per day during pairing, laying and chick rearing. All breeding pairs were checked daily for egg-laying to record laying date, egg mass and clutch size. Freshly laid eggs were weighed (±0.001 g), and individually numbered to identify laying order. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 1074B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

LPS treatment

To manipulate potential costs of reproduction, and the level of maternal antibodies in eggs, adult females (experimental, n=41; control group, n=38) were first immune challenged before egg laying. Females in the experimental group were injected intraperitoneally with 0.01 mg lipopolysaccharide (LPS; *Escherichia coli*, serotype 055:B5; Sigma) diluted in 0.1 ml phosphate buffered saline solution (PBS, concentration, 0.1 mg ml^{-1}, females in control group injected with only PBS) to initiate a primary immune response and again 10 days later to obtain a stronger, secondary immune response. Three days after the second immune challenge, male–female pairs were established and birds allowed to breed as described above (1st egg laid 10 days after the second LPS injection). To evaluate the effect of cost of reproduction on TL we blood sampled adult females at the very beginning of the experiment before the LPS challenge (T1, n=32), when the first egg was laid (T2, n=25) and at the end of chick growth 21 days after hatching (T3, n=31). Plasma (Torres et al., 2019) and yolk (Gasparini et al., 2002) antibody levels have been previously found to be increased following such an immune challenge.

Testosterone treatment

We used a split design manipulating yolk testosterone within clutches of LPS-treated and control females [i.e. producing eggs with high or low levels of maternal antibodies (MAb)] using *in ovo* egg injection of testosterone on day 3 after the eggs were laid, as described in Torres et al. (2019). Eggs from each clutch were randomly assigned to either the testosterone or the control group (to control for variation among females and genetic background). Eggs in the testosterone-treated group were injected with 500 pg testosterone (Fluka) dissolved in 2 µl sesame oil (von Engelhardt et al., 2006). Before injection, the side of the egg was cleaned with 100% ethanol, and the egg was held vertically with the apex at the top and the cap (air cell) at the bottom, until the yolk floats to the top of the egg. The vehicle was injected into the yolk using a 10 µl removable needle Hamilton syringe (gastight 1700 series) and 26½ G small hub removable needle with a bevel tip. To reach the yolk, eggs were candled with a high-powered LED flashlight (900 lumens) and the needle was pushed through the shell at an upward angle. The hole in the shell was closed with a drop of cyanoacrylate glue (Loctite gel control) and eggs were placed back in their nest once the glue was dry (∼10 min). Eggs in the control group were injected with 2 µl sesame oil, but otherwise were treated in a similar way to eggs in the testosterone treatment.

At hatching, nests were monitored daily to identify hatching order. Hatchlings were marked by uniquely clipping down plumage for individual identification and at 8–12 days post-hatching all birds were banded with a numbered aluminium ring. Body mass (±0.01 g) and the length of tarsus (±0.01 mm) were recorded at hatching (day 0) and independence (30 days post-hatching). Juveniles (30 days of age) were then removed from their natal cages and were housed in same-sex communal cages with visual and acoustical contact with birds of the opposite sex, until sexual maturity at 90 days of age. Chicks were sexed based on their sexually dichromatic plumage. To compare TL of offspring and mothers, and effects of treatments and growth on chick TL, we used blood samples from chicks at the end of their growth period (day 26) and again close to sexual maturity on day 82 (Torres et al., 2019).

Telomere length assay using quantitative PCR

TL was measured on DNA obtained from frozen red blood cells from zebra finch adult females and chicks. Red blood cells of birds are nucleated and DNA can be easily extracted (Nucleospin Blood QUIckPure kit, Macherey-Nagel) in adequate quantity (assessed by spectrophotometric absorbance, Nanodrop 1000 Thermo Scientific) and quality (ratio A260/280 and A260/230 and by checking for DNA degradation after gel-migration on a sub-sample, see Fig. S1) for qPCR amplification. DNA was then diluted using sterile distilled water at 5 ng µl^{-1} for amplification using telomere and control gene (GAPDH) primers previously designed and used in the same species (Reichert et al., 2015a), with a BRYT Green fluorescent probe (GoTaq qPCR Master Mix, Promega). The samples were

amplified on a 384 wells thermocycler (CFX-384, BioRad Hercules), in a final volume of 10 µl containing 200 nmol l^{-1} forward and reverse primers (0.4 µl of each diluted at 5 µmol l^{-1}), 5 µl SYBR Green PCR mix (Promega), 2 µl pre-prepared diluted DNA (i.e. 2 ng) and 2.2 µl sterilised distilled water. The amplification conditions were set as 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s for GAPDH. The conditions for the telomere sequence amplification were set at 95°C for 2 min followed by 30 cycles of 15 s at 95°C, 30 s at 56°C and 1 min at 72°C. Each amplification ended by a melting curve to check for non-specific signals. On each plate, a dilution curve was run, using a randomly chosen sample serially diluted from 20 to 1.25 ng $µl^{-1}$, to establish the efficiency of the qPCR amplification. Because amplification temperatures were different for the telomere and the control gene (56°C and 60°C), the runs were conducted on different plates. Thus, our samples were measured (in duplicate) using 2 runs of 2 plates (telomere and control gene). Efficiency of the qPCR was 99.3 and 100.3% (telomere) and 100.6 and 99.1% (control gene). TL was finally calculated as described in Pfaffl (2001) and expressed as the T/S ratio (roughly the ratio between telomere and control gene amplifications, corrected for amplification efficiencies, thereafter z-transformed, see below). Intra-class coefficients for T/S ratio estimating intra-run repeatability was 0.989, and 0.901 for inter-run repeatability (based on 10 repeated samples over the 2 runs).

Statistical analysis

For adult females, we used generalized linear mixed models (using female ID as random factor) to test how TL was influenced over (a) the egg production period (T1 as initial length and T2 as final length); (b) the chick rearing period (T2 to T3); and (c) then we verified how this is corroborated by statistical analysis over the entire reproduction period (T1 to T3). Repeated female TL values (at T1, T2 and T3) were used as response variable. As fixed factors, time (T1, T2, T3), clutch size or brood size at hatching and fledgling, and female (maternal) treatment (LPS injection) were used, as well as the interaction term Time×LPS, which was kept in the final models since we were interested in the timing of the putative effect of maternal treatment on telomeres. Using the methods of Simons et al. (2014), we distinguished telomere elongation from measurement error within our longitudinal data.

Resemblance of mother–offspring TLs was tested using a generalized linear mixed model with TL of chicks as the response variable and mother TL, chick sex and the interaction as fixed factors, and nest ID as a random factor. Models were successfully run to test the mother–offspring TL relationship at different ages (mother TL at egg laying, end of chick rearing, chick TL at day 26, day 82) and within experimental groups (control, LPS, all groups together).

For chicks, we used a generalized linear mixed model to test for the influence of maternal LPS treatment and testosterone treatment (testosterone) on TL at fledging (day 26, nest identity was included as a random factor to control for chicks raised in the same nest), or on TLs measured at fledging and at sexual maturity (day 26, day 82). We kept these two separate analyses in the study since we were interested in the effect of our experimental treatments on TL during growth and during sexual maturity. In this case, nest identity was included as a random factor as well as chick age (day 26, day 82), but none of the age interactions were kept in the final model since none were showing strong effects and also to reduce the number of fixed factors included, owing to sample size constrains. The following fixed factors were included: chick sex, brood size at fledging, growth rate in body mass (g day^{-1}), maternal treatment (LPS) and testosterone treatment (T). Growth rate, brood size at fledging (model at fledging) and the interaction terms concerning LPS×T×Chick sex (model until adulthood) were conserved in the final models, since we were looking for synergistic effects between treatments and we knew that males from LPS mothers were bigger at 30 days (Torres et al., 2019).

All variables were z-transformed beforehand (Verhulst, 2019), analyses were performed on R v.3.5.1, and standardized marginal effects plot with 95% confidence intervals were obtained using the 'sjPlot' package in R (https://CRAN.R-project.org/package=sjPlot). Maternal investment was evaluated by computing a principal component analysis (PCA) using clutch size, brood size at hatching and brood size at fledging (Fig. S2) to get independent variables on maternal investment in reproduction that can be used in the mixed model testing variation in female TLs over the entire reproduction period (i.e. that encompasses egg production and chick rearing). We checked for variance inflation factor (VIFs; Zuur et al., 2010) when using maternal investment in egg production (PCA2) and chick rearing (PCA1) in the same model. Results were interpreted based on significance ($P<0.05$) and on effect sizes and their 95% intervals using benchmarks proposed by Nakagawa and Cuthill (2007) ($r=0.1$, 0.3, 0.5 correspond to small, medium and large effects, respectively). We present models containing more explanatory variables with the lower AICc values.

RESULTS

Telomere length in adult females as a cost of reproduction

TL in adult females at egg laying (T1) was found to be negatively affected by clutch size and LPS treatment, the interaction Clutch size×LPS having an effect size of 0.66 (Table 2A). Regression analysis by treatment suggested that clutch size explained TL variation in control females ($r^2=0.45$, estimates: −1.483±0.492, $t=-0.381$, $P=0.012$) but not in LPS females ($r^2=0.01$, estimates: −0.180±0.474, $t=-0.381$, $P=0.711$). A similar trend was found for changes in TL of females over the egg production period (measured at T1 and T2; Table 2B, Fig. 1A). Again, the Clutch size×LPS interaction had an effect size=0.45, underlying that clutch size was negatively related to female TLs in control ($r^2=0.18$, estimates: −1.048±0.357, $t=-2.937$, $P=0.006$), but not in LPS females ($r^2=0.01$, estimates: −0.176±0.317, $t=-0.557$, $P=0.582$). Time had a small effect size, i.e. no significant changes in TLs were observed over the egg production period in adult females.

For female telomere dynamics during the chick rearing period (i.e. from the egg stage to day 25 post-hatching), females challenged with LPS before pairing lost significantly less TL during the rearing period than control females (Table 2C; Time×LPS, $r^2=0.05$, estimates: −0.340±0.276, $t=-1.233$, $P=0.228$; control females, $r^2=0.314$, estimates: −1.157±0.343, $t=-3.379$, $P=0.002$) and had on average shorter telomeres. Change in TL (T2–T3) was found to be positive in LPS females (Fig. 1B) although lengthening was not significant (comparison of variance test, $F=0.935$, $P=0.795$). Results are consistent when controlling for clutch size instead of brood size at fledging (LPS, $P=0.015$; Time×LPS, $P=0.010$). Again, time had a small impact per se on adult female change in TL (Table 2C).

Considering telomere dynamics over the whole reproduction period (egg production plus chick rearing periods, T1–T3), laying and rearing investments had the largest effects on female TL, both being negatively related to TL (Table 2D). While effects of female treatment were not significant, there was still a moderate effect size of the LPS×Rearing investment, but regressions were non-significant in LPS ($r^2=0.02$, estimates: −0.139±0.156, $t=-0.886$,

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Table 2. Output of the linear model and generalized linear mixed models testing the effects at egg laying and of egg production, chick rearing and reproduction investment in both periods on adult female telomere lengths

Predictors	Effect size	CI 95%	*P*-value
A. Mother TL at egg laying			
Intercept	**0.22**	**−0.30 to 0.75**	**0.020**
Clutch size	**−0.80**	**−1.39 to −0.21**	**0.015**
LPS treatment (LPS)	**−0.58**	**−1.13 to −0.03**	**0.050**
Clutch size×LPS	0.66	−0.04 to 1.36	0.078
Observations	28		
Marginal/conditional R^2	0.265/–		
B. Mother telomere changes over egg production at T1 and T2			
Intercept	0.12	−0.26 to 0.49	0.052
Clutch size	**−0.57**	**−1.00 to −0.13**	**0.017**
LPS treatment (LPS)	−0.35	−0.75 to −0.05	0.099
Time	−0.09	−0.34 to 0.15	0.459
Clutch size×LPS	0.45	−0.05 to 0.96	0.092
Random effects σ^2	1.09		
T_{00}	0.09		
ICC	0.08		
N_{id}	32		
Observations	58		
Marginal/conditional R^2	0.121/0.188		
C. Mother telomere changes over chick rearing at T2 and T3			
Intercept	−0.07	−0.52 to 0.38	0.230
Brood size	0.01	−0.30 to 0.32	0.957
LPS treatment (LPS)	**−1.30**	**−2.29 to −0.30**	**0.016**
Time	−0.20	−0.47 to 0.07	0.153
Time×LPS	**0.66**	**0.44 to 2.41**	**0.009**
Random effects σ^2	0.38		
T_{00}	0.43		
ICC	0.53		
N_{id}	32		
Observations	57		
Marginal/conditional R^2	0.077/0.566		
D. Mother telomere changes over reproduction at T1 and T3			
Intercept	0.00	−0.32 to 0.32	0.057
LPS treatment (LPS)	−0.30	−0.71 to −0.08	0.170
Time	−0.01	−0.20 to 0.18	0.915
Laying investment	**−0.39**	**−0.70 to −0.08**	**0.020**
Rearing investment	**−0.52**	**−0.92 to −0.12**	**0.018**
LPS×Laying investment	0.17	−0.13 to 0.46	0.281
LPS×Rearing investment	0.46	−0.06 to 0.47	0.094
Random effects σ^2	0.86		
T_{00}	0.08		
ICC	0.08		
N_{id}	32		
Observations	89		
Marginal/conditional R^2	0.125/0.197		

Females belong either to the control (sham injected, n=12) or LPS-challenged (n=13) group which was conducted before the formation of the breeding pairs. TLs were measured before the LPS challenge (T1), when the first egg was laid (T2) and at the end of chick growth 21 days after hatching (T3), and are expressed as z-transformed T/S ratio corrected for the regression to the mean effect. Laying and rearing investments are independent variables obtained from a principal component analysis using clutch size at laying (PC2, Laying investment), and brood sizes at hatching and at fledging (PC1, Rearing investment). Random effects are described using σ^2 and T_{00} statistics allowing evaluation of the intraclass correlation coefficient of the random factor (ICC). N_{id} indicates the number of levels for the random factor and the number of observations are also indicated. Marginal R^2 only includes the fixed factors variance while the conditional R^2 takes fixed and random factors into account. While some ICC were close to 0 (similar within and between random factor variance), mixed modelling was always presented because conditional R^2 was always higher than marginal R^2.

P=0.380) or control females (r^2=0.07, estimates: −0.468±0.279, t=−1.679, P=0.101, Fig. 1C). Time and LPS×Laying investment were not found to have important effects on TLs (Table 2C).

Mother–chick telomere length resemblance

A significant correlation was found only when mother and chick TLs were compared at the fledging stage and in the control group (13 females, 19 chicks; all other associations: 0.445>P>0.846). There was a chick sex effect {males having shorter telomeres than females, estimates −1.11±0.46, t=−2.43, P=0.028; effect sizes: −0.86 (CI_{95} [−1.12, −0.12])} and the interaction term Chick sex×Mother TL was significant {estimates −0.77±0.36, t=−2.15, P=0.048; effect sizes: −0.62 (CI_{95} [−1.65, −0.08])}. This suggests that adult females with longer telomeres at fledging raised female offspring with longer telomeres (linear regression, estimates 0.55±0.03, t=16.04, P<0.001), while the relationship was non-significant for males (estimates −0.21±0.22, t=−0.93, P=0.373, Fig. 2). There was a marginally significant positive effect of mother TL: estimates 0.56±0.30, t=1.86, P=0.083, random effect nest ID, 0.010±0.098. This denotes a large effect of mother TL on fledging TL {effect sizes: 0.75 (CI_{95} [−0.04, 1.54])}. There was no significant effect of brood size at fledging with a moderate positive effect size on chick TL {estimates 0.38±0.13, t=1.86, P=0.088; effect sizes: 0.38 (CI_{95} [−0.02, 0.50])}.

Telomere dynamics during growth in chicks

Effects of yolk testosterone and maternally derived antibodies on fledging TL

At day 25, the largest effects on fledging TL were those of LPS treatment, the LPS×Chick sex interaction and testosterone treatment (Table 3). Testosterone treatment (T) had a small negative effect on fledging TL but the interaction with sex was moderate and positive. The three-way interaction LPS×T×Chick sex was large and negative: testosterone egg injection had a positive impact on TL of fledglings but in females in the LPS group and in males in the control group (Fig. 3A).

Effects of egg and LPS treatments on telomere dynamics to sexual maturity

There was a significant but small negative effect of age on offspring TL from hatching to adulthood (Table 4). Additional factors had effects of similar small negative impact while being non-significant: offspring sex (males having shorter telomeres), and testosterone treatment. LPS treatment had a stronger, moderate negative effect, as did the interaction terms with Sex and Testosterone treatment (LPS×Sex, LPS×T). There was a moderate positive effect of the T×Sex. The three-way interaction term LPS×T×Chick sex had a medium negative effect on offspring telomeres from fledging to adulthood: female offspring from testosterone-treated eggs produced by LPS-treated mothers tended to have longer telomeres than those hatched from control eggs from LPS-treated mothers; in males, offspring from testosterone-treated eggs and control mothers had longer telomeres than those from testosterone-treated eggs produced by LPS-treated mothers (Fig. 3B).

DISCUSSION

Here, we investigated effects of reproduction on adult telomere dynamics, the relationship between mother and offspring TL, and effects of growth and maturation on chick telomere dynamics using an experimental approach where we manipulated maternal immune

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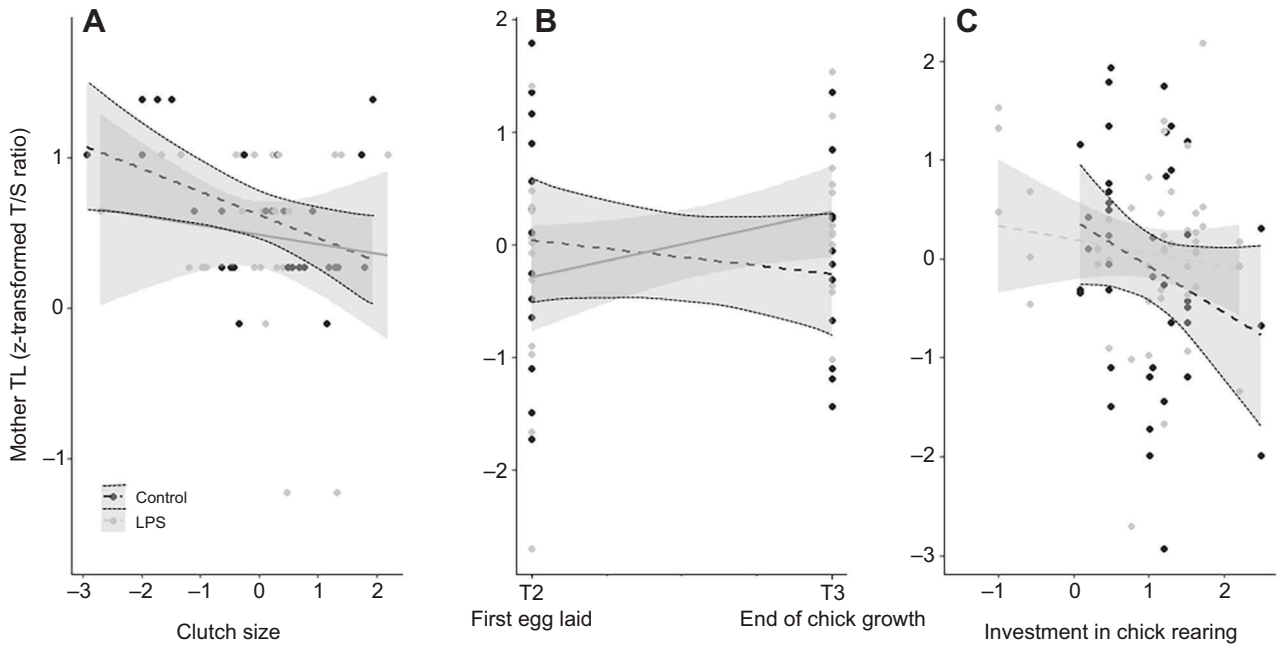

Fig. 1. Change in telomere length of adult female zebra finches after lipopolysaccharide treatment. Telomere length (TL) was assessed in birds treated with lipopolysaccharide (LPS) or in control birds before pairing. TL is shown as a function of (A) egg production period in relation to clutch size; (B) over the chick rearing period (T2 and T3); (C) the whole reproduction period in relation to investment in chick rearing (PCA1 axis including brood sizes at hatching and at fledging, see statistics). Positive changes were not found to reflect true telomere lengthening and regressions were not significant in C (see Results). TL is expressed as z-transformed T/S ratio and 0 is then an individual value equal to the mean population value.

function at laying, and MAb and testosterone *in ovo* (Torres et al., 2019). We found that production of large clutches and rearing chicks was generally associated with a cost of telomere loss in adult females, but this relationship was weaker in LPS-treated females. Adult females with longer telomeres raised female offspring with longer telomeres at fledging, but TL of male offspring was independent of maternal TL. In chicks, female offspring from testosterone-treated eggs produced by LPS-treated mothers tended to have longer telomeres than those hatched from control-eggs and produced by LPS-treated mothers whereas in males, offspring from testosterone-treated eggs and control mothers had longer telomeres than those from testosterone-treated eggs produced by LPS-treated mothers.

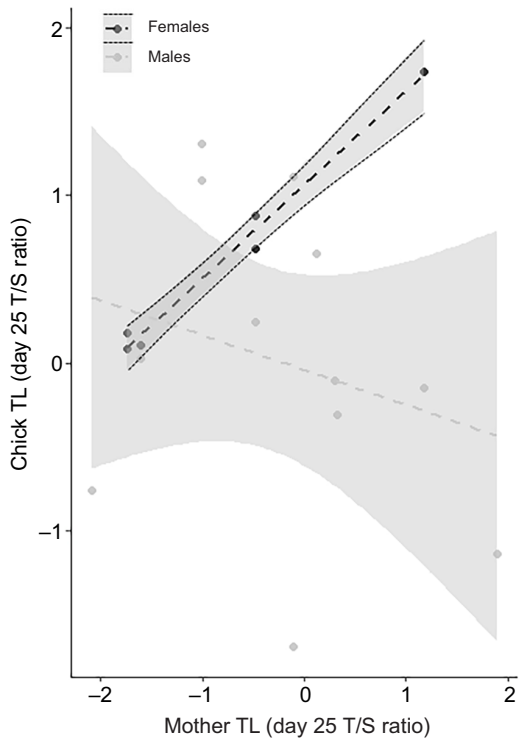

Fig. 2. Mother–offspring linear relationships of z-transformed telomere lengths (T/S ratio) measured at the fledging stage. Only adult females from the control were taken into account, which encompassed 13 females and 19 chicks (6 females and 13 males).

Table 3. Output of the generalized linear mixed model testing the effects of female immune challenge before pairing (LPS treatment) and egg testosterone injection on chick telomere length at fledging (day 25)

Predictors	Effect size	CI 95%	*P*-value
Intercept	0.65	−0.18 to 1.47	0.125
LPS treatment (LPS)	**−0.87**	**−1.59 to −0.15**	**0.021**
Testosterone treatment (T)	−0.26	−0.78 to 0.27	0.343
Sex	−0.73	−1.68 to 0.22	0.136
Growth rate	0.22	−0.04 to 0.48	0.292
Brood size at fledging	−0.14	−0.40 to 0.12	0.114
LPS×T	0.65	−0.14 to 1.45	0.112
LPS×Sex	**0.79**	**0.06 to 1.52**	**0.038**
T×Sex	0.47	−0.15 to 1.08	0.141
LPS×T×Sex	−0.68	−1.46 to 0.10	0.095
Random effects σ^2	0.92		
T_{00}	0.08		
ICC	0.08		
N_{id}	31		
Observations	68		
Marginal/conditional R^2	0.139/0.208		

The statistical model accounts for growth rate of chicks, calculated as body mass gain over the 25 days post-hatching (g day^{-1}), and brood size at fledging. Nest ID was used as random effect. TL is expressed as z-transformed TS ratio. See Table 2 for Random effects description.

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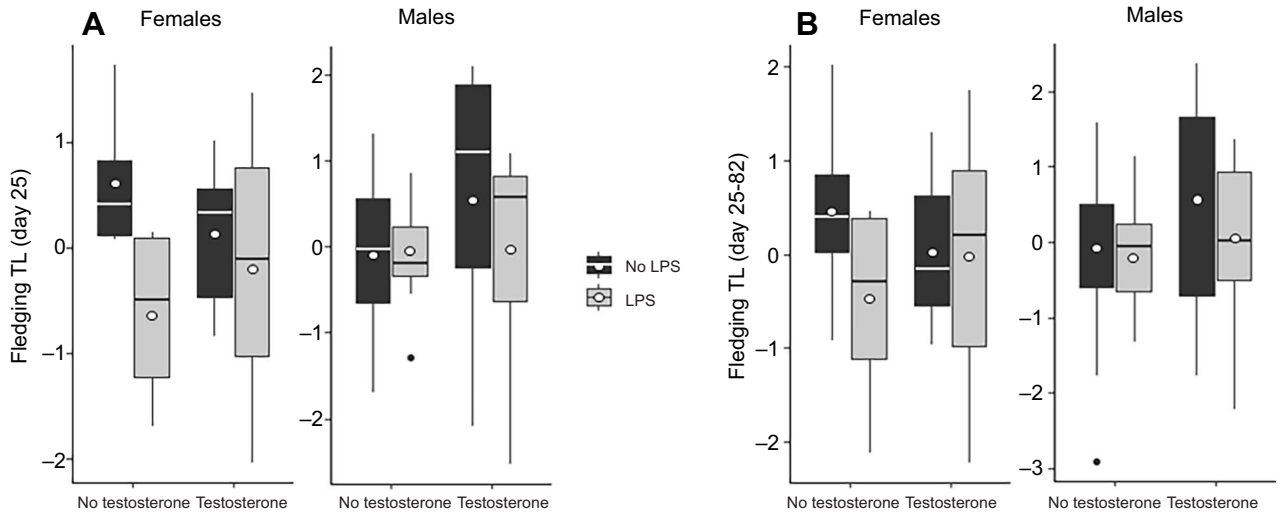

Fig. 3. Box plot of mixed model predicted values of fledging telomere length after egg (testosterone injection) and mother (LPS challenge) experimental treatments. (A) TL at fledging (day 25). (B) Fledgling TL (repeated values at day 25 and 82, sexually mature adults). Results are separated by chick sex. Mean values are represented by open circles. Error bars show s.e.m. TLs are expressed as z-transformed TS ratio and 0 is then an individual value equal to the mean population value. Boxes indicate the interquartile range distribution of the data, plus the largest and smallest value within 1.5 times the interquartile range.

Adult female telomere shortening as a cost of reproduction

Telomeres are hypothesized to shorten when individuals undergo energy-demanding activities (reviewed in Angelier et al., 2019) and in particular reproductive activities (Bichet et al., 2020; Reichert et al., 2014; Sudyka et al., 2019). There is a global consensus that higher reproductive success is achieved at the expense of an accelerated attrition of parental telomeres, both from longitudinal (Bauch et al., 2013; Sudyka et al., 2019) and experimental (e.g. Reichert et al., 2014) studies. However, of seven studies that have experimentally tested the deleterious impact of reproduction on telomeres in vertebrates and invertebrates, only four confirmed that reproduction decreased TL (Sudyka, 2019). This suggests either that parents may escape telomere loss as a cost of reproduction in certain conditions or it may be that only specific stages of the reproduction are costly for the parents. For instance, in common terns, telomere erosion is observed in parents only if their chicks do not die before the age of 10 days (Bauch et al., 2013; Bichet et al., 2020). The negative relationship we report between clutch size and female TL at laying, between TL of females over the reproduction period and laying/rearing investments confirm that reproduction shortens adult telomeres in zebra finches. We have no direct evidence for the mechanisms involved, and our observation might involve two non-exclusive explanations, i.e. energy trade-offs and/or deleterious reproductive or stress hormones effects. Indeed, corticosterone levels were observed to be higher in common terns with higher reproductive success but also shorter telomeres (Bauch et al., 2016). Interestingly, our study suggests that females immune challenged before laying (LPS treated) did not shorten TL during reproduction as much as control females did, while LPS-females did not produce lighter chicks (i.e. without apparently decreasing their reproductive investment). There may be two nonexclusive explanations. (1) We cannot exclude that LPS-treated females actually decreased their parental investment, protecting TL, which could have been compensated by males. In fact, only LPS females showed negative values of investment in chick rearing. A sex-specific telomere cost of chick rearing for the parental sex most concerned by chick provisioning was previously observed in male common terns (Bauch et al., 2016). (2) LPS females may have benefited from an energy trade-off biased towards body maintenance because of the activation of the immune activity prior to egg production. Following the concept of the hormetic response, a short term (i.e. weeks) adaptation to inflammation could be the enhancement of antioxidant defences (Costantini, 2014). While the hypothesis that oxidative stress shortens telomeres *in vivo* is still debated (Boonekamp et al., 2017; Reichert and Stier, 2017), a recent experimental supplementation of tocopherol and selenium in white stork (*Ciconia ciconia*) chicks suggested a telomere protective role of antioxidants (Bichet et al., 2020). Additional cell mechanisms involved in the mitigation of deleterious effects of inflammation, like DNA repair (Calvo et al., 2012), may also have contributed to the reduced telomere loss in our LPS-treated females.

Table 4. Output of the generalized linear mixed model testing the effects of female immune challenge before pairing (LPS treatment) and egg testosterone injection on chick telomere lengths measured at fledging (day 25) and adult maturity (day 82)

Predictors	Effect size	CI 95%	*P* value
Intercept	**0.42**	**−0.19 to −1.04**	**0.001**
Time	**−0.29**	**−0.43 to −0.15**	**<0.001**
Sex	−0.26	−0.63 to 0.11	0.180
LPS treatment (LPS)	−0.47	−0.95 to 0.01	0.058
Testosterone treatment (T)	−0.20	−0.61 to 0.20	0.331
LPS×Sex	0.39	−0.12 to 0.91	0.137
T×Sex	0.43	−0.04 to 0.90	0.080
LPS×T	0.40	−0.15 to 0.95	0.154
LPS×T×Sex	−0.46	−1.02 to 0.09	0.107
Random effects σ^2	0.64		
T_{00}	0.26		
ICC	0.29		
N_{id}	75		
Observations	137		
Marginal/conditional R^2	0.131/0.385		

The statistical model accounts for chick sex. LPS and T treatment refer to former mother challenge with LPS and testosterone or sham injections in the egg. Chick ID was used as random effect. Telomere change and length are expressed as z-transformed TS ratio. See Table 2 for Random effects description. Values in bold indicate significant effects.

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Relationship between mother and offspring TLs

The mode of inheritance of TL and the relative importance of genetic versus environmental determinants remains a hotly debated topic in telomere research (Dugdale and Richardson, 2018). The genetic basis of offspring TL results from intertwined effects of variation in TL of parental gametes, re-setting of the TL in the zygote at fertilisation, effects of parental (most often paternal) age and epigenetic or epigenetic-like inheritance (Bauch et al., 2019; Eisenberg, 2019; Entringer et al., 2018). In birds, TL inheritance was first believed to involve stronger maternal than paternal inheritance (Horn et al., 2011; Reichert et al., 2015b), possibly related to a z-linked mechanism. However, accumulated data so far has questioned this sex-specific inheritance in birds (Atema et al., 2015; Becker et al., 2015) or lizards (Olsson et al., 2011a). Additionally, environmental factors have additive effects on the resemblance of TL in parents and their offspring (e.g. Becker et al., 2015; Voillemot et al., 2012; but see Belmaker et al., 2019). For instance in king penguins (*Aptenodytes patagonicus*), offspring TL initially showed a large maternal influence (10 days after hatching), which eroded over the growing period (Reichert et al., 2015b). We found a positive relationship between mother–offspring TL but only at day 26 post-hatching. This is consistent with previous results in birds (Belmaker et al., 2019; Horn et al., 2011; Reichert et al., 2015b), although we cannot definitively test for genetic effects in the absence of paternal TL data, and the lack of relationship at sexual maturation (day 82) also suggests additional environmental effects. However, our data suggest a chick sex effect, with a positive relationship between maternal TL and that of female, but not male, offspring. Whether there is sex-specific selection for longer telomeres in female zebra finches as it has been observed in other vertebrates (Olsson et al., 2011b) is an open question. It is important to note that a previous study of telomere heritability conducted in captive zebra finches could not distinguish between maternal and paternal contributions to offspring TL but suggested high additive genetic variance (Atema et al., 2015).

Telomere length up to fledging in relation to growth, LPS and testosterone treatment

Overall, our study showed that the experimental treatment of reproductive females (LPS challenge prior to egg production) and of eggs (testosterone injection) modulated TL of chicks, with a larger effect of LPS. More importantly, we found a large effect size for the interaction of both treatments. We found that chick TL at the end of growth (day 26) was weakly affected by testosterone treatment, but largely negatively affected by the LPS treatment of reproductive females, i.e. chicks from eggs produced by LPS females had shorter telomeres. Since those chicks were not lighter than control chicks at that stage, it is unlikely that a sub-optimal parental feeding and a stronger growth–soma energy-based trade-off could explain this. Indeed, a causal relationship between higher growth rate and shorter telomeres in LPS chicks would be supported if LPS chicks were also characterized by a higher growth rate over the rearing period. Such an increased growth rate was found but only in male chicks (Torres et al., 2019) and a negative link between growth rate and TL was observed in female chicks in our current study. Therefore, a non-energy-related explanation may be more suitable to explain our sex-specific pattern of TL variation in chicks in relation to maternal LPS treatment. Since we are measuring chick TL in blood cells (including a small proportion of white blood cells), MAb derived from LPS treatment may have prematurely triggered the activation of the chicks' immune system accelerating telomere erosion of white blood cells (i.e. increased cell division rate). This may have led to variation in the ratio of white to red blood cells, which may account for LPS-induced changes in mean relative TL, owing to either large differences in TLs among white versus red blood cell populations (Olsson et al., 2020) or to a higher division rate of white blood cells leading to faster telomere shortening. Such a pre-activation of the chick immune system (i.e. in anticipation of infection) primed by LPS-induced antibodies from mothers and involving macrophages may also be associated with increased immune-derived oxidative stress (Emre et al., 2007), which is also putatively deleterious for telomeres. An alternative explanation is that the observed pattern is due to the effect of social stress on chick telomeres, following a change in maternal behaviour, as LPS immunisation may decrease nestling feeding activity by the mother (Bonneaud et al., 2003). Such a stressful environment due to increased nestling competition is known to accentuate telomere shortening in starlings (Nettle et al., 2015). Both MAb and nestling stress explanations match with short-term effects of LPS on fledging TL.

Telomere length to sexual maturity and antagonistic effects of LPS and testosterone treatment

We found some evidence for antagonistic effects of LPS-induced MAb and testosterone treatment on chick telomeres at both stages of development either when considering TL at day 26 or as a repeated variable (day 25 and day 82). This suggests that crosstalk may exist between these two components in birds (Tobler et al., 2010). Since those effects were similar at fledging (at day 26) and at adulthood (day 82), it suggests that the impact of our experimental treatments during the main growth period had persistent impacts on chick telomeres, i.e. they were not subsequently modulated during sexual maturation. Interestingly, we observed a sex-specific impact of testosterone treatment on fledging telomeres in relation to the mother's LPS exposure before egg laying. Testosterone mitigated the negative effect of LPS treatment observed in female fledglings but also negatively affected TL of female offspring hatched from control eggs, whereas it had a positive effect on TL in male offspring hatched from control eggs and no effects on LPS-treated hatched males. We currently have no explanation for these results but they suggest potentially very complex interactions between maternal effects, sex and telomere dynamics.

Evidence for synergistic/antagonistic effects of LPS and yolk testosterone treatments on telomere dynamics contrast with the general lack of interaction effects on chick growth and other phenotypic traits reported previously (Torres et al., 2019). The only correspondence between our two sister studies concerns the phenotype and TL of male offspring raised by control mothers and hatched from testosterone-injected eggs: they presented both longer telomeres and a higher breeding quality (Ardia et al., 2010), which matches well with the telomere quality hypothesis (Angelier et al., 2019): individuals with longer telomeres are those that may ensure more efficient body maintenance but also have the highest breeding success. Whether testosterone allows chicks to divert energy from costly physiological traits (immunity) towards higher investment in reproduction or simply improves the resilience of individuals to stress (social stress) need to be tested. Since pre-natal exposure to testosterone has been shown to induce an oxidative cost in male zebra finch nestlings (Tobler and Sandell, 2008), the mitigating effect of testosterone on telomeres may not be mediated by an improved oxidative balance in our birds, but rather from better access to food resources (e.g. due to higher begging activity in testosterone male chicks; von Engelhardt et al., 2006) and/or an indirect social effect such as dominance.

However, the fact that telomere dynamics are maintained from fledging to adult sexual maturity also suggests that individual differences in TL are established early in life. It would be interesting to test how our treatments are modulating the TL response of our individuals during their future reproduction or whenever they face stressful events.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.T., T.D.W.; Methodology: F.C., R.T., S.Z., T.D.W.; Formal analysis: F.C.; Resources: T.D.W.; Data curation: T.D.W.; Writing - original draft: F.C., T.D.W.; Writing - review & editing: F.C., R.T., T.D.W.; Project administration: R.T., T.D.W.; Funding acquisition: F.C., R.T., T.D.W.

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Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.232496.supplemental

Data availability
Data are available in Figshare at: https://figshare.com/s/47cc7823d5d998794644.

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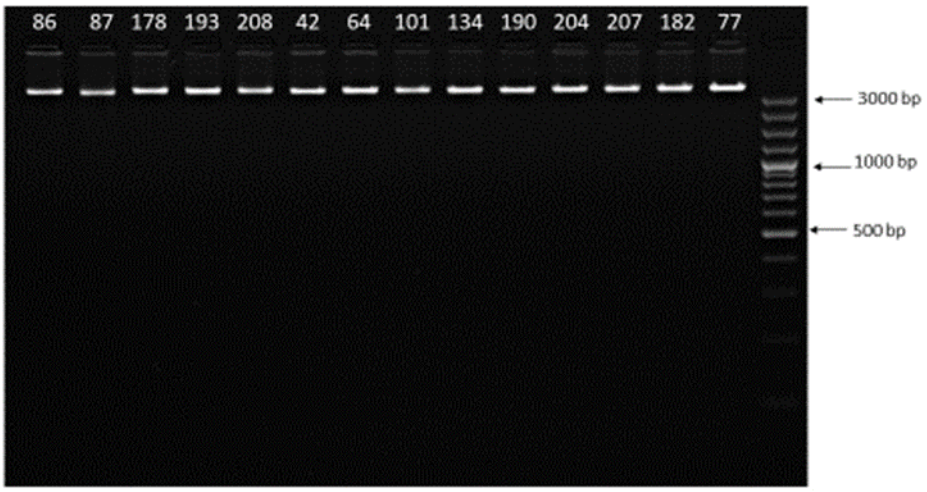

Figure S1: Digital photography of genomic DNA extracted from red blood cells of zebra finches (14 randomly chosen individuals) and exposed to UV light. The electrophoresis was conducted on an agarose gel (0.7%), at 2.5 mV/cm in 45mM Tris-borate, 1mM EDTA medium. The DNA size marker is indicated on the left. The absence of DNA smir indicated that the genomic DNA was not degraded after extraction.

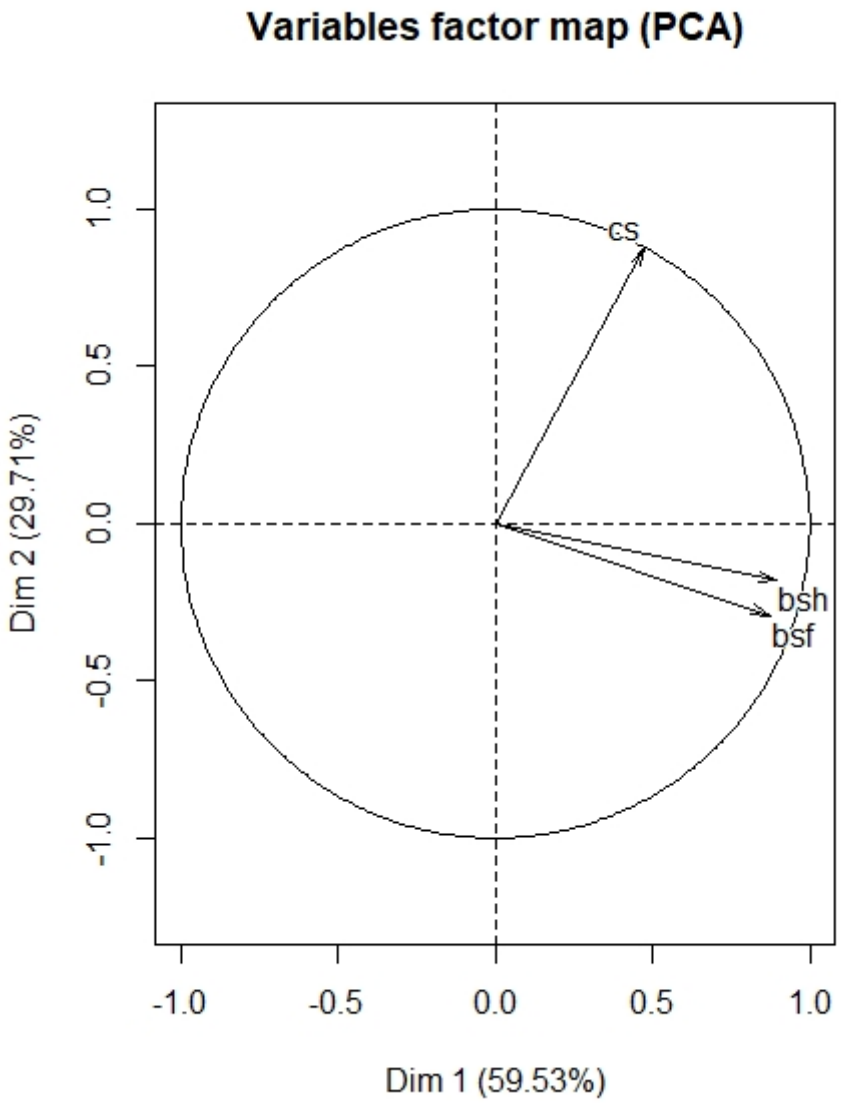

Figure S2: Principal component analysis conducted on clutch size, brood size at hatching and at fledging of 32 females zebra finches. Two axes were produced, explaining 89% of the total variance. PC1 axis (eigenvalue, 1.79) was positively loaded with brood size at hatching (0.90) and fledging (0.88), while PCA2 (0.89) only with clutch size (0.88).