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 "multivariate". Multivariate effects on offspring phenotype were evaluated in a sister study on captive zebra finches, by simultaneously manipulating maternally derived antibodies after LPS-treatment of mothers and egg-treatment using yolk testosterone injection. LPS-treatment had a positive effect on body mass growth at 30 days after hatching and immune response at sexual maturity, while egg-testosterone positively influenced immune response at fledging and courtship behaviour in sexually mature male offspring. Maternal effects are known to modulate offspring telomere length. Still, the multivariate effects of egg-derived maternal components on offspring telomere dynamics from hatching to sexual maturity are undefined. Here, using the data of the sister study completed with telomere measurements, we tested a) the effects of LPS and T treatments on telomere length (TL) from hatching to sexual maturity (day 82), b) how LPS treatment modulated telomere length over reproduction in adult females, and c) the relationship between maternal and offspring TL. We predicted, a) TL would be shorter in LPS fledglings (as a cost of faster growth), and b) TL would be longer in sexually mature adults from T-treated (as a proxy of individual quality). In adult females, there was an overall negative relationship between laying and rearing investments and telomere length, this relationship being weaker in LPS treated females. In chicks, there was an overall negative effect of LPS treatment on telomere length measured at fledging and sexual maturity (day 25 – 82). In addition, at fledging, there was a sex x LPS x T-treatment interaction, suggesting the existence of antagonistic effects of our treatments. Our data partially support the hypothesis of telomeres are proxies of individual quality and that individual differences in telomere length are set-up very early in life.

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 counter-balanced 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; Hoelzl et al., 2016b; 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 – ageing trade-off and its concomitant changes in telomere length have attracted extended interest (reviewed in (Monaghan and Ozanne, 2018)) because damaged or shortened telomeres are good candidates explaining 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, being predictive of future individual fitness (Heidinger et al., 2012; Marasco et al., 2019). Accordingly, telomere length 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 telomere length 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 telomere length (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 new born 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 Fox, 1998). For instance, pre-natal adjustments in yolk components like hormones should have additive effects on the genetic program 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 a 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 telomere length 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 telomere length of avian chicks: telomere length 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 telomere length at hatching (Stier et al., 2019) while 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 telomere length are part of the broader question of how telomere length is passed on to the next generation, *i.e.* how gene x environment interactions determine telomere inheritance (Dugdale and Richardson, 2018). Early studies suggested telomere length 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 parents / offspring telomere resemblance, like parental care-derived effects (Viblanc et al., 2020). Among those a role for epigenetic-like effects from paternal gametes have been recently underlined 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 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 *vs.* 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 telomere length 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 telomere length is only weakly affected by environmental stress or trade-offs over adult life (Bichet et al., 2020). As telomere length 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. (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 when 30 days-old (Torres et al., 2019). However, the immune response of chicks to a phytohemagglutinin test (PHA) was higher in chicks from T-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) mother's LPS challenge (*i.e.* being larger at day 30) and (ii) egg-testosterone injections, being of higher quality than control males at sexual maturity. Similarly, testosterone and LPS 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*) 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: a) changes in telomere length 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; b) the relationship of telomere length between parents (mothers) and offspring (chicks): we expect a positive relationship for mother-female offspring telomere length at least in the control groups, where inheritance has not been modulated by experimental treatments; c) effects of chick growth and age on telomere length in chicks from eggs where we manipulated both yolk T 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 (MAb) group. Based on the trade-off between growth and immune system maturation, chick and fledglings telomere lengths should be shorter in experimental groups,

except for males of the testosterone group that should have longer telomeres when reaching sexual maturity.

MATERIAL AND METHODS

Full details of our experimental protocol and sampling design are given in (Torres et al., 2019). Zebra finches were housed under controlled environmental conditions (temperature 19–23 °C; humidity 35–55%; constant light/dark schedule, 14L:10D, lights on at 07.00 AM) 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 of age (*i.e.* birds that had been paired or laid eggs previously) were randomly paired and housed in individual breeding cages (51 cm x 39 cm x 43 cm), each with an external nest box (14 cm x 14.5 cm x 20 cm). Breeding pairs had access to 6 g of egg food supplement (20.3% protein: 6.6% lipid) per day during paring, 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).

Experimental procedure

a. 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 of a lipopolysaccharide (LPS; *Escheriachia coli*, serotype 055:B5; Sigma) diluted in 0.1 mL of phosphate buffered saline solution (PBS, concentration = 0.1 mg/mL, 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 telomere length 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.

b. T-treatment. We used a split design manipulating yolk testosterone within clutches of LPS-treated and control females (*i.e*. producing high- and low-MAb eggs) using *in ovo* egg injection of T 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 T-treated group were injected with 500 pg of testosterone (T, Fluka) dissolved in 2 µl of 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 (ca. 10 minutes). Eggs in the control group were injected with 2 µl of sesame oil, but otherwise were treated in a similar way to eggs in the T-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 were recorded (± 0.01 mm) 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 telomere length of offspring and mothers, and effects of LPS/T-treatments and growth on chick telomere length, 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 Polymerase Reaction Chain (qPCR)

Telomere length was measured on DNA obtained from frozen red blood cells of adult females and chicks of zebra finch. 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 (ratios A260/280 and A 260/230 and by checking for DNA degradation after gel-migration on a sub-sample, see Electronic Supplementary Material 1) for qPCR amplification. DNA was then diluted using sterile distilled water at 5 ng/µL 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 nM of primers forward and reverse (0.4 µL of each diluted at 5 µM), 5 µL of SYBER Green PCR mix (Promega), 2 µL of pre-prepared diluted DNA (*i.e.* 2 ng) and 2.2 µL of sterilised distilled water. The amplification conditions were set as 95°C for 2 min, followed by 40 cycles of data collection at 95°C for 15 s, 56°C for 30 s and 72°C for 30 s for GAPDH. The conditions for the telomere sequences' amplification were set at 95°C for 2

min followed by 30 cycles of data collection for 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, 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 duplicates) using 2 runs of 2 plates (telomere and control gene). Efficiencies of the qPCR were of 99.3 and 100.3% (telomere) and 100.6 and 99.1% (control gene). Telomere length was finally calculated by (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 is of 0.989, and of 0.901 for inter-run repeatability (based on 10 repeated samples over the 2 runs).

Statistical analysis

We used a mixed model approach, as describe in the following steps:

(i) for adult females, we used generalized linear mixed models (using females ID as random factor) to test how telomere length was influenced over 1) the egg production period (T1 as initial length and T2 as final length), 2) the chick rearing period (T2 to T3) and then we verify how this is corroborated by statistical analysis over 3) the entire reproduction period (T1 to T3). Repeated female telomere length 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 x LPS-treatment 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.

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

(ii) for chicks, we used a generalized linear mixed model to test for the influence of maternal LPS treatment (MAb) and T-treatment (testosterone) on telomere length at fledging (day 26, Nest identity was included as a random factor to control for chicks raised in the same nest), or on telomere lengths measured at fledging and at sexual maturity (day 26 – day 82). We kept these two separated analyses in the manuscript since we were interested in the effect of our experimental treatments on telomere length during growth and during sexual maturity. In that 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 any were showing strong effects and also to reduce the number of fixed factors included, due to sample size constrains. The following fixed factors were included: chick sex, brood size at fledging, growth rate in body mass (g/day), maternal treatment (LPS) and T treatment (testosterone injection). Growth rate, brood size at fledging (model at fledging) and the interaction terms concerning maternal x T treatments x 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 (Lüdecke, 2017). Maternal investment was evaluated by computing a principal component analysis (PCA) using clutch size, brood size at hatching and brood size at fledging (see ESM) to get independent variables on maternal investment in reproduction that can be used in the mixed model testing variation in female telomere lengths 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 in the same model maternal investment in egg production (PCA2) and in chick rearing (PCA1). Results were interpreted based on significance (P value < 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). We present models containing more explanatory variables with the lower AICc values.

RESULTS

Telomere lengths of adult females as a cost of reproduction

Telomere length of adult females at egg laying (T1) was found to be negatively affected by clutch size and LPS treatment , the interaction Clutch size*LPS-treatment having an effect size of 0.66 (Table 2A). Regression analysis by treatments suggested that clutch size explained telomere length 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 telomere length of females over the egg production period (measured at T1 and T2, Table 2B, Figure 1A). Again, the Clutch size x LPS interaction had an effect size = 0.45, underlying that clutch size was negatively related to female telomere lengths 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 telomere lengths 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 telomere length during the rearing period than control females (Table 2C, Time x LPS treatment, 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 telomere length (T2 –T3) was even found to be positive in LPS females (Figure 1B) though 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 treatment, *P* = 0.015; Time x LPS treatment, *P* = 0.010). Again, Time had a small impact *per se* on adult females' change in telomere length (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 telomere length, both being negatively related to telomere length Table 2D). While effects of female treatment were not significant, there was still a moderate effect size of the LPS treatment x Rearing investment but regressions were non-significant in LPS (r^2 = 0.02, estimates: -0.139 ± 0.156, *t* = -0.886, P = 0.380) or control females (r^2 = 0.07, estimates: -0.468 ± 0.279, *t* = -1.679, P = 0.101, Figure 1C). Time and LPS treatment x Laying investment were not found to have important effects on telomere lengths (Table 2C).

Mother-chick telomere length resemblance

Correlation was found significant only when both telomere lengths 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 x 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, Figure 2). There was a marginally significant positive effect of mother telomere length: 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 telomere length on fledging telomere length (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 telomere length (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 T treatment (yolk testosterone) and LPS treatment (maternally derived antibodies (MAb)) on fledging TL

At day 25, the largest effects on fledging telomere length were those of LPS treatment, the LPS-treatment* offspring sex interaction and with T-treatment (Table 3). T-treatment had a small negative effect on fledging telomere length but the interaction with sex was moderate and positive. The three-way interaction LPS x T-treatment x sex was large and negative: testosterone egg injection had a positive impact on telomere length of fledgling but in females in the LPS group and in males in the control group (Figure 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 telomere length 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 T-

treatment. LPS treatment had a stronger, moderate negative effect, as did the interaction terms with sex and T-treatment (LPS treatment x sex, LPS x T treatments). There was a moderate positive effect of the T-treatment x Sex . The three-way interaction term LPS x T treatments x 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 produced by 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 (Figure 3B).

DISCUSSION

Here we investigated effects of reproduction on adult telomere dynamics, the relationship between mother-offspring telomere length, and effects of growth and maturation on chick telomere dynamics using an experimental approach where we manipulated maternal immune function at laying, and maternally-derived MAb and testosterone *in ovo* [31]. We found that producing large clutches and rearing chicks were 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 telomere length of male offspring was independent of maternal telomere length. 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.

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 telomere length (Sudyka, 2019). This suggests either that (i) parents may escape telomere loss as a cost of reproduction in certain conditions or (ii) 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 telomere length at laying, between telomere length of females over the reproduction period and laying/rearing investments confirm that reproduction shorten 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 / 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 telomere length over 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: (i) we cannot exclude that LPS-treated females actually decreased their parental investment, protecting telomere length, 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 males common terns (Bauch et al., 2016). (ii) 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 repairing (Calvo et al., 2012), may also have contributed to the reduced telomere loss in our LPS-females.

Relationship between mother and offspring telomere lengths

The mode of inheritance of telomere length 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 telomere length results from intertwined effects of variation in telomere length of parental gametes, re-setting of the telomere length 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, telomere length inheritance was first believed to involve stronger maternal than parental 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 in lizards (Olsson et al., 2011a). Additionally, environmental factors have additive effects on the parental-

offspring telomere length resemblance (*e.g.* (Becker et al., 2015; Voillemot et al., 2012) but see (Belmaker et al., 2019). For instance in king penguins (*Aptenodytes patagonicus*), offspring telomere length 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 telomere length 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), though we cannot definitively test for genetic effects in the absence of paternal telomere length 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 telomere length 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 telomere length but suggested high additive genetic variance (Atema et al., 2015).

Telomere length up to fledging in relation to growth, LPS and T-treatments

Overall, our study showed that the experimental treatment of reproductive females (LPS challenge prior to egg production) and of eggs (testosterone injection) modulated telomere length 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 telomere length at the end of growth (day 26) was weakly affected by T-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 telomere length 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 telomere length variation in chicks in relation to maternal LPS treatment. Since we are measuring chick telomere length 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 driven to variation in the ratio white/red blood cells which may account for LPS-induced changes in mean relative telomere length, either due to large differences in telomere lengths among white *vs.* 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-antibodies from mothers and involving macrophages may also be associated with increased immune-derived oxidative stress (Emre et al., 2007), putatively also 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 telomere length.

Telomere length to sexual maturity and antagonistic effects of LPS and T-treatments

We found some evidence for antagonistic effects of LPS-induced MAb and T-treatment on chick telomeres at both stages of development either when considering telomere length at

day 26 or as a repeated variable (day 25 – day 82). This suggests that cross talk 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 T-treatment on fledging telomeres in relation to the mother LPS exposure before egg laying. Testosterone mitigated the negative effect of LPS-treatment observed in female fledglings but also negatively affected telomere length of female offspring hatched from control eggs, while it had a positive effect on telomere length in male offspring hatched from control eggs and no effects on LPS-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 MAb and yolk T 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 telomere length 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 insure a 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. Because pre-natal exposure to testosterone has been shown to induce an oxidative cost in male nestling of zebra finch (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 like dominance. However, the fact that telomere dynamics are maintained from fledging to adult sexual maturity also suggests that individual differences in telomere length are established early in life. It would be interesting to test how our treatments are modulating the telomere length response of our individuals during their future reproduction or when they will face stressful events.

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COMPETING INTERESTS

The authors declare no competing of financial interests

AUTHORS' CONTRIBUTIONS

T.D.W. and R.T. collected the data and R.T. run all the physiological analyses, F.C. extracted the DNA and S.Z. ran the qPCR measurements of telomere length, S.Z. and F.C. analysed the qPCR data, F.C. and T.D.W. did the statistical analyses, and drafted the final manuscript on which R.T. made comments.

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DATA ACCESSIBILITY

Data will be deposited on Figshare once the paper is accepted.

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Figures

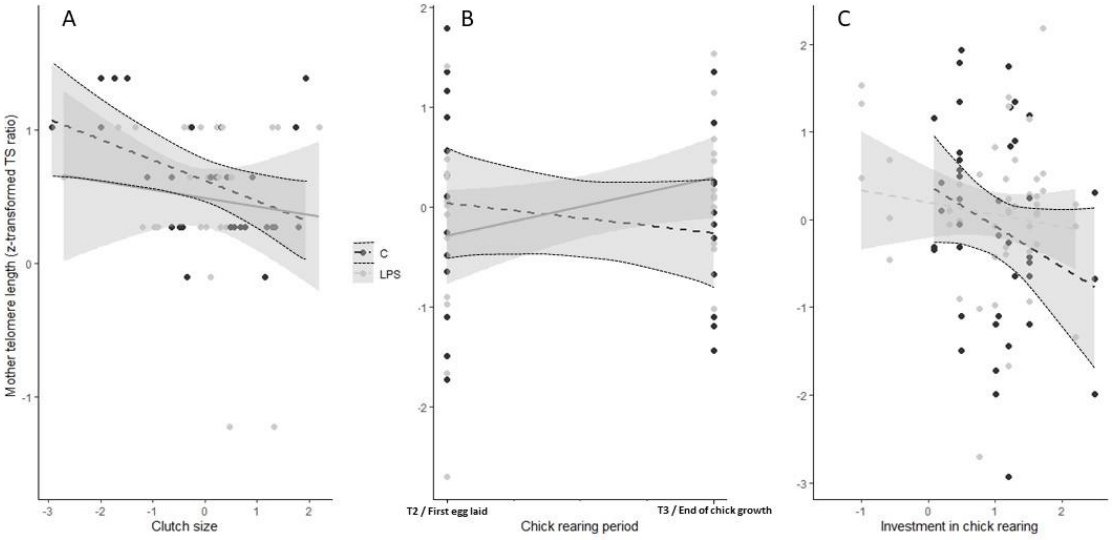


Figure 1: Change in telomere length of adult female zebra finches from the control or the experimental treatment (LPS challenge before pairing), during the **(A)** egg production period in relation to clutch size; **(B)** over the chick rearing period (T2 and T3 refer to times of sampling of adult females, see Experimental procedure); **(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 1C (see Results). Telomere length is expressed as z-transformed TS ratio and 0 is then an individual value equal to the mean population value.

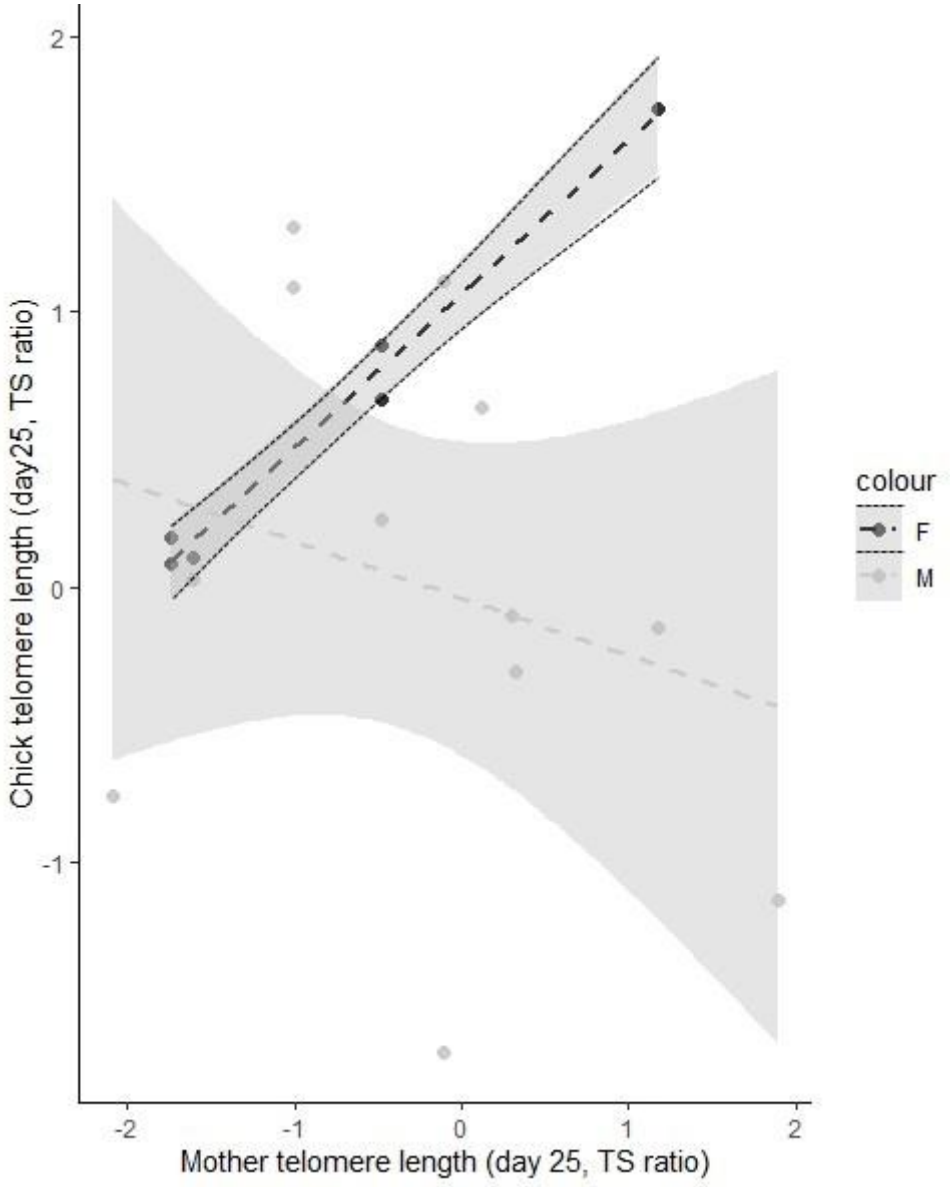


Figure 2: Mother-offspring linear relationships of z-transformed telomere lengths (T/S ratio) measured both at the fledging stage. Only adult females from the control were taken into account, which encompassed 13 females and 19 chicks (6 females and 13 males).

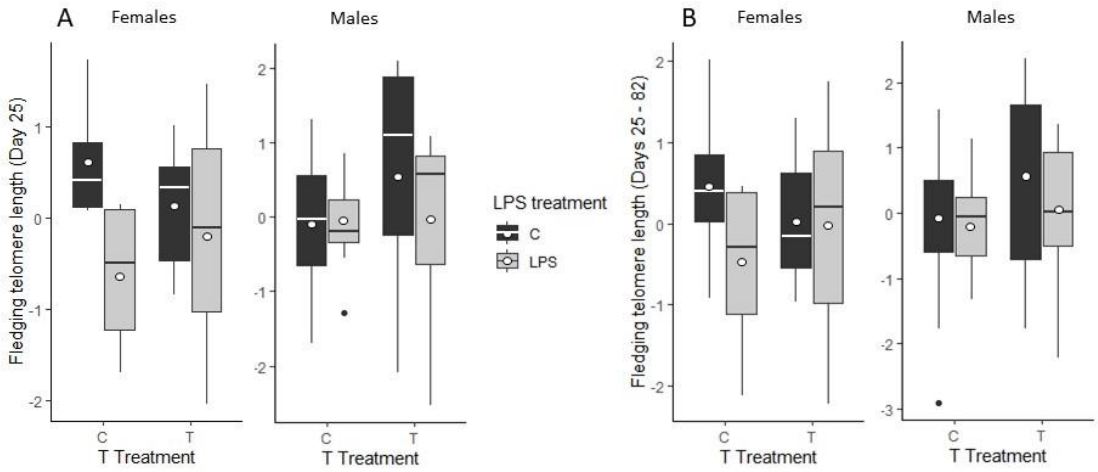


Figure 3: Box plot of mixed model predicted values of **(A)** fledging telomere length (day 25) and **(B)** fledging telomere lengths (repeated values at day 25 and 82, sexually mature adults) in relation to egg (testosterone injection) and mother (LPS challenge) experimental treatments. C indicates control groups for both treatments. Results are separated by chick sex. Means value ± SE are represented using open circles. Telomere lengths are expressed as z-transformed TS ratio and 0 is then an individual value equal to the mean population value.

Tables

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. 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.*, (Torres et al., 2019).

		Immature phenotype (day 26)			Young adult phenotype (day 82)		
Treatment	Sex	Growth	Immune function	Telomere length	Immune function	Adult quality	Telomere length (days 26-82)
Yolk testosterone (T)	M	0	+	-	0	+	+
	F	0	+	-	0	0	0
LPS-maternally derived antibodies (LPS)	M	+	0	-	+	0	-
	F	0	0	0	+	0	-
Interaction T x LPS	M	0	0	0	0	0	0
	F	0	0	0	0	0	0

Table 2. Output of the linear model (2A) and generalized linear mixed models (2B, C, D) testing the effects at egg laying (A) and of egg production (B, T1 and T2), chick rearing (C, T2 and T3) and reproduction investment in both periods (D, T1 and T3) on adult female telomere lengths. 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. Telomere lengths 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, and are expressed as z-transformed TS ratio and has been 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 to evaluate the intraclass correlation coefficient of the random factor (ICC). N_{id} indicate 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 as Conditional R^2 was always higher than Marginal R^2.

TABLE 2A. MOTHER TELOMERE LENGTH AT EGG LAYING			
Predictors	**Effect size**	**CI 95%**	**P value**
Intercept	**0.22**	**-0.30 – 0.75**	**0.020**
Clutch size	**-0.80**	**-1.39 – -0.21**	**0.015**
LPS treatment	**-0.58**	**-1.13 – -0.03**	**0.050**
Clutch size x LPS treatment	0.66	-0.04 – 1.36	0.078
Observations	*28*		
Marginal / Conditional R^2	*0.265 / -*		
TABLE 2B. MOTHER TELOMERE CHANGES OVER EGG PRODUCTION			

Predictors		**Effect size**	**CI 95%**	**P value**
Intercept		0.12	-0.26 – 0.49	0.052
Clutch size		**-0.57**	**-1.00 – -0.13**	**0.017**
LPS treatment		-0.35	-0.75 – -0.05	0.099
Time		-0.09	-0.34 – 0.15	0.459
Clutch size x LPS treatment		0.45	-0.05 – 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*		

TABLE 2C. MOTHER TELOMERE CHANGES OVER CHICK REARING

Predictors		**Effect size**	**CI 95%**	**P value**
Intercept		-0.07	-0.52 – 0.38	0.230
Brood size		0.01	-0.30 – 0.32	0.957
LPS treatment		**-1.30**	**-2.29 – -0.30**	**0.016**
Time		-0.20	-0.47 – 0.07	0.153
Time x LPS treatment		**0.66**	**0.44 – 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*		

TABLE 2D. MOTHER TELOMERE CHANGES OVER REPRODUCTION

Predictors	**Effect size**	**CI 95%**	**P value**
Intercept	0.00	-0.32 – 0.32	0.057
LPS treatment	-0.30	-0.71 – -	0.170

		0.08	
Time	-0.01	-0.20 – 0.18	0.915
Laying investment	**-0.39**	**-0.70 – -0.08**	**0.020**
Rearing investment	**-0.52**	**-0.92 – -0.12**	**0.018**
LPS treatment x Laying investment	0.17	-0.13 – 0.46	0.281
LPS treatment x Rearing investment	0.46	-0.06 – 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*		

Table 3. Output of the generalized linear mixed model testing the effects of female immune challenge before pairing (LPS treatment) and egg testosterone injection (T treatment) on chick telomere length at fledging (day 25). The statistical model account for growth rate of chicks, calculated as body mass gain over the 25 days post-hatching (g/day), and brood size at fledging. Nest ID was used as random effect. Telomere length is expressed as z-transformed TS ratio. See Table 2 for Random effects description.

TABLE 3. FLEDGING TELOMERE LENGTH			
Predictors	**Effect size**	**CI 95%**	**P value**
Intercept	0.65	-0.18 – 1.47	0.125
LPS treatment	**-0.87**	**-1.59 – -0.15**	**0.021**
T treatment	-0.26	-0.78 – 0.27	0.343
Sex	-0.73	-1.68 – 0.22	0.136
Growth rate	0.22	-0.04 – 0.48	0.292
Brood size at fledging	-0.14	-0.40 – 0.12	0.114
LPS treatment x T treatments	0.65	-0.14 – 1.45	0.112
LPS treatment x Sex	**0.79**	**0.06 – 1.52**	**0.038**
T treatment x Sex	0.47	-0.15 – 1.08	0.141
LPS treatment x T treatment x Sex	-0.68	-1.46 – 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*		

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

TABLE 4. CHICK TELOMERE LENGTH OVER EARLY LIFE			
Predictors	**Effect size**	**CI 95%**	**P value**
Intercept	**0.42**	**-0.19 – -1.04**	**0.001**
Time	**-0.29**	**-0.43 – -0.15**	**<0.001**
Sex	-0.26	-0.63 – 0.11	0.180
LPS treatment	-0.47	-0.95 – 0.01	0.058
T treatment	-0.20	-0.61 – 0.20	0.331
LPS treatment x Sex	0.39	-0.12 – 0.91	0.137
T treatment x Sex	0.43	-0.04 – 0.90	0.080
LPS treatment x T treatments	0.40	-0.15 – 0.95	0.154
LPS treatment x T treatment x Sex	-0.46	-1.02 – 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*		

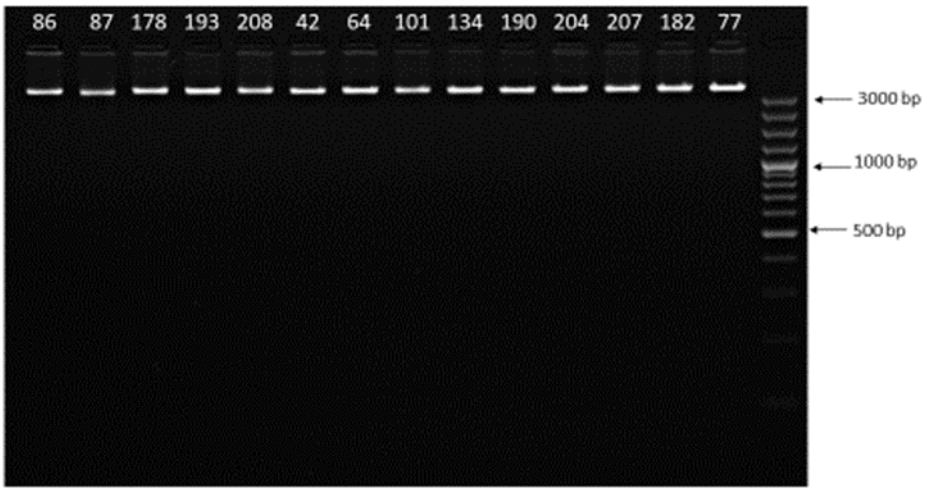


Figure S1: Digital photography of genomic DNA extracted from red blood cells of zebra finches (14 randomly chosen individuals) and exposed to UV light. The electrophoresis was conducted on an agarose gel (0.7%), at 2.5 mV/cm in 45mM Tris-borate, 1mM EDTA medium. The DNA size marker is indicated on the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

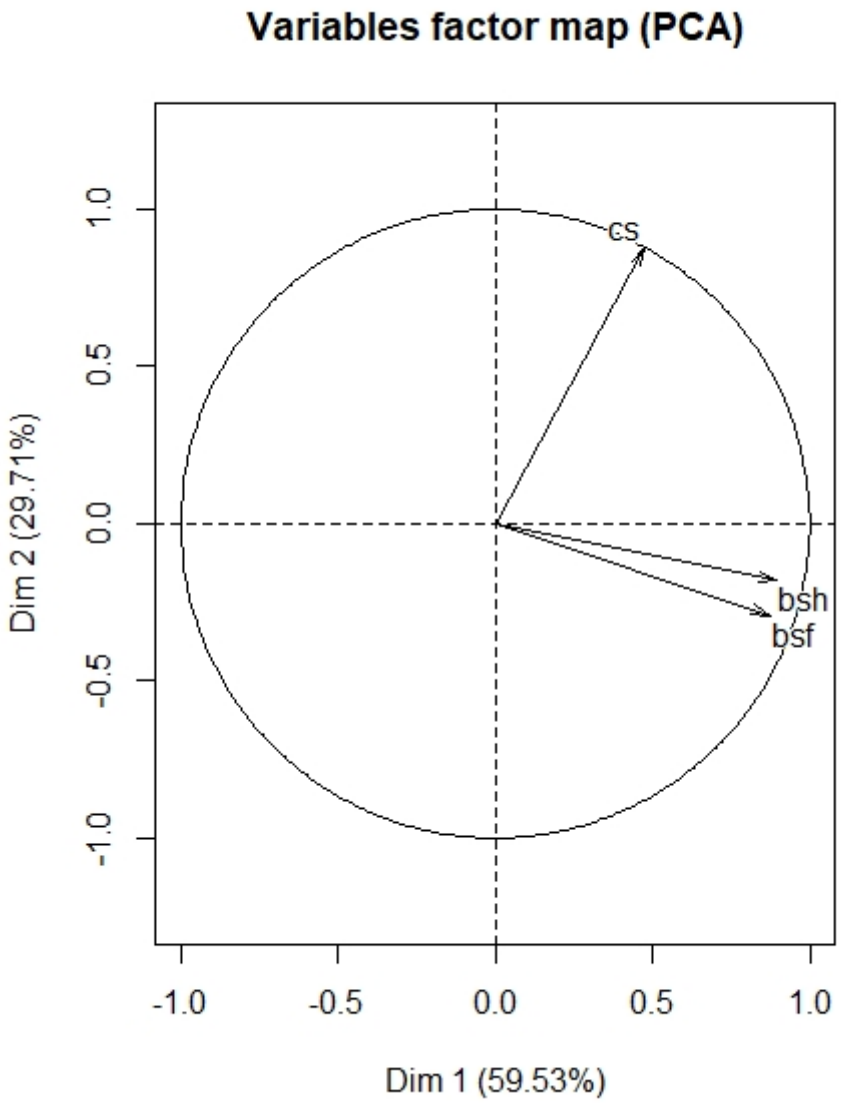
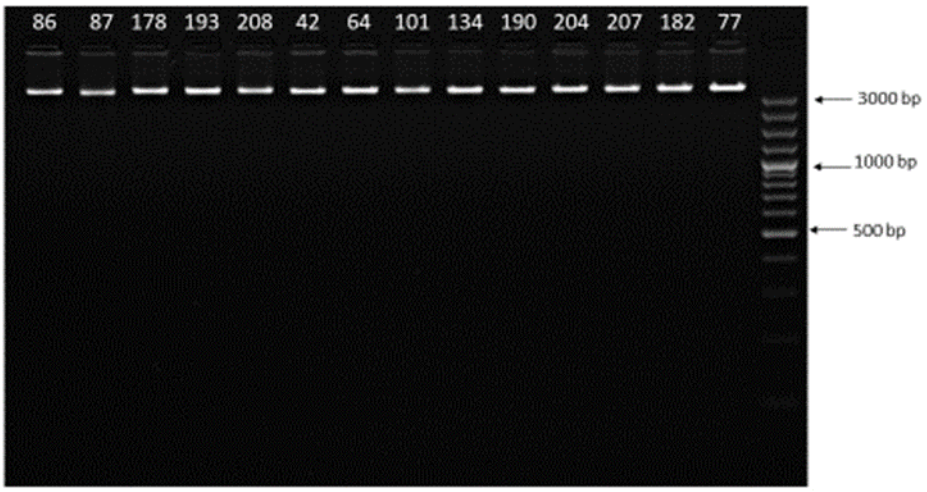


Figure S2: Principal component analysis conducted on clutch size, brood size at hatching and at fledging of 32 females zebra finches. Two axes were produced, explaining 89% of the total variance. PC1 axis (eigenvalue, 1.79) was positively loaded with brood size at hatching (0.90) and fledging (0.88),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



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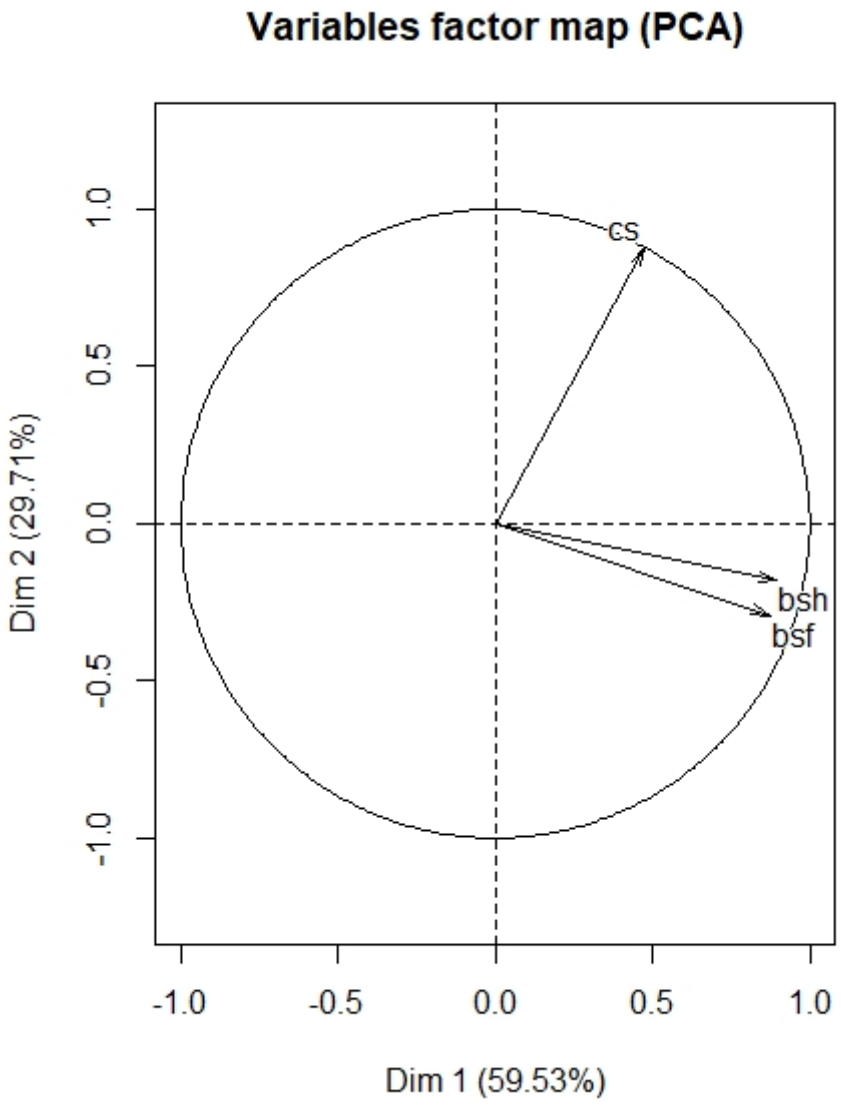

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