Received Date: 7 August 2019

Accepted Date: 9 September 2019

Article type: Original Article

Intergenerational effects on offspring telomere length; interactions among maternal age, stress exposure and offspring sex

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Abstract

Offspring produced by older parents often have reduced longevity, termed the Lansing Effect. Because adults usually have similar aged-mates, it is difficult to separate effects of maternal and paternal age, and environmental circumstances are also likely to influence offspring outcomes. The mechanisms underlying the Lansing Effect are poorly understood. Variation in telomere length and loss, particularly in early life, is linked to longevity in many vertebrates and therefore changes in offspring telomere dynamics could be very important in this context. We examined the effect of maternal age and environment on offspring telomere length in zebra finches. We kept mothers under either control (*ad lib* food) or more challenging (unpredictable food) circumstances and experimentally minimised paternal age and mate choice effects. Irrespective of the maternal environment, there was a substantial negative effect of maternal age on offspring telomere length, evident in longitudinal and cross sectional comparisons (average of 39% shorter). Furthermore, in young mothers, sons reared by challenged mothers had significantly shorter telomere lengths than sons reared by control mothers. This effect disappeared when the mothers were old, and was absent in daughters. These findings highlight the importance of telomere dynamics as inter-generational mediators of the evolutionary processes determining optimal age-specific reproductive effort and sex allocation.

Keywords: maternal age, maternal effects, stress, telomere length, Lansing Effect

Introduction

The conditions under which offspring are produced can have profound effects on their subsequent health and life histories [1]. In long lived, iteroparous species with parental care, key aspects of this are likely to be parental age and the prevailing environmental conditions. The age at which offspring are produced is a fundamental factor in the evolution of reproductive scheduling as the temporal pattern of investment is expected to be tailored to maximise individuals' lifetime fitness [2, 3]. This is because both the success of a breeding event, and the quality of the offspring produced, can be influenced by parental age at reproduction. The general relationship between age and measures of reproductive performance tends to be an inverted U-shape, showing improvement with age early in reproductive life and a decline in old age [3-6]. The late life decline in offspring production has been attributed to parental senescence [7, 8]. However, in addition to a decline in fertility with age, there is substantial evidence that parental age at reproduction also has consequences for the health, pattern of ageing and longevity of those offspring that are produced, with offspring of older parents often showing reduced probability of survival and impaired health, termed the Lansing Effect [9-14]. Thus reduced offspring production in later life could be an evolved strategy to reduce investment in less fit offspring. The stronger such late life effects, the greater the impact this will have on the evolution of reproductive schedules.

There has recently been substantial work investigating and modelling the evolutionary consequences of parental, and particularly maternal, age effects on offspring performance [15, 16]. However, the processes by which offspring are adversely influenced by the age of their parents are not well understood, and are likely to involve both environmental and genetic effects. The quality of both the pre-natal and post-natal environment provided by parents is likely to be very important. The genetic inheritance of the offspring can be influenced by parental age via, for example, increased likelihood of their inheriting adverse germ-line

mutations with advancing parental age, changes in the genome stability of germ cells or via changes in the epigenome with age [17-21].

Prevailing environmental conditions are also an important component influencing offspring fitness, and potentially also the magnitude of parental age effects, which could be masked or exaggerated under environmentally-induced stress. In line with the disposable soma theory of ageing, models have proposed the presence of interactive trade-offs between the optimal allocation of maternal investment in somatic maintenance and investment allocated to the production and rearing of the offspring [16]. Such trade-offs would result in old-mothers or mothers living under poor environmental conditions having offspring with altered biological age at birth and long-term fitness consequences, such as reduced lifespans [16, 22]. However, empirical evidence in support of such predictions is limited (but see [22, 23]). Furthermore, because rearing male versus female offspring could be associated with different costs and benefits, parental age effects on offspring might be sex-specific and vary with environmental conditions [24].

One key mechanism that could have an important inter- and trans-generational effect on offspring performance, and potentially vary with parental age and environmental conditions, is effects on offspring telomere dynamics; this could affect both the telomere length that offspring inherit from their parents, as well as the subsequent pattern of telomere loss in offspring during the period of parental dependence. Telomeres are highly conserved, protective structures that occur at the ends of the linear eukaryotic chromosomes, involving tandem repeats of DNA. Together with shelterin proteins, telomeres play a key role in genome stability, shielding genes from loss of coding sequences as cells divide and preventing end-to-end joining of chromosomes by the DNA repair machinery [25, 26]. Across many studied species, telomere length decreases with age in most somatic tissues and such a decline is especially pronounced during early development [27-30]. Telomere length has been associated with organismal fitness

proxies as individuals with shorter telomeres have shorter lifespans [28, 31-33] and can have an increased susceptibility to disease [34-37]. Telomeres are thought to be integrative markers of exposure to stress [38]. Stress exposure, induced either via direct experimental elevations of glucocorticoid stress hormones or via exposure to various stressors, including poor parental care or immune challenges, has been shown to increase telomere shortening, especially in developing individuals [39, 40]. As recently reviewed [41], accumulating evidence from studies in birds and mammals highlight that stress exposure in the parental generation, occurring primarily via the maternal route during the pre- or post-natal stages, can have a long-lasting impact on offspring telomere dynamics (e.g. [42-44]).

We still know relatively little about parental age effects on offspring telomere dynamics, or the impact that any such effects have for offspring fitness, and we know even less about the extent to which parental age effects vary depending on differing environmental circumstances. The majority of the studies of parental age effects on telomeres carried out to date have focused on testing the association between paternal age and offspring telomere length [45]. While across human populations, older fathers have offspring with longer telomere lengths (reviews: [45, 46]), in most non-human species this pattern is either reversed [13, 14, 47-49], or absent [46, 50]. Most studies of the association between maternal age and offspring telomere length have been performed in humans and found no association between these two factors when statistically controlling for the age of the fathers [review: 46]. The limited work in other vertebrate species reports variable results, with some species showing a negative association of maternal age with offspring telomere length [51] and others showing no maternal age effect [13, 46, 49, 50]. However, a multitude of factors, in addition to study design, are likely to be important in this context, including variation in maternal health status [32], the age of offspring at telomere measurement, the age of fathers [46], and the differential survival of parents with

differing telomere lengths [28]. It remains therefore unclear to which extent maternal ageing influences offspring telomere length.

Here we used an experimental manipulation to examine (i) the effect of maternal age at reproduction on offspring telomere length at the time of parental independence, and (ii) to assess to what degree challenging environmental conditions experienced by the mothers alter any such maternal age effects. We used zebra finches (*Taeniopygia guttata*), which begin to show signs of reproductive senescence between 2-3 years of age [52, 53]. We manipulated the quality of the environment by exposing our study females to unpredictable episodes of food withdrawal throughout adulthood and experimentally controlled the breeding opportunities of the birds. When the females were young, and when they were old, they were paired with a randomly assigned, relatively young adult male. Thus, we experimentally minimised the association between male and female age, and the effect of assortative mating via mate choice often occurring in correlative studies.

Materials and Methods

(a) Study subjects and housing conditions

All females used in this study (n = 180) were produced from the breeding stock at the University of Glasgow. We conducted two replicates of the experiment; replicate 1 females were produced in April-June 2011 and replicate 2 females were produced in August-September 2011. The environmental manipulations started when the focal females were fully grown, sexually mature, young adults (~ 5 months old; mean ± se: 152 ± 1 days). Prior to the start of the study, birds were kept in single-sex groups under standard housing and feeding conditions (*ad lib* supply of mixed seeds - common millet, yellow millet and canary seed in a ratio of 3:1:1 (Johnson and Jeff, UK) - oyster shell grit, cuttlefish and *ad lib* water used for stock birds in our facilities) and housed in treatment-specific cages (n = 7-10 females per 120 x 50 x 50 cm cage). The

135 photoperiod was always maintained at 14h:10h light:dark cycle and the temperature was
136 between 20-24°C. All procedures were carried out under UK Home Office Project Licence
137 60/4109.

138 ***(b) Environmental manipulation***

139 When the females were ~ 5 months of age, they were randomly allocated to one of the two
140 experimental groups: a challenging (n = 89), or control environment (n = 91). In the challenging
141 environment, food was made unavailable for a continuous period of ~ one-third of the daylight
142 period (4.9h), 4 days/week on a random time schedule. For the remaining two-thirds of the day
143 and on the remaining 3 days/week, challenged females received *ad lib* food. Challenged females
144 always experienced this food regime except during breeding when they were given *ad lib* access
145 to food from the time they were paired with a male or shortly afterwards until after they
146 completed breeding (~ two months for each breeding event). The treatment had no detectable
147 effect on female body mass [53]. Control females were always provided with *ad lib* food and
148 experienced exactly the same breeding scheduling as the challenged birds. As previously
149 shown, the simulated challenged environmental conditions led to increases in corticosterone
150 secretion, the primary avian glucocorticoid stress hormone. At the end of each food withdrawal
151 exposure, challenged females had higher corticosterone than controls (on average 1.6-fold
152 increase and within the baseline range of variation for our study species), and this physiological
153 response was consistent over a very prolonged exposure periods (up to 3 years), indicating no
154 habituation of the birds to the environmental manipulation [6, 53].

155 ***(c) Adult female breeding timeline and offspring sampling***

156 We examined the telomere length of offspring produced by mothers that bred at two time points:
157 (1) during young adulthood at six months (i.e. young-mother breeding event: mean age ± SE,
158 187.6 ± 1.0 days; range: 156-207 days, n = 172 mothers) and, (2) in old age at 3.5 years old (i.e.
159 old-mother breeding event: mean age ± SE, 1269.3 ± 1.3 days; range: 1259-1293 days, n = 52

mothers). When not breeding, the females were kept in single-sex groups and thus did not form long-term pair bonds with particular males. The reduced number of mothers in the old-mother breeding event was due to natural maternal mortality and/or breeding failure (i.e. no fledglings produced); offspring telomere length data from the same mothers in both the young- and old-mother breeding events were available for 44 females (18 controls and 26 challenged). During these two breeding events, females were paired with a different, un-related, randomly assigned male of prime breeding age. These males had always been kept in control environmental conditions (see above). While the males were similar in age to the females during the first breeding event, when the females themselves were young (age of the males at the young-mother breeding event - mean ± SE: 185.7 ± 1.2 days, range: 142-204 days), the experimental design ensured that this was not the case when the females were old; the males with which the females were paired in their old age were still relatively young, on average just over 1.2 years (age of the males at the old-mother breeding event – mean ± SE: 464.1 ± 23.9 days, range: 212-699 days). The age of the father in the old-mother breeding event, where males were substantially younger than females (t-test: t = -53.72, df = 125, $p < 0.0001$), had no effect on offspring telomere length (GLMM: $p \leq 0.85$, full statistics in Table S1, Supplementary).

Each pair was placed in individual breeding cages (60 x 50 x 50 cm) equipped with an external nest box and nest material (coconut fibres and jute, Haiths Ltd). Breeding birds were provided with a commercial seed mix (Johnson and Jeff, UK), oyster shell grit, cuttlefish, and water. Once a week, the birds were also provided with Calcivet calcium supplement (Vetafarm, Wagga Wagga, NSW, Australia), a protein conditioning supplement (J.E. Haith, Cleethorpes, UK), and fresh vegetables. Between the young- and old-mother breeding events, females in both replicate groups experienced the same breeding schedules, with two breeding events in the intervening years. They were allowed to lay, but not rear, a clutch of eggs at 1.1 years and to rear their biological or foster brood when they were 1.8 years old. Both these breeding events

185 involved pairing with similarly young males as in the young- and old-mother breeding events,
186 but the fact that the breeding regime varied from that in the young and old breeding events
187 precludes comparison of the effects on offspring. The actual number of eggs laid and young
188 reared to fledging prior to the 3.5 years breeding event were included in the analysis to check
189 whether variation among females in prior breeding effort was associated with variation in
190 offspring telomere length produced during the old-mother breeding event. Neither of these
191 estimates of breeding effort had any effect on offspring telomere length, thus excluding the
192 possibility of potential confounds between differences in prior maternal reproductive effort and
193 chronological age (Table S1, Supplementary).

194 ***(d) Blood sampling and telomere length analysis***

195 Chicks were weighed and small blood samples (~ 70 µl) were collected by venipuncture of the
196 alar vein when they were ~30 days old and feeding independently of their parents (fledgling
197 mean age ± SE : 29.6 ± 0.05 days, range: 25-32 days) during both the young-mother and old-
198 mother breeding events. Chick age was estimated from the first chick hatched within each
199 clutch; hatching order within each nest was also recorded since this can influence telomere
200 length [54]. Blood samples were immediately placed on ice after sampling. Within 4 hours, the
201 blood samples were spun to separate plasma from red blood cells, and the latter were stored at
202 -80ºC until later telomere analysis. Our cohort of experimental females was also periodically
203 sampled for telomere analysis. However, the blood sampling was never performed in close
204 proximity to or during the breeding events to minimise disturbance and potential additional
205 stress associated with handling. We found no correlations between maternal and offspring
206 telomere lengths (to be published in a separate manuscript).

207 During the first breeding event, we measured telomere length in two randomly selected
208 chicks per nest (brood sizes reaching the sampling age for mothers that produced a clutch: 3.15
209 ± 0.09 chicks, mean ± SE); there was no sampling bias in the hatching order of the selected

chicks among the differing clutch sizes (chi-squared = 47.15, df = 40, p = 0.20). At the old-mother breeding event, when brood sizes were smaller, all chicks were measured (brood sizes reaching the sampling age for mothers that produced a clutch: 2.50 ± 0.17 chicks, mean ± SE) to ensure an adequate sample size per brood similar to that during the young-mother breeding event. DNA from red blood cells was extracted using commercial kits and following the manufacture's protocol (Macherey-Nagel, USA). Relative telomere length (RTL) was quantified in the red blood cell DNA by using qPCR as described elsewhere [55]; this correlates well with measurements using TRF method [55]. Briefly, the relative telomere length of each sample was measured by determining the ratio (T:S) of telomere repeat copy number (T) to a single copy control gene (S), relative to the same DNA reference sample run on each plate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy control gene. The telomere and GAPDH reactions were carried out on separate plates, and in both reactions the number of PCR cycles (Ct) required for the products to accumulate enough fluorescent signal to cross a threshold was determined. Reaction efficiencies were always within the acceptable range (i.e. 100 ± 10%). All samples fell within the bounds of the standard curve run on every plate (6 standard dilutions, from 40ng to 125ng of DNA). All telomere assays were run between October 2015 and February 2016 and samples were randomly spread across the different plates; each plate contained a standard curve and all standards and samples were always run in triplicate. The intra-plate coefficient of variation for the telomere and GAPDH assays for the raw Ct values were 0.65% and 0.97%, respectively; the inter-plate coefficient of variation calculated using the standard dilutions that were run across each plate for both the telomere and GAPDH assays were 1.63% and 1.96%, respectively.

The raw qPCR data were analysed using the software Qbase+ [56]. Mean Ct values were used to calculate a relative measure of telomere length as a T:S ratio of telomere repeat copy number to a control, single copy gene number (GAPDH). The qBase+ software provides the

advantage of adjusting for differences in amplification efficiencies among plates (as described in [57]) and correcting for further inter-run variation by including three inter-run calibrators (i.e. the reference sample and two points from the standard curve - 10ng and 5ng of DNA). For each sample, the software produced a calibrated normalised relative telomere measurement, which is similar to the T:S ratio described by [31] but offers a greater control of inter-plate stochastic variation. The inter-assay coefficient of variation for the calibrated normalised T:S ratios calculated using the standard dilutions run across each plate was 15.25%.

(e) Data analysis

Analyses were performed in R (version 3.5.1; R core team, 2014). We used Generalised Linear Mixed Models with a Gaussian distribution - GLMMs R package "lme4" [58] and "lmerTest" [59] - to examine whether maternal age and/or the maternal environmental treatment influenced offspring body mass or offspring telomere length at fledgling, upon nutritional independence of the chicks from their parents. Telomere data were ln-transformed to improve normality of model residuals. One offspring produced during the first breeding event with a telomere length value of 4.56 was excluded from telomere analyses because this value was an extreme statistical outlier as suggested by inspection of model residuals and as exceeds the upper quartile by more than three times the interquartile range [60].

All final models included the effects of experimental design factors expected to influence the response variables either as parameters of interest integral to the questions being investigated or for the purpose of adjustment (i.e. to control for potentially confounding variables). These relevant factors were always retained in the main models rather than tested using selection procedures to avoid overfitting and inflating the type I error. Unless otherwise specified, final models always included the following main factors: maternal age (young-mother breeding event or old-mother breeding event), maternal treatment (control environment or challenging environment), replicate, offspring sex (determined by colour plumage when the

chicks were ~50 days old), brood size (i.e. number of chicks reared) at the time of sampling, and the hatching order within the clutch to control for the slight variation in age of the chicks at the time of sampling [54]. We also entered the two- and three-way interactions among maternal age, maternal treatment and offspring sex in order to test whether the potential effect of maternal age and/or treatment on offspring body mass or offspring telomere length differed between male and female offspring; non-significant interactions ($p > 0.05$) were sequentially removed using backward selection starting from the three-way interaction. In initial models of the telomere length data, we also examined whether body mass of the offspring at the time of sampling (values available for 441 out of 444 chicks) and the two-way interaction between offspring body mass and maternal age influenced offspring telomere length; but neither of these factors were significant ($p \geq 0.5$) and were consequently removed from the final models. The identities of the mothers were included as random factor to account for non-independence of offspring from the same mother. In order to assess within-mother age- and treatment-effects and to exclude bias in the results associated with the loss of specific individuals from the female population due to death or non-breeding, we also performed analyses using only those offspring telomere data from females that reared chicks during both the young- and old-mother breeding events (185 out of 444 chicks and 44 out of 180 mothers). We used the R package "lsmeans" [61] to perform pairwise post-hoc comparisons for significant outcomes in the main models (Tukey's *p* value adjustment). Multi-collinearity was examined in all models by calculating variance inflation factors; these ranged from 1.0 to 1.3 indicating acceptable degrees of multi-collinearity among the explanatory variables. All models met assumption of normality and homogeneity, which was assessed via graphical diagnostics of the residuals [62]. Unless otherwise specified descriptive statistics are provided as mean ± SE.

Results

285 ***(a) Effects of maternal age and environmental conditions on offspring body mass***

286 There was no effect of the maternal treatment, replicate, offspring sex, and hatching order on
287 offspring body mass as main factors; there were no interacting effects among maternal age,
288 maternal treatment and offspring sex on the response variable (full statistics in Table S2a,
289 Supplementary). Regardless of the maternal environment and offspring sex, fledglings
290 produced during the old-mother breeding event were lighter when compared to the fledglings
291 produced during the young-mother breeding event (maternal age: p = 0.001; Figure S1a,
292 Supplementary). We also found that lighter offspring were those reared in larger broods (p =
293 0.001, Table S2a). However, when restricting the analysis to the subset of offspring produced
294 by the mothers that bred during both the young- and old-mother breeding events, the significant
295 effects of maternal age and brood size on offspring body mass disappeared (Table S2b and
296 Figure S1b, Supplementary).

297 ***(b) Effects of maternal age and environmental conditions on offspring telomere length***

298 The strongest main effect on offspring telomere length was maternal age ($p < 0.001$, full
299 statistics in Table S3a, Supplementary) with offspring produced in the old-mother breeding
300 event having substantially shorter (39% on average) telomere lengths compared to offspring
301 produced during the young-mother breeding event (Figure 1a). However, the effect of the
302 maternal environment on offspring telomere length differed with offspring sex and with
303 maternal age (maternal treatment x maternal age x offspring sex: p = 0.02, Table S3a, Figure
304 1). For mothers living in the control conditions, the effect of maternal age was consistent in
305 both sons and daughters; daughter telomere lengths were 43.5% shorter when their mothers
306 were old compared with when mothers were young, and 49.2% shorter in sons ($p \leq 0.003$ for
307 both). There was no effect of the maternal environment on the telomere length of daughters
308 either when their mothers were young or old ($p \geq 0.8$ for both). Telomere length of sons however
309 was reduced in young mothers living in the challenging environment compared to the sons

310 produced by the young control mothers (by 27.7%, p = 0.0001). This resulted in daughters
311 produced by young mothers living in challenging conditions having longer telomeres than
312 equivalent sons (by 20.7%, p = 0.048). In contrast, when mothers were old, telomere length in
313 their sons did not differ between the two maternal treatment groups (p = 0.8), and the same was
314 true in their daughters (p = 0.9). Telomere lengths of sons produced by young mothers in the
315 challenging environment were similar to those of the sons of old challenged mothers (p = 0.2),
316 but slightly longer compared to the telomere lengths of sons of the old control mothers (p =
317 0.04). We found no effect of replicate, hatching order, or brood size as main factors on offspring
318 telomere length (Table S3a). Results were qualitatively similar (36% on average telomere
319 shortening with maternal age) when we performed the same analysis on the subset of offspring
320 reared by the same mothers during both the young- and old-mother breeding event (Figure 1b;
321 Table S3b, Supplementary).
322
323 **Discussion**
324 This is the first long-term, longitudinal study to compare changes in telomere length in offspring
325 produced by females at different ages (i.e., when young at 6 months of age, and when old at 3.5
326 years of age) and in which the age of their partners was experimentally standardised to enable
327 maternal effects to be identified. From young-adulthood and when not breeding, our focal
328 females were living either under control (*ad lib* food) or more challenging environmental
329 circumstances (random withdrawals of food, which produced repeated increases in circulating
330 glucocorticoid stress hormones). Our study therefore also enabled us to examine whether any
331 reduction in telomere length resulting from maternal age was affected by the environmental
332 conditions experienced by the mothers prior to breeding (thus excluding direct effects of the
333 environment on offspring), and whether effects differed between sons and daughters. Our data
334 clearly show that, in non-stressful environments, both sons and daughters produced by mothers

335 in old adulthood have substantially shorter telomeres than those produced by mothers in young
336 adulthood. We also found sex-specific interactive effects between the maternal environment
337 and maternal age with sons produced by the challenged females as young breeders effectively
338 having their telomere lengths equivalent to those sons produced when mothers were old; the
339 decline in telomere length in the sons was of comparable magnitude to the telomere shortening
340 associated with maternal age. These results are consistent with the results we obtain when we
341 restrict the analyses to the subset of mothers that reared chicks during both breeding events,
342 which confirms that these trans-generational effects on offspring telomere length occurred
343 within-individual mothers and were not due to selective mortality or breeding quality of the
344 females. Overall, the mean decline in offspring telomere length with maternal age was marked
345 - ~39% over the elapsed maternal age period of ~3 years – overriding any potential effect
346 associated with the maternal environmental manipulation. However, we do not know whether
347 this decline was linear, or only occurred after a particular maternal age, which warrants future
348 investigations.

349 The negative relationship between maternal age and offspring telomere length in both
350 offspring reared by either challenged or control females can be attributed to the change in
351 maternal age; other factors such as variation in previous maternal reproductive effort had no
352 significant effect on offspring telomere length. Experience and resource acquisition by older
353 individuals are also likely to be important factors, especially in the field. Our study was
354 conducted in captivity under controlled environmental conditions, thus making it easier to
355 isolate effect due to changes in maternal age as well as to challenging environmental
356 circumstances. Paternal age, independently of maternal age, has been shown to affect telomere
357 length in zebra finches as early as the embryonic developmental stages [14]. The design of our
358 study aimed at minimising variation in the father's age; the females grew older and we were
359 therefore able to examine the effect of maternal ageing in the absence of an effect of paternal

ageing on offspring telomere length. We do not however know whether the stage at which maternal effects occur differs from that of paternal effects, or indeed whether such maternal and paternal effects are additive. Clearly, the effect of maternal age was also influenced by the maternal environment and offspring sex, which could also contribute to inconsistencies in the effects found in different studies [51, 13, 49]. The lack of an effect of the maternal treatment during old-adulthood in either sons or daughters suggests that the effect of maternal age might have overridden any maternally-environmentally derived effects on offspring telomere length. It may be that there is a critical length below which offspring telomere length cannot fall and the offspring remain viable. Hence, the absence of an additive effect. All our experimental females were housed in single-sex groups and were paired with a young-adult male only during the age-specific breeding events to minimise mate familiarity. We can thus exclude the possibility that the reduction in offspring telomere length during the old-mother breeding event could be attributable to increased maternal stress due to the sudden introduction of an unfamiliar male after years being paired with the same male, thereby having formed a long-term pair bond which is broken. That the effect of the maternal treatment was observed only when the mothers were young, and only in their male offspring is also intriguing. There are several possibilities that could explain such sex-dependent sensitivity to maternal effects. For instance, it is plausible that male nestlings were simply more vulnerable to poorer maternal rearing conditions than female nestlings as has been reported in a number of studies in birds including lesser black-backed full (*Larus fuscus*)[63, 64], great tits (*Parus major*) [65], and collared flycatchers (*Ficedula albicollis*) [66]. However, studies in the zebra finch suggest that sons are generally over-produced under poor rearing conditions and so daughters may be more vulnerable [67-69]. We note however that in our study we are unable to distinguish among effects that might arise from differential survival of sons and daughters during the pre-natal or the very early post-natal stages or by shifts in primary sex ratios linked to maternal condition [63, 64]. It is also

possible that the sex-specific effect could relate to differences in telomere dynamics in the sex chromosomes, but nothing is known about this in birds.

The reduction in offspring body mass with maternal age probably reflected earlier mortality of females producing heavier offspring during early adulthood, not maternal age-specific variation in offspring body mass within individual mothers. Such effect is interesting as it occurred in the benign conditions of captivity and it might be associated with trade-offs between reproduction and survival. That adults can adopt differing patterns of reproductive investment that are related to their life span variation has also been found in other studies. For example, in the red-billed chough, parents that produced high quality offspring had reduced longevity compared to parents producing lower quality offspring [23].

Studies in humans suggest that maternal effects on offspring telomere length could occur as early as the oocyte. This may be because eggs ovulated in older women enter meiosis at a later point in foetal egg formation than eggs ovulated when women are younger [20]. These late ovulated eggs will therefore have been produced via more cell replications, which could shorten telomeres [20, 70]. Increased exposure to ROS-induced oxidative damage with storage time in the ovary may also play a role [21]. Alternatively, the decrease in offspring telomere length with maternal age in birds could occur as a consequence of differences in egg composition, including differences in yolk:albumen ratio content [71], concentrations of hormones and immune antibodies [72], yolk fatty acid profiles [73]. Such differences could be the result of adaptive age-specific adjustments or could arise because of physiological constraints associated with female reproductive senescence. Similar proposed mechanisms could also explain the shortening of telomere length in the offspring produced by the challenged mothers as mothers exposed to stress deposit higher levels of stress hormones *in ovo* [74] and this effect has been linked to faster offspring telomere loss in early life [42].

409 Rearing conditions after hatching could also play a key role in telomere shortening. The
410 latter effect was reported in the European shag (*Phalacrocorax aristotelis*), where telomere
411 length at hatching was not related to parental age, but at fledging offspring of older parents had
412 shorter telomeres. This post-hatching effect is presumably attributable to the quality of the
413 rearing environment, which could potentially be poorer and thus more challenging when parents
414 are older, and stress exposure during the rearing developmental stages is associated with faster
415 offspring telomere loss [39, 43]. Evidence suggesting that the quality of female parental care
416 might be important comes from a recent experiment in the Alpine swift, in which offspring
417 telomere length at parental independence was negatively related to the age of the cross-fostered
418 mother but not to the age of the cross-fostered father [48]. Potential age-related differences in
419 maternal and paternal care, together with associated offspring fitness consequences would be
420 important to investigate in future research in our study species.
421 That offspring longevity can be adversely affected by parental age, the so-called Lansing
422 effect, has been established in many taxa [9, 10]. It is also known that exposure to stressors can
423 accelerate cellular ageing, alter survival trajectories and increase vulnerability to diseases [41,
424 52]. A key question arising from this study is therefore whether the decline in offspring telomere
425 length in relation to maternal age and maternal challenging conditions of the magnitude we
426 observed is sufficient to modulate offspring longevity and life-history trajectories. Noguera et
427 al recently showed that increasing parental age is associated with a substantial reduction in
428 offspring longevity in zebra finches in captivity, though maternal and paternal effects could not
429 be clearly separated [14]. Heidinger et al showed that telomere length upon parental
430 independence in zebra finches is predictive of longevity [28]; the relationship observed in that
431 study suggested that the ~ 39% decline that we observed in offspring telomere length with an
432 increase in maternal age of ~ three years would be associated with some 25% reduction in
433 offspring lifespan. For mothers living in control conditions, the effect on offspring was more

marked – a 44-49% telomere reduction in daughters and sons respectively. The effect of maternal age on offspring longevity is thus likely to have substantial fitness consequences.

To conclude, our results strongly emphasize the need of more studies to improve our understanding of the role of parental age in determining optimal timing of breeding and breeding effort across the life course [15]. Such studies should be carried out under a variety of different parental environments for a greater understanding of the dynamics of such induced trans-generational phenotypic plasticity, thus determining the "fittest" genotype depending on the environment. That telomere length is reduced when mothers are old suggest that reduced offspring production at older ages reduces investment in less fit offspring and is not simply a consequence of parental ageing. This study also raises the intriguing question of why zebra finches remain fertile in old adulthood. This could be because some fitness benefits are still accrued from such offspring, provided the effects on parent and offspring survival are not too severe. The potential fitness benefits associated with different scenarios of parental effort, quality of the rearing environment, and longevity effects should be further explored in future studies.

Ethics. All procedures were carried out under Home Office Project Licence (60/4109).

Data accessibility. Data are available from Dryad Digital Repository (doi:10.5061/dryad.h8c9781).

Author Contributions. VM, WB, BH, and PM designed the experiment; VM and PM analysed the data and wrote the manuscript; all authors carried out the animal experimental procedures, WB and VM carried out the laboratory telomere analyses; all authors commented previous drafts of the manuscript.

Competing Interests

We declare no competing interests.

Funding

This work was funded by a European Research Council Advanced Investigator Award (268926) to PM, VM was supported by a Marie Sklodowska-Curie Postdoctoral Fellowship at the time of writing (704582).

Acknowledgements

We thank G. Adam, G. Anderson, A. Kirk, J. Laurie, G. Law, G. Grey, R. Philips, and A.Magierecka for excellent assistance with animal husbandry; J. C. Noguera, J. Laurie, A. Magierecka and S. Reichert for help in collecting blood samples; A. Magierecka for help with data entry, and P. Johnson for advice with the statistical analyses.

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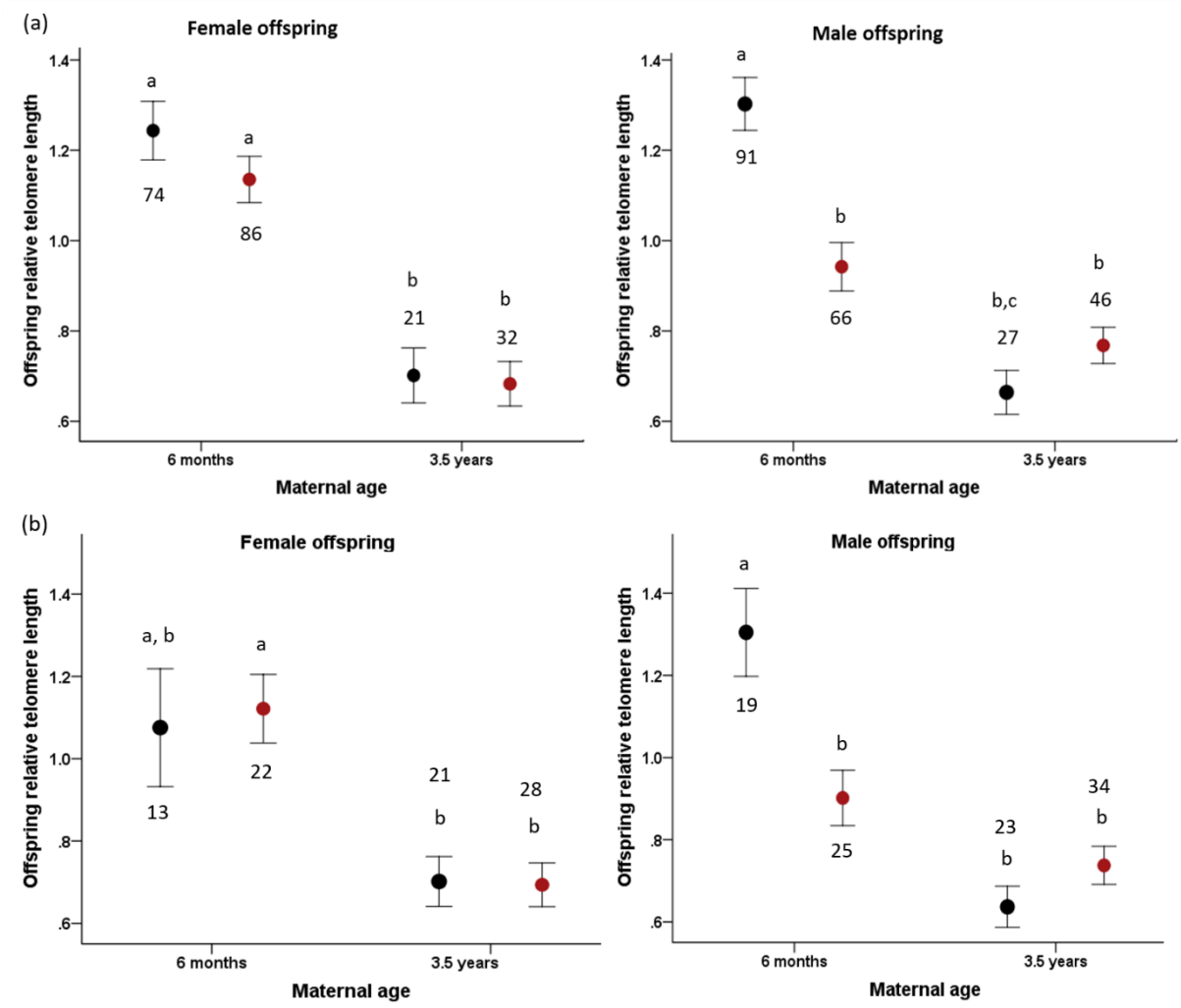

Figure 1. (a) Early life relative telomere length (~ 30 days of age – values were adjusted for plate amplification efficiencies and inter-run calibration using the software Qbase+, see "Material and Methods" for full details) of offspring produced by mothers that bred during the young-mother breeding event at 6 months of age (89 control and 83 challenged mothers) and/or during the old-mother breeding event at 3.5 years of age (20 control and 32 challenged mothers). In (b) data are shown only from the subset of females that produced offspring during both the young- and old-mother breeding event (18 control and 26 challenged mothers). Data are shown as means ± SE; black circles indicates offspring produced by control mothers and red circles indicates offspring produced by challenged mothers, numbers indicates offspring sample sizes separately by maternal treatment, maternal age and offspring sex; different letters indicate significant post-hoc pairwise contrasts after Tukey's multiple comparison adjustment.

673 **Supplementary Material**

674 **Intergenerational effects on offspring telomere length; interactions among maternal age,**
675 **stress exposure and offspring sex**

676

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Table S1. GLMM modelling with Gaussian distribution to test whether prior maternal reproductive effort ((a) total number of eggs laid, or (b) total number of chicks reared up to the first three breeding events prior the old-mother breeding event at 3.5 years of age), maternal treatment, fathers' age at reproduction, and other selected fixed parameters (see "Data Analysis", Material and Methods) influenced early life telomere length (measured at ~ 30 days of age, values ln-transformed) in the offspring produced during the old-mother breeding event; female identity was added as random factor; *indicates non-significant factor removed from the model ($p > 0.05$). Number of offspring: 126; number of mothers: 52.

(a)

Parameter	Estimate	SE	df	t	*p*
Female ring identity (r)	0.021				
Residual	0.153				
Intercept	-0.491	0.305	48.240	-1.611	0.114
Maternal treatment (Challenging environment)	0.041	0.096	39.580	0.428	0.671
Father age at reproduction	<0.0001	<0.0001	41.330	0.196	0.846
Replicate (2)	-0.046	0.100	43.060	-0.457	0.650
Hatching order	<0.0001	0.031	115.200	0.003	0.998
Sex (Male)	0.061	0.075	118.700	0.810	0.420
Prior maternal egg laying effort	0.003	0.019	41.750	0.154	0.878
Offspring Body mass*					0.9

(b)

Parameter	Estimate	SE	df	t	*p*
Female ring identity (r)	0.021				
Residual	0.153				
Intercept	-0.396	0.207	58.690	-1.918	0.060
Maternal treatment (Challenging environment)	0.024	0.095	39.480	0.257	0.798
Father age at reproduction	<0.0001	<0.0001	40.330	0.259	0.797
Replicate (2)	-0.049	0.098	42.830	-0.497	0.622
Hatching order	0.003	0.031	116.100	0.083	0.934
Offspring sex (Male)	0.056	0.076	118.600	0.747	0.457
Prior maternal chick rearing effort	-0.011	0.021	44.800	-0.527	0.601
Offspring Body mass*					0.9

Table S2. (a) GLMM modelling with a Gaussian distribution to test the effects of maternal age (young or old, 6 month or 3.5 years of age respectively), maternal treatment (control environment or challenging environment), offspring sex, and selected fixed parameters (see "Data Analysis", Material and Methods) on offspring body mass at nutritional independence (~ 30 days of age); female identity was added as random factor. Fixed factor estimates are indicated in parenthesis; r indicates random factor and its associated variance. The non-significant interactions (*$p > 0.05$) were removed from the final model, significant factors are in bold. In (b) the same model as in (a) was performed using only the data from offspring produced by experimental females that bred at both breeding events.

(a)

Parameter	**Estimate**	**SE**	**df**	**t**	***p***
Female ring identity (r)	0.631				
Residual	0.942				
Intercept	**15.232**	**0.234**	**337.464**	**65.072**	**<0.0001**
Maternal age (old)	**-0.420**	**0.131**	**415.380**	**-3.199**	**0.001**
Maternal treatment (Challenging environment)	-0.176	0.155	173.151	-1.130	0.260
Replicate (2)	-0.156	0.158	174.240	-0.987	0.325
Hatching order	-0.023	0.050	324.012	-0.459	0.647
Offspring sex (Male)	-0.148	0.104	345.678	-1.422	0.156
Brood size	**-0.204**	**0.060**	**420.947**	**-3.399**	**0.001**
Maternal treatment x Offspring sex*					0.8
Maternal treatment x Maternal age*					0.1
Maternal age x Offspring sex*					1.0
Maternal treatment x Maternal age x Offspring sex*					0.5

(b)

Parameter	**Estimate**	**SE**	**df**	**t**	***p***
Female ring identity (r)	0.528				
Residual	1.052				
Intercept	**14.475**	**0.449**	**95.708**	**32.206**	**<0.0001**
Maternal age (old)	-0.120	0.161	147.352	-0.750	0.455
Maternal treatment (Challenging environment)	-0.174	0.288	40.693	-0.602	0.551
Replicate (2)	0.098	0.304	40.796	0.320	0.750
Hatching order	-0.017	0.078	146.588	-0.225	0.822
Offspring sex (Male)	-0.072	0.162	150.828	-0.443	0.659
Brood size	-0.137	0.085	176.518	-1.598	0.112
Maternal treatment x Offspring sex*					0.3
Maternal treatment x Maternal age*					0.1
Maternal age x Offspring sex*					0.8
Maternal treatment x Maternal age x Offspring sex*					0.5

Table S3. (a) GLMM modelling with a Gaussian distribution to test the effects of maternal age (young or old, 6 month or 3.5 years of age respectively), maternal treatment (control environment or challenging environment), offspring sex, and selected fixed parameters (see "Data Analysis", Material and Methods) on offspring telomere length measured at ~ 30 days of age (ln-transformed values); female identity was added as random factor. Fixed factor estimates are indicated in parenthesis; r indicates random factor and its associated variance. The non-significant factors (*$p > 0.05$) were removed from the final model; significant factors are in bold. In (b) the same model as in (a) was performed using only the data from offspring produced by experimental females that bred at both breeding events.

(a)

Parameter	**Estimate**	**SE**	**df**	**t**	***p***
Female ring identity (r)	0.006				
Residual	0.202				
Intercept	**0.203**	**0.085**	**358.831**	**2.401**	**0.017**
Maternal age (old)	**-0.481**	**0.116**	**392.819**	**-4.145**	**<0.0001**
Maternal treatment (Challenging environment)	-0.063	0.073	407.101	-0.872	0.384
Replicate (2)	-0.0814	0.0468	179.522	-1.740	0.084
Hatching order	-0.013	0.022	402.148	-0.613	0.540
Offspring sex (Male)	0.068	0.071	415.943	0.952	0.342
Brood size	-0.010	0.022	361.482	-0.444	0.657
Maternal treatment x Offspring sex	**-0.262**	**0.103**	**418.401**	**-2.545**	**0.011**
Maternal treatment x Maternal age	-0.026	0.150	402.226	-0.175	0.861
Maternal age x Offspring sex	-0.130	0.152	424.265	-0.857	0.392
Maternal treatment x Maternal age x Offspring sex	**0.461**	**0.199**	**430.575**	**2.311**	**0.021**

(b)

Parameter	**Estimate**	**SE**	**df**	**t**	***p***
Female ring identity (r)	0.012				
Residual	0.153				
Intercept	0.223	0.157	151.372	1.421	0.157
Maternal age (old)	**-0.358**	**0.142**	**164.605**	**-2.515**	**0.013**
Maternal treatment (Challenging environment)	0.038	0.145	169.338	0.265	0.791
Replicate (2)	-0.1547	0.0774	46.969	-1.998	0.051
Hatching order	-0.016	0.029	161.353	-0.563	0.574
Offspring sex (Male)	0.246	0.144	166.811	1.700	0.091
Brood size	-0.028	0.029	163.263	-0.960	0.339
Maternal treatment x Offspring sex	**-0.473**	**0.186**	**167.086**	**-2.541**	**0.012**
Maternal treatment x Maternal age	-0.153	0.183	166.252	-0.837	0.404
Maternal age x Offspring sex	-0.348	0.191	173.492	-1.816	0.071
Maternal treatment x Maternal age x Offspring sex	**0.648**	**0.249**	**173.256**	**2.606**	**0.010**

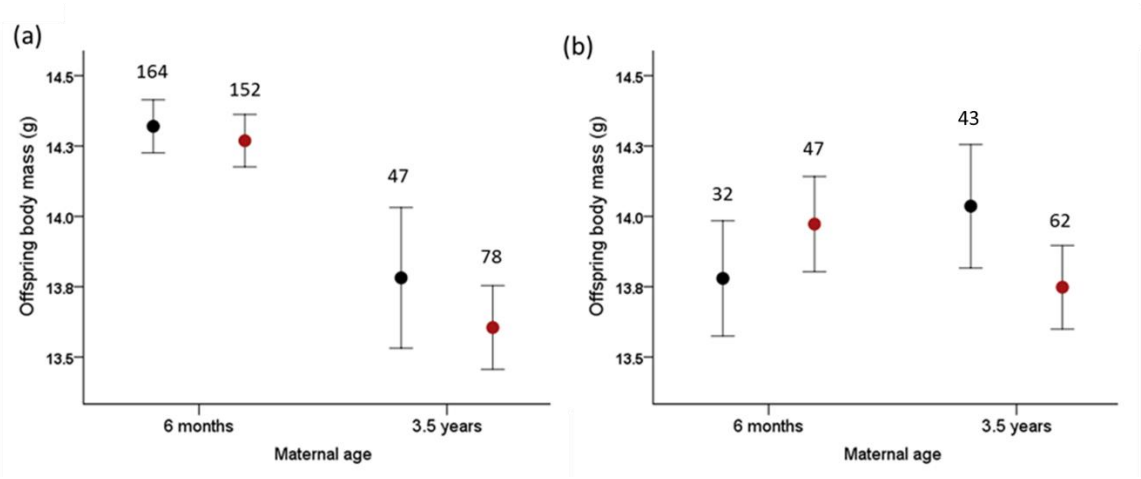

Figure S1. (a) Body mass at nutritional independence (~30 days old) of zebra finch offspring produced by mothers that bred during the young-mother breeding event at 6 months of age (89 control and 83 challenged mothers) and/or during the old-mother breeding event at 3.5 years of age (20 control and 32 challenged mothers). In (b) data are shown only from the subset of females that produced offspring during both the young- and old-mother breeding event (18 control and 26 challenged mothers). Data are shown as means ± SE; black circles indicates offspring produced by control mothers and red circles indicates offspring produced by challenged mothers, numbers indicates offspring sample sizes separately by maternal treatment and maternal age.