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Harsh conditions during early development influence telomere length in an altricial passerine: Links with oxidative stress and corticosteroids

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Abstract

Stress during early development can induce substantial long-term effects in organisms. In the case of birds, despite growth compensations, nestlings reared under harsh conditions typically show reduced survival chances in adulthood. It has been proposed that environmental early-life stressors could affect longevity via effects on telomere length, possibly mediated through oxidative stress. However, the link between these processes is not clear. In this study, we experimentally manipulated brood size in spotless starlings (*Sturnus unicolor*) to test the causal relationship between early stress, oxidative and corticosterone-mediated stress and telomere shortening. Our results show that experimentally enlarged brood sizes led to a reduction in morphometric development on nestlings, the effect being stronger for females than males. Additionally, basal corticosterone levels increased with increasing brood size in female nestlings. Neither plasma antioxidant status nor malondialdehyde levels (a marker of lipid peroxidation) were affected by experimental brood size, although the levels of a key intracellular antioxidant (glutathione) decreased with increasing brood size. We found that the treatment showed a quadratic effect on nestling telomere lengths: these were shortened either by increases or by decreases in the original brood size. Our study provides experimental evidence for a link between developmental stress and telomere length, but does not support a direct causal link of this reduction with corticosterone or oxidative stress. We suggest that future studies should focus on how telomere length responds to additional markers of allostatic load.

KEYWORDS

corticosterone, early-life stress, oxidative stress, starlings, *Sturnus unicolor*, telomeres

1 | INTRODUCTION

Several evolutionary theories, not mutually exclusive, have been proposed to explain ageing and the evolution of senescence (Shefferson, Jones, & Salguero-Gómez, 2017). Specially compelling are those that propose that ageing is caused by deleterious pleiotropic effects of genes at the end of life. These genes would cause in turn positive effects on fitness in an earlier reproductive age, thus escaping selection against (Hamilton, 1966). However, most empirical studies on ageing are mostly based on a few laboratory animal models, which may not be generalizable to other organisms

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in the wild (Ricklefs, 2008). Research in wild populations is necessary to uncover the physiological mechanisms by which variation in life history influences longevity (Haussmann et al., 2003). For instance, important variation in longevity, as shown by substantial heritability estimates of ageing, suggests the existence of mechanisms that trade-off early vs. late effects, possibly based on repair and stress resistance mechanisms (Ricklefs, 2008; Zera & Harshman, 2001). Notably, adverse effects in early age have been shown to shape longevity across different organisms (Lindström, 1999) and may be particularly relevant for the study of ageing patterns in natural populations. Early-life adverse conditions can permanently shape individual life history traits through their influence in phenotypic development and individual condition, often leading to adaptive state-dependent life histories (Costantini, 2008; McNamara & Houston, 1996; Monaghan, 2008). Recent research in natural conditions suggests that molecular markers may translate early information into long-term effects. A likely candidate for this role is telomere length and its shortening pattern, which has been shown to be a good predictor of survival in several free-living vertebrates (Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Boonekamp, Mulder, Salomons, Dijkstra, & Verhulst, 2014; Heidinger et al., 2012; Monaghan, 2014; Monaghan & Haussmann, 2006). However, the mechanism by which telomeres end up reflecting these organismal processes and influence longevity (Haussmann & Marchetto, 2010), and transgenerational effects (Burton & Metcalfe, 2014; Criscuolo, Zahn, & Bize, 2017), is still not fully understood.

Telomeres are noncoding DNA regions located at the end of eukaryote chromosomes (Blackburn, 1991), which protect the chromosome from deterioration and fusion with other chromosomes. Telomeres are involved in many cellular functions, but their leading role is the maintenance of chromosome stability (Monaghan & Haussmann, 2006). They also act as 'mitotic clocks', shortening with each round of cell division (Watson, 1972). The rate of telomere shortening can be accelerated by environmental stressors (Angelier, Vleck, Holberton, Marra, & Blount, 2013; Asghar et al., 2015), for instance by the effect of high oxidative stress and active immune processes (Epel et al., 2004; von Zglinicki, 2002). High levels of oxidative stress and telomere shortening during early development have been recognized as potential negative consequences of fast growth (Alonso-Alvarez, Bertrand, Faivre, & Sorci, 2007; Kim, Noguera, Morales, & Velando, 2011; Monaghan & Haussmann, 2006). Compensatory growth rate has also been associated with short telomeres and increased telomere attrition under laboratory (Tarry-Adkins, Martin-Gronert, Chen, Cripps, & Ozanne, 2008) and natural conditions (Geiger et al., 2012). Previous studies have shown that telomere loss is particularly strong during early life (Salomons et al., 2009), probably because of the high levels of rapid cell proliferation that occur at this time (Frenck, Blackburn, & Shannon, 1998). Rearing environments can thus be a main driver of telomere shortening (Nettle et al., 2015; Reichert et al., 2015). Additionally, this dependence on stress levels suggests that differences in telomere shortening could be considered an indicator of resistance and/or exposure to oxidative stress (Epel et al., 2004; von Zglinicki, 2002, but see: Boonekamp, Bauch, Mulder, & Verhulst, 2017; Reichert & Stier, 2017).

Altricial birds are good models for studying the link between environmental conditions, telomere dynamics and ageing because of their developmental immaturity at hatching, their considerable longevity and somatic down-regulation of telomerase (Nettle, Monaghan, Boner, Gillespie, & Bateson, 2013). Furthermore, although it varies among chromosomes and tissues, telomere length during embryonic development in vertebrates is similar in the cells of most tissues (Youngren et al., 1998). In birds, telomere dynamics are typically measured in red blood cells (RBC), and research has shown that reductions in telomere length are repeatable across different tissues (Asghar et al., 2016).

Some previous studies have examined the effects of within-brood competition on telomere shortening (Boonekamp et al., 2014; Nettle et al., 2013; Reichert et al., 2015; Voillemot et al., 2012) or the role of oxidative stress on telomere dynamics by manipulating antioxidants (Badás et al., 2015; Kim & Velando, 2015; Noguera, Metcalfe, Boner, & Monaghan, 2015) or corticosterone (Haussmann, Longenecker, Marchetto, Juliano, & Bowden, 2012) during development. In this study, we experimentally manipulated developmental stress by means of a brood size manipulation to assess its effect in telomere length in a wild passerine, the spotless starling, *Sturnus unicolor*. Our hypothesis was that telomere length would decrease with increasing levels of nutritional stress brought about by the brood size manipulation and that this stress would result in parallel modifications of physiological (basal corticosterone) and oxidative stress markers (i.e., increased oxidative damage and/or depleted antioxidant defences). Furthermore, if oxidative stress is responsible for telomere shortening, we expected a negative relationship between telomere length and physiological and oxidative stress levels. We included nestling sex in the analyses, since stress levels may differ between sexes because of differences in competitive ability and physiology (Giordano, Costantini, & Tschirren, 2015; Jimeno, Briga, Verhulst, & Hau, 2017; Jones, Nakagawa, & Sheldon, 2009).

2 | MATERIALS AND METHODS

2.1 | Study area and species

This study was conducted between April and June 2013 in a free-living nest box population of spotless starlings (*Sturnus unicolor*) located in central Spain (Soto del Real, Madrid). The study area is a *dehesa* ecosystem used by grazing cattle, covered by a woodland of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolia*). The spotless starling is a relative long lived, colonial and sedentary passerine that exhibits a facultative polygynous breeding system, that breeds in natural tree holes and artificial cavities, and shows sexual dimorphism (Moreno, Veiga, Cordero, & Mínguez, 1999). Average brood size in our population is 4.72 ± 0.57 (*SD*) nestlings per brood

(Muriel et al., 2015), and fledglings leave the nest around 22 days of age (Cramp, Simmons, & Perrins, 1982–1994). In our study area, most pairs can lay up to two clutches per season; the first one between mid-April and the beginning of May, and the second one at the end of May. First clutches are typically characterized by higher food availability, and brood size and offspring fitness decrease as the season advances (López-Rull, Salaberria, & Pérez, 2010). A total of 723 nestlings originated from 172 different broods were cross-fostered into 168 experimental broods (the number of nests per manipulation was 27 [+2 chicks], 33 [+1 chick], 32 [same clutch size], 36 [−1 chick], 37 [−2 chicks] and 3 [−3 chicks]). The final sample size of 14-day-old nestlings for which biometrical data were obtained was 469. However, blood samples were only taken from a random sample of nests balanced between broods: 264 nestlings (133 males and 131 females) from 72 different experimental nests.

2.2 | Cross-fostering and field procedure

From mid-April onwards, we monitored daily nest box occupancy and egg laying to identify pairs of nests that could be used for cross-fostering. Nestlings were cross-fostered around their third or fourth day of age (day 1 being the hatching day). Nest swaps were conducted by choosing pairs of nests with nestlings of similar size and weight. A random nest within each pair was randomly assigned to a new brood size within the natural range observed in the population (2–7 nestlings per brood), and we removed nestlings to or added nestlings from the other nest to achieve this brood size. We assigned to the second nest a brood size that would match the number of remaining nestlings from the previous exchange. We strived to keep in each nest at least one nestling from the original nest together with cross-fostered chicks, in order that each experimental nest was composed of nestlings from at least two different origin nests. In all analyses, we used the change in brood size as a measure of the magnitude of the manipulation, which ranges from −3 to +2, and added original brood size as a covariate to control for differences in parental rearing capacity.

Nestlings were weighed (to the nearest g) three times during development: at manipulation day (3 days of age) and at 7 and 14 days of age. We define growth as the increase in weight between manipulation day and 14 days of age. When nestlings were 14 days old, tarsus and wing length were also measured to the nearest 0.1 and 1 mm, respectively, and a 600 μl blood sample was collected from the jugular vein with heparinized syringes. As circulating corticosterone quickly increases from basal levels, in order to reduce handling time, blood sampling was performed in each nest by a team of three people: nestlings were removed from the nest box one at a time, and the time elapsed from nest box handling to blood sampling (always <3 min) was carefully recorded with a stopwatch. Blood samples were kept cold (ca. 4°C) until arrival to the laboratory and centrifuged around 5 hr after collection (9,500 g, 10 min). Plasma and RBCs fractions were stored separately at −80°C for further analyses of telomere length, corticosterone levels, oxidative stress markers, plasma metabolites (triglycerides and uric acid) and for molecular sexing. Nests were visited again when nestlings were 19 days old, and four great covert feathers from each individual (in this case N = 208 individuals because of mortality and logistic limitations to visit all nests) were taken and kept in individual small plastic bags at room temperature for feather corticosterone assays.

2.3 | Genomic DNA extraction

Genomic DNA was extracted from RBC samples using DNeasy Blood & Tissue Kit (Qiagen©, Hilden, Germany), following the manufacturer's protocol. DNA quantification was performed using Take3 Micro-Volume Plate in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc, Winooski, Vermont, USA).

2.4 | Molecular sexing

Nestlings were sexed on the basis of a sex-specific gene, that was amplified through PCR using the primers P2 and P8, which amplify part of the CHD-W gene in females and CHD-Z in both sexes (Griffiths, Double, Orr, & Dawson, 1998). Amplified products were visualized in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, California, USA).

2.5 | Telomere length measurements

Telomere length was quantified in RBCs by quantitative real-time polymerase chain reaction (hereafter qPCR). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). We used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. We designed species-specific primers from a spotless starling publicly available sequence (Genbank Accession No. EU551897), using PRIMER3 (Rozen & Skaletsky, 1999). Telomere and GAPDH reactions were carried out on different plates, and specific amplifying conditions were optimized separately. Individuals with relative short telomeres are characterized by high 'threshold cycle' (C_t) values, whereas those with relative long telomere are characterized by low C_t values (Cawthon, 2002). The relative expression ratio (R) of a target gene is calculated based on efficiency (E) and the crossing points (CP, point at which the fluorescence rises appreciably above the background fluorescence), deviation of an unknown sample versus a control, and expressed in comparison with a reference gene (Pfaffl, 2001). E was calculated by the equation $E = 10^{(-1/\text{slope})}$. The primers used to amplify the telomere region were as follows: Tel 1b (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel 2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′), and for the amplification of the GAPDH, we used two specific GADPH-F (5′-GGTAGATGGGAGTTCAGTTGTG-3′) and GAPDH-R (5′-AGAAACAAAGCACTGTCAGGG-3′). Quantitative real-time PCR (qPCR) for both telomere and GAPDH was performed using 2 μl of DNA at 5 ng/μl final concentration, Tel1b/Tel2b (or GAPDH-F/GAPDH-R) set of primers at a concentration of 200/200 nM, in a final volume of 20 μl containing 10 μl of SYBR Green PCR Master

Mix (Bio-Rad, Hercules, CA, USA). qPCR telomere conditions were 10 min at 95° followed by 30 cycles of 15 s at 95°, 30 s at 54.5° and 30 s at 72°. For the GAPDH amplification, we used 10 min at 95° followed by 40 cycles of 15 s at 95°, 30 s at 60.5° and 30 s at 72°. Samples were run in duplicate on each plate. Telomere and GAPDH plates included serial dilutions (0.2, 0.4, 2, 10, 30 and 50 ng) of DNA of the same reference bird to generate a reference curve to control for the amplifying efficiency of the qPCR (all telomere and GAPDH efficiencies plates fell between 2.052 and 2.101). Mean intraplate coefficient variation was 0.31% for the GAPDH C_t values and 1.65% for the C_t values of the telomere assays. The interplate coefficient of variation was calculated on a 'golden sample' repeated in all the different plates and was 1.11% for the GAPDH C_t values and 5.38% for the telomere C_t values assays.

Telomere length was quantified using the formula: (E telomere)$^{\Delta Ct}$ telomere (control-sample)/(E GAPDH)$^{\Delta Ct}$ GAPDH (control-sample), in which control corresponds to the value obtained for the reference sample (Pfaffl, 2001). We also analysed telomere length using the Cawthon (2002) procedure, used in some previous studies in birds (Criscuolo et al., 2009), but the results were almost identical and we kept the original analysis (r^2 = 0.99).

2.6 | Plasma corticosterone assays

In a pilot study, we verified whether direct assay of plasma without extraction provided a reliable method of assay. To this end, we compared corticosterone levels from a subsample with and without extraction. Briefly, for corticosterone extraction in plasma, 5 ml of diethyl ether extra pure (Scharlab, Barcelona, Spain) was added to 150 μl of plasma in a glass tube, vortexed for 2 min and centrifuged for 5 min (4°C, 300 g) in a centrifuge equipped with safety capsules to avoid concentration of ether fumes. Tubes were then frozen in a bath of dry ice with propanol, the etheric phase transferred to clean tubes and subsequently dried in a heated block (50°C). Extracts were resuspended in 150 μl of steroid-free serum (DRG, Marburg, Germany). Samples were assayed in duplicate following kit instructions using a commercial corticosterone ELISA (DRG, Marburg, Germany), and optical density was measured with a plate spectrophotometer (BioTek Instruments, Inc, Winooski, Vermont, USA). Serial dilutions of a pooled sample over the range of assay values met linear predictions. The regression between corticosterone values obtained by extraction and directly assayed from plasma was very strong and confirmed that plasma could be directly used ($F_{1,8}$ = 152.4, p < 0.001; r^2 = 0.98). The rest of the samples were then assayed directly in plasma aliquots. Mean intraplate CV was 9.99%, and interplate CV was 17.62%.

2.7 | Feather corticosterone assays

We followed a published methodology for steroid extraction from feathers (Bortolotti, Marchant, Blas, & German, 2008) with minor modifications. Feather vanes were separated from the calamus, cut in small pieces (<5 mm) with scissors and weighed in a tared glass tube to the nearest 0.001 g with an analytical scale (Sartorius, Gottingen, Germany). Since the feather mass obtained for each individual was too small to allow us to quantify corticosterone reliably (Lattin & Romero, 2014), we pooled the feathers of two to three individuals from each nest in each tube (average = 2.39, SD = 0.59). Average mass of feather material per pool was 19.6 mg (SD = 14.1). We added 6 ml of methanol to the tube (HPLC gradient grade, Prolabo (VWR), Pennsylvania, USA) and left the tubes for 30 min in an ultrasound water bath. Then, tubes were capped with parafilm and incubated for 8 hr in an uncovered shaking water bath at 50°C. After this step, samples were left at 4°C overnight. Samples were then filtered with the aid of a syringe and a nylon plug filter (0.45 μm). We added two additional ml of methanol to the tube to wash the feather remains, and this methanol was similarly filtered and added to the previous 6 ml. Tubes were then placed in a heated tube rack (50°C) under a stream of nitrogen until evaporation (Techne, Germany). Dried extracts were suspended in 150 μl of steroid-free serum (DRG, Marburg, Germany) and vortexed for 10 min. Extracted samples were then analysed in duplicate following kit instructions using a commercial corticosterone ELISA (DRG, Marburg, Germany), and optical density was measured with a plate spectrophotometer (BioTek Instruments, Inc, Winooski, Vermont, USA). Serial dilutions of a pooled sample over the range of assay values met linear predictions. Mean intraplate CV was 5.22%, and interplate CV was 14.01%.

2.8 | Measurement of oxidative stress markers (MDA, tGSH and TAS)

Detailed procedures of the techniques used for analysing plasma malondialdehyde (MDA), intracellular total glutathione (tGSH) levels in RBC and plasma total antioxidant status (TAS) have been given in López-Arrabé, Cantarero, Pérez-Rodríguez, Palma, and Moreno (2014) and López-Arrabé et al. (2015). MDA is a widely used marker of lipid peroxidation levels in plasma (Halliwell & Gutteridge, 2007; Mateos & Bravo, 2007). It was quantified by high-performance liquid chromatography (HPLC) following published protocols (Agarwal & Chase, 2002; López-Arrabé et al., 2014, 2015). Briefly, 20 μl of plasma samples was mixed in 50 μl of butylated hydroxytoluene solution (0.05% w/v in 95% ethanol), 400 μl phosphoric acid solution (0.44 M) and 100 μl thiobarbituric acid solution (0.42 mM) and heated at 100°C on a dry bath incubator. MDA-TBA adducts were extracted by adding 250 μl n-butanol and quantified by fluorescence (excitation: 515 nm; emission: 553 nm) in an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, California, USA). The repeatability was calculated through twelve different samples ran in duplicate (r = 0.92, p < 0.001).

tGSH is the main intracellular antioxidant (Halliwell & Gutteridge, 2007), and its levels in RBC were quantified by homogenizing samples in a stock buffer and mixing it with 10% trichloroacetic acid. After centrifuging, the supernatant was mixed with 0.3 mM NADPH, 6 mM DTNB and glutathione reductase (50 units/ml), and absorbance kinetics were measured in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc, Winooski, Vermont,

USA). Variation in absorbance was used to determine the intracellular concentration of tGSH (expressed as micromoles per gram of RBCs) by comparing this value with a serial dilution of the GSH standard curve. Repeatability, calculated from a subset of 15 samples processed and measured twice, was high ($r = 0.78$, $p < 0.001$).

TAS estimates the combined action of nonenzymatic antioxidants in a plasma sample, and it was measured following Cohen (2007), with minor modifications described in López-Arrabé et al. (2014, 2015). Plasma samples (5 μl) were mixed with 15 μl metmyoglobin and 250 μl ABTS (2,2-azino-di-[3-ethylbenzthiazolinesulphonate]) metmyoglobin and 50 ml hydrogen peroxide. Changes in absorbance of the mixture were monitored using the same microplate reader mentioned before by taking measurements at 660 nm every 10 s while keeping the plates at 37°C. A water-soluble α-tocopherol derivative, trolox (1.7 mM), was used as standard. All samples were ran in duplicate (CV 4.89%).

Plasma levels of triglycerides and uric acid may affect MDA and TAS measurements, respectively, making advisable to statistically control for the metabolites in the analyses (Cohen, Klasing, & Ricklefs, 2007; Pérez-Rodríguez et al., 2015). Thus, in all plasma samples, we also quantified these two blood metabolites by using methods involving uricase/peroxidase (for uric acid) and glycerol phosphate oxidase/peroxidase (for triglycerides), following commercial kits (refs. 11,522 and 11,529; Biosystems, Barcelona, Spain). A subset of 15 samples assayed in duplicate showed high repeatability for both variables (uric acid: $r = 0.896$, $p < 0.001$; triglycerides: $r = 0.943$, $p < 0.001$).

2.9 | Statistical analysis

All analyses were performed in R v.3.3.3 (R Development Core Team, 2016). We fitted linear mixed models with Gaussian distribution. Models were run using the package *lmerTest*, and *p*-values and degrees of freedom were based on Satterthwaite approximation for denominator degrees of freedom (Kuznetsova, Brockhoff, & Christensen, 2017).

We first checked for the effect of the treatment on morphometric measures and early development of nestlings. We used mass at days 7 and 14, the growth rate of the nestlings from days 3 to 14, as well as wing and tarsus length at day 14 as our dependent variables. We included experimental brood size manipulation and original brood size as covariates in the model, and brood (first or second) and sex as fixed factors. To control for potential initial differences, we included weight at day 3, which is the weight taken before the brood manipulation took place, as a covariate. We included the interaction term between treatment and sex, and whenever it was nonsignificant, it was removed and the final model was recalculated (Engqvist, 2005). In all models, we included nest of origin and cross-fostered nest as random factors.

Secondly, we looked at the effects of treatment on telomere length, corticosterone and oxidative stress variables (MDA, tGSH and TSA). In initial models, we included quadratic effects of treatment (experimental brood size), as well as sex and brood as fixed factors. We added original brood size to control for parental rearing capacity.

Collinearity among variables was inspected by checking variance inflation factors (<5 in all models). Nestling handling time was included in the analysis of plasma corticosterone and expressed as the time (in seconds) since the nest box was taken down to blood sample collection. We checked for normality of variables and applied Box-Cox transformations when necessary. We made sure that residuals of all models followed a normal distribution and that variances were homogeneous by visually checking Q–Q plots and by plotting residuals versus fitted values, respectively.

We explored the causal conditional relationship between treatment, stress variables and telomere length using path analysis. Path analysis is a statistical tool to explore the direct and indirect causal relationships among variables, allowing a balanced test of complex relationships among variables. We applied d-separation to the set of independent claims following a direct acyclic graph, DAG (Pearl, 2009). D-separation allows the presence of hierarchical models with random factors. We calculated linear and nonlinear paths. For that, we centred the variable treatment by subtracting the population mean from each value (Scheiner, Mitchell, & Callahan, 2001). The path was oriented by computing the causal dependencies and independencies of triplets of variables (Scheines, Spirtes, Glymour, & Meek, 1994). The final DAG was tested with a d-sep test $[C = -2 \sum_{i=1}^{k} \ln(pi)]$. This statistic tests whether the basic set of independence claims follows a chi-square distribution with $k \times 2$ degrees of freedom, where k is the total number of independence claims specified in the DAG (Shipley, 2009). We analysed females and males separately to account for the purported sexual differences in physiology. Once we had the final DAG, we calculated the standardized regression coefficients between pairs of variables. All models were linear mixed models with nest of origin and cross-fostered nest included as random factors.

3 | RESULTS

3.1 | Nestling development

There were no differences in original or in experimental clutches with respect to experimental brood size at day 3, when the manipulation took place ($F_{1,257} = 3.023$, $p = 0.083$; $F_{1,82.2} = 1.131$, $p = 0.291$, respectively). However, from that age (3 days) onwards, experimental brood size had strong consequences on nestling development. Weight at days 7 and 14, as well as growth (increase in weight between manipulation and day 14), strongly decreased with increasing experimental brood size (Table 1). Structural size, measured by tarsus and wing length, also decreased with increasing brood size (Table 1). Males were heavier than females at days 7 and 14, also having larger tarsi. Interestingly, brood size affected more severely female than male weight at day 14 (males: $\beta = -0.181 \pm 0.04$ *SE*, $n = 132$, $p < 0.001$; females: $\beta = -0.311 \pm 0.04$ *SE*, $n = 131$, $p < 0.001$). A similar effect was found for wing length (males: $\beta = -0.461 \pm 0.41$ *SE*, $n = 132$, $p = 0.271$; females: $\beta = -2.348 \pm 0.48$ *SE*, $n = 131$, $p < 0.001$)

TABLE 1 Estimates, standard error (*SE*), *F*-values and *p*-values of brood size manipulation (treatment) on morphometric variables and growth

Measurement	Estimate	*SE*	*F*	*p*
Weight day 7				
Terms in the model				
Original brood size	−0.802	0.532	1.506	0.136
Treatment	−0.955	0.302	3.166	0.002
$Sex_{females}$	−1.321	0.470	2.808	0.005
Weight day 3	1.592	0.091	17.564	<0.001
$Brood_{second}$	−1.916	0.975	1.965	0.053
Weight day 14				
Terms in the model				
Original brood size	−0.104	0.059	3.16	0.079
Treatment	−0.196	0.039	56.79	<0.001
$Sex_{females}$	−0.221	0.050	19.32	<0.001
Weight day 3	0.057	0.010	34.69	<0.001
$Brood_{second}$	−0.012	0.106	0.01	0.907
Treatment* $sex_{females}$	−0.116	0.037	9.76	0.002
Wing day 14				
Terms in the model				
Original brood size	−0.424	0.67	0.39	0.531
Treatment	−1.609	0.42	25.63	<0.001
$Sex_{females}$	−0.684	0.47	2.15	0.144
Weight day 3	1.164	0.09	156.24	<0.001
$Brood_{second}$	4.799	1.21	14.77	<0.001
Treatment* $sex_{females}$	−0.690	0.35	3.96	0.048
Tarsus day 14				
Terms in the model				
Original brood size	0.051	0.11	0.23	0.635
Treatment	−0.353	0.06	33.71	<0.001
$Sex_{females}$	−0.368	0.11	11.21	<0.001
Weight day 3	0.090	0.02	18.67	<0.001
$Brood_{second}$	0.138	0.20	0.46	0.499
Growth				
Terms in the model				
Original brood size	−1.764	0.94	3.51	0.065
Treatment	−3.297	0.62	59.82	<0.001
$Sex_{females}$	−3.464	0.78	19.94	<0.001
Weight day 3	−0.068	0.15	0.20	0.653
$Brood_{second}$	−0.268	1.71	0.02	0.876
Treatment* $sex_{females}$	−1.718	0.57	8.97	0.003

and for growth (males: $\beta = -3.065 \pm 0.62$ *SE*, $n = 132$, $p < 0.001$; females: $\beta = -4.992 \pm 0.60$ *SE*, $n = 131$, $p < 0.001$; Figure 1). No differential effects were found between first and second broods, but nestlings developed larger wings in second broods (Table 1).

3.2 | Corticosterone levels

Plasma corticosterone levels were significantly and positively related to brood size manipulation. This effect depended on the sex of the nestling, as shown by the significant interaction between treatment and sex (Table 2; Figure 2). While we did not find a significant relationship between treatment and corticosterone in males ($\beta = 0.078 \pm 0.07$ *SE*, $n = 127$, $p = 0.253$), brood size manipulation significantly increased corticosterone levels in females ($\beta = 0.280 \pm 0.07$ *SE*, $n = 129$, $p < 0.001$). Nestlings in first broods had higher corticosterone levels than nestlings in second broods (Table 2). As expected, corticosterone levels increased with increasing handling time (Table 2).

We also examined how feather corticosterone levels reflected stress. However, since the amount of feathers collected per individual was too small for the assay, we were forced to pool several brood mates together (mean = 2.39 ± 0.59 *SE*, range: 2–5), thus losing information on sex and specific growth. For these analyses, we averaged weight and plasma corticosterone values from those nestlings whose feathers were pooled. In order to validate corticosterone feather estimates, we firstly checked its relationship with plasma corticosterone. Since we were using pools of feather samples for feather corticosterone, we also calculated means of plasma corticosterone. For this, we calculated the average of residuals of the regression of plasma corticosterone (log-transformed) on manipulation time for each nestling within the pool ($\beta = 0.007 \pm 0.001$ *SE*, $F_{1,200} = 30.27$, $p < 0.001$; $r^2 = 0.13$). The relationship between feather and plasma corticosterone measures was positive and significant ($\beta = 0.05 \pm 0.01$ *SE*, $\chi^2 = 13.49$, $p < 0.001$; Figure 3). Mixed models revealed that feather corticosterone values also increased with increasing brood size ($\beta = 0.110 \pm 0.03$ *SE*, $\chi^2 = 14.51$, $p < 0.001$; Figure 4).

3.3 | Oxidative stress

The effect of brood size manipulation was only marginally detected in the levels of tGSH, which showed a tendency to decrease with increasing brood enlargement (Table 2; Figure 5). Lipid peroxidation, estimated as concentration of MDA in plasma, and plasma TAS did not vary with manipulated brood size; however, they were both higher in first than in second broods (Table 2).

3.4 | Telomere length

We found a significant quadratic effect of treatment (Table 2; Figure 6). In addition, second brood nestlings had longer telomeres than nestlings from the first brood. We did not find an effect of sex on telomere length (Table 2).

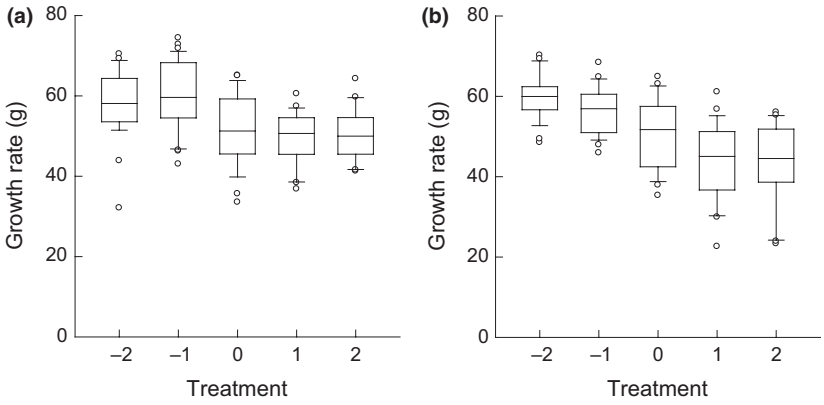

FIGURE 1 Effect of brood size manipulation on nestling growth between 3 and 14 days of age for males (a) and females (b). Both negative regression lines are significant, but the slope for the relationship in the case of females is steeper (see text for statistics). Medians with 25th/75th percentiles (box) and 10th/90th percentiles (whiskers) are shown

3.5 | Relationship between treatment, stress and telomere length

Both for males and females, the DAGs were supported by the data (for females: $C = 32.904$, $df = 28$, $p = 0.260$; for males: $C = 7.795$, $df = 12$, $p = 0.801$).

For females, treatment had both direct and indirect effects on telomere length (Figure 7a). One indirect effect was via a strong and negative effect of treatment on nestlings' growth and a positive effect of growth on the redox balance (tGSH and MDA, which are positively correlated), which in turn affected negatively telomere length (indirect effect 1: +0.056 ± 0.12 *SE*). Moreover, there was a second indirect path via MDA and tGSH. Brood enlargement affected negatively tGSH, via MDA, which in turn had a strong negative effect on telomere length (indirect effect 2: +0.019 ± 0.14 *SE*). We found a third indirect effect, a nonlinear one, through tGSH (indirect effect 3: −0.065 ± 0.13 *SE*). The total indirect effect accounted for 0.010, whereas the direct effect was stronger (−0.312 ± 0.11 *SE*). Globally, treatment had a negative effect. The total effect of treatment on telomere length was −0.302.

Corticosterone values were affected directly and nonlinearly by treatment (+0.215 ± 0.09 *SE*). The effect size was similar to the indirect effect of treatment on corticosterone via growth (indirect effect: +0.212 ± 0.11 *SE*). Nestlings from enlarged broods had lower growth rates, and those with lower growth rates had higher corticosterone values. The total effect of treatment on corticosterone was +0.427± 0.14 *SE*.

In males, the path was very simple (Figure 7b). There was a direct and nonlinear effect of treatment on telomere length, with similar effect size as in females (−0.322 ± 0.10 *SE*). However, we did not find a causal relationship between stress responses and telomere length. Interestingly, unlike females, corticosterone values in males were not affected by treatment or growth.

4 | DISCUSSION

We found evidence that differences in rearing conditions during early development induced by a brood size manipulation had significant effects on nestling development, telomere length, physiological stress and one marker of oxidative status in spotless starling nestlings. Our results are consistent with prior studies showing that brood size enlargement affects growth negatively (Bourgeon, Guindre-Parker, & Williams, 2011; Merilä, 1997; Naguib, Riebel, Marzal, & Gil, 2004; Pettifor, Perrins, & McCleery, 2001; Sanz, 1997; Verhulst, Holveck, & Riebel, 2006), as well as reducing telomere length during the nestling stage (Boonekamp et al., 2014; Voillemot et al., 2012). These effects are most likely due to nestlings in enlarged broods facing increased sibling competition (Neuenschwander, Brinkhof, Kölliker, & Richner, 2003), lower access to food and consequently significantly lower growth and poorer condition (De Kogel & Prijs, 1996; Naguib, Nemitz, & Gil, 2006). However, a possible indirect link between telomere shortening and physiological stress could only be observed in females, whereas in the case of males this mediation was missing, suggesting distinctive differences between sexes in dealing with environmental stress.

Our manipulation had repercussions on several morphological measurements, including weight, body condition, tarsus and wing length, as well as on growth (increase in mass during nestling development). Females were more affected than males in all these variables except in tarsus length, suggesting that females lose out disproportionally in competition for resources, acquiring weight at a lower rate than males (Alonso, Martín, Morales, & Alonso, 2018; Oddie, 2000).

Similarly, we found that our manipulation led to increased basal corticosterone plasma levels in females, whereas this effect was absent in males. The pattern is similar to other studies in which increasing the number of chicks in the nest resulted in increased corticosterone levels in females (Jimeno et al., 2017) or in the two sexes (Ilmonen, Hasselquist, Langefors, & Wiehn, 2003; Saino, Suffritti, Martinelli, Rubolini, & Møller, 2003). However, in a previous study conducted in our species, no effect was detected after correcting for manipulation time (Gil, Bulmer, Celis, & Puerta, 2008b), possibly because of the stronger manipulation performed in the present study. Increased corticosterone secretion may induce catabolic effects in nestlings, allowing undernourished chicks to fuel their increased activity levels, including begging rates or competitive behaviours (Axelrod & Reisine, 1984; Cherel, Robin, Heitz, Calgari, & Le Maho, 1992; Kitaysky, Kitaiskaia, Wingfield, & Piatt, 2001). The

TABLE 2 Estimates, standard error (*SE*), *F*-values and *p*-values of the effect of brood size manipulation (treatment) on telomeres and stress variables (corticosterone, tGSH, MDA and TAS)

Measurement	Estimate	*SE*	*F*	*p*
Corticosterone				
Terms in the model				
Treatment	0.114	0.06	17.45	<0.001
Original brood size	0.183	0.08	4.67	0.034
$Sex_{females}$	0.251	0.10	6.07	0.014
$Brood_{second}$	−0.660	0.15	20.26	<0.001
Manipulation time	0.005	0.01	22.90	<0.001
Treatment* $sex_{females}$	0.184	0.07	6.25	0.013
tGSH				
Terms in the model				
Treatment	−0.104	0.05	3.92	0.052
Original brood size	−0.016	0.09	0.03	0.862
$Sex_{females}$	−0.056	0.10	0.31	0.580
$Brood_{second}$	−0.854	0.17	25.05	<0.001
MDA				
Terms in the model				
Treatment	−0.062	0.05	1.79	0.186
Original brood size	0.058	0.08	0.50	0.480
$Sex_{females}$	0.003	0.10	0.01	0.978
$Brood_{second}$	−1.073	0.16	43.80	<0.001
Triglycerides	0.308	0.06	26.41	<0.001
TAS				
Terms in the model				
Treatment	−0.016	0.04	0.14	0.710
Original brood size	0.028	0.08	0.13	0.716
$Sex_{females}$	−0.120	0.07	3.11	0.079
$Brood_{second}$	−0.064	0.14	0.22	0.638
Uric acid	0.095	0.01	179.36	<0.001
Telomeres				
Terms in the model				
Treatment	−0.001	0.01	0.020	0.888
$Treatment^2$	−0.019	0.01	4.16	0.045
Original brood size	0.032	0.02	2.89	0.094
Sex_{female}	0.019	0.02	0.67	0.413
$Brood_{second}$	0.134	0.04	13.20	<0.001

fact that only females responded with higher corticosterone levels could be explained by their smaller size, which probably results in a lower competitive ability to access food from parents (Oddie, 2000). It is often found that sexes differ in environmental sensitivity to stressors (Muriel, Perez-Rodriguez, Ortiz-Santaliestra, Puerta, & Gil, 2017), but a large meta-analysis has shown that there are no general patterns across animals in relation to sex or size (Jones et al., 2009). Most likely, the phenomenon is specific to different taxonomic groups and is possibly explained by differences in their developmental mode. Although a larger body size is expected to imply higher costs, in our case it was the smaller sex, females, which suffered the strongest costs. The contrasting paths of females and males found in this study (Figure 7) also illustrate this pattern: females showed a more complex network of direct and indirect effects than males, suggesting a more systemic and integral impact of our experimental manipulation. Given that nestlings in altricial species need to compete for food with nest mates, it is possible that the bigger size of males could be an advantage in this respect. Indeed, studies in several species of the *Paridae* family have shown that male nestlings have an enhanced competitive ability in poor environments (Nicolaus et al., 2009; Oddie, 2000; Råberg, Stjernman, & Nilsson, 2005). In agreement with our results, studies in the zebra finch show that daughters have a greater susceptibility to nutritional stress than sons (De Kogel, 1997) and that female offspring (but not male) present a long-term increase in basal metabolic rates and basal corticosterone as a consequence of increases in brood size (Jimeno et al., 2017; Verhulst et al., 2006). Of two previous studies manipulating breeding stress in the closely related European starling (*Sturnus vulgaris*), one found that negative effects were restricted to female offspring (Rowland, Love, Verspoor, Sheldon, & Williams, 2007), whereas the other study found negative effects in males (Nettle et al., 2015). Although this discrepancy does not allow us to derive a general conclusion, it does underline the fact that the two sexes tend not to respond equally to developmental stressors (Muriel et al., 2017).

Early development is a phase of life where individuals are prone to high levels of oxidative stress (Metcalfe & Alonso-Alvarez, 2010). This is because the high metabolic activities required for growth lead to increased production of reactive oxygen species, and these can hardly be buffered by the relatively immature antioxidant system of the individual (Metcalfe & Alonso-Alvarez, 2010). Our results showed a tendency for brood size manipulation to decrease the amount of RBC tGSH, which is one of the most important intracellular antioxidants and plays a key role in peroxide detoxification (Anderson, 1998; Suttorp, Toepfer, & Roka, 1986; Wu, Fang, Yang, Lupton, & Turner, 2004). However, the other markers that we examined were not affected by the manipulation, and thus, neither MDA nor TAS levels in plasma varied with brood size. Path analysis showed that, in females, tGSH and MDA levels affected each other mutually, being also altered by the simultaneous effect of treatment and growth rate. Such complex causal network suggests that the brood size manipulation challenged the redox balance of females via different and nonexclusive mechanisms, such as modified growth patterns (Metcalfe & Alonso-Alvarez, 2010; Metcalfe & Monaghan, 2003; Nettle et al., 2015) or direct sibling competition (Bourgeon et al., 2011; Moreno-Rueda et al., 2012). In males, a similar effect of growth on MDA was observed, but we did not find a direct effect

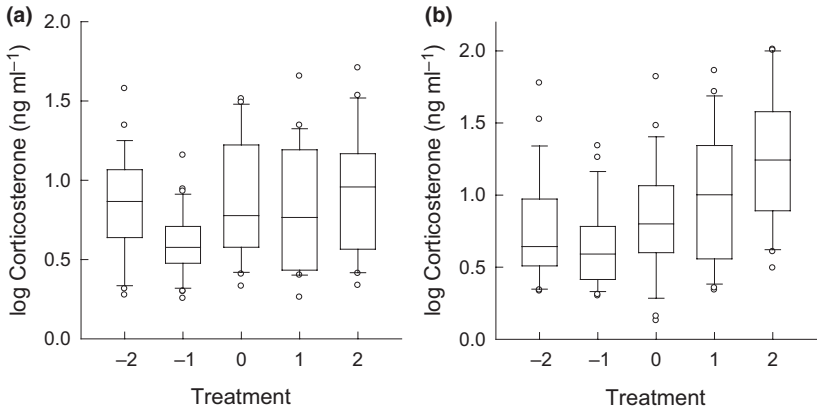

FIGURE 2 Plasma corticosterone levels in (a) males and (b) females. Corticosterone levels were not related to brood size manipulation (treatment) in males. However, females from enlarged broods had significantly higher corticosterone values. Medians with 25th/75th percentiles (box) and 10th/90th percentiles (whiskers) are shown

of brood size manipulation on MDA (Figure 7), thus reinforcing the idea that this sex was less affected by direct sibling competition. Nevertheless, nestlings of both sexes apparently managed to control effectively the oxidative challenge associated to our treatment, keeping the levels of oxidative damage at bay at the cost of suffering a marginal decrease in some key antioxidants (i.e., tGSH) in the process.

We found that MDA and TAS levels were higher in first than in second broods. This is consistent with the higher levels of corticosterone found in these first broods and can be explained by the unusually harsh conditions experienced during that event. Although second broods are typically characterized by harsher meteorological conditions than first broods in our population (Gil, Bulmer, Celis, & Lopez-Rull, 2008a), in the year where this manipulation was made (2013), an unusual cold wave with snowfall during the nestling period of the first brood caused an unusual high mortality in many nests. The percentage of eggs that produced fledglings was a mere 59%, in contrast to usual levels (e.g., 88% in 2014), and numerous nests were abandoned or remained without parental visits for prolonged periods of time. This is the most likely explanation for our finding of higher levels of corticosterone and MDA in first than in second broods. The concomitant higher levels of TAS in first as compared to second broods can be explained by a release of nonenzymatic antioxidants to the bloodstream as a response to increased MDA levels (Costantini & Verhulst, 2009).

Our treatment affected telomere length, but this effect was quadratic instead of linear positive, as expected (Figure 6). Longer telomeres—and hence, lower rates of cellular ageing—were experienced by nestlings raised in control broods (i.e., those matching the initial clutch size laid by the mother), and deviations above or below the average brood size let to reduced telomere lengths. However, the causes of telomere attrition are likely to differ for the two extremes. The negative effect of increased brood size on telomere length is in line with previous studies (Boonekamp et al., 2014; Costanzo et al., 2016; Nettle et al., 2013), probably as a consequence of developmental stress caused by increased sibling competition and reduced access to food. By contrast, nestlings raised in reduced broods were not constrained by these factors and therefore maximized their growth rates. A possible explanation for telomere shortening in these small broods may be explained by possible costs of early thermoregulation (Andreasson, Nord, & Nilsson, 2016), which may exert a negative impact in telomere shortening. Irrespective of the proximate mechanism, this quadratic effect is interesting as it would suggest the existence of a cellular ageing-based trade-off affecting optimal brood size.

In addition to the effect of treatment, we found that nestlings raised in second broods had larger telomeres than those raised in first broods. This was unexpected, because first broods are typically characterized by higher growth rates and less mortality than second broods in our population, due to higher food availability and milder weather (Muriel et al., 2015; Salaberria, Celis, López-Rull, & Gil, 2014). However, given the particularly harsh weather conditions during first broods in the year of study (see above), it is likely that developmental stress levels were higher for first broods, as shown by the higher levels of corticosterone and MDA found in these broods. The reduced telomere length in first broods could thus be explained by the fact that nestlings experienced difficulties to thermoregulate (Andreasson et al., 2016).

It should be noted, however, that in our study, we did not measure attrition per se, but a single measure of telomere size at 14 days of age. However, since we used a cross-fostering procedure, our design allows us to control for family differences and obtain a relevant proxy of telomere shortening. Similarly, previous studies where just one sample was taken to estimate telomere length have reported significant and relevant changes in telomere length in relation to stressors and nesting conditions (Brown, Dechow, Liu, Harvatine, & Ott, 2012; Epel et al., 2004; Olsen, Bérubé, Robbins, & Palsbøll, 2012; Soler et al., 2017; Voillemot et al., 2012). Previous studies have confirmed that both telomere shortening and telomere length can predict subsequent survival in numerous organisms (Bize et al., 2009; Haussmann, Winkler, & Vleck, 2005; Heidinger et al., 2012; Salomons et al., 2009; Vleck, Haussmann, & Vleck, 2007). Salomons et al. (2009) found that recruiting first-year jackdaws (*Corvus monedula*) had a much lower telomere attrition rate than birds that did not return to breed. In addition, individuals with long telomeres during early life have been shown to maintain longer telomeres than the rest of the population throughout their life (Monaghan, 2010; Monaghan & Haussmann, 2006). There is a debate as to whether

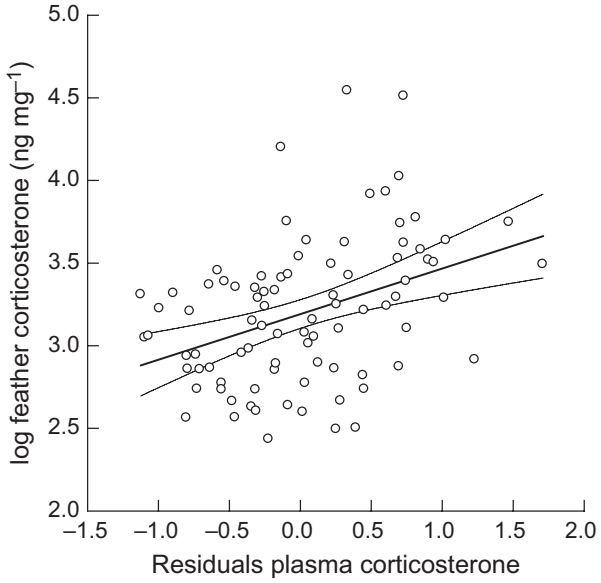

FIGURE 3 Correlation between glucocorticoids from feathers and residual plasma corticosterone and 95% confidence interval of the regression line. Note that samples were pooled per nest

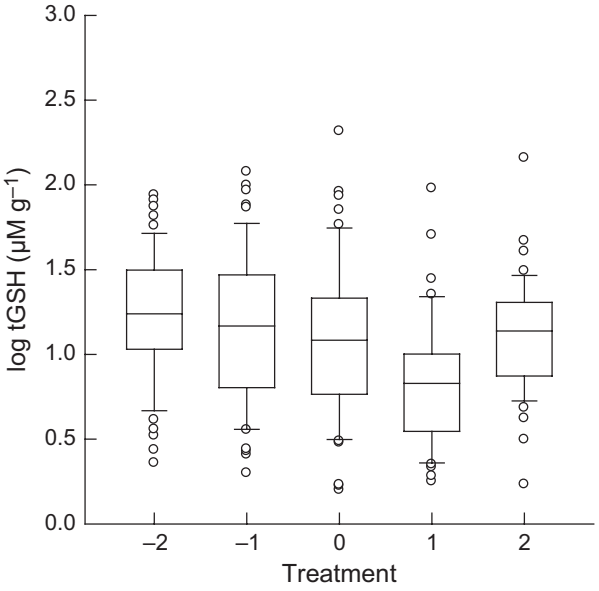

FIGURE 5 Relationship between brood size manipulation (treatment) and glutathione levels (tGSH). The effect is marginally significant (see text for statistics). Medians with 25th/75th percentiles (box) and 10th/90th percentiles (whiskers) are shown

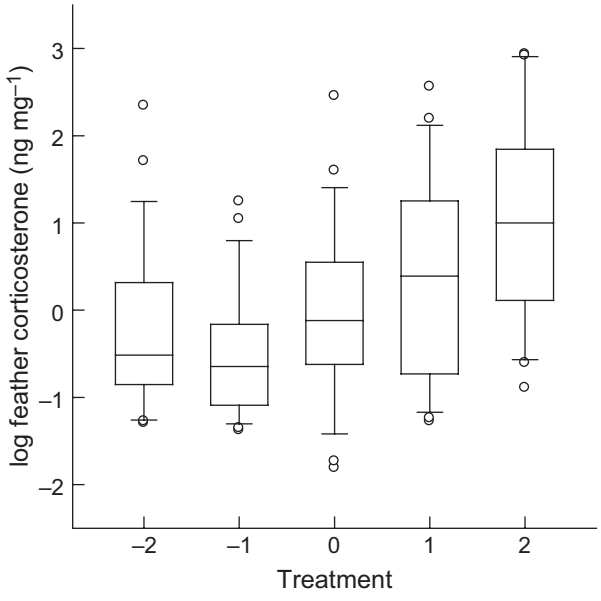

FIGURE 4 Biological validation of feather corticosterone values. Similar to the results with plasma corticosterone values, feather glucocorticoids were correlated with brood size manipulation treatment. Medians with 25th/75th percentiles (box) and 10th/90th percentiles (whiskers) are shown

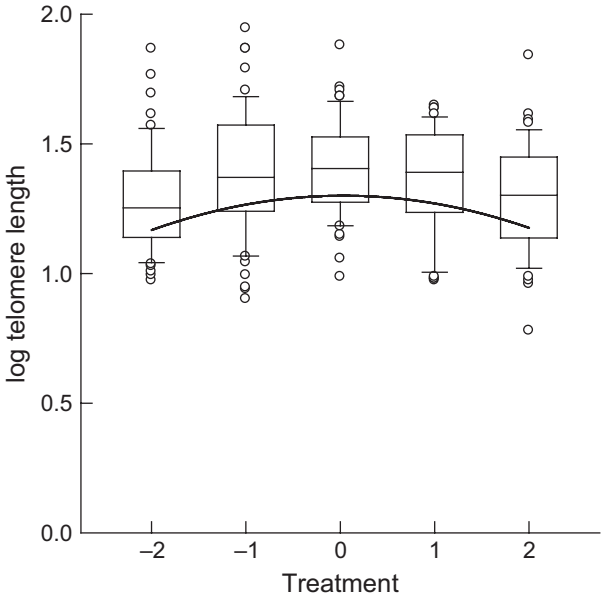

FIGURE 6 Brood size manipulation had a quadratic effect on telomere length. The graph represents the medians with 25th/75th percentiles (box) and 10th/90th percentiles (whiskers). The line represents predicted values after model selection

it is telomere length or telomere shortening the variable that best predicts survival, and evidence varies depending on which species is considered. For instance, in jackdaws, the effects of rearing condition on telomere shortening are stronger than those for absolute telomere length, and it is indeed telomere shortening the variable that best predicts survival (Boonekamp et al., 2014). In the case of starlings, a recent study found that both variables covaried positively and were equally useful in detecting effects of rearing conditions in nestlings (Nettle et al., 2015).

It is usually assumed that oxidative stress is a key factor negatively affecting telomere integrity (Kurz et al., 2004; von Zglinicki, 2002). However, our results are not consistent with this claim. Changes in telomere length with treatment were not mirrored by changes in oxidative damage among treatments, and path analyses

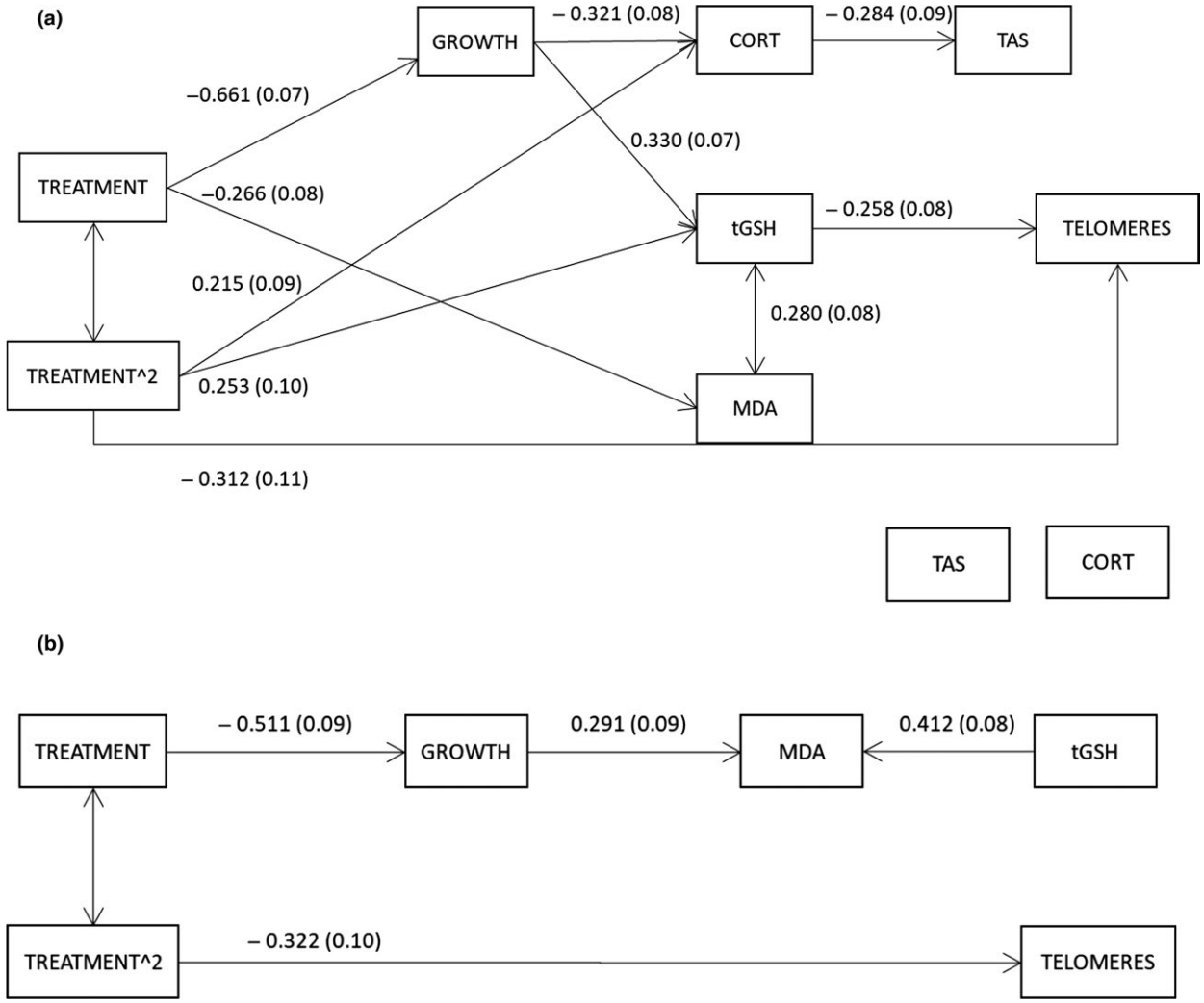

FIGURE 7 Path diagrams for (a) females and (b) males. Only statistically significant relationships are shown. The standardized coefficients and standard errors are depicted in the diagram. The double arrow between treatment and squared treatment represents that both traits are correlated (no causal link between them), as well as the double arrow between MDA and tGSH

showed a rather indirect connection between oxidative damage and telomere length. A previous study using a similar species to ours also found that MDA levels failed to predict telomere shortening (Nettle et al., 2015). Indeed, recent studies have also questioned the role of oxidative stress as a direct causal factor on telomere shortening in vivo (Boonekamp et al., 2017, but see: Reichert & Stier, 2017). It has been suggested that among-individual variation in telomere attrition during early development can be largely caused by differences in cell proliferation rate, with only a modulating effect of oxidative stress (Boonekamp et al., 2017; Reichert & Stier, 2017). However, our path analyses did not reveal a direct effect of growth rates on telomere lengths in either males or females. In any case, the lack of connection that we found between oxidative damage and telomeres should be taken with caution, as it is based on correlational evidence (Reichert & Stier, 2017). Also, it could be argued that we measured MDA in plasma, whereas telomeres were measured in the cellular fraction of the blood, precluding our capacity to detect a significant association between both. However, in our study, we tested a wider range of oxidative markers, including tGSH levels in erythrocytes, as well as plasma TAS, and only tGSH in females was marginally related to telomere length. Another possibility is that oxidative stress is not the main agent behind telomere shortening in nestlings, and that a more general measure of allostatic load, such as that reflected in the corticosteroid response, could be more relevant to this process (Epel, 2009; Speakman & Selman, 2011). In our study, however, we did not find a link between corticosterone levels and telomere length, in contrast with other studies (Haussmann et al., 2012; Quirici, Jimena Guerrero, Krause, Wingfield, & Vasquez, 2016). We are confident that this pattern is robust, because we found that a single estimation of corticosterone levels at 14 days of age was correlated with the more integrative measure of basal corticosterone found in growing feathers. However, basal and stress-induced corticosterone levels are different phenomena (Haussmann et al., 2012), not necessarily correlated, and some studies in humans show that it is the response of corticosterone to stressors, and not basal corticosterone, the variable that best covaries with telomere shortening (Gotlib et al., 2015;

Tomiyama et al., 2012). Clearly, more research is needed to clarify the proximate mechanisms determining telomere lengths in developing organisms (Boonekamp et al., 2017; Reichert & Stier, 2017).

To summarize, in this study, we have shown that spotless starlings experiencing harsh rearing conditions showed a wide range of morphological, physiological and chromosomal effects during the nestling stage. Although many of the negative effects of brood size manipulation on condition-related traits can disappear in adulthood (Dijkstra et al., 1990; Metcalfe & Monaghan, 2001; Naguib & Gil, 2005; Neuenschwander et al., 2003), some modifications result in long-term phenotypic effects (Metcalfe & Monaghan, 2001; Monaghan, 2008), lasting several generations (Naguib & Gil, 2005; Naguib et al., 2006). Some studies have found that even though organisms may compensate these initial effects, compensation is costly and might reduce fitness (Metcalfe & Monaghan, 2001). Finally, our study suggests that rearing conditions do not affect all aspects of phenotype in the same fashion. Further research should try to identify the chain of causation behind these effects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

DG, RM and LP-R wrote the original project; DG, JM, RM and LP-R collected the data; RM, DG and SA-I analysed the data; DG led the writing of the manuscript; and all authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data will be available in DRYAD.

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