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RESEARCH ARTICLE

Oxidative status and telomere length are related to somatic and physiological maturation in chicks of European starlings (*Sturnus vulgaris*)

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ABSTRACT

Telomere length can be considered as an indicator of an organism's somatic state, long telomeres reflecting higher energy investment in self-maintenance. Early-life is a period of intense investment in somatic growth and in physiological maturation but how this is reflected in telomere length remains unclear. Using European starling chicks we tested: (i) how telomere length measured at asymptotic mass is related to proxies of somatic growth and physiological maturity in 17-day-old nestlings; (ii) how telomere length measured at 17 days then predicts the changes in somatic and physiological maturity occurring in fledglings (between 17 and 21 days); (iii) how growth and telomere length co-vary when chicks are under experimentally good (fed) growth conditions. Depending on environmental conditions, our data suggest links between somatic growth, physiological maturation and body maintenance parameters (positive with oxidative stress and negative with telomere length) in nestlings. Telomere length measured at day 17 predicted a subsequent change in physiological maturation variables observed in fledglings, but only in second-brood chicks: chicks with shorter telomeres had a higher pre-fledging rate of increase in haematocrit and haemoglobin content and a greater decrease in reticulocyte count. Finally, food supplementation of chicks did not change telomere length compared with that in control siblings. Our results suggest that physiological maturation prior to fledging may occur at the expense of telomere length but only when environmental conditions are sub-optimal.

KEY WORDS: Growth, Body mass, Physiological traits, Telomere, Bird

INTRODUCTION

Early-life development is a critical period for newborn organisms because it is when the future functioning of the organism is set up to sustain maximized fitness in adulthood (Monaghan, 2008; West-Eberhard, 2003). Regulation of somatic growth is believed to be shaped by life-history trade-offs through the optimized allocation of available resources to growth and self-maintenance (Stearns, 1992). Thus, growth rate is subject to within-species plasticity in relation to context-specific environmental conditions (Dantzer et al., 2013; Dmitriew, 2011) and consequences for the future organism may be substantial: e.g. faster growth trades off with individual lifespan (Dmitriew, 2011; Metcalfe and Monaghan, 2003), even when controlling for any confounding effects of resource availability (Lee et al., 2013). In this context, evaluating the effects of growth trade-offs on the soma has been an important objective for evolutionary biologists (Monaghan and Ozanne, 2018). Rapid growth in mammals and birds has been associated with pleiotropic effects of signalling or hormonal pathways involved in both growth and ageing (Flatt and Heyland, 2011) or to an imbalance in oxidative status (Monaghan et al., 2009; Speakman et al., 2015). For example, rapid growth could trigger a rise in either oxidative damage or reactive oxygen species production (Christensen et al., 2016; Geiger et al., 2012; Rollo et al., 1996), or a decrease in antioxidant capacities (Alonso-Alvarez et al., 2007; Blount et al., 2003).

Another marker of ageing that is a component of mechanisms underlying the cost of growth is telomere erosion: maintenance of telomere length is impaired in fast-growing individuals (Geiger et al., 2012; Pauliny et al., 2015; Tarry-Adkins et al., 2009; Vedder et al., 2018) or when growth conditions are sub-optimal, energetically or socially (Nettle et al., 2015, 2017; Reichert et al., 2015). Telomeres are repeats of T_2AG_3 sequences (in vertebrates) protecting the linear ends of telomeres and may be considered as a proxy of cell maintenance costs experienced over the long term. Telomere length and rate of telomere erosion have been linked to fitness-related traits such as reproductive success or lifespan in numerous vertebrate species (Bize et al., 2009; Fairlie et al., 2016; Heidinger et al., 2012; Olsson et al., 2011; Rollings et al., 2017; Seeker et al., 2018; Wilbourn et al., 2018). Since telomeres shorten at each cell division (Blackburn, 1991) or because of a putative effect of oxidative stress (Boonekamp et al., 2017a; Reichert and Stier, 2017), this predicts a causal relationship between the rate of growth and the rate of telomere erosion during development, and thus with life-history trade-offs at adulthood (Monaghan and Ozanne, 2018). In fact, telomeres are lost more rapidly during early life than they are later on (Daniali et al., 2013; Frenck et al., 1998; Hall et al., 2004). However, the link between growth and telomere length remains debated (Monaghan and Ozanne, 2018) and needs further study notably when growth takes place under variable environmental conditions (Vedder et al., 2017).

Early-life development is not restricted to somatic growth (e.g. increases in body mass or body size) but also involves a gain of functionality, via physiological maturation processes. Functional, or physiological, maturation involves more than simple somatic changes and interpreting growth–telomere relationships based on mass alone may lead to incorrect conclusions (Durant et al., 2008). In addition, if physiological maturation is a determinant of individual fitness later in life, it may have evolved to be uncoupled from most environmental influences to avoid pervasive long-term negative

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effects. Such a canalisation phenomenon [i.e. resilience of traits to environmental variations due to robust relationships with fitness (Waddington, 1942)] has previously been observed for feather development in free-living jackdaws (*Coloeus monedula*) (Boonekamp et al., 2017b). Still, the nature of trade-offs when the canalisation of developmental maturity takes place needs to be determined. Maturation of tissues includes the development of physiological traits encompassing aerobic capacity, muscular performance or metabolic pathways that are key components of adaptations to adult life (Ricklefs and Starck, 1998). Physiological maturation at fledging in birds thus represents a key life-history transition as chicks shift from a mostly non-active nestling to an active adult life. Complex patterns of somatic and physiological maturation have recently been described in the European starling *Sturnus vulgaris* (Cornell and Williams, 2017). This study showed that poor environmental conditions do not affect the ultimate physiological maturation processes of fledglings but rather induce a cost of maintaining the programmed maturation trajectory, i.e. increased oxidative stress (Cornell and Williams, 2017). Interestingly, such a canalisation phenomenon has been recently proposed for telomere length in another bird species, the common tern (*Sterna hirundo*) (Vedder et al., 2017). In this species, between-individual variance in telomere loss during growth was low and not correlated to variance in somatic growth (body mass). This suggests that, at least when looking at somatic growth, telomere length is a cellular trait that needs to be preserved because of its potential importance in defining the adult organism's fitness (Heidinger et al., 2012; Le Vaillant et al., 2015; Salomons et al., 2009).

In the present study, we analysed co-variation between telomere length, somatic growth and physiological maturation at the nestling (day 17) and/or fledging (day 21) stages of development in European starling chicks. We assessed somatic growth through body mass/size measurements and physiological maturity as the aerobic capacity of the chicks (haemoglobin blood concentration, haematocrit and reticulocyte blood count), which has been previously shown to change just before the chicks enter their active, volant lifestyle upon leaving the nest (Cornell et al., 2017; Cornell and Williams, 2017). We then measured the dynamics of change of somatic growth and aerobic capacity during the last days before fledging (day 17–21), and tested whether nestling telomere length (day 17) predicts those growth changes. If oxidative stress reflects the cost of growth in nestlings, we expected to see negative relationships with fledging somatic growth and physiological maturation status. Similarly, as a proxy of past-investment in growth, telomere length at the nestling stage should negatively influence the ultimate growth patterns in fledglings, between day 17 and 21. In contrast, based on the canalisation hypothesis, we predicted that fledging maturation and telomere length would show low variance among individuals, and that both would be unaffected by sub-optimal environmental conditions. This could be achieved at a higher cost for the chicks, here evaluated by the measurement of the chicks' oxidative status. We tested this hypothesis further by comparing somatic and physiological maturation, telomere length and oxidative status in chicks provided with supplemental food between days 4–17 post-hatching, i.e. under 'good' growth conditions.

MATERIALS AND METHODS

Species and area of study

Field work was conducted on a free-living population of European starling *Sturnus vulgaris* Linnaeus 1758 at Davistead Farm, Langley, BC, Canada (49°08′ N, 122°37′ W), which includes ∼150 nest boxes used by ∼75 breeding pairs each year (see Cornell and Williams, 2017). In this study, we measured telomere length for a sub-sample of chicks used in studies previously reported in Cornell and Williams (2017) and Cornell et al. (2017). Specifically, we analysed telomere length in relation to somatic growth and physiological maturition in: (a) unmanipulated chicks from 1st and 2nd broods in 2 years of differing productivity (see below). Unmanipulated chicks from 2015 are hereafter referred to as 'control' chicks in comparison to their siblings in the fed treatment (see the experimental approach); and (b) chicks from a supplemental feeding experiment (Cornell and Williams, 2017) conducted in 2015. Breeding productivity of pairs [brood size at fledging (BSF) calculated from non-manipulated nests] was 2.5 chicks in 2013 (n=75) and 2.9 chicks in 2015 (n=34) including birds fledging zero chicks; both values were lower than the long-term average for our population (3.2 chicks, n=510). Breeding productivity of successful birds (BSF≥1 chick) was 3.5 chicks in 2013 (n=54) and 4.5 chicks in 2015 (n=22; long-term average, 4.3 chicks, n=380). So, based on these data we categorised 2013 as a 'poor' year and 2015 as a 'good' year in terms of offspring survival to fledging within broods.

Measurement of somatic and physiological maturity and cost of growth

We measured somatic growth using body mass, tarsus and wing lengths, physiological maturation using haematocrit (Hct), haemoglobin (Hb) and reticulocytes (measures of aerobic capacity) and potential costs of growth as oxidative damage [derivatives of reactive oxygen metabolites (d-ROMs)] measured concomitantly with antioxidant capacity (OXY). Chicks were sampled at 17 days and 21 days post-hatching, body mass (±0.01 g) and wing length (±0.01 mm) recorded and blood samples (>200 µl) were taken from the brachial vein using a 26.5 gauge needle. Chicks are at asymptotic mass at day 17 and fledge at day 21, following pre-fledging mass recession. All blood samples were obtained within 3 min of chicks being handled. Fresh blood was used for Hct and Hb measurements and two blood smears were prepared for reticulocyte counts (following Cornell and Williams, 2017). Remaining blood was transferred to heparinized tubes and kept at 4°C until centrifugation in the laboratory (3000 ***g*** for 10 min). Separated plasma and red blood cells were immediately frozen (−20°C) until further assays were run.

Hct was measured as packed cell volume (PCV) divided by total volume with digital callipers (±0.01 mm) following centrifugation of whole blood for 3 min at 13,000 ***g*** (Microspin 24; Vulcon Technologies, Grandview, MO, USA). Hb concentration (g dl^{-1} whole blood) was measured using the cyanomethemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941; Sigma-Aldrich, Oakville, ON, Canada) with absorbance measured at 540 nm. Intra-assay CV% was 0.7%, based on duplicate measurements and inter-assay CV% was 1.6%. Reticulocytes (% immature red blood cells) were calculated as number of immature red blood cells/total red blood cells counted from whole blood smears after supravital staining with new Methylene Blue (R4132, Sigma-Aldrich). A total of 1000 red blood cells was counted per slide, and reticulocytes were identified following Fowler and Williams (2017).

We assessed chicks' oxidative status based on plasma levels of oxidative damage (d-ROMs) and of antioxidant capacity (OXY) tests (following Tissier et al., 2014; Cornell and Williams, 2017). All samples were measured in duplicate to calculate coefficient of variation (OXY: 5.1%; d-ROMs: 6.4%) as a measure of intra-assay variation. To determine inter-assay variation we used a single

pooled sample each year across all plates to calculate average inter-assay coefficient of variation (OXY: 5.9%; d-ROMs: 11.6%).

Experimental manipulation: supplemental feeding of chicks

In 2015, we measured telomere length in a sub-sample of chicks from a supplemental feeding experiment described in Cornell and Williams (2017). Mean brood size at the start of the experiment was 4.2±0.1 chicks per brood. Briefly, two chicks per nest were provided with supplemental food between days 4 and 17 post-hatching; two chicks in each nest were handled as controls but did not receive food. Chicks were weighed at the end of each day (after supplementation) to monitor body mass and fed chicks received supplemental food twice a day with the daily total amount of food per day (summing both meals) equivalent to 10% of predicted daily mass gain in European starling chicks, as reported in Westerterp (1982) (see Cornell and Williams for further details).

Telomere length assay

Telomere length was measured in nestling chicks at 17 days post-hatching on DNA extracted from frozen red blood cells using a quantitative PCR technique (qPCR) initially developed by Cawthon and collaborators (Cawthon, 2002) (see also Criscuolo et al., 2009) for a full description of the principle and general methodology applied to two bird species). Specific amplification of a reference gene that was non-variable in copy number among individuals (see Smith et al., 2011) in European starlings was obtained using recombination-activating 1 (RAG 1) sequences (accession number XM_014873522). This gene is used to control for small DNA quantity variation among samples (hereafter referred as the S value) used during the qPCR amplification.

Chicks' telomere length was measured on 12 separate 96-wells plates (6 for RAG1 amplification and 6 for telomere sequence amplification due to different amplification conditions, see below). Total reaction volume in each well was 10 µl, i.e. 5 µl of SYBR green mix, 10 ng of DNA and 500 nmol l^{-1} or 200 nmol l^{-1} of primers of telomere and RAG1, respectively. Reverse and forward primer sequences for both telomeric and RAG1 genes were, respectively: Tel1b: 5′-CGGTTTGTTTGGGTTTGGGTTTGG-GTTTGGGTTTGGGTT-3′; Tel2b: 5′-GGCTTGCCTTACCCTT-ACCCTTACCCTTACCCTTACCCT-3′; RAG1-F: 5′-TGCAAA-GAGATTTCGATATGATG-3′; RAG1-R: 5′-TCACTGACATC-TCCCATTCC-3′. Amplification of telomere sequences was done using the following programme: 2 min at 95°C, followed by 30 cycles of: 15 s at 95°C, 30 s at 58°C and 30 s at 72°C. RAG1 amplification thermal profile was 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 56°C and 1 min at 72°C. All qPCR runs encompass a dilution curve (40 ng to 1.25 ng) to calculate each plate's qPCR efficiencies, two negative control wells (to check for non-specific amplification due to water contamination) and were ended by a melting curve (to check for single peak amplification and primer–dimer artefacts). qPCR amplifying efficiencies varied from 94 to 109% for RAG1 and from 99 to 104% for telomeres (an efficiency of 100% corresponded to a doubling of DNA at each cycle). Samples were randomly assigned over the plates and assayed in duplicates and each plate contained 4 different samples chosen randomly and repeated over all runs were used to calculate repeatability in amplification values. Intra-plate and inter-plate coefficients of variation (based on C_q values) were 0.34±0.03% and 0.64±0.07% for the RAG1 gene assay and 1.92±0.22% and 2.10 ±0.71% for the telomere assay, respectively. Telomere length was finally calculated as a telomere/single copy gene (T/S) ratio as stated in Pfaffl (2001). qPCR telomere length, as a ratio (T/S), is a relative and not absolute evaluation of a mean telomere length of chromosomes and red blood cell populations. Intra-plate and inter-plate coefficients of variation (based on T/S ratio) were 10.64 ±0.84% and 11.77±4.59%, respectively.

Statistical analysis

PCA analysis of growth and physiological variables

The multivariate nature of our dataset led us to use a principal component analysis (PCA) to reduce the number of response variables, and in order to get summary variables that take into account the inter-correlations between all the initial response variables. Two distinct PCAs were run using all individuals (n=98) with: (i) the variables measured at day 17 (body mass, tarsus, d-ROMs, OXY, Hct, Hb content, reticulocyte count) and (ii) the dynamics of change on variables between day 17 and day 21 (body mass loss, wing length growth, Hct, Hb content, reticulocyte count changes). The PCA approach produced 4 PC axes (see Results).

Sample sizes and composition

The dataset was split in two for the statistical analysis. First, only unmanipulated chicks from 2013 and 2015 were considered (n=70; one chick of unknown gender was excluded) to test how telomere length was related to year and brood type: n=28 from 1st brood and n=11 from 2nd brood in 2013, n=15 from 1st brood and n=15 from 2nd brood in 2015 (see below). In a second step, only chicks from 2015 were used [n=59: 30 controls (10 from 1st brood) and 29 fed chicks (14 from 1st brood)], to assess how our supplemental feeding experiment affected growth patterns and telomere length. These chicks originated from 28 nests, and pairs of fed and control chicks were present in 21 nests. We checked for brood effects since, in our population: (i) the incidence of second clutches is relatively high (∼40% of the females) but (ii) chicks from 2nd clutches are generally of lower quality. Double brooding is independent of timing of breeding and female individual quality, but second broods have lower rates of success (Cornell and Williams, 2016). Sex, brood size at day 17 and brood number (1st or 2nd) were always included as fixed factors, with their interactions and with PC axes or telomere length in all models. The final models presented in the tables were selected on the basis of the lowest Akaike information criterion (AIC) value using the null model (with only random factors) as a reference. Model choice was done following an ANOVA on the outputs of all the possible fitted models. Statistics were performed using R v.3.5.1 (http://www.R-project.org.) and the packages lme4 (function lmer) (https://arxiv.org/abs/1406.5823) and Factominer (Lê et al., 2008). We tested for normality with both a Shapiro–Wilk's test and QQ plots distribution (fitdistrplus package in R). Only telomere length data were $\log_{10}$ transformed (T/S ratio) to reach normality. Plots were generated using ggplot2 (Wickham, 2016) and sjPlot (https://rdrr.io/cran/sjPlot/). P<0.05 was considered as significant.

General growth patterns and telomere length in starlings

Analysis of variation in growth was done based on a sub-sample (i.e. individuals for which telomere lengths were measured) of a data set previously analysed by Cornell et al. (2017). Reanalysis of our sample of telomere chicks produced the same key results: overall, being born as 1st brood chicks and in 2015 seemed to favour growth and maturation processes.

Cost of nestling somatic growth and physiological maturation

Using a mixed model approach on unmanipulated chicks from 2013 and 2015, we first sought to explain variation in telomere length across nestling chicks at the age of 17 days. We thereby developed 3 models

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with telomere length as the response variable, and either chick's PCA1 (mass, tarsus, physiological variables at day 17), PCA2 (day 17 oxidative status, plasma d-ROMs and OXY levels) as fixed explanatory variables. Using a multivariate approach did not change most of the output of the analysis (data not shown), but was not adopted because of the uncontrolled random effect. In each mixed model, the year (2013–2015), the brood (1st or 2nd), the brood size at day 17, the sex and the interactions with PCA values were included as covariates to explore the context dependency of the respective relationships evaluated. Finally, the nest identity was included as a random factor in each of our models to control for the non-independence of the chicks (i.e. nestlings within the same brood or from 1st and 2nd broods that have been raised by the same parents). In addition, given the existing links between oxidative damage and telomere dynamics, we tested the significance of an oxidative cost of growth using another mixed model (random factor: nest ID), with the chick's oxidative status (PCA2) as a response variable and PCA1 as one of the explanatory factors (year, brood, brood size, sex and interactions).

Nestling telomere length as a predictor of fledging growth and physiological maturation

The second objective of our statistical analysis was to test whether telomere length at 17 days predicts the fledging changes in physiological/somatic maturation (PCA3 and PCA4), i.e. changes between day 17 and day 21 (fledging day). To do so, we used 2 mixed models with PCA3 and PCA4 as response variables. For each model, year, brood, brood size and sex were included as explanatory variables in addition to telomere length, PCA1 and PCA2, and nest identity was added as random factor. We also check for regression to the mean effect for all variables (Kelly and Price, 2005), and we found no significant effect in any cases.

Supplemental feeding experiment and consequences for costs of growth

The third objective was to test how an experimental manipulation of food availability influences growth and maturation patterns, and what the outcome would be for the chick's oxidative status and telomere length. To do so, we used the same mixed model approach with explanatory factors as above (except the year effect, since only 2015 chicks were considered), and with experimental treatment as an additional factor. The experiment involved 59 chicks followed in 2015 (see statistics above), distributed as follows: fed group, n=29; control group (unmanipulated siblings), n=30. In all models, nest identity was considered as random factor.

RESULTS

PCA analysis of growth and physiological variables

Principal component analysis (PCA) on somatic and physiological maturation variables was conducted: in nestlings at day 17 (body mass, tarsus length, d-ROMs and OXY plasma levels, and reticulocyte count, Hct and Hb content) (Fig. 1A) and in fledglings between day 17 and day 21 (changes in body mass, wing length, reticulocyte count, Hct and Hb content) (Fig. 1B). Day 17 PCA resulted in two principal axes (eigenvalues: PCA1, 1.315; PCA2, 1.245; others <0.989), explaining 57.7% of the total variance. PCA1 was positively loaded with day 17 body mass (correlation with PCA1, 0.595, P=1.1×10^{-10}), tarsus length (0.591, P=1.5×10^{-10}), Hct (0.678, P=1.7×10^{-14}) and Hb content (0.601, P=5.9×10^{-11}), and negatively with reticulocyte count (−0.668, P=5.8×10^{-14}). PCA2 was positively loaded with both d-ROMs and OXY plasma levels (0.746, P=1.3×10^{-18} and 0.722, P=5.0×10^{-17}, respectively). Kaiser–Meyer–Olkin (KMO) supported the adequacy of the data with PCA analysis (0.66) and Bartlett's test of sphericity supported high correlation among variables for PCA (χ^2_{21}=151.1, P<0.001). Individual PCA1 and PCA2 scores were subsequently used as somatic and physiological maturation index (PCA1) and oxidative stress index (PCA2).

Day 21 PCA was also defined by two principal axes (eigenvalues: PCA3, 2.537; PCA4 1.502; others <0.878), explaining 51.2% of the total variance. PCA3 was positively loaded with change in Hb content between day 17 and day 21 (correlation with PCA3, 0.685, P=7.71×10^{-15}), change in Hct (0.569, P=1.0×10^{-09}),

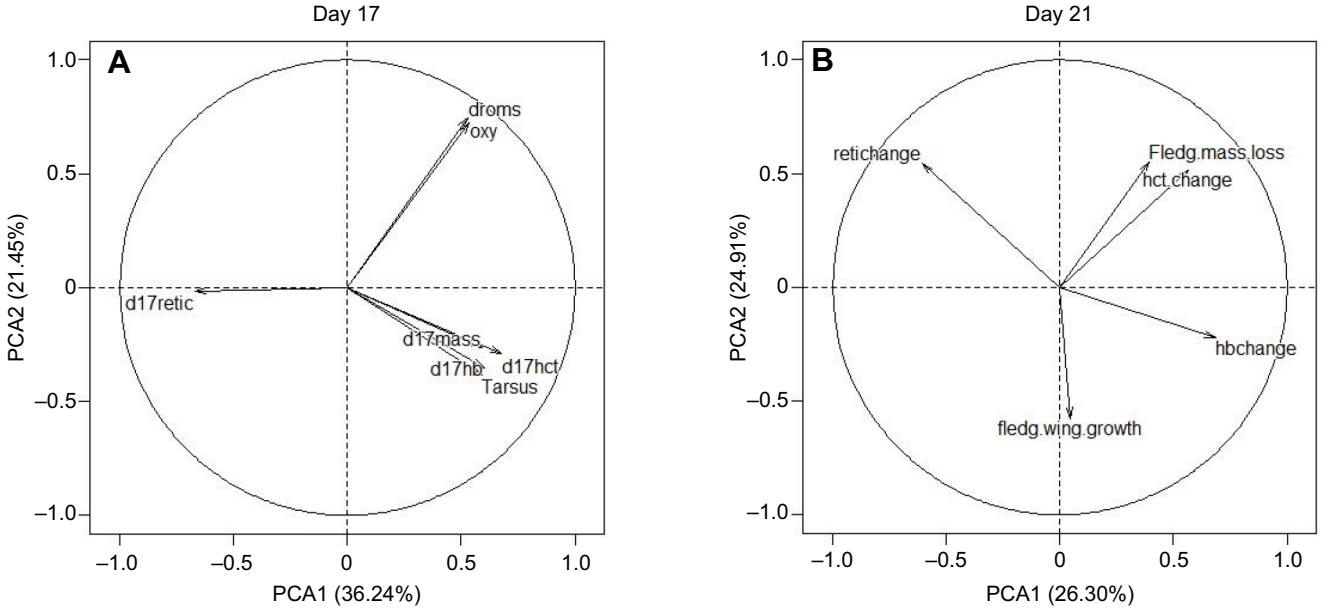

Fig. 1. Principal component analysis conducted on starling chicks. PCA of chicks (n=98) showing the distribution over two principal component axes of nestling growth variables measured at day 17 (A) and fledging variable dynamics between day 17 and day 21 (B). d17, day 17; droms, derivatives of reactive oxygen metabolites; fledg., fledgling; hb, haemoglobin; hct, haematocrit; oxy, antioxidant capacity; retic, reticulocytes.

and negatively with change in reticulocyte count (−0.605, P=4.0×10^{-11}). PCA4 was positively loaded with body mass loss between day 17 and day 21 (0.549, P=4.7×10^{-09}) and negatively with increase in wing length (−0.576, P=5.5×10^{-10}). The KMO value was 0.46 and the Bartlett's test was significant (PCA, χ^2_6=13.6, P=0.034). Individual PCA scores were subsequently used as pre-fledging physiological (PCA3) and pre-fledging somatic (PCA4) maturation index.

To summarize, PCA analysis conducted on 17-day-old chicks (nestlings) resulted in two axes: (i) a somatic and physiological maturation axis (PCA1), a positive value reflecting an advanced maturation status of chicks; (ii) an oxidative stress axis (PCA2), positively loaded with high d-ROMs and OXY plasma levels. PCA analysis conducted on changes in variables between day 17 and day 21 (fledglings) also uncovered two axes: (i) fledging physiological maturation axis (PCA3), high values characterising large changes in physiological variables; (ii) fledging somatic maturation axis (PCA4) with positive scores indicating lower body mass loss and slower wing growth in fledging chicks.

General growth patterns and telomere length in starlings

We first repeated the analysis presented in Cornell et al. (2017) to confirm patterns of growth and maturation for the subset of nestlings for which we obtained telomere data. Separated mixed models (using PCA1 and nest ID as random factor) were mostly consistent with the description of changes of somatic and physiological traits previously presented for starling chicks (Cornell et al., 2017; Cornell and Williams, 2017). In short, there was a year and brood effect on PCA1, chicks born in 2013 and in a 2nd brood having lower somatic and physiological maturation (random factor Nest ID: 0.931±0.965; year, estimates −0.610±0.172, $t_{1,49.9}$=−3.541, P<0.001; brood, −1.415±0.272, $t_{1,52.5}$=−5.199, P<0.001). Looking more closely at each variable, on average chicks reared in 1st broods and in 2015 had higher body mass, longer tarsus, higher Hct and Hb content than those born in 2013 or as 2nd brood chicks. In addition to Cornell et al. analysis, the lower PCA1 value in 2015 was also driven by higher reticulocytes count at day 17 (negative load of PCA1).

Variability in growth patterns and telomeres in 2013–2015

Cost of nestling somatic growth and physiological maturation

Results of the mixed models are presented in Table 1A. Chicks with higher nestling growth (PCA1) values showed longer telomeres at day 17 (Fig. 2A). Oxidative stress axis (PCA2) was also significantly related to telomere length at day 17, but in relation to chick's gender (significant interaction Sex×PCA2): females with higher d-ROMs and OXY values had shorter telomeres, while the relationship was positive in males (Fig. 2B). However, none of the regressions were found to be significant (males: 0.089±0.079, $t_{1,32}$=1.131, P=0.267; females: −0.080±0.041, $t_{1,33}$=1.982, P=0.056). Finally, telomere length and oxidative status of chicks did vary with years, telomeres being longer and oxidative stress being lower in 2015 (Table 1A and B, respectively).

The oxidative status of chicks at day 17 (PCA2) varied between years, being lower in 2015 than in 2013 (Mixed model, random effect: nest ID 0.321±0.550; −1.043±0.148, $t_{1,44.3}$=−7.043, P<0.001) while it did not significantly vary with sex (0.251±0.202, $t_{1,36.4}$=1.244, P=0.221) or brood number (−0.387±0.258, $t_{1,62.9}$=−1.503, P=0.138). PCA2 was negatively related to PCA1 (−0.373±0.095, $t_{1,54.1}$=−3.917, P<0.001), indicating that chicks with higher mass and size at day 17, and higher Hct and Hb content, but lower reticulocyte count, had lower d-ROMs and OXY plasma levels.

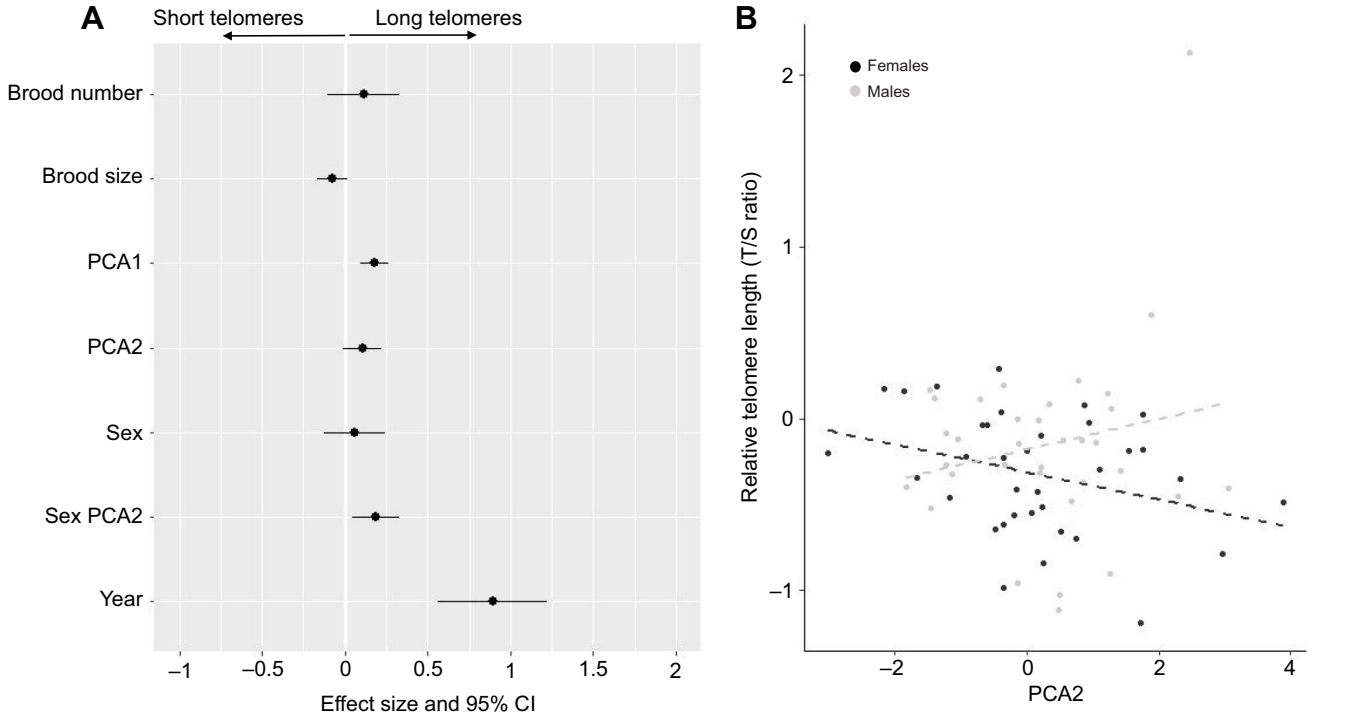

Fig. 2. Effects of growth and maturation on telomere length in starling nestlings at day 17. (A) Effect sizes and 95% confidence intervals (CI) of the selected mixed model. Effect sizes with CI that do not overlap zero are significant (see Table 1A). (B) Linear relationships between nestlings' oxidative status after 17 days of post-hatching development (PCA2) and telomere length (log_{10}-transformed T/S ratio), in relation to sex. The dashed lines represent the linear regressions for females (black) and males (grey): none of them was found to be significant (males, P=0.267; females, P=0.056). See Table 1A for statistics.

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Table 1. Summary of mixed models testing for relationships between log_{10}-transformed telomere length (T/S ratio), oxidative status of starling chicks and growth variables measured either at the nestling stage (17 days) or at the fledging stage (21 days)

	Estimates	d.f.	*F*	*P*
A. Nestling telomere length. Response variable: log_{10}(T/S ratio)				
Random effect: nest ID	0.007±0.084			
Residual	0.141±0.376			
Intercept	**−898.50±169.67**	**1,58.7**	**−5.296**	**>0.001**
Year (2015 versus 2013)	**0.446±0.084**	**1,58.7**	**5.294**	**>0.001**
Brood (2nd versus 1st)	0.113±0.111	1,51.9	1.023	0.311
Brood size at day 17	−0.077±0.048	1,59.8	−1.611	0.113
Sex (M versus F)	0.058±0.096	1,52.3	0.611	0.544
PCA1	**0.180±0.044**	**1,48.9**	**4.069**	**>0.001**
PCA2	*0.106±0.060*	*1,60.5*	*1.764*	*0.083*
Sex×PCA2 (M versus F)	**0.188±0.072**	**1,50.8**	**2.632**	**0.011**
B. Nestling oxidative cost of growth. Response variable: PCA2				
Random effect: nest ID	0.303±0.550			
Residual	0.530±0.728			
Intercept	2101.15±298.01	1,44.2	7.051	**<0.001**
Year (2015 versus 2013)	**−1.042±0.148**	**1,44.3**	**−7.043**	**<0.001**
Brood (2nd versus 1st)	−0.387±0.258	1,62.9	−1.503	0.138
Brood size at day 17	−0.139±0.111	1,61.9	−1.260	0.213
Sex (M versus F)	0.251±0.202	1,36.4	1.244	0.221
PCA1	**−0.373±0.095**	**1,54.1**	**−3.917**	**<0.001**
C. Fledging physiological maturation (days 17–21). Response variable: PCA3				
Random effect: nest ID	0.046±0.213			
Residual	0.701±0.838			
Intercept	160.34±451.23	1,59.8	0.355	0.724
Year (2015 versus 2013)	−0.079±0.224	1,59.8	−0.354	0.724
Brood (2nd versus 1st)	*−0.513±0.284*	*1,54.6*	*−1.809*	*0.076*
Brood size at day 17	0.035±0.110	1,59.6	0.319	0.751
Sex (M versus F)	0.279±0.212	1,48.2	1.316	0.194
log_{10}(T/S ratio)	**2.167±0.720**	**1,57.1**	**3.009**	**0.004**
PCA1	**−0.465±0.112**	**1,49.3**	**−4.170**	**<0.001**
PCA2	0.065±0.133	1,59.8	0.488	0.627
log_{10}(T/S ratio)×Brood (2nd versus 1st)	**−1.610±0.489**	**1,55.4**	**−3.290**	**0.002**
D. Fledging somatic maturation (days 17–21). Response variable: PCA4				
Random effect: nest ID	0.543±0.737			
Residual	0.487±0.698			
Intercept	**−1353.05±534.83**	**1,61.0**	**−2.530**	**0.014**
Year (2015 versus 2013)	**0.671±0.265**	**1,61.0**	**2.526**	**0.014**
Brood (2nd versus 1st)	**0.724±0.303**	**1,60.5**	**2.393**	**0.020**
Brood size at day 17	0.162±0.131	1,60.9	1.231	0.223
Sex (M versus F)	0.114±0.229	1,34.8	0.497	0.622
log_{10}(T/S ratio)	−0.185±0.315	1,55.9	−0.585	0.561
PCA1	**0.364±0.136**	**1,61.0**	**2.678**	**0.010**
PCA2	*0.266±0.156*	*1,60.4*	*1.706*	*0.093*

Only non-manipulated chicks in 2013 and 2015 were considered. Telomere length in relation to physiological maturation/somatic growth (PCA1) and oxidative status (PCA2); oxidative status (PCA2) in relation to physiological maturation/somatic growth (PCA1); physiological maturation at day 21 (PCA3) in relation to previous growth patterns (PCA1 and PCA2); somatic growth at day 21 (PCA4) in relation to previous growth patterns (PCA1 and PCA2). Brood number, brood size at day 17, year and sex were added as fixed factors. Nest identity (ID) was used as a random factor to control for the fact that some chicks were raised in the same nest. Significant results are indicated in bold ($P<0.05$), and results which $P<0.1$ are indicated in italics. The presented models were those selected using the AIC criteria.

Nestling telomere length as a predictor of fledging growth and physiological maturation

Telomere length of nestling chicks (at 17 days old) predicted the fledging PCA3 values (i.e. the changes in physiological variables measured between day 17 and 21 days; Table 1C): long telomeres were positively associated with high PCA3, i.e. to a higher increase in Hct and Hb content, and to a decrease in reticulocyte count. However, this relationship was mainly driven by an interaction with brood number (Fig. 3A). The slope of the linear regression between telomere length and PCA3 was different between broods, being positive for 1st brood chicks and negative in 2nd brood chicks (Fig. 3B). Only in the latter case was the regression significant (2nd brood: −1.114±0.054, $t_{1,24}=-2.108$, $P=0.046$; 1st brood: 0.416±0.310, $t_{1,41}=1.341$, $P=0.187$). There was a trend for 2nd brood chicks to have lower PCA3 values (i.e. a lower increase in Hct, Hb and larger decrease in reticulocyte count between day 17 and 21) than 1st brood chicks (Table 1C, $P=0.076$). There was also a negative effect of PCA1 on PCA3 values (Table 2C): the chicks with higher values of Hct, Hb and lower reticulocyte count at day 17 had the lowest change in Hct and Hb content, but a greater change in reticulocyte count at day 21.

Somatic maturation between day 17 and 21 before fledging (PCA4, i.e. body mass loss and wing length growth) was significantly greater in 2015 (year effect, Table 2D), and in 2nd brood chicks than in 1st brood chicks (brood effect, Table 2D). There was also a relationship with the nestling growth patterns, with PCA1 positively influencing PCA4: larger chicks at day 17, but also those having higher Hct and Hb (and lower reticulocyte count) lost less body mass and had slower wing length growth in the last days before fledging (PCA1 effect, Table 2D).

Supplemental feeding experiment and consequences for costs of growth

At day 4 (before the beginning of the feeding/mother stress experiments), all chicks were of similar body mass. At 17 days of age, food provisioned nestlings did not show any significant differences in somatic growth or physiological patterns (PCA1) compared with their control siblings (mixed model, estimates −0.027±0.279, $t_{1,31.8}=-0.110$, $P=0.913$). There was no significant effect of supplemental feeding on telomere length or the oxidative status of nestlings at day 17 (Table 2A and B, respectively). In both cases, PCA1 was found to be the only significant factor of the model: nestlings that were more somatically and physiologically mature had longer telomeres (Table 2A) and lower oxidative status (PCA2, Table 2B).

In fledglings (between day 17 and day 21), supplemental feeding also had no effect on changes in physiological variables before birds left the nest (PCA3, Table 2C) or on somatic growth (PCA4, Table 2D). There was a significant effect of PCA1 on PCA3 (Table 2C): fledglings with high levels of somatic and physiological maturation at the nestling stage (PCA1, day 17) presented low values of PCA3 at day 21. There was a tendency for 1st brood chicks to have a higher PCA3 value than 2nd brood chicks (Table 2C), underlying a greater increase in their blood Hb concentration and in their Hct, and a larger decrease in their reticulocyte count during the last days before fledging.

DISCUSSION

Our paper focused on potential maturation costs, in terms of oxidative stress and telomere length, of natural variation in developmental trajectories of chicks prior to fledging over 2 years of contrasting environmental conditions, and in 1st and 2nd broods for both somatic and physiological developmental traits. In agreement with previous studies, we found that when growth takes place in a good year (2015), nestlings grew faster and suffered from less oxidative stress. Also, oxidative status and telomere length were negatively related in nestling females at that stage (day 17). However, we found a positive relationship between growth patterns

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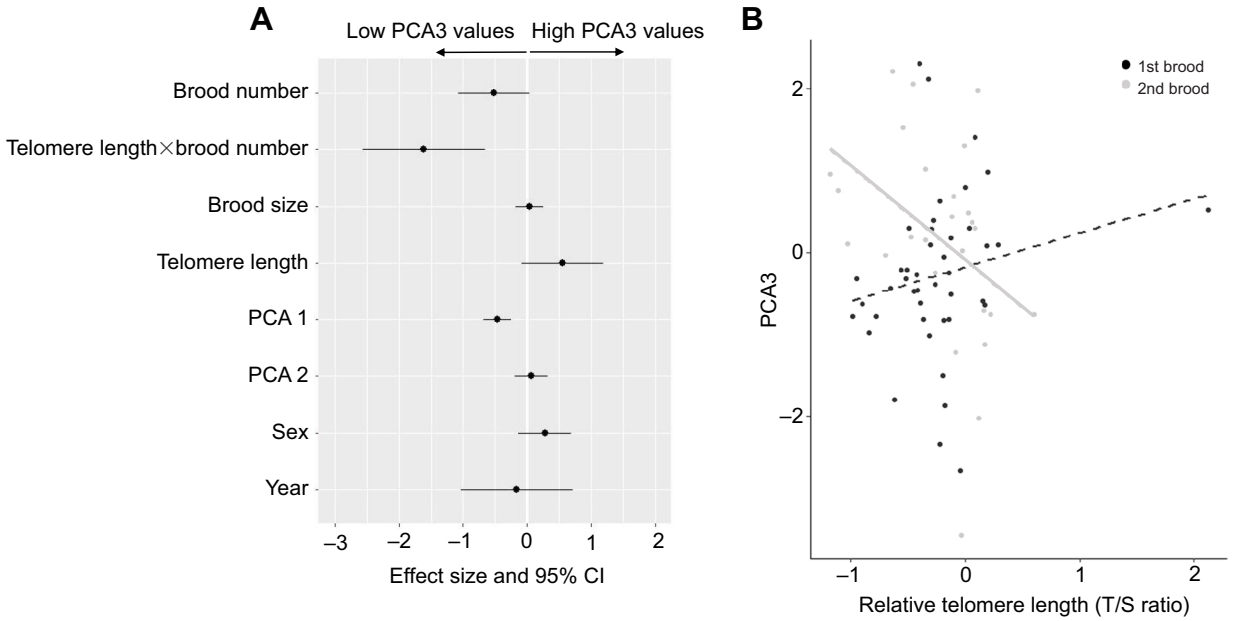

Fig. 3. Telomere length at day 17 as a preditor of physiological maturation in starling fledglings between day 17 and day 21. (A) Effect sizes and 95% confidence intervals (CI) of the selected mixed model explaining physiological maturation in fledging chicks. Effects size with CI that do not overlap zero are significant (see Table 1C). (B) Linear relationships between starling nestling telomere length measured at day 17 (log_{10}-transformed T/S ratio) and their physiological maturation undergone at fledging stage, between day 17 and day 21 (PCA3). The relationship was dependent on the interaction with brood number (filled circles, chicks raised in a first brood; open circles, in a second brood). The dashed (non-significant) and plain (significant) lines represent the linear regressions for 1st (black) and 2nd brood (grey) chicks (P=0.187 and 0.046, respectively). See Table 1C for statistics.

and telomere length, suggesting that chicks that grew faster did not pay any immediate costs in terms of telomere erosion. When looking at the final developmental changes prior to fledging (days 17–21), we found that telomere length measured at day 17 significantly predicted subsequent physiological maturation: 2nd brood chicks had short telomeres at day 17, had larger increases in Hb content and Hct, and the lowest decrease in reticulocyte count. Keeping in mind that (i) good quality chicks should exhibit a slighter body mass loss and greater wing length growth before leaving the nest, as well as an improved aerobic capacity (larger increase in Hb, Hct and, consequently, a larger decrease in reticulocytes), and (ii) that our supplemental feeding experiment had no significant effects on any measure of growth, our results suggest that developmental trajectories are mostly resilient to both positive and negative environmental factors, and that they are maintained even if they incur costs of shorter telomere length at the end of growth.

Nestling growth and maturation cost

Our first objective was to discriminate any relationships that may exist between somatic growth and ageing from those of physiological maturation and ageing in starlings at 17 days of age (asymptotic mass). Since our PCA1 axis did not discriminate between somatic and physiological maturations (both projected on PCA1 axis), this question unfortunately remains open. However, several putative interpretations are possible. The first one relies on the fact that our somatic and physiological maturation axis was positively loaded with body mass and tarsus length: high individual PCA1 values corresponded to structurally large and heavy nestling chicks. Life-history trade-offs are most frequently based on the idea of allocation of limited resources among competitive traits, and natural growth trajectories are generally expected to be derived from such trade-offs (Monaghan and Ozanne, 2018). In support of the idea of telomere length as a cellular indicator of an individual's biological state (Monaghan, 2014), numerous previous studies have reported that telomere length or telomere loss are negatively correlated with body mass at the end of the growth period (Boonekamp et al., 2014; Herborn et al., 2014; Noguera et al., 2015). In contrast, our data failed to confirm that shorter telomeres reflect the cost of sustained growth rate. Similarly, there was no direct oxidative cost of growth in our study since a negative relationship was found between growth patterns and oxidative status at the age of 17 days, suggesting that larger chicks that grew faster had lower oxidative stress. Therefore, in our population, it may be that optimal growth conditions deriving either from genetic, parental or environmental effects may allow good quality nestlings to grow better without incurring a growth–ageing trade-off.

The second interpretation of our data for day 17 chicks relates more to the physiological proxies that were, indeed, the most significant in defining the growth and maturation axis (PCA1). High PCA1 values indicated higher Hb content and Hct, and lower reticulocyte count, all variables that characterized individuals in an advanced stage of physiological maturation. This is corroborated by yearly differences in body mass, because we do know that chicks in 2013 were lighter than in 2015 (2013 being a 'poor' year; Cornell and Williams, 2017). Thus, higher PCA1 values in 2013 may underline a faster physiological maturation. In that case, fast physiological maturation (during the first 17 days of development) was associated with shorter telomeres, suggesting that there might be additional maturation costs when growth takes place in a sub-optimal environment. The oxidative status of nestlings was lower in 2013 compared with 2015 ('good' year), and independently of year effect, high levels of oxidative stress were associated with shorter telomeres. The year effect matches well with the idea of an increased energy allocation to physiological maturation and away from telomere maintenance because of food shortage. However, the energy-based trade-off on which physiological maturation may be based remained elusive, and our result may also derive from non-energy-related

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Table 2. Results of mixed models testing for the effect of experimental treatment (additional feeding) conducted on starling chicks in 2015

	Estimates	d.f.	*F*	*P*
A. Nestling telomere length (day 17). Response variable: $\log_{10}$(T/S ratio)				
Random effect: nest ID	0.004±0.062			
Residual	0.045±0.213			
Intercept	−0.013±0.054	1,55.0	−0.236	0.815
Treatment	−0.075±0.056	1,55.0	−1.338	0.186
PCA1	**0.060±0.024**	**1,55.0**	**2.530**	**0.014**
PCA2	0.062±0.037	1,55.0	1.764	0.104
B. Nestling oxidative cost of growth (day 17). Response variable: PCA2				
Random effect: nest ID	0.135±0.368			
Residual	0.445±0.667			
Intercept	−0.981±0.221	1,50.4	−4.440	**<0.001**
Treatment	0.198±0.181	1,38.2	−1.094	0.281
Brood (2nd versus 1st)	−0.272±0.255	1,48.6	−1.069	0.290
Sex (M versus F)	0.255±0.200	1,52.5	1.277	0.207
PCA1	**−0.390±0.088**	**1,53.0**	**−4.467**	**<0.001**
C. Fledging physiological maturation (days 17–21). Response variable: PCA3				
Random effect: nest ID	0.543±0.737			
Residual	0.487±0.698			
Intercept	0.506±0.820	1,51.3	0.618	0.593
Brood (2nd versus 1st)	*−0.686±0.353*	*1,51.7*	*−1.941*	*0.058*
Brood size at day 17	−0.060±0.159	1,49.2	−0.375	0.710
Sex (M versus F)	−0.232±0.237	1,42.8	−0.980	0.332
Treatment	0.015±0.202	1,39.5	0.073	0.943
PCA1	**−0.652±0.128**	**1,50**	**−5.111**	**<0.001**
PCA2	0.050±0.163	1,47.1	0.306	0.761
D. Fledging somatic maturation (days 17–21). Response variable: PCA4				
Random effect: nest ID	0.618±0.786			
Residual	0.633±0.796			
Intercept	0.130±0.340	1,53.0	0.383	0.703
Sex (M versus F)	0.455±0.267	1,46.8	1.731	0.090
Treatment	0.261±0.226	1,35.0	1.155	0.256
PCA1	0.191±0.117	1,53.3	1.631	0.109
PCA2	0.233±0.181	1,50.2	1.291	0.203

Explanatory variables were telomere length and oxidative status (PCA2) 17-day-old nestlings; fledging physiological maturation (PCA3) and pre-fledging somatic growth between the ages of 17–21 days. Nest identity (ID) was used as a random factor to control for the fact that some chicks were raised in the same nest. The models that are presented are those corresponding to the best AIC value. Significant results are indicated in bold (P<0.05) and results for which P<0.1 are indicated in italics.

costs, as a result of higher chick competition or social stress within broods (Nettle et al., 2017; Reichert et al., 2015). The deleterious effects of stress hormones, like corticosterone, on oxidative stress and telomere length may then be invoked (Choi et al., 2008; Quirici et al., 2016). Competition among nestlings has been shown previously to impact telomere variation in starling chicks (Nettle et al., 2016). However, brood size did not directly impact ageing parameters in our study, and the social modulation of the cost of growth remains to be properly tested in our population. Our data also suggest that putative costs of growth may vary among the sexes: females with higher oxidative stress tended to have shorter telomeres while the reverse was observed in males. Interestingly, a recent study conducted on spotless starlings (*Sturnus unicolor*) showed that experimental manipulation of growth before day 14 affected telomere length of female chicks, especially via an increase in oxidative stress, while this modulation was indirect in males (Gil et al., 2019). Whether this reflects a sex-specific consequence of cell-level trade-offs leading to faster cell division, higher metabolic rate or red blood cell maturation remains to be defined.

As suggested previously (Arendt, 1997), growth trajectories may have evolved in relation to intrinsic developmental constraints, e.g. the need to reach functional maturity of tissues and organs early in life. Our results suggest that (i) developmental trajectories (for both somatic growth and physiological maturation) have evolved in such a way that they are maintained even when environmental conditions are poor (Cornell and Williams, 2017), probably because the ultimate cost of impaired development is high (i.e. decreased survival; Bowers et al., 2014); (ii) sustaining developmental trajectories (and long-term fitness) under sub-optimal conditions occur with a potential maturation cost (higher oxidative stress, shorter telomere length or both towards the end of growth). While oxidative stress has previously been suggested to be a conserved mechanism mediating the somatic growth/lifespan trade-off (Carney Almroth et al., 2012; Kim et al., 2010), the cost of somatic and physiological maturation processes has been less well studied. In amphibian larvae, growth but not development (here transition in life stages which is also related to physiological maturation) has been found to trade-off with oxidative stress (Burraco et al., 2017). In our study system, while oxidative stress at fledging has been previously characterized as an overall cost of growth, none of the physiological parameters of maturation was found to be correlated with oxidative damage (Cornell and Williams, 2017). Accordingly, we did not fully characterise a direct cost of somatic/physiological maturation at day 17, but only a link between oxidative stress and telomere length. However, our data support the idea that there may be a strong selection for rapid maturation of physiological mechanisms. This should be advantageous, and selected for, because of functional requirements of active fledglings outside the nest (i.e. a flying metabolism; Riera et al., 1983), which partly relies on red blood cells (Hct and Hb) for oxygen transport. This process is likely to be sustained by an increase in the rate of division of the stem cells from the haematopoietic tissue (Orkin and Zon, 2008), and then to translate into a parallel reduction in red blood cell telomere length. Accordingly, the significance of this relationship between maturation and telomere length should be more pronounced when chicks are preparing themselves for active flight, i.e. at the fledging stage.

Nestling (day 17) telomere length and subsequent somatic and physiological maturation

Hct and Hb content increase (while reticulocytes count decreases) during physiological maturation in fledglings (between day 17 and day 21) in starling chicks. Somatic traits (tarsus, mass) were already close to adult values by day 17, and then mass declined, although wing length continues to increase, to fledging at day 21 (Cornell et al., 2017). We evaluated whether telomere length measured at day 17 predicted subsequent somatic and physiological changes that are observed in chicks immediately prior to fledging (day 21). Fledging somatic maturation (PCA4) was independent of telomere length, which may be due to the fact that the final body mass loss and continuing wing growth are either uncoupled from trade-offs with other traits (and then from maturation costs) or do not require any substantial additional energy investment (Cornell et al., 2017). The second possibility is supported by our experimental approach, showing that food provisioning did not significantly alter patterns of somatic growth before fledging. Therefore, it may not be energy per se that modulates the cost of somatic maturation at fledging, but rather the investment of energy over the entire growth period that has consequences for final somatic maturation. The fact that nestling and fledgling maturation axes are positively related (larger chicks at day 17 lost less body mass before fledging) suggests that the way energy modulates fledging maturation is related to the intrinsic control of how somatic growth and maturation are traded off over the entire nestling period.

Telomere length at day 17 did predict the subsequent change in physiological variables, but this was dependent on brood number. In 2nd broods, chicks with short telomeres had a larger increase in Hb and Hct, and a larger decrease in reticulocyte count, just before fledging. First brood chicks generally benefit from better seasonal food availability, supporting higher growth rates, and fledge at higher mass, both probably favouring higher survival prospects (Cornell et al., 2017; Naef-Daenzer et al., 2001). Better environmental conditions probably allow them to escape fledging 'emergency' maturation patterns. Accordingly, nestling maturation axis (PCA1) was negatively related to fledging maturation axis (PCA3). This may suggest that physiological maturation immediately prior to fledgling is less critical if a maturation threshold was reached earlier in the nestling stage. Whether this applies only to physiology of aerobic capacity (Hct etc.) rather than other components of physiological phenotype will need deeper mechanistic approaches. Still, given that Hb content is a predictor of fledging and post-fledging survival in others passerines (Bowers et al., 2014; Nadolski et al., 2006), our results support the hypothesis that chicks with short telomeres which adopt a slow developmental trajectory may have to catch up to fulfil the maturation requirements associated with fledging. Such a catch-up response has been previously shown to come from both an energy and/or time-constrained window of optimal growth (Mangel and Munch, 2005; Metcalfe and Monaghan, 2001). Evolution of faster-than-normal growth has attracted extended interest for several years, and several studies both correlative and experimental suggested that such catch-up growth can be associated with oxidative stress and/or shortened telomeres (Smith et al., 2016; Tarry-Adkins et al., 2009). Our experimental data, by showing that physiological maturation at fledging (day 17–21) does not respond to food provisioning, confirms that physiological maturation has been under strong selection, probably to promote survival in the immediate post-fledging period. Still, the costs of ultimate physiological maturation may take place later, since in many altricial birds chicks are somatically mature (close to adult size/mass) before fledging, but are still physiologically immature (Cornell et al., 2017). It would be interesting to examine how cell division rate on the one hand and the process of Hb production per se (which in birds may take place both in reticulocytes and in mature nucleated red blood cells) on the other hand have the same impact on telomere maintenance. Perhaps, when conditions are optimal, sufficient energy is available to sustain both Hb production and telomere maintenance, while reticulocyte precursors division and reticulocyte maturation in erythrocytes is traded off with telomere shortening. In fact, since reduced Hb levels or lower Hct are associated with reproductive costs in adult starlings (Fowler and Williams, 2017), it is understandable that those variables may also mediate, under some conditions, the cost of maturation. Our paper provides correlative evidence that physiological maturity may be traded off with telomere length and are in accordance with the idea that development (growth and maturation) has been canalized because of large effects on fitness (Boonekamp et al., 2018). Previous studies conducted in European starlings have stressed that adverse early-life conditions of growth induced both precocious telomere shortening and have delayed impact on physiological condition at adulthood (i.e. inflammation status; Nettle et al., 2017). If telomere length at fledging is of key importance in defining the fitness prospects of fledging, we may expect that canalisation has also taken place for telomere maintenance (Vedder et al., 2017). Therefore, how the ultimate maturation process is actually reflected in telomere length during the last days before fledging and when chicks enter their active post-fledging lifestyle needs to be evaluated.

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Competing interests
The authors declare no competing or financial interests.

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

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Data availability
Data are available from figshare: https://figshare.com/articles/ES2019_csv/9944165/1.

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