Telomere length vary with sex, hatching rank~~order~~ and year of birth in little owls, *Athene noctua*

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Abstract

Telomeres are non-coding DNA sequences located at the end of linear chromosomes, protecting genome integrity. In numerous taxa, telomeres shorten with age and telomere length (TL) is positively correlated with longevity. Moreover, TL is also affected by environmental stressors and/or resource-demanding situations particularly during early-life. Thus, TL has been used as a physiological marker of individual quality and also as an indicator of population trend in conservation physiology. In this study, we investigated the effects of hatching rank, year of birth (2014 to 2017), sex and nest environment on TL of 137 little owls nestlings (*Athene noctua*). Little owls' populations in Europe showed a marked declined in the end of the 20th century. Nowadays, in the studied Alsatian population, the population is increasing. In this study, our results indicated that telomeres are longer in females and, independently of sex, in nestlings with the highest body condition. There was also a negative effect of hatching rank but only for last-hatched nestlings in large clutches of 5 nestlings. We did not find ~~a marked~~any effect of the environmental covariates on nestlings' TL. Finally, we found that nestlings' TL were shorter the last year of the study~~decreased over years~~, while nestlings' body condition stayed unchanged over the same period. This result is intriguing given the local positive population dynamics and is further discussed in the context of physiological conservation. Future studies should investigate the link between reduced TL and survival prospects in this species.

Introduction

Telomeres are non-coding DNA structures, located at the end of the linear chromosomes, serving as a safe-keeper for preservation of coding DNA over cell duplication (Blackburn, 1991). Thanks to the formation of a capped structure with specific shelterin proteins, telomeres help the cell to distinguish real chromosome ends from DNA breaks, thereby avoiding unappropriated cell emergency responses. Still, this telomere status is degrading over time, due to the progressive loss of telomere sequences at each cell division, affecting its functionality and triggering cell senescence (Blackburn, 2000). In addition, telomere sequences are enriched in GC bases, making them highly sensitive to a well-known ageing mechanism, the oxidative stress (von Zglinicki, 2002; Reichert & Stier, 2017) (but see Boonekamp *et al.*, 2017). Such a stress-related property triggered the interest of evolutionary biologists to study how telomeres (length or dynamics) may vary with age and thus be used as a proxy to address the question of the existing variance in ~~explain~~ inter-specific longevity (Haussmann *et al.*, 2003; Dantzer & Fletcher, 2015; Tricola *et al.*, 2018; Criscuolo *et al.*, 2021) ~~and the link between environmental stress or life-history trade-offs and~~or inter-individual differences in lifespan and fitness (Beaulieu *et al.*, 2011; Foote *et al.*, 2011; Boonekamp *et al.*, 2014; Nettle *et al.*, 2017; Bichet *et al.*, 2020; Chatelain *et al.*, 2020; Fitzpatrick *et al.*, 2021; Sheldon *et al.*, 2021; Salmón & Burraco, 2022).

The importance of how early life conditions affect inter-individual telomere length quickly appears as a key question to understand how somatic growth may shape individual life trajectories in the context of life history trade-offs~~pleiotropy~~ (Metcalfe & Monaghan, 2003; Monaghan & Ozanne, 2018). This is based on the observation that growth is a period of high energy metabolism (2-6 times basal metabolic rate, e.g. Kirkwood, 1991) to fuel intense rate of cell division, which is~~both physiological traits~~ likely to be costly in terms of telomere

erosion (Vedder *et al.*, 2017; Spurgin *et al.*, 2018). ~~Short telomeres in fledgling may then reflect accumulated stress that impaired investment in cell maintenance of the growing organism, due to deleterious effects of sub-optimal nutritional, social and/or hormonal environments~~ Studies have shown juveniles exposed to challenging conditions in early life to have shorter telomeres. This could be due to reduced investment in somatic maintenance as a consequence of low resource availability when conditions are harsh (Herborn *et al.*, 2014; Nettle *et al.*, 2015, 2017; Reichert *et al.*, 2015; Angelier *et al.*, 2017; Quque *et al.*, 2021). Interestingly, telomeres may also be affected during the pre-hatching developmental period. For instance, temperature instability during egg development triggers shorter telomere length at hatching in Japanese quail (*Coturnix Japonica*, Stier *et al.*, 2020), and decreasing incubation temperature in the common tern (*Sterna hirundo*) slows down growth rate and preserve~~save~~ telomere length in matched-body sized hatchlings (Vedder *et al.*, 2018). Yet, telomere dynamics are not only affected by stress effects. Producing eggs is costly for the female, and depending on maternal characteristics and environmental conditions, we can expect an adjustment of egg characteristics that will shape consequent embryonic traits (Williams, 1994; Groothuis & Schwabl, 2008). As such, a large diversity of egg components (like yolk and hormones), that may be positively or negatively correlated with each other, may vary and modulate ~~the~~ future offspring phenotype ~~in a synergistic or antagonistic ways, leading to the concept of multivariate egg~~ (Postma *et al.*, 2014; Williams & Groothuis, 2015). In addition, because an entire clutch is produced over sequential laying of consecutive eggs, intra-clutch variability in ~~multivariate~~ egg traits may be part of a mother strategy of adaptation of the chick's phenotype, and is then expected to follow the laying order (Groothuis *et al.*, 2005). In particular, according to the brood reduction hypothesis, it is expected that the probability of survival of last hatched nestlings (from last laid eggs) will be smaller than that of first hatched

ones in case of harsh conditions (Lack, 1947; Amundsen & Slagsvold, 1996). Thus, we can expect maternal investment to decrease over the laying sequence. Telomere length is not an exception, and progressive shortening has been observed within clutch laying order in captive zebra finches (*Taeniopygia guttata*, Noguera *et al.*, 2016) ~~as well as inter-individual variation within the multivariate egg concept (Criscuolo *et al.*, 2020)~~. In this~~e former~~ study, the astonishing result is that the difference in embryonic telomere lengths between the 1st and the last laid eggs represents 60% of the telomere loss an offspring will show over its first year of life. This source of variation in telomere length may be important to consider since many studies have shown negative consequences of telomere erosion on future individual fitness~~Given that the negative consequences of fast telomere erosion during growth on future individual fitness prospects are legions~~, *e.g.* jackdaws (*Corvus monedula*, Boonekamp *et al.*, 2018), king penguins (*Aptenodytes patagonicus*, Geiger *et al.*, 2012) or in wild purple-crowned fairy-wrens (*Malurus coronatus coronatus*, Eastwood *et al.*, 2019), to name a few.~~, variability in telomere length within clutch is likely not an epiphenomenon.~~ Still, we lack data on the effect of laying order in many~~other~~ bird species and on how laying order effect on telomere length may vary in relation to additional stress sources, like environmental conditions in the wild (but see Kärkkäinen *et al.*, 2021).

Our study is based on 4 years of data from a wild population of Little Owl (*Athene noctua*) reproducing in artificial nestboxes. All nestlings are ringed and measured before fledging. After checking for hatching rank and environmental effects on chick phenotype, ~~First, we tested whether individual characteristics (sex and body condition) are dependent on hatching rank and on environmental characteristics around the nest. Second, using~~ we used telomere length measurements made on individual feather sampling to test ~~, we tested~~ how nestling telomere length varied with hatching rank and with the local characteristics of nest

environment. To do so, we ~~,~~controlled~~ing~~ for nestling sex, age, body condition, clutch size and year of birth~~,~~ ~~and with the local characteristics of nest environment.~~ To estimate nest environment characteristics, we calculated the proportion of orchards, meadows, crops, buildings, water and forests around each nest box from land use maps. In central Europe, the Little Owl is a bird species associated with traditional farmlands and its optimal habitat should provide cavities, perches for hunting and short herbage with invertebrates and small rodents (herbage size is linked to prey accessibility and availability, van Nieuwenhuyse *et al.*, 2008). In particular, meadows and orchards are supposed to be food-rich habitats (Michel *et al.*, 2017).

We predicted last hatched nestlings to be in worse condition (body mass, telomere length) than first hatched nestlings according to the brood size reduction hypothesis. We also predicted shorter telomeres in broods raised in unfavourable environments, *i.e.* more proportion of buildings, water and forests around the nest box.

Material and Methods

Model species and data collection

The Little Owl is a small nocturnal raptor living in open or semi-open areas, such as farmland or orchards (van Nieuwenhuyse *et al.*, 2008). The Little Owl is territorial and breeds in cavity, including artificial nestboxes. In Alsace (France), numerous ringers and volunteers from the French league for the protection of birds (LPO) installed and maintained more than 1,500 nest boxes since 2006, thereby monitoring the yearly reproductive success of the local population. Females lay 2-6 eggs in April, hatching occurs *ca.* 1 month later and nestlings are ringed between 15-35 days of age. At ringing, nestlings' body mass was measured with an electronic balance to the nearest 0.1 g, as well as tarsus length with a calliper to the nearest 0.1 mm, and the length of the third primary feather with a ruler to the nearest mm. The measure of the

feather allows us to approximate the age of the nestling with the formula: age=(length of the feather+36)/3.3, where the age is in days and the length of the feather is in mm (Juillard, 1984; Hameau *et al.*, 2015). This formula is valid between age 15 and 35 when there is a linear growth of the feather. Using the age of each nestling in a nest, the hatching rank~~order~~ was deduced. When two nestling had the same estimated age, we assigned them the same hatching rank. We also collected 3-6 ventral ~~covert~~ feathers that are stored in ethanol 70% at ambient temperature during fieldwork and then at 4°C in the lab.

For this study, we used data collected on 142 nestlings from 39 broods from 2014 to 2017. All those broods had more than 1 chick. We included in our study only broods with more than 1 chick i~~I~~n order to estimate the effect of hatching rank ~~we used only broods with more than 1 chick~~ (n=3, n=14, n=16, n=6 for broods with respectively 2, 3, 4 and 5 chicks).

Land use around the nestbox

To determine the land use around the nest boxes, we used a land cover database for Alsace (Source: BdOCS CIGAL v2 2011/2012, www.geograndest.fr) which categorizes all the habitats found in our study area. We used the software QGIS version 3.4.14 (QGIS Development Team, 2020) to map the active nest boxes and create a circular buffer zone of a 150 m radius around each one of them. This radius was established thanks to data on home range size (Exo, 1992; Génot, 2005) and the field observations made during the breeding season. Due to the high number of habitats, we made groupings based on the environmental characteristics of each variable to calculate the area (m^2) covered by each land type within the buffer zones. Our final nest environment included six categories: (1) buildings, (2) meadows, (3) crops (crop fields, hedges, and vineyard), (4) orchards, (5) forest and (6) water. Because of the rarity of the last two categories, forest and water were pooled together. The surface of habitat of the different categories were correlated with each other and thus we used in the model only the proportion

of surface of favorable habitat defined as the proportion of meadows and orchards in the buffer.

Relative telomere length (RTL) measurement and sexing

Genomic DNA was extracted from feathers using an adapted protocol of the NucleoSpin Tissue kit (Macherey Nagel, Düren, Germany). RTL was measured in the 142 nestlings in one 384-wells plate, using the quantitative PCR (qPCR) methodology (see Electronic Supplementary Material, ESM). Intra-plate repeatability of RTL (ICC, see (Eisenberg *et al.*, 2020)) was of 0.769. Molecular sexing of nestlings was determined using the same extracted DNA (following Griffiths *et al.*, 1998). Briefly, the technique is based on the existence of two conserved CHD (chromo-helicase-DNA-binding) genes that are located on the sex chromosomes. The CHD-W gene is located on the W chromosome (only in females) and the CHD-Z gene is located on the Z chromosome (both in males and females). For technical reasons, sex could not be determined in 5 nestlings. All the statistical analyses were performed on the remaining 137 nestlings with known sex.

Statistical analyses

We used R version 4.3.1 (R Core Team, 2023) to compute mixed models (package lme4 version 1.1-33 and lmerTest version 3.1-3). In all statistical models, brood identity was included as a random factor to account for the non-independence of nestlings of the same brood. We checked models' assumptions (homoscedasticity, normal distribution of residuals) graphically using the package DHARMa (version 0.4.6). We assessed multicollinearity among predictors by calculating variance inflation factor, VIF (package car, version 3.1-2).

Individual phenotypic characteristics

~~We tested whether sex is dependent on hatching rank. We computed a generalized mixed model with binomial family and with sex as a dependent variable and hatching rank and~~

~~nestling number as fixed effects. The significance of the effects was tested with type III Wald chisquare tests.~~

To test for inter-individual variation in body condition, we first calculated the Scale Mass Index (SMI) following the formula of Peig & Green (2009): $SMI = M_i [L_0/L_i]^b$ where M_i and L_i are the body mass and size measurements of individual i, b is the slope of the standardised major axis (SMA) regression of log-transformed M on log-transformed L and L_0 is the arithmetic mean of L for the study population. We then computed a linear mixed model with SMI as a dependent variable and hatching rank, sex, nestling number, nestling age, cohort, the proportion of meadows and orchards, the interaction between hatching rank and sex, and the interaction between hatching rank and the proportion of meadows and orchards as fixed effects. From this global model, we fitted every possible model and then selected a set of top models (AICc threshold of 2). Then, if the null model was not the best model, w~~W~~e ~~then~~ averaged the models from these top models set (conditional average, package MuMIn, version 1.47.5).

~~Then, we computed a linear mixed model with SMI as a dependent variable and environmental covariates (proportion of buildings, meadows, crops, orchards and of water and forest around the nest box) as fixed effects. The environmental covariates were scaled before the analysis. Model selection was similar as described above.~~

Inter-individual variation in Relative Telomere Length

RTL were log-transformed before analyses. ~~First, w~~We computed a linear mixed model with individual covariates (hatching rank, sex, the interaction between hatching rank and sex, nestling number, nestling age, SMI and cohort) and environmental covariates (the proportion of meadows and orchards, the interaction between hatching rank and this proportion) as fixed effects~~)~~. ~~For both models, T~~the model selection procedure was the same as described above.

Results

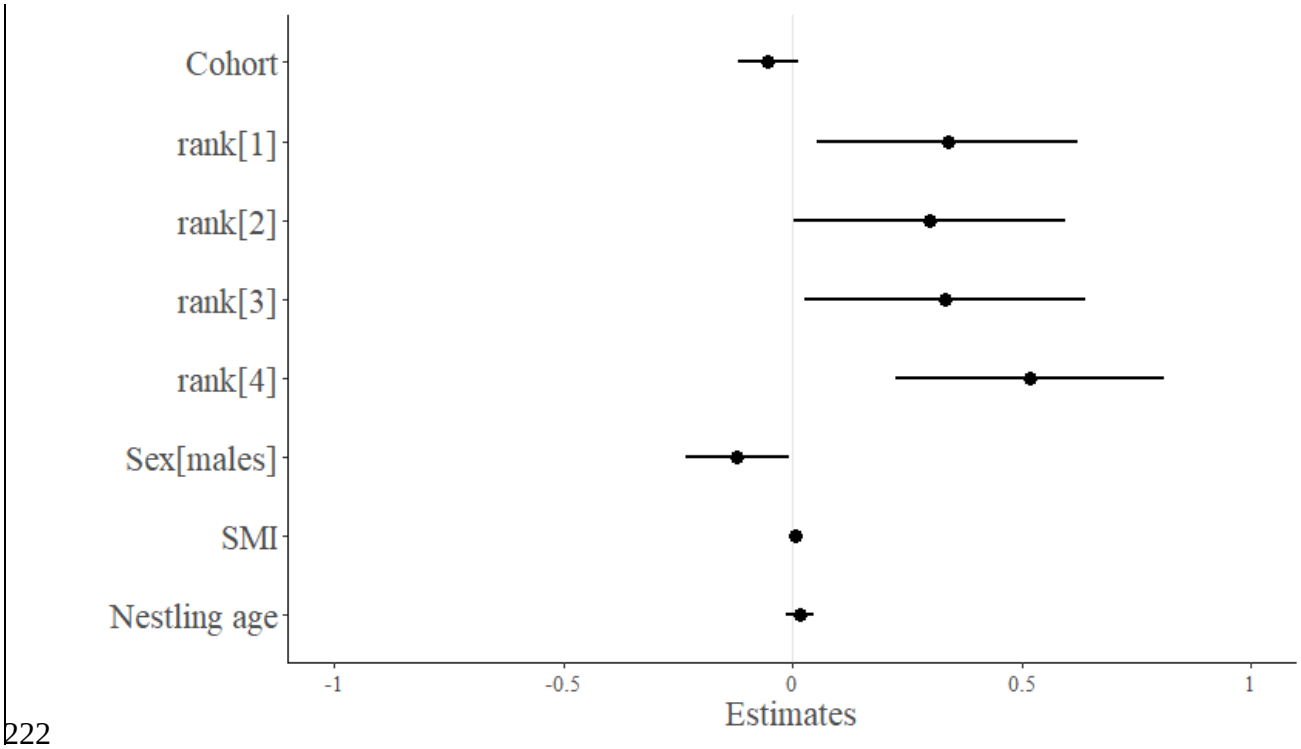
Individual phenotypic characteristics

~~The sex of the offspring was not significantly correlated with hatching rankorder (chi^2=4.6145, P=0.335) or nestling number (chi^2=0.48, P=0.49).~~

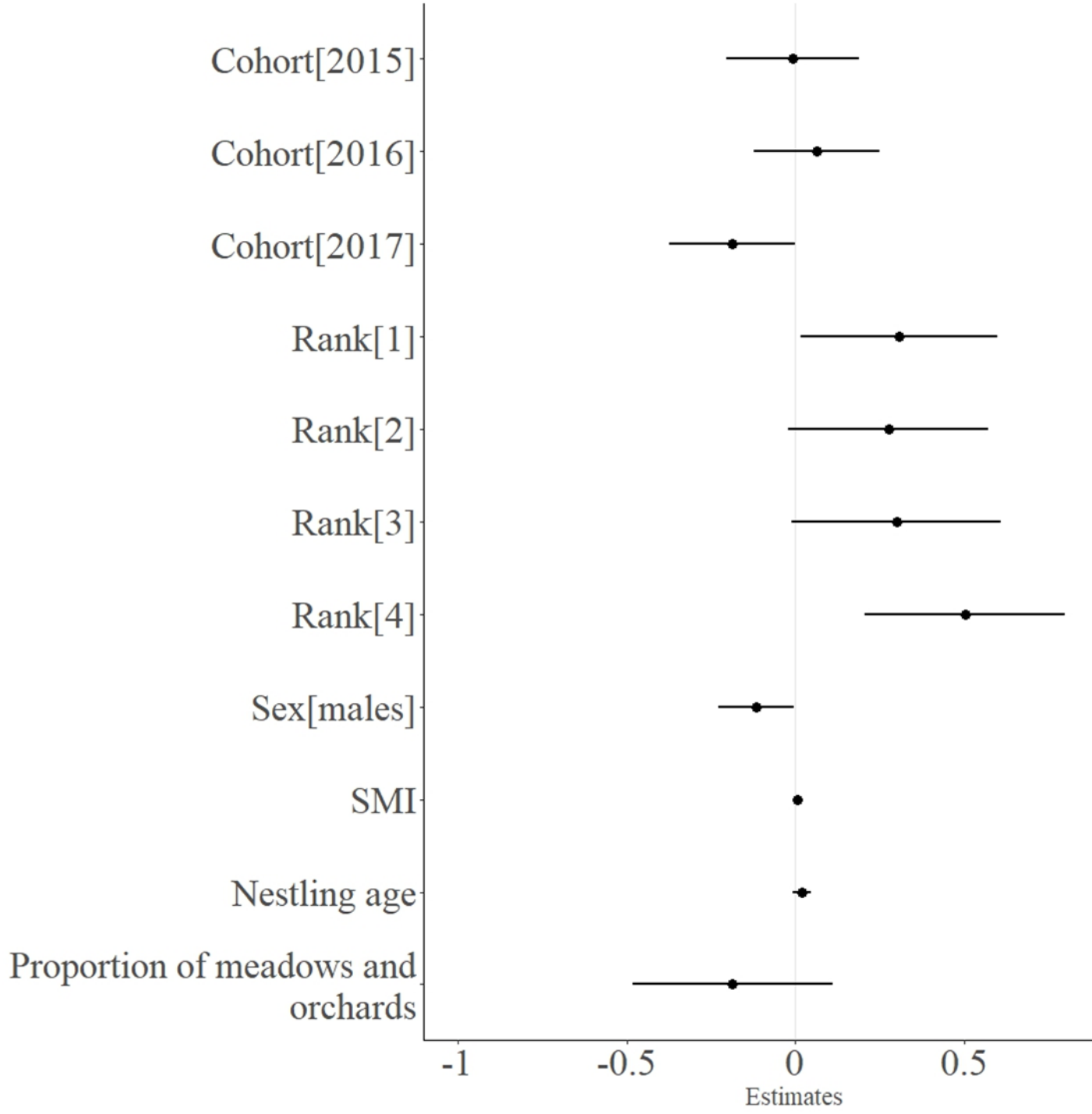
Concerning individual covariates, there were no significant variables that explained variation in SMI in our models. The fixed effects retained in the top models set (5 models) were ~~nestling age,~~ the proportion of meadows and orchards, nestling number and sex (see Table S1) but their effects were not significantly different from 0 (see Figure S1). This is consistent with the fact that the null model was in the top models set (see Table S1).

~~Concerning environmental covariates, the proportion of buildings, crops, meadows and orchards around the nest box were kept in the best models (Table S2). The increase of buildings and of crops has a marginally negative effect on the SMI of little owls (Figure S2).~~

Figure 1. Forest-plot of estimates for the average model of relative telomere length and individual covariates (see Table S3). Reference level for sex is females, for cohort is 2014 (the first year of the study) and for rank is 5 (last hatched chicks). Significance levels are annotated with asterisks: *** p<00.1,**p<0.01,*p<0.05, . p<0.10


Cohort
rank[1]
rank[2]
rank[3]
rank[4]
Sex[males]
SMI
Nestling age
-1
-0.5
0
0.5
1
Estimates

222



Inter-individual variation in Relative Telomere Length (RTL)

Concerning individual covariates, RTL was not dependent on nestling number and there was no interaction between rank and sex, or between rank and the proportion of meadows and orchards. The variables in the top models set (6 models) were rank, sex, SMI, cohort, nestling

age and the proportion of meadows and orchards (Table S3, Figure 1). Males have significantly shorter telomeres than females ~~(Figures 1 and 2)~~ and there is a small significant positive effect of SMI on RTL (Figure 1). In addition, last hatched nestlings have shorter telomeres but only in the largest brood of 5 nestlings (Figures 1 and 2~~3~~). The effect of the year of birth is significant for the last year of study, meaning that individuals born in 2017 have shorter telomeres than individuals born earlier ~~marginally significant and is negative, meaning that RTL are decreasing in recent years~~ (Figures 1 and 3).

Concerning environmental covariates, the proportion of meadows and orchards was kept In the best model but has no significant effect on RTL~~buildings, crops, orchards and forest and water around the nest box were kept in the best models (Table S4). There is a marginal negative effect of the proportion of forest and water around the nest box on nestlings RTL~~ (Figure 1~~S3~~).

~~Figure 2. The effect of sex on the relative telomere length before fledging.~~

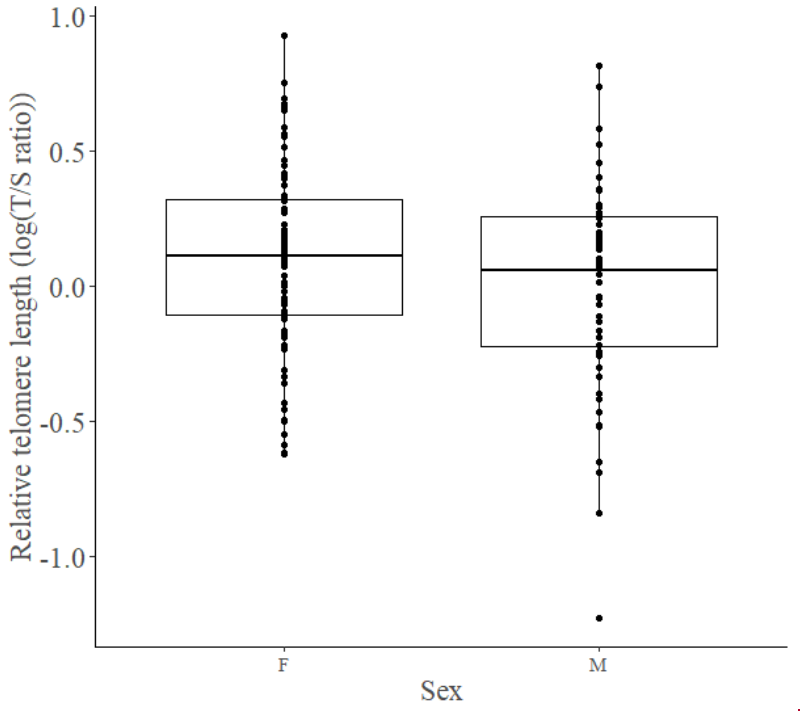


Figure 2~~3~~. The effect of hatching rank~~order and year of birth~~ on the relative telomere length before fledging.

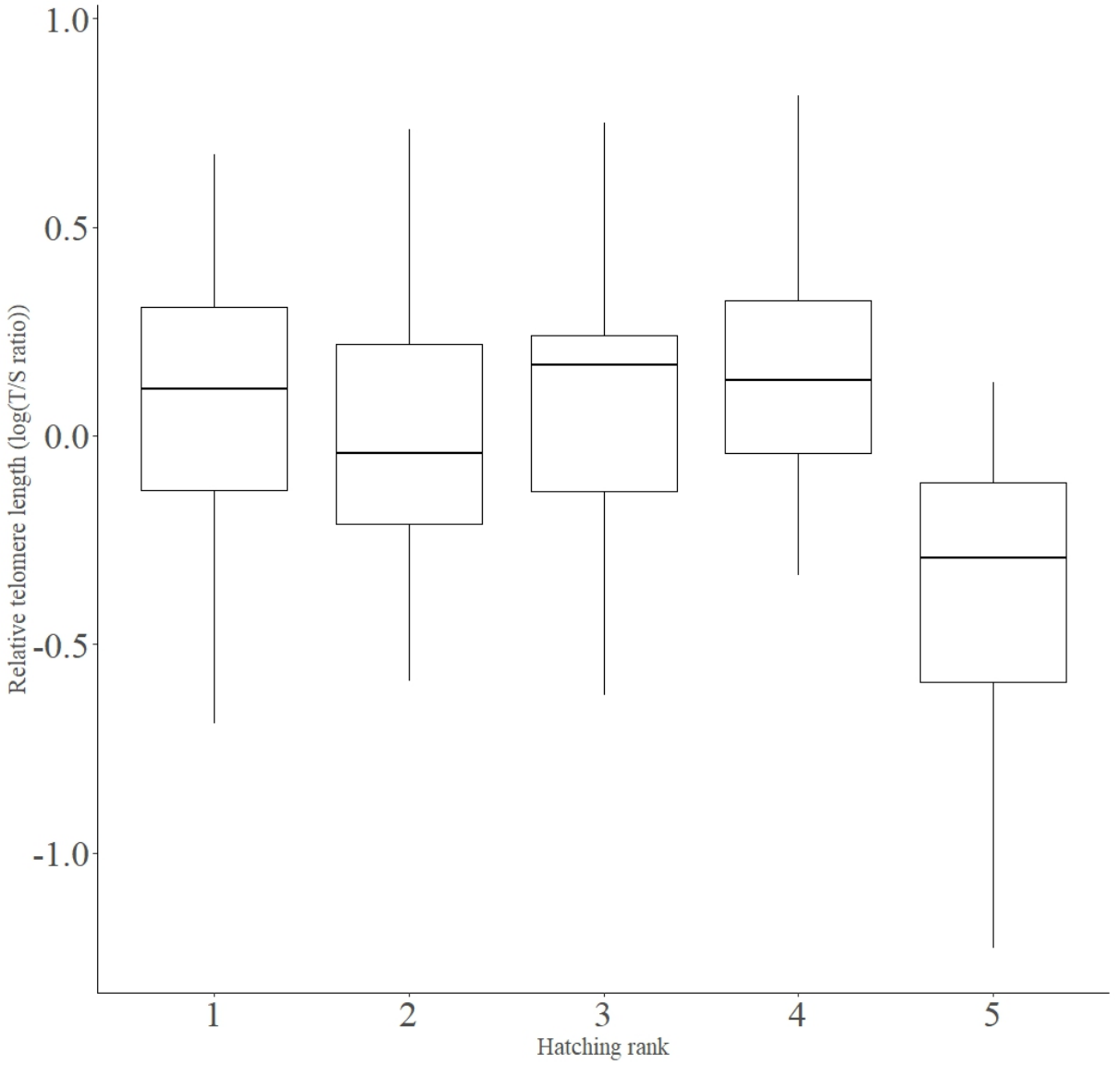
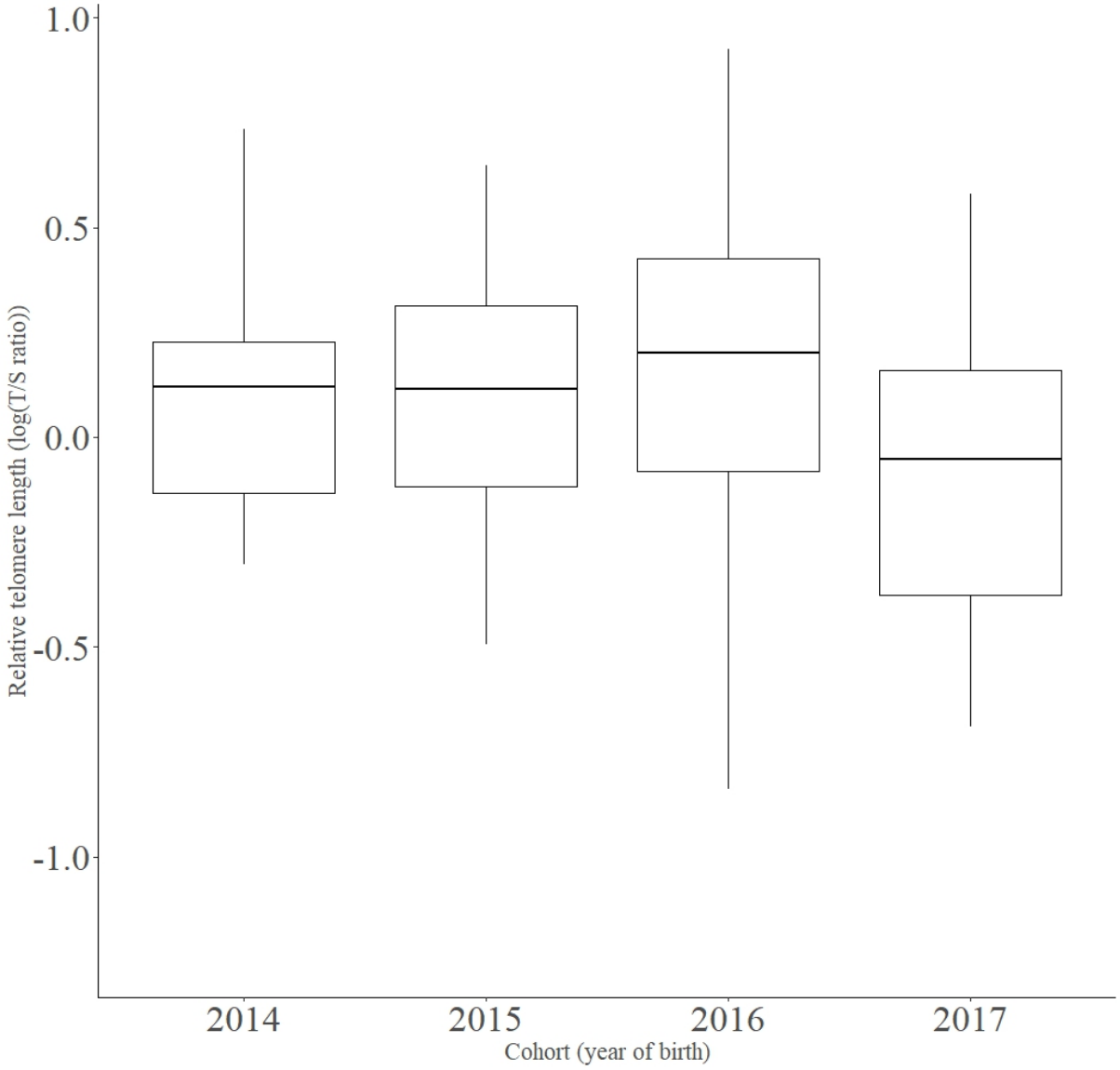


Figure 3. The effect of the cohort on the relative telomere length before fledging.



Discussion

Based on the current knowledge on growth and telomeres in bird nestlings, we initially predicted that RTL of little owl nestlings will be: (i) negatively related to the hatching rank~~order~~ and (ii) negatively affected by the unfavourable nature of the nest surroundings. Our results indicated that RTL are longer in females and, independently of sex, in nestlings with the highest body condition. They also supported a mixed negative effect of hatching rank~~order~~ and intra-brood competition on little owl nestlings' RTL, i.e. detectable only in the largest brood size, suggesting that the effect of hatching rank on telomeres is dependent on a threshold effect in this species. We did not find an ~~clear~~ effect of the environmental covariates

on nestlings' RTL. Finally, our ~~longitudinal~~ scan of nestlings' RTL over years surprisingly underlined a possible progressive shortening, independent of any changes in body condition.

~~Our indication of an erosion of l~~Little owl nestlings' RTL were shorter in the last year of the study (2017) in comparison to previous ~~over~~ years (201~~4~~ onwards). Both telomere data and such year effect ~~need to be replaced in the emerging~~are of great interest in the context of conservation physiology aiming at developing physiological markers of individual quality to infer consequences at the population level (Beaulieu & Costantini, 2014; Lea *et al.*, 2018). Telomeres are good candidate to be such marker because~~However, Telomere~~ telomere length at a given age is not reflecting only the negative effects of time on the cells (i.e. chronological age), it also points out the cumulative effects of stressors encountered over time that may accelerate the rate of loss of telomere ends over the expected rate at a given age for a given species (Asghar *et al.*, 2015; Louzon *et al.*, 2019; Chatelain *et al.*, 2020; Salmón & Burraco, 2022). Thus, the use of telomere assay is potentially providing data that are useful to establish survival rates at specific age stages, like the nestling period. Since deleterious environmental conditions can affect negatively telomere length, the period of growth is supposed to be the life stage where telomere sequences can be the most impacted ~~Because the rate of cell division and/or the oxidative metabolism are higher in a growing organism, the period of growth is supposed to be the life stage where telomere sequences are the most impacted by environmental stressors~~ (Salomons *et al.*, 2009; Young *et al.*, 2013; Monaghan & Ozanne, 2018). Beside the classical explanation that the growing period is particularly sensitive to environmental stressors because the rate of cell division and/or the oxidative metabolism are higher in a growing organism, it is likely that chicks can just hardly escape the trade-off between growth and survival. As such, sustaining a fast (but not too fast, see below) rate of growth to shorten as much as possible the nestling period may be done at a cost for

telomere length. Thus, depending on the harshness of early life environment, erosion of telomeres can be accelerated for a given age (e.g. Boonekamp *et al.*, 2014; Stier *et al.*, 2015), leading the ~~nestlings~~ fledglings to be grown ~~, prematurely,~~ physiologically old. In addition, variation in growth rate, due to changes in food availability, may affect telomere length and not body mass or body condition. As an example, growth rate may accelerate after a stunt when optimal feeding conditions are re-established, which are known to trigger transient over-optimal compensatory growth rate and faster telomere erosion (Metcalfe & Monaghan, 2001; Geiger *et al.*, 2012). This has, theoretically, obvious consequences for the individuals in terms of survival prospects and recruitments as adult breeders in the population, as early life telomere length or rate of telomere loss have been shown to predict future individuals' survival (Boonekamp *et al.*, 2014; Watson *et al.*, 2015; Wood & Young, 2019). Consequently, it also has the potential to affect the population dynamics. First conceptualized few years ago (Stindl, 2004), such a hypothesis was recently supported by studies conducted on ectotherms' populations (Dupoué *et al.*, 2017, 2022). In the common lizard populations studied, analysis of telomere length in yearlings of populations showing different risks of collapsing due to local global warming pointed out reduced mean telomere length in the most endangered populations (Dupoué *et al.*, 2017). Thereafter, the same group showed that short telomeres were already inherited in neonates of declining populations, thereby suggesting (epi)genetic roots, i.e. progressive telomere shortening being not only the result of bad early life conditions (Dupoué *et al.*, 2022). We cannot draw the same conclusions in our case, particularly because (i) our data indicate that 2017 was the only year with shorter telomeres and (ii) we lack data on inter-generational variation of telomere length. It can be noted that in vertebrates, heritability estimates are moderate (Chik *et al.*, 2022), but this recent meta-analysis has no data on raptors (Chik *et al.*, 2022). In addition, as low rates of recruitments of juveniles as first-

breeders is an important determinant of population decline in the little owl (Le Gouar *et al.*, 2011), the link between reduced telomere length and survival prospects of nestlings needs to be established. Finally, this result is counter-intuitive in our study population of little owl since the population is expanding and not decreasing (Bersuder & Wassmer, 2020), contrary to other populations (Andersen *et al.*, 2017). Whether 2017 is a transient year with unknown bad conditions for chicks or is actually the start of a longer adverse period for our population is currently unknown. Thus, the effects of yearly variations in food availability, intra-nest competition or density on telomere length need to be addressed in future studies.

Little owl female nestlings had longer telomeres than male ones. This has several implications for our understanding of sex-differences in telomere dynamics and of its meaning in terms of sex-biased life history. Differences in telomere length in relation to ~~gender~~sex has been previously illustrated in several taxa (reviewed in Barrett & Richardson, 2011), and particularly in birds with sex-biased body size or investment in reproduction, ~~with no consensual general pattern~~producing no consistent male-female differences (e.g. Caprioli *et al.*, 2013; Remot *et al.*, 2020; Saulnier *et al.*, 2022 for no sex differences) (e.g. Bauch *et al.*, 2020 for sex differences). In our study, sex-differences in RTL were observed at the nestling stage, with longer telomeres in the females. A previous study showed that females were slightly but consistently of bigger size (Tschumi *et al.*, 2019), however it is not the case in our population. Yet, we did not investigate nestlings growth rates, which can be different even~~t~~ if the final size and/or body mass is similar (e.g. Criscuolo *et al.*, 2008). Higher growth rates are usually associated with shorter telomeres (Geiger *et al.*, 2012; Monaghan & Ozanne, 2018) and generally the larger sex is growing at a slower rate in sexually dimorphic bird species (e.g. Teather & Weatherhead, 1994)~~.~~, This may potentially account for our sex-difference in telomere length as females may dilute the growth-body maintenance trade-off over a longer

period. ~~.~~ However, we also found that, independently of sex, nestlings in better body condition had in general longer telomeres. Thus, it is either unlikely that little owl nestlings had to face such a growth-body maintenance trade-off, or that our result is driven by high quality individuals that can sustain growth without showing any associated cost in terms of telomere loss. Given that body mass is a determinant of survival from hatching to fledging in little owl (Tschumi *et al.*, 2019), nestling telomeres rather acts as a proxy of individual quality (Angelier *et al.*, 2019). In addition, our results do not match with the idea that the heterogametic sex (*i.e.* females) would be more prone to telomere erosion than the homogametic one (*i.e.* males) due to the unguarded expression of deleterious alleles of sex chromosomes for telomere maintenance (see Barrett & Richardson, 2011; Remot *et al.*, 2020 for a deep discussion related to telomere dynamics). One alternative explanation lies on optimal parental care towards the offspring sex with the highest chance of survival (Hasselquist & Kempenaers, 2002). It has been shown previously that females have a higher survival probability from hatching to fledging, independent of any variation in body mass (Tschumi *et al.*, 2019). However, it is not known whether this sex-difference persists in older individuals. In that context, the parents would favour female individuals, meaning that within little owl broods females may, on average, benefi~~cia~~t~~e~~ from better access to food resources due to specific parental investment. This may lead to an attenuated body maintenance (*i.e.* telomere length) and growth rate trade-off. Still, further study in our case is needed to determine whether adaptive brood sex ratio actually occurs, since it may result from non-adaptive additional effects (e.g. sex specific mortality, see Bortolotti, 1986; Hasselquist & Kempenaers, 2002).

The hypothesis that RTL is an indicator of quality is further supported by the fact that, in the largest clutches, the last hatchling of little owl presented the shortest telomeres. Even if our sample size is small (i.e., 6 clutches with 5 eggs), ~~This~~ our data ~~is~~ are~~also~~ in accordance

with the brood size reduction hypothesis that predicts a lower investment with laying order. Still, our data would restrict such an effect to the last laid egg. We cannot distinguish between effects of the laying order *per se* on RTL (see introduction) and postnatal effects. Postnatal effects may arise from selective parental care as discussed above. Last-hatched nestling may also suffer from intra-brood competition. Indeed, in a brood, larger nestlings have a competitive advantage compared to smaller nestlings for feeding ("Competitive advantage hypothesis", Anderson *et al.*, 1993). A previous experiment testing the effect of competitive disadvantage within a brood, based on the size of the nestlings cross-fostered among clutches, highlighted an interesting increased telomere attrition of less competitive nestlings without affecting body mass growth (in European starlings, Nettle *et al.*, 2015).

Finally, our study only suggested non-significant effects of nest surroundings~~, with shorter telomeres in nests with higher proportion of water and forest areas, and with worse body condition in nests with higher proportion of buildings and crops~~. In other studies, local habitat types around nests and also the heterogeneity of habitats available have been shown to affect reproductive output in our species (Thorup *et al.*, 2010; Michel *et al.*, 2017). Moreover, it has been shown that the home range size is dependent on the environment around the nest and also is different between males and females (Michel *et al.*, 2017). Thus, it may be important to consider the habitat at a fine scale. Future studies should explore how environmental quality, food resources, parental care, chick growth, intra-brood competition and sex-specific susceptibility to stressors are intertwined factors that determine offspring telomere length and how all these factors affect population dynamics of little owls.

Ethics statement. This work is in accordance with the French legislation concerning the capture and the biological sampling of wildlife. All the ringers of the project had received

ringing licenses and authorizations for feather sampling from the CRBPO (National Museum of Natural History, Paris, France) as part of a program led by Bertrand Scaar (PP N°454).

Data accessibility. Datasets used in this study are openly available on zenodo (doi: 10.5281/zenodo.770153~~10.5281/zenodo.7701531~~).

Authors' contributions. JB and FC conceived the study. BS and volunteers collected the data. SZ developed and performed the sexing and qPCR measurements. IF sorted the samples and calculated the land use around nest boxes. JB and FC ran the statistical analyses and, with SZ for the ESM, wrote the first draft of the manuscript. All authors provided comments on the manuscript and agreed on the final version of the manuscript to be submitted for publication.

Conflict of interest disclosure. The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

~~**Competing interests.** We declare we have no competing interests.~~

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ESM

Amplification of telomere repeats using q-PCR methodology

The protocol for DNA extraction from feathers provided us with sufficient amount of DNA to run both sexing and telomere determinations. One to three feathers per individual were selected and a 0.5-1 cm piece from each feather were cut in small pieces with a sterilized scissor. After digestion, feather quills will remain unlysed. For samples containing unlysed quills, we centrifuge briefly and we transfer the supernatant to another tube before proceeding with step 4 of the standard protocol.

Individual relative telomere length (RTL) were obtained following the qPCR methodology previously used in several bird species by our group (*e.g.* Criscuolo et al. 2009, Bize et al. 2009, Criscuolo et al. 2020, Chatelain et al. 2021). DNA quantity and quality were assessed based on spectrophotometer absorbance (Nano-Drop 1000, Thermo Fisher Scientific, Waltham, MA, USA, ratios A260/280 and A260/230) and gel migration. Individual DNA were all diluted to a concentration of 5.0 ng/µL, and further used for RTL determination by qPCR. To control for variation in DNA concentrations among diluted samples (due to potential pipetting errors), which may induce a methodological bias to the final RTL values, we amplified, for each individual, a genomic DNA sequence, defined so far as non-variable in copy numbers. The gene used in our species was RAG-1 gene (recombination activating protein 1 gene, NCBI number EU348872.1). Amplifications were conducted in two 384 wells-plates filled by a calibrated automated liquid handling workstation (Epmotion, Eppendorf, Montesson, France), using one distinct plate for control gene and telomere amplifications, due to the different qPCR conditions due to primers sequences properties. Conditions of amplification were 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 (control gene) and of 2 min at 95°C followed by 30 cycles of 15 s at 95°C, 30 s at 56°C and 30 sec at 72°C, (telomere sequence). Reactions were done in a master mix prepared for each primer set, with 5 µL GoTaq QPCR Mix (Promega, Madison, WI, USA). We used 10 ng of DNA (in a volume of 2 µL), to which we added the telomere primers at a concentration of 200 nM or the control gene primers at 400 nM (for a final reaction volume of 10 µL in each well, completed with ultra-pure water). In both plates (control gene and telomere sequences) we amplified individuals' DNA samples plus three quality control references. A DNA golden sample (as a mix of22 individual samples randomly chosen) that was used as the reference value of 1 for RTL calculations. A dilution curve obtained from the amplification of a randomly chosen reference sample that was serially diluted (from 10 to 0.625 ng/mL). Dilution curves enable us to assess quality of control gene and telomere sequences qPCR amplifications (i.e. efficiency values (control gene 0.999; telomere sequences 0.993) and r^2 (0.993 and 0.995, respectively) of the dilution curves). A negative control sample (ultra-pure water) to control for putative contaminations of non-bird DNA. All runs ended by a fusion curve to verify the absence of non-specific amplifications. RTL values were calculated following Pfaffl (2001), shortly as the ratio between Telomere (T) and Control gene (S) Cq values, controlled for their respective amplification efficiencies and expressed relatively to the golden sample T/S value of 1. All samples were run in duplicates and intra-individual repeatability of RTL, evaluated using the Intra Class Coefficient (Eisenberg *et al.*, 2020), was of 0.769.

Table S1. Top models set for models of SMI. For continuous variables, each value represents the estimate of the effect; for categorical variables, there is a "+" when the variable is retained in a model.
df = degree of freedom. delta = difference of AICc with the model with the lowest AICc.

Intercept	Nestling number	Proportion of meadows and orchards	Sex	df	AICc	delta
125.8		14.44		4	1057.3	0.00
145.3	-3.52			4	1058.0	0.70
136.7	-2.66	11.93		5	1058.1	0.82
132.3				3	1058.3	0.93
125.3		14.32	+	5	1058.9	1.59

Figure S1. Forest-plot of estimates for the average model from Table S1. Reference level for sex is females.

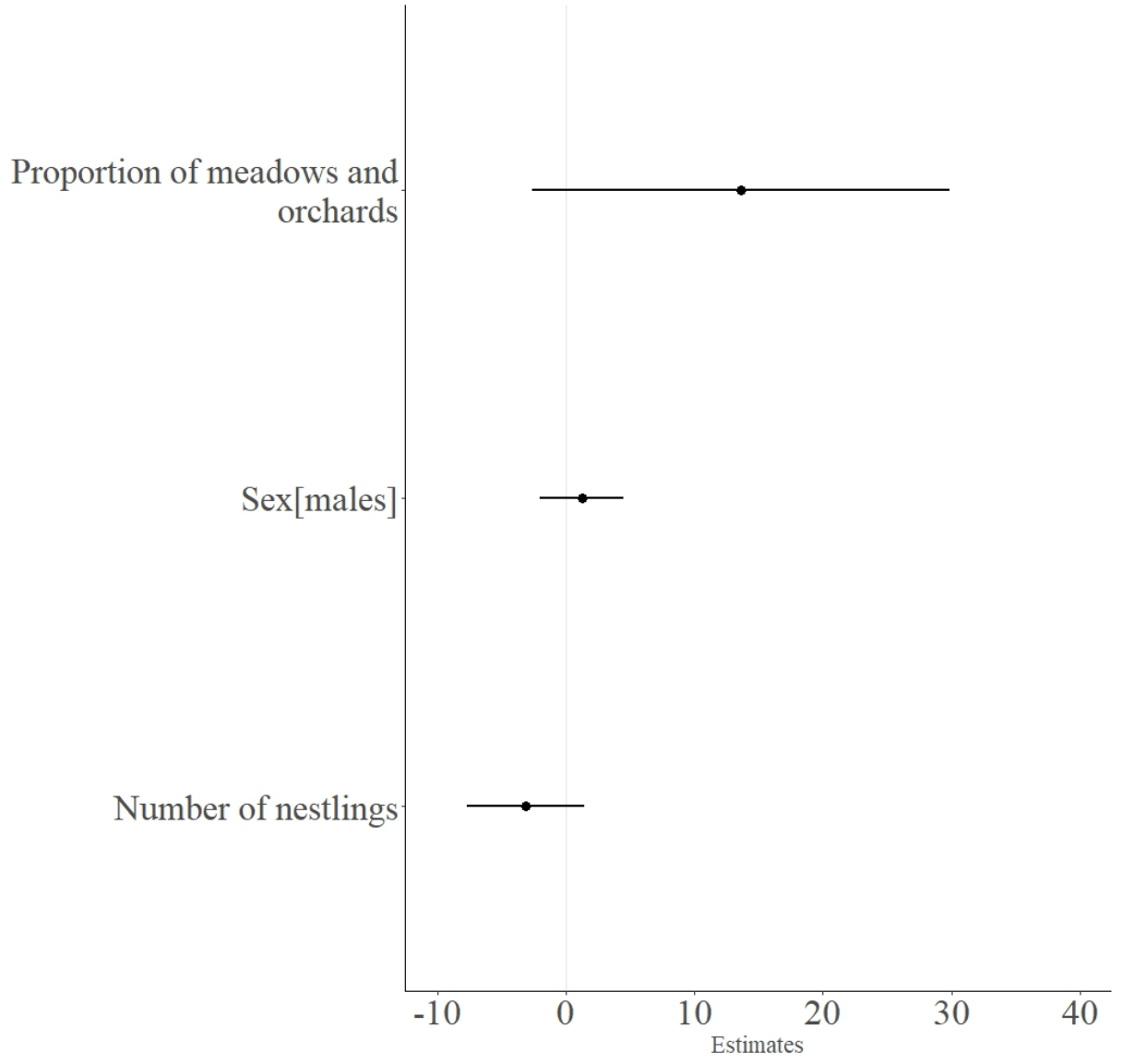

Table S3. Top models set for models of RTL. For continuous variables, each value represents the estimate of the effect; for categorical variables, there is a "+" when the variable is retained in a model.
df = degree of freedom. delta = difference of AICc with the model with the lowest AICc.

Intercept	Proportion of meadows and orchards	Nestling age	Cohort	Rank	Sex	SMI	df	AICc	delta
-0.82			+	+	+	0.0049	12	103.8	0.00
-0.86				+	+	0.0046	9	104.6	0.81
-1.16		0.019	+	+	+	0.0047	13	104.6	0.83
-0.84	-0.17		+	+	+	0.0055	13	104.9	1.12
-0.86			+	+		0.0046	11	105.3	1.46
-1.23	-0.20	0.021	+	+	+	0.0054	14	105.3	1.48

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