**Telomere length and dynamics of spotless starling nestlings
depend on nest-building materials used by parents**

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Nest materials used by animals can have profound effects on developing offspring. They can modify the bacterial and parasitic environment of the nest, and can influence parental investment through sexual signalling processes. In spotless starlings, *Sturnus unicolor*, green plants and feathers are known nest materials with such functions. The aim of our study was to experimentally assess their influence on nestlings' telomere length and attrition, which are good predictors of their survival prospects. In a full-factorial experiment, we explored these effects in two different populations, together with the potential effects of hatching date, ectoparasitism, bacterial environment and nestling growth. Telomere length and attrition largely depended on population identity and hatching date. After correcting for these effects, the addition of feathers resulted in higher rates of telomere attrition. The addition of plants did not affect nestling telomeres in general, but did in interaction with location: in Hueneja, the experimental addition of green plants resulted in longer telomeres. Feather pigmentation also did not affect telomere length or attrition in general, but did in interaction with location: in Hueneja, the experimental addition of unpigmented feathers resulted in nestlings with longer telomeres and lower attrition rates. Moreover, prevalence of staphylococci on the skin of 8-day-old nestlings was negatively related to telomere lengths of fledglings. Taken together, these results suggest a direct link between nest material composition and nestling telomere length and dynamics. This relationship could be partially mediated by the antimicrobial and/or antiparasitic properties of nest materials or by sexual signalling processes. We discuss possible roles of maternal effects, parasites, immunity and nestling growth in explaining these experimental effects.

Keywords: antimicrobial properties, *Carnus* flies, feathers, green plants, hatching date, nest-building behaviour, nest-dwelling ectoparasites, sexual selection, telomere dynamics

Environmental conditions that offspring experience during growth have important consequences for their survival and reproductive prospects (Monaghan, 2008). These environmental conditions include indirect genetic effects of parents on offspring phenotypes through parental behaviours that in birds include, among others, nest building, nest defence against predators, nest sanitation, incubation, brooding and feeding effort (Mousseau & Fox, 1998). From an evolutionary perspective, research on the effects of nest-building behaviour on offspring survival prospects and recruitment is of particular interest because nests are extended phenotypes of builders (Dawkins, 1982) on which natural and sexual selection operate (Collias & Collias, 1984; Hansell, 2000; Moreno, 2012; Palomino, Martín-Vivaldi, Soler, & Soler, 1998; Soler, Møller, & Soler, 1998). On the one hand, nest-building behaviour may have indirect consequences for developing offspring because nests may signal the phenotypic quality of builders (Collias, 1964; Moreno, 2012). This would affect reproductive decisions of their mates through differential investment (Burley, 1986; Sheldon, 2000) in a typical postmating sexual selection process (Soler et al., 1998). On the other hand, nests may directly influence nestling phenotypes by their structures and materials having thermoregulatory, antipredator, antimicrobial or antiparasitic properties (Dubiec, Gózd, & Mazgajski, 2013; Heenan, 2013; Moreno, 2012).

The use of nest materials with antimicrobial and/or antiparasitic properties is considered a form of self-medication (Clayton & Wolfe, 1993; de Roode, Lefèvre, & Hunter, 2013). Most green plants used for nest building are aromatic plants that contain

volatile compounds or essential oils with repellent or toxic effects on blood-sucking arthropods and microorganisms and, therefore, could play a role in minimizing the effects of pathogenic bacteria and nest parasites on developing offspring (Clark, 1990; Clark & Mason, 1985; Tomás et al., 2012). Evidence of green plants reducing the risk of bacterial and parasitic infection of nestlings is compiled in Dubiec et al. (2013). More recently, the use of feathers as nest material has also been proposed to have antimicrobial effects (Soler, Martín-Vivaldi, Peralta-Sánchez, & Ruiz-Rodríguez, 2010). Evidence of this function has accumulated during the last few years. We know, for instance, that bacterial colonies from unpigmented feathers have higher antimicrobial activity than those from pigmented feathers in some nest environments (Peralta-Sánchez et al., 2014). This property would explain the reduced bacterial loads and hatching failures of barn swallow, *Hirundo rustica*, eggs in nests with experimentally supplied unpigmented feathers (Peralta-Sánchez, Møller, Martín-Platero, & Soler, 2010; 2011). More recently, experimental addition of feathers led to a reduction in eggshell bacterial loads in nests of spotless starlings, *Sturnus unicolor*, and in artificial nests without parental influence (Ruiz-Castellano, Tomás, Ruiz-Rodríguez, Martín-Gálvez, & Soler, 2016). Thus, effects related to the antimicrobial and antiparasitic properties of nest materials are key candidates to explain the expected associations between nest-building behaviour of parents and variables related to survival prospects of nestlings (Dubiec et al., 2013; Gwinner & Berger, 2005; Mennerat et al., 2009a; 2009b; Polo, Rubalcaba, & Veiga, 2015).

Nest building, however, is a costly activity (Mainwaring & Hartley, 2013) and, thus, nest-building effort should be adjusted to environmental characteristics. Selection pressure due to parasitism varies greatly both geographically (Ardia, 2007; Freeman-Gallant, O'Connor, & Breuer, 2001; Martin II, Pless, Svoboda, & Wikelski, 2004;

Møller, Garamszegi, Peralta-Sánchez, & Soler, 2011; Møller, Martín-Vivaldi, Merino, & Soler, 2006) and temporally (i.e. laying date; Merino, Møller, & de Lope, 2000; Sorci, Soler, & Møller, 1997) and, thus, antiparasitic and antimicrobial effects of experimental manipulations of nest material will also depend on geographical and temporal variation in selection pressures. In fact, the expected beneficial effects of nest materials in terms of probability of nestling recruitment (e.g. body mass, immunocompetence) have not been detected in several experimental studies (see review in Dubiec et al., 2013).

Recently, telomere length and dynamics have been proposed as measures that encapsulate the effects of stressful environmental conditions on nestlings' development and survival prospects (Monaghan, 2014; Monaghan & Haussmann, 2006). Telomeres are specialized structures at the end of the chromosome, consisting of short repeats of the noncoding DNA sequence TTAGGG, which protect the integrity of genetic information during cell division (Blackburn, 1991). Recent studies have reported negative associations between telomere length and baseline corticosterone levels (Quirici, Guerrero, Krause, Wingfield, & Vásquez, 2016), oxidative stress (Badás et al., 2015; Kim & Velando, 2015) and parasite infection (Asghar et al., 2015b). Moreover, early life telomere length has strong maternal effects (Asghar, Bensch, Tarka, Hansson, & Hasselquist, 2015a) and telomere dynamics depend on abiotic (e.g. altitude, laying date, Soler et al., 2015; Stier et al., 2016) and biotic environmental conditions (e.g. nestling competition for food, Nettle et al., 2015; Reichert et al., 2014; Soler et al., 2015), including those related to parental behaviour (Sudyka et al., 2014). Thus, telomere length and dynamics in nestlings are appropriate target variables for testing the effects of nest-building behaviour (e.g. nest material used) on nestling development.

Here, we experimentally explored the effects of green plants and/or feathers on telomere length and dynamics in spotless starling nestlings, while considering temporal and geographical variation. We expected to detect the beneficial effects of these nest materials in terms of telomere length and reduced telomere attrition in nestlings from nests with experimentally added plants and/or feathers, especially in the area with higher ectoparasitism. We also quantified ectoparasitism and bacterial loads on the skin of nestlings, and explored the expected negative relationship with telomere length and positive relationship with telomere shortening in nestlings close to fledging.

<H1>Methods

<H2>Study area and species

The study was performed in Hoya de Guadix, southeast of Spain, a high-altitude plateau 1000 m above sea level with a semiarid climate, during the 2012 breeding season. The spotless starling populations under study breed in cork nestboxes (internal dimensions: 180 x 210 mm and 350 mm high, 240 mm from the bottom to the hole) attached to tree trunks or walls 3–4 m above ground. The two populations breed in the old railway stations of La Calahorra (37°15'N, 3°01'W) and Hueneja (37°13'N, 2°56'W), 8 km apart. Ecological conditions of the study areas are similar except for colony size and ectoparasitism level (see Results). Approximately 80 pairs breed per year at La Calahorra and 35 pairs in Hueneja. Ectoparasitism by the fly *Carnus hemapterus* and occupation of nestboxes was higher in La Calahorra than in Hueneja, although empty nestboxes were available in both populations during the study.

The hole-nesting spotless starling mostly breeds in colonies and uses a variety of nesting materials, including feathers and green plants, for both the structural and the lining layer of the nest (Peralta-Sánchez et al., 2012; Ruiz-Castellano et al., 2016;

Veiga, 2002). In the studied populations, starlings usually lay four or five eggs per clutch in mid-April. Full incubation starts with the penultimate egg resulting in asynchronous hatching, which usually takes place from early May onwards (Soler, Navarro, Pérez-Contreras, Avilés, & Cuervo, 2008). At the beginning of April, before breeding started, nestboxes were checked every second or third day until eggs were detected. Only first breeding attempts were considered in this study. Hatching date (age 0), defined as the day when half or more of the brood was hatched (Tomás, 2015), was established by visiting nestboxes daily close to the expected dates (incubation lasts for 7–12 days after clutch completion). The nestling period ranges from 18 to 25 days (Veiga, 2002).

<H2>Experimental design

Our experiment followed a full-factorial design with feather or plant treatments (see below) starting on day 3 of nestling age. We first recorded the number of feathers and whether plants were present in nests and, subsequently, all plants and feathers were removed. Each nest was randomly assigned to one treatment. Since feathers of different colours may also differ in antimicrobial properties (Peralta-Sánchez et al., 2010), the feather treatment consisted of adding (1) 15 pigmented or (2) 15 unpigmented feathers to the nest or (3) leaving the nest without feathers. This number of feathers is within the range and close to the modal interval of number of feathers found in starling nests in the study area (from 55 nests, 17%, 33% and 17% of nests had 0–10, 10–20 and 20–30 feathers, respectively). Feathers were marked on the quill with a permanent marker to distinguish them from feathers introduced by the parents. The plant treatments consisted of (1) adding 1.6 g of a mixture of aromatic plants (the maximum quantity that starlings

add to the nests during the nestling stage in our population) or (2) leaving the nest without plants (see details below).

At 3 days old, all nestlings in the nest were individually marked by cutting some of their down feathers from the head, back or wings, weighed to the nearest 0.1 g and their tarsus length measured to the nearest 0.01 mm. Moreover, the belly of one randomly selected chick was sampled to characterize the bacterial environment of the nest (see below) before the experimental treatment. In addition, from each hatchling we collected a drop of blood by brachial venipuncture with the aid of a needle for estimating telomere length. Because of the difficulties and risks associated with blood sampling recently hatched nestlings, we just punctured their brachial vein and collected a small drop of blood on blotting paper, which was kept dry at 4 °C until DNA isolation in the following months (see below).

Two days after the first visit (day 5 of nestling age), we removed all green plants and any feathers added by birds, and refreshed green plants in the plant treatment nests. At 8 days old, all nestlings were ringed and we sampled one chick belly per nest (not sampled during the first visit) to estimate bacterial load. Moreover, we quantified parasitism by *C. hemapterus* flies, a 2 mm blood-sucking fly found in nests of an extremely wide diversity of birds (Brake, 2011; Grimaldi, 1997). It feeds exclusively on birds while in the nests, mainly on nestlings (Vaclav et al., 2016), but also on incubating birds (Avilés, Pérez-Contreras, Navarro, & Soler, 2009; Lopez-Rull, Gil, & Gil, 2007). Briefly, on day 8, nestlings were carefully taken from the nest and put inside a white cloth bag thus minimizing the possibility that flies would jump into the nest material. We counted parasites on the body surface of each chick as well as the remaining flies in the bag to estimate parasite load for every brood (Avilés et al., 2009).

On day 14, we collected a second blood sample for estimating telomere length in 75 µl heparinized capillary tubes after puncturing the brachial vein. Blood was later stored in an Eppendorf tube with absolute ethanol and maintained at 4 °C until DNA isolation 3 months later (i.e. September 2012). During this visit, we also recorded body mass, tarsus and wing length of all nestlings.

Preparation of experimental nest lining feathers and aromatic plants has been explained previously (Ruiz-Castellano et al., 2016). Briefly, experimental unpigmented and pigmented body feathers were collected from chickens on small farms close to the study area. In the laboratory, all experimental feathers were sterilized using a UV sterilizer chamber (Burdinola BV-100), and sprayed with approximately 84 ml of an overnight culture of *Bacillus licheniformis* D13 per m². This is one of the commonest feather-degrading bacteria (Burt & Ichida, 1999) that also produces antimicrobials (Callow & Work, 1952; Gálvez et al., 1994; Lebbadi, Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1994) and, thus, we ensured our experimental feathers harboured similar amounts of antimicrobial-producing bacteria. We added to each nest 1.6 g of a mixture of fragments of the four plant species most used by starlings in the studied population (*Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*). These species produce volatile compounds or essential oils with known antimicrobial activity (Ruiz-Castellano et al., 2016). We used sterile gloves to collect and manipulate feathers and plants. For further information on experimental protocols see Ruiz-Castellano et al. (2016).

Bacterial sampling and laboratory work

For each nest visit and sampling we wore new gloves sterilized with 96% ethanol to prevent contamination of bacterial samples among nests. For bacterial

sampling of nestlings, we cleaned the complete belly surface of nestlings with a sterile rayon swab (EUROTUBO DeltaLab, Barcelona, Spain) slightly wetted with sterile sodium phosphate buffer (0.2 M; pH = 7.2). The swab was kept in an Eppendorf tube with the buffer solution and preserved at 4 °C in a portable refrigerator until processed in the laboratory within 24 h after collection. Once in the laboratory, 100 µl of solution containing bacteria were cultivated in four different solid media (Scharlau Chemie S.A. Barcelona, Spain): Tryptic Soy Agar (for aerobic mesophilic bacteria), Hektoen Enteric Agar (for Enterobacteriaceae), Vogel-Johnson Agar (for *Staphylococcus*) and Kenner Fecal Agar (for *Enterococcus*). Plates were incubated at 37 °C for 72 h, when the colonies on each plate were counted. For more details, see Peralta-Sánchez et al. (2010).

Belly bacterial density was estimated by standardization of the number of colonies per cm² of sampled surface (CFU, colony forming units). Belly surface was estimated from measurements of length and breadth of the nestling's belly, obtained with a digital calliper to the nearest 0.01 mm, assuming that the belly is half of an ovoid (Narushin 2005). We measured the surface area of 12 3-day-old and seven 8-day-old nestlings. Bacterial counts of these samples depend on the sampled surface area and, thus, on nestling age. However, for nestlings of the same age, the relationships between bacterial counts and belly area sampled at day 3 (mesophilic bacteria: $F_{1,10} = 1.38$, $P = 0.266$; *Enterococcus*: $F_{1,10} = 1.56$, $P = 0.240$) or at day 8 (mesophilic bacteria: $F_{1,3} = 2.74$, $P = 0.454$; *Enterococcus*: $F_{1,3} = 2.74$, $P = 0.666$) were far from statistical significance. Thus, we standardized bacterial density to average belly area for different nestling ages (3-day-old nestlings: 7.24 cm²; 8-day-old nestlings: 9.36 cm²).

Characterization of bacterial environments by traditional culture techniques produces a relatively narrow picture of bacterial communities (Lee et al., 2013), but it has been shown to be an appropriate method for exploring effects of skin bacterial

density on nestlings (González-Braojos et al., 2012a; 2012b, 2012c; Gwinner & Berger, 2005; 2009a) and, thus, for our purposes.

<H2>Telomere length estimations

We extracted DNA from blood samples using a standard chloroform-isoamyl alcohol-based protocol (see Ferraguti, Martinez-de la Puente, Ruiz, Soriguer, & Figuerola, 2013; Soler et al., 2015). Using distilled water, we adjusted DNA concentration to 20 ng/μl and samples were conserved frozen (-20 °C) until further analyses. Following Criscuolo et al. (2009), telomere length was estimated by q-PCR as the quantity of telomere sequences in the q-PCR reaction of the sample relative to that of a single copy of the gene that encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The final PCR volume was 20 μl containing 10 μl of LightCycler 480 SYBR Green I Master (Roche) and 1 μl of DNA at 20 ng/μl. Because of the different PCR conditions, the reactions for telomeres and GAPDH were done in different plates in a LightCycler 480 RT-PCR System (Roche). Telomere PCR conditions were 10 min at 95 °C followed by 30 cycles of 1 min at 56 °C and 1 min at 95 °C. GAPDH PCR started with 10 min at 95 °C followed by 40 cycles of 1 min at 60 °C and 1 min at 95 °C. We ran each sample in duplicate and those with a coefficient of variation higher than 5% were removed from the analyses (3.05% of the 262 samples). To generate the standard curves, and a blank control with no DNA, we ran in triplicate each 96-well plate that included serial dilutions of DNA (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) from a reference pool (the internal control). Quantification cycle values (Ct) were transformed into normalized relative quantities (NRQs) following Hellemans et al. (2007). This procedure controls for the amplifying efficiency of each qPCR which ranged between 1.858 and 2.143 for telomere products and between 1.893

and 2.007 for GAPDH products. The slope of the calibration curve ranged between -3.718 and -3.021 for telomere products and between -3.608 and -3.163 for GAPDH products. The melting curves of the control gene cycles confirmed no evidence of primer dimer or nonspecific amplification. Although a variety of techniques for measuring telomere length in wildlife are available (reviewed in Nussey et al., 2014), the method employed here is adequate for comparing patterns of variation within species based on repeated measures of the same individuals across time, as we have done here (for a similar approach see Asghar et al., 2015b).

Values of telomere length estimated by these techniques largely depend on the method of blood conservation and DNA isolation (Tolios, Teupser, & Holdt, 2015). Mainly because of difficulties and risks associated with bleeding recently hatched nestlings, and the amount of blood needed to collect samples within capillaries, methods for conservation of samples from recently hatched nestlings and from nestlings close to fledging differed. Thus, although estimates of telomere length of hatchlings were much lower (NRQ = 0.776, SE = 0.020, $N = 131$, only nestlings with information from both nestling stages) than those of fledglings (NRQ = 1.00, SE = 0.039, $N = 131$), differences can be due to different methodologies of blood conservation. We statistically accounted for differences due to different protocols by using ranked values (i.e. rank 1 was assigned to the smallest value, while ranks for ties were the mean) of NRQs for hatchlings and for fledglings (see below).

<H2>Ethical Note

We performed the study following relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. The ethics committee of the Spanish National Research Council (CSIC) approved the protocol, and the Consejería de Medio Ambiente de la

Junta de Andalucía, Spain, provided all necessary permits for nest and nestling manipulations (Ref: SGYB/FOA/AFR/CFS). The spotless starling is not an endangered or protected species. The time spent at each nest was the minimum necessary for bacterial and blood sampling, measuring of nestlings and treatment application. The protocols adhered to the ASAB/ABS Guidelines for the Use of Animals in Research. This experiment did not show detectable effects on adult nest attendance behaviour or nestling condition and survival.

<H2>Statistical analyses

Tarsus length, body mass, hatching date and log₁₀-transformed numbers of feathers in the nests prior to the experimental addition approached normal distributions (Kolmogorov–Smirnov tests for continuous variables: $P > 0.2$). Abundance of *Carnus* flies and bacterial loads estimated for mesophilic bacteria and for *Enterococcus* were log₁₀-transformed before the analyses to approach normal distributions. Estimates for enterobacteria and *Staphylococcus* included many zero values and thus were analysed as binomial distributed variables (i.e. presence/absence). The presence of green material in the nests was included in the models as a binomial independent factor. We managed to collect information from 52 nests, 137 hatchlings and 135 fledglings. Table 1 gives the number of nests in the different experimental treatments from the two populations. For 131 nestlings from 52 nests, we obtained information from both stages, as hatchlings and as fledglings. Differences in ranked values between fledglings and hatchlings were used as an estimation of changes in telomere length. These values did not differ from normal distributions (Kolmogorov–Smirnov tests for continuous variables: $P > 0.2$). Statistically significant associations between independent factors and telomere length of hatchlings or fledglings were detected independently of whether ranked or raw values of

telomere length were analysed (results not shown). However, because telomere dynamics during the nestling period were estimated as the difference in ranked values for each nestling, for consistency we only show results using ranked values.

To reduce the number of independent factors included in models exploring relationships with telomere lengths or dynamics, we separately analysed the effect of variables describing nest materials and nestling measurements on the one hand and bacterial loads on the other. One of the models explaining telomere length of hatchlings therefore included information on hatching date, tarsus length, body mass and number of feathers as continuous predictors, and study area and presence of plants in the nest as categorical predictors. The other model included information on hatching date and bacterial loads as continuous predictors and study area as a categorical predictor. Nestlings sharing the same nest have identical values for some of these variables (e.g. nest materials, hatching dates) and, to account for nonindependence of data, we adjusted degrees of freedom to the number of sampled nests.

Experimental effects on body mass, tarsus length, *Carnus* flies, bacterial loads, and telomere length and dynamics in fledglings were analysed by means of general linear mixed models (GLMM) with study area, plant and feather treatments and their interactions as fixed categorical factors. Because nestlings within the same nest were in the same experimental treatments, nest identity nested within the major order interaction among fixed factors was included in the models as the random factor. The experimental effects on prevalence of Enterobacteriaceae and of *Staphylococcus* were analysed in generalized linear models with binomial distribution and logit link function while correcting for overdispersion. Feather and plant treatments, study area and their interactions were included as fixed effects in the models. To reduce the number of factors in these models, we used residual values of telomere length and dynamics after

controlling for hatching date and the square of hatching date (see Results) as dependent variables.

Our feather experiment included three treatments that represent two different factors, one of them nested within one of the other two treatments. The first factor deals with the effect of feathers (i.e. removed feathers versus feathers added), while the second factor deals with the effect of pigmentation (i.e. pigmented versus unpigmented feathers added). Because the effect of pigmentation can only be tested by comparing nests with pigmented and unpigmented feathers, only nests with added feathers can be used to answer this question. Thus, we analysed the experimental effects of feathers and those of feather pigmentation in two different sets of models. First, we explored the effects of feathers as a whole (i.e. factor with two levels: with versus without feathers) and thus considered together nests in the pigmented and unpigmented feather treatments. Second, we explored the effects of feather pigmentation (two-level factor: pigmented versus unpigmented feathers) and thus considered only nests with experimentally added feathers. Analyses considering these three treatments in the same model, while exploring predictions by post hoc tests, result in identical conclusions. The rationale for exploring the effects of feather pigmentation is based on previous results suggesting differential effects of pigmented and unpigmented feathers in nests of barn swallows (Peralta-Sánchez et al., 2010) and spotless starlings (Ruiz-Castellano et al., 2016).

Associations between bacterial or *Carnus* loads and telomere length and dynamics of nestlings at the age of fledging were also explored in separate models. We used residuals of telomere length and attrition on hatching date as dependent variables and bacterial loads of nestlings 3 and 8 days after hatching as continuous predictors, and

study area as categorical predictor. Again, the degrees of freedom were adjusted to the number of sampled nests.

For final model selection, fixed factors with the largest P values were removed one by one up to $P = 0.1$, starting from the two-level interactions. Full and reduced models are shown except for those showing bacterial influence for which only reduced models are discussed. Statistically nonsignificant main effects were retained in the models when the interaction with other factors reached statistical significance. All statistical tests were performed with the software Statistica (Statsoft-Inc., Tulsa, OK, U.S.A.).

<H1>Results

<H2>Nest materials, biometry, bacterial loads and ectoparasitism

Neither body mass (linear terms: $\text{Beta}(\text{SE}) = -0.860(1.225)$, $F_{1,51} = 0.492$, $P = 0.486$; quadratic term: $\text{Beta}(\text{SE}) = 1.029(0.225)$, $F_{1,51} = 0.705$, $P = 0.405$) nor tarsus length (linear terms: $\text{Beta}(\text{SE}) = 0.086(1.241)$, $F_{1,51} = 0.005$, $P = 0.944$; quadratic term: $\text{Beta}(\text{SE}) = 0.005(1.241)$, $F_{1,51} = 0.001$, $P = 0.996$) of 14-day-old nestlings depended on hatching date. Nestlings in La Calahorra had larger tarsi, and, within each study area, nestlings in nests that received experimental plants had shorter tarsi (Table 2, Fig. 1a). Those from nests that received pigmented or unpigmented feathers had similar tarsus length (GLMM: $F > 0.97$, $P > 0.33$).

Experimental treatments, study area and their interactions failed to explain a significant proportion of variance in nestling body mass (GLMM: $F = 2.43$, $P > 0.126$). However, nestlings from nests with experimental unpigmented feathers were heavier than those from nests with experimental pigmented feathers, but only when plants were also added; the opposite effect occurred in nests when plants were removed (Fig. 1b;

GLM: reduced model included feather ($F_{1,39.7} = 0.06$, $P = 0.80$) and plant treatments ($F_{1,39.7} = 0.04$, $P = 0.84$), their interaction ($F_{1,39.7} = 6.71$, $P = 0.01$) and the random effect of nest identity ($F_{32,59} = 1.23$, $P = 0.25$)).

Hatching date was not associated with bacterial loads of 8-day-old nestlings (MANOVA, linear term: Wilks's $\lambda = 0.93$, $F_{4,46} = 0.94$, $P = 0.45$; quadratic term: Wilks's $\lambda = 0.923$, $F_{4,46} = 0.961$, $P = 0.44$). Experimental treatments, study area and their interactions failed to explain mesophilic and enterococci bacterial loads (GLM: $F_{1,49} < 2.49$, $P > 0.12$), and prevalence of enterobacteria and staphylococci (GLZ: $\chi^2 < 2.75$, $P > 0.097$). However, in nests with experimental plants added (i.e. interaction between feather pigmentation and plant treatment), mesophilic bacterial density (GLM: $F_{1,31} = 7.37$, $P = 0.011$) and enterobacteria prevalence (GLZ: $\chi^2 = 4.76$, $P = 0.029$) were lower in nests with unpigmented feathers than in those with pigmented feathers (Fig. 1c, d). The opposite or no trend was detected for nestlings in nests with plants removed (Fig. 1c, d).

Abundance of *Carnus* flies on 8-day-old nestlings tended to decrease as the season progressed (linear term: Beta(SE) = -3.63 (2.00), $F_{1,48} = 3.31$, $P = 0.08$; quadratic term: Beta(SE) = 3.22 (2.00), $F_{1,48} = 2.59$, $P = 0.11$). Abundance of *Carnus* in nests was not significantly affected by experimental treatments or interactions among them (GLM: $F_{1,49} < 2.30$, $P > 0.114$), but was larger in La Calahorra (log-transformed average parasite abundances (SE) = 2.53(0.18)) than in Hueneja (1.45(0.36); GLM: $F_{1,49} = 8.13$, $P = 0.006$). Pigmentation of experimental feathers also failed to explain a significant proportion of variance in parasite loads (GLM: $F_{1,33} < 0.25$, $P > 0.62$).

<H2>Telomere length and dynamics

<H3>Telomere length of hatchlings

Prior to the experimental manipulation, telomere length of hatchlings was not related to biometrical variables (tarsus length and body mass) and did not differ between study areas, but was positively associated with the number of feathers in the nest and with hatching date (Table 3). Nestlings that hatched at intermediate to late dates in nests harbouring a higher number of feathers prior to the experimental removal had longer telomeres (Fig. 2). In addition, those harbouring a higher density of *Enterococcus* had longer telomeres (Beta (SE) = 0.17 (0.08), $F_{1,49} = 4.13$, $P = 0.048$) even after controlling for the significant linear and negative quadratic effects of hatching date and total nest lining feathers in their nests ($F_{1,45} > 5.23$, $P < 0.024$; sign of the estimates are identical to those shown in Table 3).

<H3>Telomere length and nest material manipulation

Telomere length of fledglings decreased as the season progressed (Beta(SE) = -3.01(1.26), $F_{1,132} = 5.70$, $P = 0.02$), showing minimum values for fledglings hatched at intermediate dates (i.e. quadratic term: Beta(SE) = 2.94(1.26), $F_{1,132} = 5.44$, $P = 0.02$; Fig. 3a). After controlling for this effect, telomere length varied with study area, and the experimental addition of plants did not explain a significant proportion of additional variance (Table 2, Fig. 4). However, in Hueneja, the experimental group with added plants had shorter telomeres than those with plants removed, while the opposite effect was detected in La Calahorra where nestlings had the shortest telomeres at this age (Table 2, Fig. 4a). The inclusion of biometrical information (body mass and tarsus length) in the model did not affect the detected experimental effects of plant treatments on telomere length (interaction between areas and plant experimental treatments: $F_{1,52.9} = 9.09$, $P = 0.004$), while none of the biometrical variables reached statistical significance (all $F_{1,79} < 0.44$, $P > 0.50$).

The addition of feathers did not affect final telomere length (Table 2). Feather pigmentation also did not affect telomere length of nestlings (final GLM model: $F_{1,27.8} = 2.21$, $P = 0.149$), but in the Hueneja population, fledglings in nests with unpigmented feathers tended to have longer telomeres than fledglings in nests with pigmented feathers (final GLM model, interaction between study area and feather treatment: $F_{1,22.3} = 3.61$, $P = 0.068$; Fig 4b). For this subset of nests (i.e. with feathers added), study area ($F_{1,22.3} = 6.36$, $P = 0.019$) and the interaction with plant treatment ($F_{1,21.3} = 8.59$, $P = 0.008$) also reached statistical significance.

<H3>Telomere dynamics and nest material manipulation

Telomere attrition experienced by nestlings during development increased with hatching date (Beta(SE) = 3.79(1.26), $F_{1,128} = 9.09$, $P = 0.003$), with the highest values at intermediate dates (quadratic term: Beta(SE) = -3.68(1.26), $F_{1,128} = 8.57$, $P = 0.004$; Fig. 3b). After controlling for these effects, the experimental addition of feathers, but not that of plants, resulted in a higher rate of telomere attrition (Table 2, Fig. 4c). We also detected a significant effect of location, with nestlings in La Calahorra experiencing more telomere attrition. The inclusion of biometrical information (growth in body mass and tarsus length) in the model did not modify the detected effects of study area ($F_{1,45.4} = 7.68$, $P = 0.008$) or feather treatment ($F_{1,48.6} = 13.60$, $P = 0.0006$). None of the biometrical variables or the experimental addition of plants reached statistical significance ($F_{1,76} < 0.42$, $P > 0.51$). However, telomeres of nestlings that developed in nests with unpigmented feathers shortened at a lower rate than those of nestlings in nests with pigmented feathers (final GLM model: $F_{1,29.6} = 9.08$, $P = 0.005$). This effect was more pronounced in the Hueneja population (final GLM model, interaction between feather treatment and population: $F_{1,28.5} = 5.442$, $P = 0.027$; Fig. 4d).

<H3>Telomeres and nestling biometry, parasitism and bacterial loads

<H4>Telomere length. Body mass ($F_{1,50} = 0.61$, $P = 0.434$) and tarsus length ($F_{1,50} = 0.01$, $P = 0.959$) failed to explain telomere length of 14-day-old nestlings (i.e. residual values after controlling for hatching date, see above) after controlling for the effect of study area ($F_{1,50} = 9.99$, $P = 0.003$) and the random effect of nest identity nested within study area ($F_{50,81} = 1.43$, $P = 0.075$). The abundance of *Carnus* also failed to predict telomere length of nestlings ($F_{1,36} = 0.05$, $P = 0.826$) after controlling for the effect of study area ($F_{1,36} = 1.91$, $P = 0.175$). Staphylococci prevalence on 8-day-old nestlings was negatively associated with telomere length (Beta(SE) = -0.25(0.09), $F_{1,48} = 7.48$, $P = 0.009$; Fig.5) after controlling for study area ($F_{1,48} = 18.91$, $P < 0.0001$) and prevalence of enterobacteria in nestlings of 3 (Beta(SE) = -0.15(0.09), $F_{1,48} = 3.01$, $P = 0.089$) and 8 days old (Beta(SE) = 0.16(0.09), $F_{1,48} = 3.44$, $P = 0.070$).

<H4>Telomere dynamics. Body mass and tarsus length did not explain telomere dynamics experienced by nestlings (body mass: $F_{1,50} = 0.12$, $P = 0.731$; tarsus length: $F_{1,50} = 0.29$, $P = 0.591$), after controlling for the effect of study area ($F_{1,50} = 6.25$, $P = 0.016$) and the random effect of nest identity nested within study area ($F_{50,77} = 2.00$, $P = 0.003$). Abundance of *Carnus* also failed to predict telomere attrition of nestlings after controlling for the effect of study area ($F_{1,36} = 0.38$, $P = 0.541$). Finally, nestlings with a lower density of mesophilic bacteria (Beta(SE) = -0.36(0.12), $F_{1,48} = 9.06$, $P = 0.004$) and higher density of *Enterococcus* (Beta(SE) = 0.32(0.12), $F_{1,48} = 7.49$, $P = 0.009$) before the experimental treatment experienced the largest reduction in ranked values of telomere length from hatching to fledging. That was the case after controlling for the effects of study area ($F_{1,48} = 15.90$, $P = 0.0002$) and the nonsignificant positive effects

of prevalence of enterobacteria (Beta(SE) = 0.17(0.09), $F_{1,48} = 3.89$, $P = 0.054$) and of *Staphylococcus* (Beta(SE) = 0.16(0.09), $F_{1,48} = 3.40$, $P = 0.07$). Reduced models did not retain any of the variables reflecting bacterial loads of nestlings in the second sampling.

<H1>Discussion

We obtained experimental evidence supporting the hypothesis that nest materials affect growth and telomere length and dynamics of spotless starling nestlings. These results point to direct effects of nest materials on survival prospects of nestlings, mainly due to the known association of telomere length with survival (Asghar et al., 2015b). However, interestingly, the strength of these relationships, and even the sign of some of these effects, depended on study area. Nestlings with the shortest final telomere length and the most telomere attrition were from the area with the highest ectoparasite abundance and nest density, which suggests a link between parasitism and nestling telomeres. We also detected curvilinear associations between hatching date and telomere length and dynamics, which may reflect the effects of territory and/or parental quality on telomere characteristics of nestlings. Finally, we also found a negative relationship between the presence of staphylococci on the skin of 8-day-old nestlings and telomere length of fledglings, which suggests a role for bacteria in determining telomere dynamics during the nestling phase. Below, we discuss possible mechanisms that could explain these relationships and, therefore, the hypothesis that nest-building behaviour affects cellular senescence and offspring development.

Evidence supporting the assumed close relationship between telomere length and/or dynamics and survival prospects of nestlings has been accumulating during recent years. Laying or hatching dates are usually considered as variables reflecting territory or parental quality (Brinkhof, Cavé, Hage, & Verhulst, 1993; De Neve, Soler,

Soler, & Pérez-Contreras, 2004; Moreno, 1998; Tomás et al., 2012; Verhulst & Nilsson, 2008) and, thereby, availability of resources for developing offspring. Thus, laying or hatching dates should be related to telomere dynamics of nestlings (Foote, Gault, Nasir, & Monaghan, 2011; Soler et al., 2015). We detected these linear and quadratic patterns of association between telomere dynamics of nestlings and hatching date. Telomere length of hatchlings and telomere attrition experienced during development increased as the season progressed with maximum values at intermediate dates. However, telomere length of fledglings decreased as the season progressed, reaching minimum values at intermediate dates. These results suggest an adjustment between telomere length at hatching and telomere attrition during development. This may be, for instance, explained by differential maternal investment or genetic quality of offspring of intermediate hatching dates. Mechanisms underlying this relationship should be further explored. For the hypothesis tested, it highlights the importance of considering hatching dates when exploring associations between telomere dynamics and life history traits. Accordingly, we used residuals of telomere length and attrition after correcting for phenology.

We also found a strong influence of study area on telomere length and dynamics. Nestlings from the area with larger colony size and ectoparasite abundance (La Calahorra) had shorter initial telomeres and experienced more attrition than those from the area of smaller colony size and lower *Carnus* abundance (Hueneja). These results may suggest a link between risk of parasitism and telomere dynamics. However, we failed to identify any significant direct association between *Carnus* load and telomere dynamics. We know very little about geographical variation in telomere length and dynamics, but geographical variations in factors affecting growth or oxidative stress of nestlings, including any parasites or pathogens, or contaminants or pollution, not

considered here, may be the cause of the detected differences (Asghar et al., 2015b; Ilmonen, Kotrschal, & Penn, 2008; Salmón, Nilsson, Nord, Bensch, & Isaksson, 2016). Further research is in any case necessary to elucidate factors explaining the differences between study areas.

Several mechanisms including direct effects of nest material, or indirect genetic effects, related to sexual selection processes or maternal effects, could explain the associations between nest materials and telomere length and dynamics. The positive association between number of feathers in the nest at the time of hatching and telomere length of hatchlings could be explained by the thermoregulatory or antimicrobial properties of feathers during the incubation period. However, indirect effects due to sexual selection seem a more plausible explanation. In spotless starlings, feathers are carried to the nests mainly by females as a postmating sexually selected signal of quality (Polo & Veiga, 2006; Veiga & Polo, 2005) in response to the green plants carried by males, which potentially are another sexually selected trait (Tomás et al., 2013; Veiga, Polo, & Viñuela, 2006). Telomere length is a heritable character, but only on the maternal side (Asghar et al., 2015a; Reichert et al., 2015). Thus, hatchlings with longer telomeres may be those from high-quality mothers that carried a lot of feathers to the nest. The finding that presence of green plants in the nest, which are mainly carried by males as stated above, did not predict telomere length of hatchlings is in agreement with the hypothetical stronger influence of females determining telomere length of hatchlings. Further studies are, however, necessary to explore this possibility.

Indirect genetic associations seem less likely to account for the experimental effects of nest materials on telomere length and dynamics of fledglings. The results could only be explained by direct effects of nest materials on nestling growth and/or by differential parental effort in response to the experimental manipulations. Interestingly,

we know that both green plants (Clark & Mason, 1985; Dubiec et al., 2013) and feathers (Mennerat et al., 2009a; Peralta-Sánchez et al., 2010; Ruiz-Castellano et al., 2016) employed as nest materials influence the probability of ectoparasitic and bacterial infections, respectively, with potential benefits in nestling development (Mennerat et al., 2009b; Sanz & García-Navas, 2011). These effects, however, may vary between populations and/or seasons (Mennerat et al., 2009b), and are expected to be larger under higher selection pressures. Thus, the predicted positive effects of green plants should be mainly detected in nestlings from the population that experienced the highest *C. hemapterus* parasite loads (see above), which is partially in accordance with our results. We failed, however, to detect a direct effect of experimental green plants on *Carnus* load, although other blood-sucking insects (e.g. biting midges, blackflies) not considered in this study could play a role in this respect. In fact, the effect of plant-derived repellents on blood-sucking insects may differ between groups, with no significant effects on more permanent insects in avian nests as in the case of *Protocalliphora* larvae (Martínez-de La Puente et al., 2009) or *Carnus* in this study (but see Tomás et al., 2012).

The negative effect of experimental green plants on telomere length of nestlings in the Hueneja population is more difficult to explain. Interestingly, telomere length of nestlings from nests with added plants did not differ between the populations. We could speculate that, because green plants apparently boost nestling growth (Mennerat et al., 2009b), the reduced telomere length of nestlings in nests with added green plants at Hueneja was just the result of the higher rates of cell divisions experienced by these nestlings. Adult birds may transport green plants to the nests mainly when risk of parasitism at the nest is high, and offspring may adjust growth rate to risk of parasitic infections or related cues (Saino, Calza, & Møller, 1998). In agreement with a possible

adjustment of growth to risk of parasitic infection, nestlings from the area with the highest parasite load and shorter telomeres had longer tarsi than those from the area with longer telomeres. Increasing growth as a response to parasitism is, however, costly in terms of poor development of the immune system (Saino et al., 1998; Soler, De Neve, Pérez-Contreras, Soler, & Sorci, 2003). Immunity is a good predictor of nestling recruitment (Cichon & Dubiec, 2005; Moreno et al., 2005). Consequently, if nestlings (or parents) use an abundance of green material as a cue to adjust differential investment in development at the cost of immunity, the experimental addition of green plants would result in reduced survival prospects. This would be the case especially in areas experiencing lower levels of selection pressure by parasites. Recently published experimental results by Polo et al. (2015) support this prediction in our species model. Although, in our opinion, this is the most likely explanation, further research is necessary to determine the factors explaining population differences in growth trajectories of nestlings that depend on green nest materials.

The presence and pigmentation of the experimental feathers did not influence the telomere length of nestlings in general, but did so in interaction with study area. In contrast to pigmented feathers, the experimental addition of unpigmented feathers resulted in nestlings with relatively longer telomeres, but only in the Hueneja population. Unpigmented feathers collected from barn swallow nests have superior antimicrobial properties to pigmented feathers (Peralta-Sánchez et al., 2014) and are preferred as nest materials by swallows (Peralta-Sánchez et al., 2010) and starlings (Ruiz-Castellano, Tomás, Ruiz-Rodríguez, & Soler, 2017a). Thus, the detected effects may be the consequence of antimicrobial properties of unpigmented feathers. In fact, for nestlings in experimental nests with green plants added, those in nests with unpigmented feathers tended to be heavier and experienced lower bacterial loads than

nestlings in nests with pigmented feathers. Thus, it is possible that the differential antimicrobial properties of unpigmented feathers explain the effects on telomere length. However, if that was the case, the effects would be more evident in the area with the highest risk of infection, a prediction that our results do not support since the experimental effect was detected in the population possibly experiencing the lowest risk of infection (i.e. smaller colony size). Another possible explanation is that unpigmented feathers were more attractive for males which may have invested in feeding the offspring differentially in nests with the unpigmented, most conspicuous, experimental feathers (Veiga & Polo, 2005), which may have direct effects on telomere dynamics in nestlings (Badás et al., 2015; Kim & Velando, 2015). Further investigation of the mechanisms explaining these links is necessary.

When considering telomere attrition, we detected significant effects of feather treatment but not of green plant treatment. Contrary to our prediction, we found that telomere attrition was higher in nestlings from nests with added feathers. However, this was mainly due to the effects of pigmented feathers because telomere attrition of nestlings under this treatment was significantly higher than that of nestlings in nests with unpigmented feathers. We did not find significant associations between telomere attrition and body mass or tarsus length of nestlings, but bacterial load in nestlings was higher in nests with the highest telomere attrition (i.e. with experimental pigmented feathers). In addition, recent research has detected a positive effect of feather material on nestling immune response (Ruiz-Castellano, Tomás, Ruiz-Rodríguez, & Soler, 2017b). Here we also found a negative association between prevalence of staphylococci on nestling skin and telomere length, which suggests a role of bacteria in driving telomere dynamics, but also that nestlings with weaker immune defence are those with shorter telomeres as suggested above. Thus, it is possible that the differential level of

telomere attrition experienced by nestlings in nests with added feathers was due to differential investment in immunity, mainly in nests with pigmented feathers. Experimental manipulation of the developing immune system is necessary, however, to examine this idea.

In conclusion, whatever the relative importance of bacteria, parasites, immune system and resource availability in telomere length and dynamics, our results show a direct link between nest materials and telomere dynamics in developing offspring. The strength and even the sign of the experimental effects differed for the two populations which varied in ectoparasitism loads and telomere length and dynamics of nestlings, suggesting that the effects of nest material on predictors of survival prospects of nestlings will depend on ecological characteristics of the birds' populations. We hope these results encourage further research to clarify the mechanisms and ecological conditions underlying these associations.

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Statement of authorship

J.J.S., G.T., M.M.V. and J.F. designed the study with considerable assistance from J.M.P. and C.R.C. C.R.C. performed all bacterial and molecular analyses with considerable assistance from J.M.P. G.T. and C.R.C. performed most of the field work with assistance from J.J.S., M.M.V. and M.R.R. J.J.S. performed all the statistical analyses and wrote the manuscript with substantial contribution from all authors.

Data Accessibility

Data used in this paper can be found in the CSIC Institutional Repository, with the accession numbers (<http://hdl.handle.net/10261/143025>).

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949 Table 1: Number of nests under different experimental treatments for the Calahorra/Hueneja
 950 populations
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Plant treatments	Feather treatments			Total
	Unpigmented	Pigmented	No feathers	
Plants	8/3	6/2	6/1	20/6
No plants	8/1	7/1	6/3	21/5
Total	16/4	13/3	12/4	41/11

Table 2: Experimental effects of feathers and plants as nest materials on tarsus length and telomere length and dynamics of starling nestlings close to fledging

Effect	<i>df</i>	Tarsus length			Telomere length			Telomere dynamics		
		<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	
Study area (1)	Fixed	1,44.8	6.71	0.013	1, 43.9	12.96	0.001	1,43.0	8.87	0.005
Plant treatment (2)	Fixed	1,45.6	4.87	0.032	1, 45.0	0.00	0.965	1,43.7	0.30	0.589
Feather treatment (3)	Fixed	1,45.5	1.46	0.233	1, 45.0	1.67	0.202	1,43.6	10.20	0.003
(1)*(2)	Fixed	1,45.4	0.05	0.816	1, 44.7	4.21	0.046	1,43.5	0.40	0.532
(1)*(3)	Fixed	1,45.6	2.30	0.136	1, 44.9	0.28	0.600	1,43.5	2.72	0.107
(2)*(3)	Fixed	1,46.3	5.04	0.030	1, 46.3	0.28	0.596	1,44.5	1.42	0.240
(1)*(2)*(3)	Fixed	1,46.2	1.17	0.285	1, 45.9	0.91	0.345	1,44.2	0.00	0.978
Nest ((1)*(2)*(3))	Random	43,90.0	2.23	0.001	43, 83.0	1.29	0.157	44,79.0	1.92	0.006
Reduced model										
Study area (1)	Fixed	1,43.3	5.82	0.020	1, 41.9	14.73	< 0.001	1,43.6	8.87	0.005

Plant treatment (2)	Fixed	1,49.6	8.47	0.005	1, 42.8	0.24	0.628			
Feather treatment (3)		1,44.1	1.98	0.166				1,43.6	13.19	0.0007
(1)*(2)	Fixed				1, 41.8	10.35	0.003			
(1)*(3)	Fixed	1,43.3	3.06	0.087				1,43.9	3.55	0.066
(2)*(3)	Fixed	1,49.5	3.76	0.058						
Nest ((1)*(2))	Random	46,90.0	2.24	0.001	48, 83.0	1.23	0.200	1,43.4	1.89	0.006

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957 Results from full and reduced generalized linear mixed models explaining experimental effects of feathers and plants as nest materials on tarsus
958 length and telomere length and dynamics of starling nestlings close to fledging (residuals after controlling for the effects of hatching date, see
959 text). Study area, treatments and their interactions were included as fixed factors and nest identity nested within the interaction between the three
960 factors was included as the random effect. The reduced model resulted from backward stepwise selection up to $P = 0.1$. Significant values are in
961 bold.

Table 3: Telomere length of hatchlings depending on characteristics of nestlings and nest materials

	Beta	SE	<i>F</i>	<i>P</i>	Corrected <i>P</i>
Hatching date	2.645	1.307	4.10	0.045	0.049
Hatching date ^{^2}	-2.363	1.303	3.29	0.072	0.076
Tarsus length	0.274	0.177	2.39	0.124	0.129
Body mass	-0.218	0.181	1.45	0.231	0.235
Number of feathers	0.265	0.102	6.69	0.011	0.013
Green plants	-0.058	0.087	0.45	0.503	0.505
Study area	0.113	0.112	1.01	0.316	0.576
Reduced model					
Hatching date	2.638	1.245	4.49	0.036	0.039
Hatching date^{^2}	-2.461	1.238	3.95	0.049	0.052
Number of feathers	0.244	0.096	6.48	0.012	0.014

Results from full and reduced models explaining telomere length of hatchlings depending on characteristics of nestlings (hatching date, tarsus length and body mass), nest materials (log-transformed number of feathers and presence or absence of green plants) and study area. The reduced model resulted from backward stepwise selection up to $P = 0.1$. Corrected P values by adjusting degrees of freedom ($df = 128$) to number of sampled nests ($df = 48$ and 50 for full and reduced model, respectively) are also shown. Significant results are in bold.

Fig 1: Effects of experimental green plants (added versus removed) as nest material on (a) tarsus length, (b) body mass, (c) mesophilic bacterial loads and (d) prevalence of enterobacteria, in relation to (a) study area (Calahorra and Hueneja) and (b, c, d) feather treatment (unpigmented and pigmented). Average values ($\pm$ 95% confidence intervals) are shown.

Fig 2: Surface plot showing the relationship between telomere length of spotless starling hatchlings with hatching date and number of feathers in the nest (log10-transformed). Different colours indicate different values for telomere length.

Fig 3: Relationship between hatching date and (a) telomere length and (b) dynamics (i.e. difference in rank values of telomere length between hatching and fledging) of 14-day-old spotless starling nestlings.

Fig 4: Effects of the green plant and feather treatments on (a, b) telomere length and (c, d) telomere dynamics (i.e. difference in rank values of telomere length between hatching and fledging) of spotless starling fledglings in relation to study area (Calahorra and Hueneja). (a) Green plants added versus removed. (b) Unpigmented versus pigmented feathers. (c) Feathers added versus removed. (d) Unpigmented versus pigmented feathers. Telomere length and dynamics show average ($\pm$ 95% confidence interval) residual of ranked values after controlling for the curvilinear association with hatching date.

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1000 **Fig 5:** Effect of *Staphylococcus* presence on the skin of 8-day-old spotless starling
1001 nestlings on telomere length. Telomere length shows average ($\pm$ 95% confidence
1002 interval) residuals of ranked values close to fledging after controlling for the curvilinear
1003 association with hatching date.

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