**Interspecific variation in deterioration and
degradability of avian feathers: The evolutionary role
of microorganisms**

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Abstract

Feathers are essential for avian life, and factors affecting their integrity are important to understand their evolution. These factors should depend on, among other traits, species-specific bacterial environments and life-history characteristics. However, interspecific variation in feather deterioration, feather susceptibility to degradation by keratinolytic bacteria (degradability), and bacterial environment, have rarely been quantified. Here, we did so by measuring deterioration and degradability of wing feathers of fledglings in 16 bird species, and characterizing the bacterial environment where they developed. We found statistically significant interspecific variation for all considered variables. On average, non-melanised were more deteriorated than melanised feathers, but differences depended on the species. Moreover, nest bacterial loads were related to feathers wear, but the sign of the association depended on the bacterial group considered and on feather pigmentation. We also found a positive association of feather degradability with wear of non-melanised feathers, and with bacterial loads. These results suggest that bacterial environments determine the integrity of fledgling feathers as well as their resistance to bacterial degradation, which implies a preponderant role of bacteria in driving the evolution of avian feathers.

Keywords: Feather degrading bacteria, Feather wear, Interspecific comparisons, Keratinolytic bacteria, Nest bacterial environment.

Introduction

The presence of feathers is one of the defining characteristics of the Class Aves and has historically attracted the attention of evolutionary biologists trying to understand the extremely high variation in avian morphology, structure and coloration (Hanson 2011). Classic adaptive explanations for the existence of feathers include insulation (i.e., thermoregulation or waterproofing) and flight (Rayner 1988, Stettenheim 2000), but they also function in different scenarios of social communication and sexual selection (Andersson 1994, Møller et al. 1998, Senar 2006, Hanson 2011, Roulin 2016). Thus, feather characteristics providing the holder with advantages in the above mentioned scenarios have been studied as possible key adaptations driving the evolution of birds (Rayner 1988, Stettenheim 2000).

Factors affecting the integrity of feathers are therefore important to understand their evolution. Feather integrity might for instance depend on physiological conditions of individuals during feather growth, which could result in feather malformations (e.g. fault bars) that enhance feather deterioration and breakage (Jovani and Rohwer 2017). Among factors directly promoting feather damage, the physical abrasive wear due to friction while flying or diving, or deterioration due to contacts with distinct materials while walking or perching, are well known since many years (Francis and Wood 1989, Swaddle et al. 1996). Chemical abrasion of feathers has also received attention, especially during the last decade, focussing on that induced by keratinase-producing bacteria (Gunderson 2008). All these factors negatively affect feather integrity and, thus, their functioning in thermoregulation, communication and flight, resulting in severe fitness consequences. Risk of feather deterioration would in any case depend on life style or life history characteristics such as migratory or foraging behaviours (Møller et al. 2012). This scenario therefore raises the expectation that the

degree of feather deterioration should be a species-specific character, a prediction that to the best of our knowledge has never been explored.

Feather degradation by keratinolytic bacteria occurs in wild birds (Leclaire et al. 2014, Kent and Burt 2016, but see Cristol et al. 2005), which for instance, might have consequences in scenarios of sexual selection (Shawkey et al. 2007, 2009, Ruiz-Rodríguez et al. 2015) and survival (Møller et al. 2012). Moreover, bacterial communities of avian feathers vary interspecifically (Javůrková et al. 2019). Detecting evidence of interspecific variation in feather wear in standard conditions could suggest the existence of interspecific variation in environmental factors associated with the risk of feather deterioration, including bacterial environment (Kent and Burt 2016). Moreover, since these environmental factors likely vary among species (see above), natural selection could have favoured the evolution of characteristics that counteract or reduce feather degradation by keratinolytic bacteria in environments that are distinctive of each species (Burt 2009, Burt et al. 2011, Javůrková et al. 2019). If this was the case, we should find interspecific variation in traits functioning in preventing feathers' wear (Burt 2009) such as feather susceptibility to degradation by keratinolytic bacteria (hereafter, feather degradability) (Ruiz-de-Castaneda et al. 2015, Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2016).

The structure and chemical composition of feathers are known to affect feather wear of wild birds (Ruiz-de-Castañeda et al. 2012). Although the main chemical component of feathers is β -keratine, a slight variation in pigment composition may influence feather degradability. We know for instance that indentation hardness of melanic keratin is greater than that of non-melanic keratin (Bonser 1995), and that rates of wear (Burt 1979) and of breakage (Kose and Møller 1999) are lower in melanic than in non-melanic feathers. Furthermore, melanised feathers resist bacterial

degradation better than pale, unmelanised feathers (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012). Moreover, in comparisons with pale birds, dark birds often live in humid habitats (Delhey 2017), where more active feather degrading bacilli are abundant (Burt and Ichida 2004), which suggests a link between habitat-related risk of feather degradation and feather characteristics that reduce such a risk. This scenario therefore suggests that, because birds should compensate by strengthening their feathers (Schreiber et al. 2006, Ruiz-de-Castañeda et al. 2012), feather degradability should vary between melanised and unmelanised feathers within the same species, and that a considerable interspecific variation should exist in such feather characteristics. Although variation in degradability has been studied intraspecifically in relation to feather pigmentation (Burt et al. 2011, Ruiz-de-Castañeda et al. 2012) and habitat characteristics (Ruiz-Rodríguez et al. 2016), interspecific variation in feather degradability has only been explored for 13 parrot species (Burt et al. 2011).

Here, we have quantified intra and interspecific variation in feather wear of nestlings' birds close to abandoning the nest (hereafter fledglings). We did so by microscopically quantifying deterioration of wing feathers, mostly secondary-covert-wing feathers, in 16 species of birds. We also estimated feather degradability by quantifying keratin degradation by the well-known feather degrading bacterium *Bacillus licheniformis* (Kent and Burt 2016). We expected to find significant interspecific variation in feather wear and in feather degradability, and that these two traits would positively covary interspecifically. By using feathers of nestlings we restricted (i.e., standardized) factors that could have affected feather deterioration state to those occurring within the nest environment. Moreover, independently of the altricial bird species, feathers of nestlings close to abandon the nests are at a similar state of development and, thus, are interspecifically comparable.

Nestling feathers are often of lower quality than adult feathers (Callan et al. 2019). Nestling feathers might play pivotal roles in social communication during nestling (Morales et al. 2019) and post-fledging periods (Moreno and Soler 2011), and even explain probability of predation (Callan et al. 2019) and body condition (Minias et al. 2015) during the post-fledging and first winter periods. Thus, although post-juvenile moult is likely related to quality of fledgling feathers (Minias et al. 2015), exploring factors affecting integrity of nestling feathers is important to understand factors affecting their survival.

Within nests, the bacterial environment would largely determine feather bacterial loads (Jacob et al. 2018) and, thus, risk of bacterial degradation of feathers. Nest bacterial environment are largely determined by the behaviour of parents including those related to nest building (e.g., nest materials used) (Ruiz-Castellano et al. 2016, 2017, 2019), nest sanitation (e.g., nestling faeces removal) (Soler et al. 2015, Azcárate-García et al. 2019) and incubation (Ruiz-Castellano et al. 2019). Consequently, selection pressures acting on feather degradation during the nestling phase differ from those acting after abandoning the nest. Previous studies have detected interspecific variation in nest bacterial environments by sampling the microbiome of the eggshells of different species (Peralta-Sánchez et al. 2018). Moreover, interspecific variation in eggshell bacterial load is associated with life history characteristics (Peralta-Sánchez et al. 2012) and, thus, it is possible that nest bacterial environments determine wear and degradability of nestling feathers. Here, we characterised bacterial loads of the same nests where we collected nestling feathers to evaluate their deterioration state. However, the effects of nest bacterial environments on feather wear is not straightforward. It for instance depends on the presence of bacteria with keratinolytic activity (i.e. able to degrade feather tissue) that will positively affect feather deterioration. Antimicrobial activity (against

feather-degrading bacteria) of bacteria within the nest community would also determine feather degradation; the higher the density of these bacteria the lower the effect on feather deterioration. Finally, it is also possible that the physical structure of feathers was adapted to species-specific nest bacterial communities, which might result in these two variables being not related to each other at the interspecific level. Thus, we explored the association of nest bacterial density with feather deterioration and degradability by distinguishing different groups of bacteria.

Material and Methods

Study areas and fieldwork

Fieldwork were carried out in the Hoya de Guadix (37° 18' N; 3°11' W), southern Spain, and in the forest of Valsaín (40°54'N, 04° 01'W), central Spain, during the 2015 and 2016 breeding seasons. For a detailed description of the southern and central Spain study areas see Martín-Vivaldi et al. (2006), Soler and Avilés (2010), and González-Braojos et al. (2017). We collected information for 16 species that included hole and non-hole nesting species (Table 1). The nests of hole-nesting species were mostly located within nest boxes installed in the study areas, while nests of the other species were found by systematic search of appropriate habitats within the study areas. Samples (wing feathers and nest bacterial samples) were collected at a standard relative age during their ontogeny when less than 10% of the primary feathers were covered by the cylindrical sheaths (i.e., when they were in the last fifth of its normal nestling development, close to abandoning the nest). Moreover, for a subsample of species, we captured adults during the breeding season and collect the same kind of feathers than in nestlings. We used these samples to validate the use of nestling feathers to explore interspecific differences in feather characteristics.

We collected a minimum of two wing feathers of at least two randomly selected nestlings in each sampled nest. For most sampled species, we collected the third secondary covert feathers. Whenever possible, we collected wing feathers that had both dark (i.e., with relatively high concentration of eu- and/or pheo-melanin) and pale (i.e., with relatively low, if any, concentration of eu- and/or pheo-melanin) areas, or different feathers that were either dark or pale. Feather darkness is usually related to presence of melanin (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012) and, thus, we call them as melanised and non-melanised feathers, respectively. To cut the feathers at the upper part of the quill (to avoid bleeding) we used scissors previously washed with 95% ethanol and disinfected with a Bunsen burner. Feathers of each nestling were kept in new zipper plastic bags and stored in the lab at dark and 4 °C until the analyses within the following ten months. The same day of feather collection, during the last days of the nesting period, we sampled nest bacterial loads by gently rubbing the nest-cup material, or nest surface where nestlings were located, with a sterile swab (Sterile R, Nuova Aptaca S.R.L.) slightly wetted with sterile sodium phosphate buffer (0,2 M; pH = 7,2) during 10 seconds. Meanwhile, nestlings were kept in a cotton bag. Afterwards, we kept the swab in a microcentrifuge tube with 1.2 ml of phosphate buffer at 4-6 °C, in a portable refrigerator until being processed in the lab within the next 4 days.

Feather wear

The level of feather wear was estimated following the protocol described in Ruiz-Rodríguez et al. (2015). Briefly, feathers were examined and photographed (2x) under a binocular lens (Nikon SMZ1500, Melville, NY, USA) connected to a camera (Nikon Digital Sight DS Fi1). Measurements from pictures were taken using the software NIS Elements F 3.1. Following

Ruiz-Rodríguez et al. (2015), we considered: (i) whether or not the tip of the feather was incomplete; (ii) the length (mm) of the feather tip that showed clear signs of wear (apical part); and (iii) the number of barbules that were degraded in 20 randomly chosen barbs of the basal part (see Figure 3 in Ruiz-Rodríguez et al. (2015)). Wear levels were ranked from 0 (no degradation) to 3 (more than 2/3 of the feather damaged). Two lab technicians, who were unaware of the feather origin (i.e., species) or hypotheses tested, evaluated all photographs. Whenever possible, we distinguished wear of melanised and of unmelanised feathers (or parts of the same feather). Repeatability of visual estimations was calculated by comparing both assessments in a one-way ANOVA. Repeatability was relatively high ($F = 7.32$, $df = 2599, 2600$, $P < 0.0001$, repeatability index = 0.76). Feather wear varied significantly among nests of the same species (GLM, effect of nest identity nested within species identity, $F = 1.88$, $df = 151, 177.8$, $P < 0.001$), but not among nestlings within the same nest (GLM, effect of nestling identity nested within nest and species identities, $F = 0.99$, $df = 227, 1648$, $P = 0.53$). Thus, we used average values of evaluated feathers from the same nest in subsequent analyses.

Feather degradability

Feather degradability was explored by using complete feathers and following the protocol described elsewhere (Ruiz-Rodríguez et al. 2015, Ruiz-Rodríguez et al. 2009, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012). It is worth mentioning here that because degradability was estimated for complete feathers, we were not able to distinguish between degradability of the pigmented and non-pigmented parts of feathers. Moreover, because pigmentation (melanised vs non-melanised) of the collected wing feathers does not vary within species, we did not contemplate pigmentation as a factor

when analysing possible interspecific differences in feather degradation. Finally, a single feather per sampled nests were analysed.

A brief description of the protocol is as follows: Previously separated and weighed feathers were sterilized in the autoclave before the experiment. Afterward, each feather was included in a previously sterilized experimental glass tube containing 4 mL of a PBS buffer (9.34 mM NH_4Cl , 8.55 mM NaCl , 1.72 mM K_2HPO_4 , 2.92 mM KH_2PO_4 , 0.49 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.01% yeast extract in 100 mL of distilled water). Moreover, a colony of *Bacillus licheniformis* D13 previously isolated from TSA plates was introduced in each of the experimental glasses with a sterile loop. After vortexing, we collected 1 mL from each tube as a basal measurement, and kept it at 4 °C until measurement in the spectrophotometer. Experimental tubes were incubated at 37 °C in constant agitation at 120 rpm in an orbital agitator (VWR, Spain). 1 mL was collected from each experimental tube after 21 days of incubation. Collected samples were centrifuged to remove bacterial cells, and absorbance of the supernatant was then estimated using a spectrophotometer (Helios Zeta UV-Vis, Thermo Scientific, United Kingdom) at 230 nm (Goldstein et al. 2004). The oligopeptide concentration in the supernatant mainly originates from keratin degradation and thus it is directly related to the amount of feather degraded. Calibration curves of absorbance and oligopeptide concentration (from 0 to 300 $\mu\text{g} \times \text{mL}^{-1}$) were obtained by using bovine serum albumin (BSA) ($R^2 = 0.98$ in both curves), which allowed us to extrapolate the absorbance to values of oligopeptide concentration.

To assure that oligopeptide measurements were due to the degradation of feathers by *Bacillus licheniformis* exclusively, we also prepared the following control samples: (i) tubes containing the buffer, (ii) tubes containing the buffer and the bacteria, but without the feathers, and (iii) tubes

containing the feathers but without the bacteria. After 21 days of incubation, oligopeptide concentrations increased significantly (Repeated Measures ANOVA, effect of incubation, $F = 803.2$, $df = 1,360$, $P < 0.0001$), mainly in experimental tubes (Repeated Measures ANOVA, interaction between incubation and treatment effect, $F = 140.3$, $df = 1,1080$, $P < 0.0001$). Although intermediate level of oligopeptide concentrations were detected in tubes containing feathers without bacteria that were incubated during 21 days, control tubes had significantly less oligopeptide concentration than experimental tubes (Repeated Measures ANOVA, effect of experimental treatments, $F = 206.2$, $df = 1,1080$, $P < 0.0001$, ESM Fig 1). Thus, for subsequent analyses, as a measure of feather degradability, we conservatively used differences in oligopeptide concentration between experimental tubes containing feather and bacteria and control tubes containing feathers but not bacteria. These measures were standardized by feather weight (precision scale Mettler AB135-5/FACT, accuracy 000001g).

Nest bacterial loads

In the lab, tubes containing the bacterial samples were vigorously shaken in a vortex (Boeco V1 Plus) to remove bacteria from swabs. Afterward, we spread homogeneously 100 μ l of serially diluted samples until 10^{-4} in Tryptic Soy Agar (TSA) and 10^{-2} in the others media (see below). Plates were incubated at 37 °C during 72 hours, and then the number of colonies on each plate was counted. Following the method of Peralta-Sánchez et al. (2010), we used four different solid media (Scharlau Chemie S.A., Barcelona) to grow bacterial samples. We used TSA, a broadly used general medium to grow mesophilic bacteria that provide a good estimation of bacterial density (Peralta-Sánchez et al. 2010, 2012, 2018, Soler et al. 2011). We also used three differential and selective media: Kenner Fecal

Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. *Enterobacteriaceae* and *Staphylococcus* sp. are saprophytic and opportunistic bacteria (Singleton and Harper 1998, Houston et al. 1997, Cook et al. 2005) that live on skin, hair and feathers of mammals and birds (Krieg and Holt 1984). *Enterococci* are also opportunistic pathogens (Franz et al. 1999), although some species may also have beneficial effects (Soler et al. 2010, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2012). Bacterial load of nests for each bacterial group was then expressed as the number of colony forming units (CFU) per ml (N° colonies $\times 10^{\text{dilution factor}}$) / 100 μ l spread).

Sample sizes and statistical analyses

We considered 16 species for which we collected information of feather wear for more than two nests (Table 1). For 11 of these species we successfully collected information of wear of pigmented (melanised) and unpigmented (parts of) feathers. However, because of malfunctioning of the spectrophotometer determining keratin degradation, we lost all feathers collected for this purpose during 2015. Thus, during 2016, we again collected feathers of most (11) species with information of feather wear. Nest bacterial loads were estimated for the 16 species with information of feather wear; most of the samples were in fact from the same nests where we collected the feathers to analyse their deterioration state. Nest bacterial loads were log₁₀-log₁₀ transformed ($\log_{10}(\log_{10}(X+1)+1)$) before the analysis to approach normal distribution. Used Statistical models assumed Gaussian distributions and residuals were visually checked for normality.

Interspecific variation in feather wear of melanised and non-melanised feathers was explored in separate univariate ANOVAs with average values per nest as dependent variable and species identity as the unique independent factors. Possible differential wear between melanised and non-melanised feathers was explored with a subset of species with information for both kinds of feathers in a Repeated Measures ANOVA. Average values of feather wear per nest of melanised and non-melanised were used as repeat measures. Thus, feather pigmentation was the within independent factor and species identity as the between independent factor.

Interspecific variation in feather degradability was explored by using average nest values as the dependent variable and species identity as the only independent factor. Interspecific variation in nest bacterial loads ($\log(\log(X))$ transformed data) were explored in a MANOVA, with bacterial loads of mesophilic bacteria, enterobacteria, staphylococci and enterococci as multiple dependent variables and species identity as the independent factor.

The association between feather wear and nest bacterial loads was explored in General Linear Models (GLM) that did include species identity as additional independent factor to those reflecting nest bacterial load (i.e., the four bacterial groups). The expected associations were explored separately for melanised and non-melanised feathers, but also for the subset of species with information on degradation of melanised and non-melanised feathers in a repeated measures design. In this case, values for pigmented and unpigmented feathers were included as dependent variables in a Repeated Measures ANOVA (i.e. pigmentation as repeated measure), and bacterial loads of mesophilic bacteria, staphylococci, enterococci and enterobacteria as independent covariables. This model also included species identity as an additional independent factor. For all these models trying to

explore the expected association between bacterial loads and feather wear, we adopted a backward stepwise procedure for model selection where factors with the largest p-values were removed one by one up to a p-value lower than 0.1. Final reduced models coincide with best models from AIC criteria (results not shown).

To explore the associations between feather degradability and feather wear, and between feather degradability and nest bacterial loads, we performed regression analyses. Since feather degradability and feather degradation were estimated from samples collected in different nests, but bacterial environment were estimated from the nests where feathers for estimating degradation were collected, we used average species values of feather degradability, but nest values for bacterial environment and feather wear. The analyses including information of feather degradability did not include species identity as independent factor because no within species variance exists in feather degradability.

The effects of age (fledging *vs* adults) (independent factor) on feather degradability (dependent variables) were explored in General Linear Models that also included species identity as additional independent factor. Feather wear was estimated for several nestling within the same nest and, thus, the effects of age on this variable were analysed in General Linear Mixed Models that included species identity as additional independent fixed factor and nest identity nested within the interaction between species identity and age as the random factor. We estimated the interaction between age and species identity in separate models that also included main affects, while main effects were estimated in models that did not include interactions.

Finally, expected association between feather deterioration or feather susceptibility to degradation and nest bacterial loads were also explored in weighted phylogenetic linear models (wPGLS) with average per species

values. As far as we know, available methodologies to consider within species variance into the PGLS models is restricted to one of the predictor variables (Garamszegi and Møller 2010, Garamszegi 2014) and, thus, degrees of freedom were reduced to those reflecting number of species. Briefly, we used consensus phylogenetic trees from 1000 downloaded trees from birdtree.org (Jetz et al. 2012) and used wPGLS following recommendations by Garamszegi (2014) in R (version 3.6.1) with libraries ape (Paradis and Schliep 2018) and nlme (Pinheiro et al. 2019).

All statistical tests were performed in Statistica V13 (Dell-Inc 2015).

Results

Feather wear; interspecific comparisons

We found interspecific differences in wear of secondary covert feathers of nestling birds, both for melanised ($F = 7.24$, $df = 14, 146$, $P < 0.0001$) and non-melanised feathers ($F = 11.50$, $df = 10, 132$, $P < 0.0001$). Blue tits (*Cyanistes caeruleus*) and black wheatears (*Oenanthe leucura*) were the species with most damaged melanised feathers, while those of pied flycatchers (*Ficedula hypoleuca*) and magpies (*Pica pica*) were the least damaged (Fig. 1A). When considering non-melanised feathers, the blue tit had again the highest feathers wear, while non-melanised feathers of hoopoes (*Upupa epops*) were by far those least damaged (Fig 1A). Finally, degradation of non-melanised feathers (mean (SE) = 1.57 (0.04)) did not differ significantly from that of melanised feathers (mean (SE) = 1.51 (0.04); Repeated measures, pigmentation effects: $F = 1.15$, $df = 1, 128$, $P = 0.28$). However, in some species the melanised feathers were more degraded than the unpigmented ones, while in some other species the tendency was the

opposite resulting in a statistically significant interaction between feather pigmentation and species identity (Fig 1A; Repeated measures, $F = 7.15$, $df = 9, 128$, $P < 0.0001$). These differences are mainly due to hoopoes and pied flycatchers (Fig 1A).

Feather degradability; interspecific comparisons

We found interspecific differences in feather degradability ($F = 5.26$, $df = 11, 107$, $P < 0.0001$), even after controlling for the positive influence of feather mass used (Beta (SE) = 0.83 (0.18), $F = 21.78$, $df = 1, 107$), $P < 0.0001$). Covert feathers of nestlings of little owls (*Athene noctua*), pied flycatchers, great tits (*Parus major*), hoopoes and blue tits, were the most easily degraded, while those of choughs (*Pyrrhocorax pyrrhocorax*), jackdaws (*Corvus monedula*) and magpies (*Pica pica*) were the most resistant to bacterial degradation (Fig. 1B).

Nest bacterial loads; interspecific comparisons

Estimated nest bacterial loads of different groups of bacteria covaried with each other ($R(\text{Max-Min}) = 0.65\text{-}0.46$, $N = 161$, $P < 0.0001$). We detected a large interspecific variation (MANOVA, Wilks' $\lambda = 0.26$, $F = 3.80$, $df = 60$, 556.5 , $P < 0.0001$; univariate results: $F > 3.018$, $df = 15, 145$, $P < 0.0003$) that depended on the considered bacterial group (Fig. 1C). However, independently of the bacterial group considered, nests of some species consistently harboured the lowest or the largest densities. For example, little owls, jackdaws and hoopoes were those with higher mesophilic bacterial loads, while nests of swallows (*Hirundo rustica*), choughs, and magpies harboured the lowest densities of this bacterial group. When considering *Enterobacteriaceae* and *Staphylococcus*, nests of hoopoes were those with

the highest density, while those of swallows harboured the lowest densities of these bacterial groups. Finally, when considering *Enterococcus*, nests of rollers (*Coracias garrulus*) and of hoopoes were those with the highest bacterial density, and nests of swallows again showed the lowest densities (Fig. 1C).

Feather wear and nest bacterial loads

Nest bacterial loads predicted wear of nestling feathers, (mainly those melanised). After controlling for the effect of species identity, the density of enterobacteria resulted positively associated with melanised feathers wear (Table 2, Fig. 2). Interestingly, in final-reduced models, density of mesophilic bacteria resulted negatively associated with melanised feather wear (Table 2, Fig. 2). When considering non-melanised feathers, no bacterial counts explained a significant proportion of variance of feather wear ($F < 2.16$, $df = 1,124$, $P > 0.14$), even after correcting for the effect of species identity ($F = 10.15$, $df = 10,124$, $P < 0.0001$).

Differences in feather wear between non-melanised and melanised feathers (i.e. only species with these two kinds of feathers considered) depend on nest bacterial environment (enterobacteria (negatively, Fig. 2) and mesophilic bacterial loads (positively, Fig. 2)). Moreover, after controlling for nest bacterial environment, within-nest differences in degradation of non-melanised minus melanised feathers resulted significantly different from zero; non-melanised were more degraded than melanised feathers (see within nest repeated measures in Table 2). That was the case after correcting for the effect of species identity (Table 2)

Feather degradability, feather wear and nest bacterial loads

Average degradability of feathers of different species tended to be positively related to wear of non-melanised feathers (Beta(SE) = 0.16(0.09), $F = 3.27$, $df = 1,126$, $P = 0.073$), but not to that of melanised (Beta(SE) = 0.01(0.08), $F = 0.01$, $df = 1,138$, $P = 0.958$). Moreover, in the final model, feather degradability resulted negatively related to staphylococci bacterial loads and tended to be positively related to mesophilic and enterobacteria loads of sampled nests (see feather degradability in Table 2).

When considering both factors in the same models, feather degradability and nest bacterial environment explained feather wear. Feather degradability did not explain significant proportion of variance of wear index of melanised feathers (Beta(SE) = -0.01(0.08), $F = 0.02$, $df = 130$, $P = 0.895$), but bacterial environment did after controlling for species identity as showed in Table 2 (see wear of melanised feathers). On the other hand, wear of non-melanised feathers was positively associated with feather degradability and negatively, but not significantly, with staphylococci and enterococci bacterial loads of the nests (see wear of melanised feathers and feather degradability in Table 2).

All statistically significant associations disappeared when considering mean values per species of feather degradation, feather degradability and nest bacterial environment, and controlled for phylogenetic association among them (PGLS, $P > 0.07$, ESM, Table 1).

Wear and degradability of adults and nestling feathers

For the seven species with information on feather wear, interspecific variation of adults and nestlings followed a similar pattern (see the effect of age in Table 3). Only in hoopoes, feather wear of nestlings was higher than that detected in adult hoopoes (Fig 3A). Moreover, feathers of adult

individuals were more resistant to bacterial degradation than those of nestlings. However, rank positions of feather degradability of adults and nestlings coincided except for hoopoes (Fig. 3B), which are the responsible of the significant interaction between species and age (Table 3).

Discussion

We here estimated feather wear and degradability in fledglings and nest bacterial loads in nests of 16 species of Palaearctic birds, and detected statistically significant interspecific variation for all considered variables. On average, non-melanised feathers were more deteriorated than melanised feathers, but it was only detected when comparing feathers of nestlings within the same nests, and after controlling for bacterial environment. Moreover, our results suggest that the feather deterioration state was explained by feather degradability (only for non-melanised feathers) and nest bacterial environment. Feather degradability was also explained by nest bacterial environment, suggesting that the nest microbiome determines the integrity of fledgling feathers as well as their strength to resist bacterial degradation. These last results were however not controlled for phylogenetic effects. Below we discuss and offer several possible explanations for the sign of the detected associations.

Consistent with previous papers claiming that non-melanised feathers are more easily degraded by bacterial activity than melanised feathers (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012), we found that non-melanised feathers of fledglings were more deteriorated than melanised feathers of the same individual. Feather deterioration was estimated for wing cover feathers and, thus, we assumed that melanised and non-melanised parts of feathers should have experienced a similar risk of deterioration. Moreover, the pigmentation effect on feather

deterioration was only detected when comparing melanised and non-melanised feathers within the same nest (i.e. repeated measures). These results suggest that the detected differences are due to differential effects of nest bacterial environment on melanised and non-melanised feathers, which might be explained by melanin conferring resistance (Bonser 1995) to feathers or/and different feather microstructure, which would confer different resistance to melanised and non-melanised part of feathers (2012, Ruiz-Rodríguez et al. 2015). This general pattern of non-melanised feathers being more deteriorated does not occur in all studied species, with hoopoes showing the opposite patterns, and some other species showing no apparent differences. We did not consider the possibility that different species varied in the quality and quantity of pigments in analysed feathers or in the microstructure of feathers of different colouration (Schreiber et al. 2006, Ruiz-de-Castañeda et al. 2012), which might partially explain interspecific differences in the effect of feather pigmentation. Future work directed to explore interspecific differences in pigmentation and microstructure of feathers would clarify the importance of such characters in explaining detected interspecific differences in feather wear.

The most robust result is the detected interspecific differences in all considered variables. Interspecific differences in nest bacterial environment estimated as eggshell bacterial loads were first detected by Peralta-Sánchez et al. (2012), whom also demonstrated associations with life history characteristics and hatching success (Peralta-Sánchez et al. 2018). Here, we directly sampled nest materials and for the first time confirm interspecific variation in nest bacterial environment that should affect probability of bacterial contamination of eggs, nestlings and adults. Moreover, we also found significant interspecific variation in feathers' susceptibility to bacterial degradation, which was first suggested by Burt et al. (2011) in

association with interspecific differences among feathers' pigmentation of 13 parrot species. Interestingly, these authors suggested that interspecific differences in feather degradability should be explained by the risk of feather degradation typically experienced by each species. If this was the case, interspecific differences in degradation state of feathers collected from the same body parts (i.e. wing feathers) and at similar stage (similar nestling developmental stage) should be close to zero. However, our results did not fit this prediction, but pointed out interspecific differences in feather susceptibility to degradation. Our results, therefore, suggest that feather resistance do not completely adapt to risk of bacterial degradation, and that some other factors will play a role.

Interspecific differences in factors affecting feather wear could explain the detected differences in deterioration of fledgling feathers. We characterized interspecific differences in feather degradability and in nest bacterial environment to explore whether these characteristics were responsible of the wear variability of fledgling feathers, and it resulted associated with bacterial load of nests where sampled fledglings grew. However, the sign of the association depended of feather pigmentation and of the bacterial group considered. Negative associations between bacterial loads of nests and feather deterioration can be explained in several ways. First, feathers of species developing in nests with higher bacterial densities could be more resistant to bacterial degradation. Partly in accordance with this *a posteriori* explanation, we found that feather degradability was lower in species with a high density of staphylococci in their nests. However, the association turned to be positive when considering mesophilic bacteria or enterobacteria, which suggest that the possible influences of nest bacterial environment on the evolution of feather resistance to bacterial deterioration would depend on the bacterial group considered and of characteristics of the

bacterial community in relation to the risk of feather deterioration by microorganisms. This speculative inference links the first to the second possibility explaining the detected negative association between bacterial loads of nests and feather wear index. This second explanation posits that characteristics of the bacterial community, or that some bacteria in avian nests, could protect nestling feathers from feather-degrading microorganisms. If that was the case, feathers of species with protecting bacteria in their nests should be more susceptible to bacterial degradation. On the one hand, nest environments with higher bacterial densities might harbour keratinolytic bacteria in lower proportion than those with lower bacterial density. This possibility is however unlikely since mesophilic and keratinolytic bacterial densities are positively correlated, at least in starling feathers (Ruiz-Rodríguez et al. 2015). It might also be possible that bacteria producing antibiotics against keratinolytic ones occur at higher relative density in nests with higher bacterial densities. Bacterial antimicrobials function by reducing competition for space and resources against other bacterial strains (Ji et al. 1997, Riley and Wertz 2002), and bacterial density is one of the factors known to enhance antimicrobial production (Riley and Wertz 2002). Thus, it is possible that high-density bacterial communities also produce high level of antimicrobials against keratinolytic bacteria able to colonize feather tissues, resulting in lower levels of feather degradation. However, we did not quantify the density of keratinolytic bacteria in nest environments nor in nestling feathers, which is necessary to test and discuss this possibility.

Another possibility that could explain the negative association between nest-bacterial density and wear of unpigmented feathers is that this part of nestling feathers function honestly reflecting (i.e., signalling) individual abilities of nestlings to maintain plumage integrity during the last

days of the nestling stage (Morales et al. 2019). Reliability of signals partly depends on costs associated with their production, showiness and/or maintenance (Maynard-Smith and Harper 2003), and fledglings showing non-degraded plumage, or plumage of a particular coloration, might signal its phenotypic quality to conspecifics (López-Idiáquez et al. 2018). Following the handicap principle (Zahavi and Zahavi 1997), individuals will show their ability to resist bacterial infection more honestly if they use traits that, as it occurs for unpigmented feathers, are more easily infected but are revealed with no or reduced sign of bacterial infection. The role of bacteria in the evolution of feather characteristics that honestly signal phenotypic quality of holders has been previously demonstrated in adult males of the spotless starling (*Sturnus unicolor*), which wear delicate, long sexual feathers that are easily degraded by bacteria and that harbour bacteria at a higher density than non-sexual feathers (Ruiz-Rodríguez et al. 2015). Only males with a higher capacity to prevent feather wear by bacteria will show non-deteriorated feathers. Similarly, only good quality fledglings would show non-degraded white patches on their feathers. Signalling roles of fledgling plumage in different scenarios of social interaction have been recently proposed to explain juvenile plumage patterns (Moreno and Soler 2011, Ligon and Hill 2013, Fargallo et al. 2014, Romano et al. 2016, Galván 2017, Morales et al. 2019). Our results show that feather degradability explains feather wear, but mainly that of non-melanised feathers, which is in accordance with the proposed scenario. However, melanised feathers were less deteriorated than non-melanised feathers in some, but not all species, which is contrary to the proposed scenario. Future work should concentrate on exploring this hypothesis intra- and interspecifically.

Detected associations that included feather degradability should be considered cautiously since they are not controlled by species identity. The

impossibility of controlling these analyses for species identity was because information on feather degradability, and nest bacterial loads or feather wear, were not available for the same individuals and nests, which impede the estimation of intraspecific covariance matrices. Moreover, the effects disappeared when using mean values per species in phylogenetically controlled analyses. These analyses however included only 11 species, which implies a reduced statistical power that prevent to reach strong conclusions. Thus, further investigations estimating feather wear, feather degradability and nest bacterial density in the same individuals and nests, or increasing the number of species with this kind of information, are necessary to reach firmer conclusions.

Summarising, we found clear interspecific differences in the deterioration state of fledgling feathers that tended to parallel interspecific differences in feather resistance to bacterial degradation, and in nest bacterial environment, which suggest a role of bacteria in determining such interspecific variation. The signs of the association between nest bacterial loads and feather deterioration depend on the bacterial group considered so the different possible explanations offered should be considered as preliminary and further tested both at the intra- and interspecific levels.

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Author contributions

Conceived and designed work: JJS, MMV, JM, MMB and MRR. Performed the field and laboratory work: MAG, SGB, SDL, JJS, MMV and MRR. Analysed the data: MAG and JJS. Contributed reagents/materials/analysis tools: MAG, MMB, SDL and MRR. JJS and MAG wrote the manuscript with substantial contribution from all authors.

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Table 1: Sample sizes to estimate degradation of pigmented and unpigmented feathers, feathers degradability and nest bacterial loads of different species.

	N = Nests (feathers)		N = Nests	N = Nests
	Feather wear		Feather	Bacterial
	Melanised	Non-melanised	Degradability	loads
<i>Athene noctua</i>	13	13	9	13
<i>Clamator glandarius</i>	9	9	15	9
<i>Coracias garrulus</i>	6	6		6
<i>Corvus monedula</i>	6		6	6
<i>Cyanistes caeruleus</i>	5	5	10	5
<i>Ficedula hypoleuca</i>	23	25	10	24
<i>Hirundo rustica</i>	3	0		3
<i>Oenanthe leucura</i>	3	0		3
<i>Otus scops</i>	12	12	5	11
<i>Parus major</i>	19	18	18	17
<i>Passer domesticus</i>	8	8		8
<i>Petronia petronia</i>		3	4	3
<i>Pica pica</i>	17	17	15	17
<i>Pyrrhocorax pyrrhocorax</i>	5		11	5
<i>Sturnus unicolor</i>	5		9	5
<i>Upupa epops</i>	26	26	8	26

Table 2: Results from GLM models exploring the effects of nest bacterial environment (estimated as density of mesophilic bacteria, enterobacteria, staphylococci and enterococci) on (1) wear of melanised feathers, on (2) differences in wear of melanised and non-melanised of the same individual (repeated measures), after controlling for the effect of species identity. The effects of nest bacterial environment on feather degradability (average species values) were also explored in (3) GLM models. Finally, Results from GLM models exploring the effect of both bacterial environment and feather degradability (average species values) on wear of melanised feathers are also showed (4). We show statistics associated with full and final models (i.e., after a backward procedure, eliminating one by one the independent variable with the larger p-value up to 0.1. Variables associated with p-values smaller than 0.1 are showed in bold fonts to facilitated interpretation to readers.

	Full models				Final models			
	Beta(SE)	F	df	P	Beta(SE)	F	df	P
<i>Wear of melanised feathers (1)</i>								
Mesophilic bacteria	-0.179(0.098)	3.31	1,137	0.07	-0.238(0.092)	6.75	1,139	0.010
Enterobacteria	0.206 (0.099)	4.30	1,137	0.04	0.157(0.095)	2.75	1,139	0.099
Staphylococci	-0.094(0.095)	0.97	1,137	0.33				
Enterococci	-0.132(0.108)	1.49	1,137	0.22				
Species identity		7.19	14,137	< 0.0001		7.00	14,139	< 0.0001
<i>Feather wear in relation to feather pigmentation (within-nests Repeated Measures) (2).</i>								
Pigm		3.32	1,120	0.071		4.34	1,122	0.039
Pigm x Mesophilic bacteria		4.27	1,120	0.041		6.99	1,122	0.009
Pigm x Enterobacteriaceae		6.03	1,120	0.015		5.52	1,122	0.020
Pigm x Staphylococci		1.78	1,120	0.184				
Pigm x Enterococci		0.05	1,120	0.823				
Pigm x Species identity		6.22	9,120	< 0.0001		6.70	9,122	< 0.0001
<i>Feathers degradability (3)</i>								
Mesophilic bacteria	0.175(0.110)	2.53	1,136	0.114	0.190(0.106)	3.17	1,137	0.077
Enterobacteria	0.181 (0.112)	2.60	1,136	0.109	0.196(0.109)	3.24	1,137	0.074
Staphylococci	-0.229(0.103)	4.94	1,136	0.028	-0.211(0.096)	4.79	1,137	0.030
Enterococci	0.056(0.111)	0.26	1,136	0.614				
<i>Wear of melanised feathers and feather degradability (4)</i>								
Mesophilic bacteria	0.590(0.432)	1.86	1,119	0.175				
Enterobacteria	-0.191(0.193)	0.98	1,119	0.325				
Staphylococci	-0.345(0.193)	3.18	1,119	0.077	-0.328(0.185)	3.15	1,121	0.078
Enterococci	-0.373(0.209)	3.20	1,119	0.076	-0.355(0.188)	3.54	1,121	0.062
Feather degradability	0.238(0.102)	5.47	1,119	0.021	0.241(0.098)	5.98	1,121	0.016

Table 3: Results from General Linear Mixed Models exploring the effects of age (fledglings vs adults) and species identity explaining feather wear and degradability by keratinolytic bacteria. The main effects were estimated in models that did not include the interaction, and the interaction was estimated in models that also included main effects. Since feather wear was estimated from several nestlings of the same nest, we included nest identity nested within the interaction between species and age as a random factor. Moreover, since estimates of feather degradability depend of feather mass, we included such information as a covariable in the model.

Factors	Effect	Ms effect	MS error	df	F	P
<u>Feather wear</u>						
Species	F	15.96	0.62	6, 211.4	25.64	< 0.0001
Age	F	0.01	0.57	1, 280.9	0.01	0.923
Species * Age	F	0.81	0.56	6, 313.0	1.45	0.197
Nest id(Species*Age)	Rnd	0.66	0.37	168, 1706	1.99	< 0.0001
<u>Feather degradability</u>						
Feather mass	F	4.28	0.27	1, 101	15.89	0.0001
Species	F	2.12	0.27	5, 101	11.99	< 0.0001
Age	F	3.14	0.27	1, 101	30.85	0.0009
Species * Age	F	0.98	0.27	5, 96	4.23	0.0016

Figure legends:

Fig. 1: Wear of melanised and non-melanised parts of secondary-covert wing feathers (A), feather degradability in terms of feather keratine degradability (B), and nest bacterial environment (C) estimated as density of mesophilic bacteria (TSA), enterobacteria (HK), staphylococci (VJ) and enterococci (KF). Values are averages $\pm$ 95% CI and species are ordered from the highest to the lowest value of each of the considered variables (density of mesophilic bacteria in the case of nest bacterial environment).

Fig 2: Association between feather wear of melanised feathers and nest bacterial environment estimated as densities of mesophilic bacteria and enterobacteria. Bacterial load and wear values are corrected for species identity. Lines are regression lines.

Fig 3: Wing feathers wear and degradability of 7 and 6 species, respectively, with information for fledging (filled dots) and adult (open circles) birds. Values are Least square means $\pm$ 95% CI. Species are ordered from the largest to lowest fledglings' values of the considered variable.

Fig. 1

Fig 2.

Fig. 3

ESM Table 1: Results from PGLM models exploring the effects of nest bacterial environment (estimated as density of mesophilic bacteria, enterobacteria, staphylococci and enterococci) on (1) wear of melanised feathers (2), and feather degradability (3) after correcting for phylogeny. We also explored the effect of both bacterial environment and feather degradability on wear of melanised (4) and non-melanised (5) feathers. For all the analyses, we used average species values, which were weighted by sample size (1/N). We estimated effect sizes (Cohen's *d*) and power associated to independent factors in each of the models.

	Full models				Effect size	Power
	B (SE)	t	df	P		
<u>Wear of melanised feathers (1)</u>						
Mesophilic bacteria	3.887(2.612)	1.49	10	0.17	0.112	0.217
Enterobacteria	-0.617(1.166)	0.53	10	0.61	0.015	0.072
Staphylococci	1.001(0.960)	1.05	10	0.32	0.255	0.424
Enterococci	-0.656(1.000)	0.66	10	0.53	0.205	0.354
<u>Wear of non-melanised feathers (2)</u>						
Mesophilic bacteria	-1.975(3.268)	0.60	6	0.57	0.136	0.179
Enterobacteria	1.926(1.435)	1.34	6	0.23	0.393	0.417
Staphylococci	-2.298(1.046)	2.20	6	0.071	0.665	0.619
Enterococci	0.814(1.235)	0.66	6	0.53	0.511	0.512
<u>Feather degradability (3)</u>						
Mesophilic bacteria	1.527(4.270)	0.358	7	0.73	0.008	0.058
Enterobacteria	1.594(1.925)	0.828	7	0.44	0.098	0.156
Staphylococci	-2.330(1.726)	1.35	7	0.22	0.378	0.451
Enterococci	0.959(1.909)	0.50	7	0.63	0.059	0.113
<u>Wear of melanised feathers and feather degradability (4)</u>						
Mesophilic bacteria	3.226(3.101)	1.04	5	0.35	0.056	0.090
Enterobacteria	0.569(1.456)	0.39	5	0.71	0.066	0.098
Staphylococci	-0.096(1.492)	0.06	5	0.95	0.004	0.053
Enterococci	-0.361(1.429)	0.25	5	0.81	0.000	0.050
Feather degradability	-0.324(0.278)	1.16	5	0.30	0.195	0.192
<u>Wear of non-melanised feathers and feather degradability (5)</u>						
Mesophilic bacteria	2.099(4.159)	0.50	3	0.65	0.226	0.130
Enterobacteria	1.765(1.801)	0.98	3	0.40	0.327	0.164
Staphylococci	-2.372(1.809)	1.31	3	0.28	0.176	0.113
Enterococci	0.881(2.033)	0.43	3	0.69	0.392	0.185
Feather degradability	0.036(0.342)	0.10	3	0.92	0.047	0.067

ESM Fig 1. Means $\pm$ CI 95% of oligopeptide concentration after 21 days of incubation of control tubes containing only buffer, the buffer plus the degrading bacteria *Bacillus licheniformis*, the buffer plus a piece (1 cm²) of feather, or the buffer plus the bacteria plus the piece of feather.

Fig. 1