Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study

Juan M. Peralta-Sanchez*,[1], Anders P. Møller[2], Antonio M. Martin-Platero[3] and Juan J. Soler[1,4]

[1]*Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas, CSIC, Carretera de sacramento s∕n, Cañada de San Urbano, E-04012 Almería, Spain;* [2]*Laboratoire d'Ecologie, Systématique et Evolution, CNRS UMR 8079, Université Paris-Sud, Bâtiment 362, F-91405 Orsay Cedex, France;* [3]*Departamento de Microbiología, Universidad de Granada, C∕ Fuentenueva s∕n, E-18075 Granada, Spain; and* [4]*Grupo Coevolución, Unidad asociada al CSIC, Granada, Spain*

Summary

1. The use of feathers as nest lining material has traditionally been explained by the thermo-regulatory properties of feathers. Feather nest lining could additionally affect nest detectability by predators, or play a role in a sexually selected context. Furthermore, feather nest lining harbours microorganisms that may influence environmental conditions where eggs and nestlings develop.

2. Microorganisms growing on nest lining feathers could affect the bacterial load of eggshells because they occupy space and∕or produce antimicrobial substances against other bacteria, including egg pathogens. Feathers of different colours are known to differ in their bacterial community (i.e. feather degrading bacteria) and, thus, colour composition of nest lining feather could also affect the bacterial environment of avian nests.

3. Here we tested this hypothesis in the barn shallow (*Hirundo rustica*) by exploring the relationship between eggshell bacterial loads and number of feathers, and the effect of experimentally modified colour composition of nest lining feathers on eggshell bacterial load.

4. In agreement with the hypothesis we found that, before treatment, the number of nest lining feathers (mainly that of unpigmented-white colour) predicted eggshell bacterial load, and that, at the end of the incubation period, eggshells of experimental nests with white feathers had a lower bacterial density than those in experimental nests with black feathers.

5. We failed to detect a relationship between bacterial load and hatching success. However, since evidence of that relationship exists for other species, these results would explain the previously detected experimental effect of colour composition of nest lining feathers on hatching success of swallows.

6. Nest design in general, and the use of nest-lining white feathers in particular, may therefore have important consequences for reproductive success of birds. The reduced eggshell bacterial loads of experimental white nests would explain preferences by barn swallows for feathers of white colour.

Key-words: eggshell bacterial load, feather colour, incubation, nest building, nest lining feathers

Introduction

Many birds use feathers as lining material (Harrison 1975; Cramp 1998; Hansell 2000) and, although it has commonly been associated with nest insulation (Møller 1984; Hilton *et al.* 2004), other hypotheses are possible. Feathers, for instance, affect nest detectability by predators (Møller

1987), but also could be a sexually selected signal of nest builders (Veiga & Polo 2005). However, most evidence is in accordance with the use of feathers as insulating nest material. For example, it has been experimentally demonstrated that in nests of barn swallows (*Hirundo rustica*) feathers affected cooling and warming rate of eggs, duration of recess periods, duration of incubation bout periods and nest attendance (Møller 1991). Therefore, an association between nest lining feathers and hatching success should

*Correspondence author. E-mail: peralta@eeza.csic.es

exist. However, hatching success could be explained not only by thermoregulatory properties of feathers, but also by incubation behaviour of adult and bacterial environmental conditions of nests (Cook *et al.* 2003, 2005a; Soler *et al.* 2009).

Nest lining feathers harbour microorganisms that in most cases are feather degrading bacteria or fungi (e.g. Pugh & Evans 1970; Shawkey, Pillai & Hill 2003; Cristol *et al.* 2005) that could infest incubating adults or growing nestlings. These microorganisms associated with feathers could also affect the probably of trans-eggshell pathogenic infection of embryos if for instance they occupy space and/or produce antimicrobial substances against egg pathogens. Growth of feather-degrading bacteria is mainly controlled or prevented by uropygial secretions that birds spread on feathers during preening (Shawkey, Pillai & Hill 2003). However, feathers, carried to nests as lining material, are not preened and consequently microorganisms would grow more quickly in nest lining feathers.

Most bacteria detected on feathers belong to the genera *Enterococcus*, *Staphylococcus* (Parisien *et al.* 2008), *Streptomyces* (Omura *et al.* 2001) and *Bacillus* (Burtt & Ichida 1999; Gunderson 2008). These bacteria are known as producers of antibiotic substances and, therefore, if present in feathers transported to the nest they could play a role in preventing the establishment of other bacteria within the nest environment. However, mainly *Enterobacteriacea*, but also *Staphylococcus* are frequently found inside dead-in-shell eggs of ducks, waterfowl, hens and turkeys, and, therefore, might also act as pathogenic bacteria (Bruce & Drysdale 1994). Among the many strains of bacteria that degrade feathers *Bacillus licheniformis* is particularly common and abundant (Burtt & Ichida 1999; Gunderson *et al.* 2008). *Bacillus licheniformis*, apart from its keratinolytic activity on avian feathers, is known to produce antimicrobial substances (Simlot, Specht & Pfaender 1972) active not only against different strains belonging to the genera *Bacillus*, *Corynebacter*, *Enterococcus* and *Mycobacterium*, but also against amoebae (Galvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel, Tendulkar & Chattoo 2004). Thus, microorganisms from nest-lining feathers and antimicrobial chemicals producers might be transferred or migrate to the eggshell preventing colonization of pathogenic bacteria of embryos by directly coming in contact with incubated eggs. If that was the case, the use of feathers as nest lining material could at least partially be interpreted by adult birds as a growing culture of bacteria (i.e. *B. licheniformis*) that diminish the probability of embryo infection (Soler *et al.* 2009) (for a similar hypothetical effect of feathers of incubating birds in contact with eggshells see Cook *et al.* 2005a; Shawkey *et al.* 2009).

Growth of *B. licheniformis* depends on feather colour (Goldstein *et al.* 2004; Grande, Negro & Torres 2004; Gunderson *et al.* 2008). The feather colour that *B. licheniformis* more easily degrades is however controversial, and although Goldstein *et al.* (2004) demonstrated that white feathers are better degraded by *B. licheniformis* (apparently because of the absence of melanin that makes feather degradation especially easy), Grande, Negro & Torres (2004) found the opposite pattern. Gunderson *et al.* (2008) trying to resolve the apparent controversy, repeated the experiments and concluded that white feathers are more easily degraded by *B. licheniformis* than melanized feathers. However, these studies used different *B. licheniformis* strains, which could explain the contradictory results. In any case, these results indicate that feathers of different colours (i.e. white vs. black), in the absence of preening (i.e. those used for nest lining), would vary in bacterial density. Therefore, if feathers in the nest affect the bacterial community of eggshells, it can be predicted that feather coloration should also affect the bacterial community on eggshells.

Here we tested this hypothesis by experimental transfer between nests of coloured (black hereafter) and white (i.e. unpigmented) nest lining feathers between nests of barn swallows. Briefly, soon after the start of incubation, we removed all feathers of a target colour (i.e. white or black) from a nest and replaced them with feathers of the other colour from previously visited nests. Thus, we had experimental nests with all lining feathers of the same colour. In order to estimate eggshell bacterial density, we sampled eggshells before and after (i.e. few days before hatching) manipulations, and also counted feathers of different coloration at the time of sampling. The hypothetical role of nest-lining feathers as a source of bacteria predicts a relationship between the number of feathers and estimates of bacterial density of the eggshells that could differ for feathers of different colour.

Materials and methods

FIELD WORK

We performed our experiment in 2008 during the breeding season of the barn swallow at Kraghede, Denmark (57° 12′ N; 10° 00′ E). For a detailed description of the study area, see Møller (1987). We visited nests twice a week to determine laying date and clutch size, and once a clutch was complete we took a sample of bacteria from the eggshell and performed the feather experiment.

The experiment was performed 2–3 days after clutch completion and consisted of randomly removing all white or black feathers from finished nests of barn swallows after clutch completion. Briefly, we first removed and counted all white and black feathers in the nest cup and, if for instance the nest was randomly assigned to become a 'white nest', we removed all black feathers and replaced them with white feathers collected from a previously sampled nest that was assigned to the 'black nests' treatment. Transfer of feathers from one nest to another, was made using single-use-sterilized paper towels to prevent further bacterial contamination. The removed black feathers were transfer to the subsequently sampled nest that was then assigned to the other experimental treatment ('black nest'), and so on. We wore latex gloves sterilized by ethanol 70% to prevent bacterial contamination between nests. Furthermore, because barn swallow nests harboured more black than white feathers, experimental black nests had almost twice the number of feathers than in white nests. Consequently, our experiment not only modified the colour composition of lining material, but also the number of feathers. A few days before hatching, we again visited the nests and counted white and black feathers present in the nest lining material. Subsequently we visited nests at the day of hatching to determine hatching success.

We have shown in another paper with this set of nests that (i) white and black nests did not differ in the number of white, black or total feathers before the experiment, (ii) that birds counteract the experimental manipulation of feather colour composition, but not that of number of feathers (i.e. black nests harboured more feathers, but a similar percentage of each colour than white nests), (iii) that number of feathers decreased during incubation, and (iv) that rate of feather renewal (number of feathers of the removed colour found in nests close to hatching time) did not differ for experimental black and white nests (J.M. Peralta-Sanchez, A.P. Møller & J.J. Soler, unpublished data). Therefore, possible experimental effects on the bacterial community on eggshells could not be exclusively related to experimental feather numbers or feather colour composition, but also to feather number and colour composition that experimental nests harboured during incubation. Consequently, in our analyses we include treatment as a fixed factor, but number of feathers as a covariate.

BACTERIAL PROTOCOL

Bacterial communities on eggshells of experimental nests were sampled twice, before the experiment and 1 or 2 days before hatching. We sampled eggshells in sterile conditions mainly to prevent between nest contaminations. We wore sterilized latex gloves with ethanol and took bacterial samples by cleaning eggshells with a sterile swab slightly wet with sterile sodium phosphate buffer (0·2 M; pH 7·2). The complete clutch was cleaned with the same swab, which was preserved in an eppendorf tube at 4 °C containing the sterile buffer until lab analyses. Estimates of bacterial load were standardized to total eggshell surface sampled by taking into account number and surface of eggs in the nests. Eggshell surface was estimated according to the formula:

$$S = 3 * L^{0.771} * W^{1.229} \text{ (Narushin 1997)}$$

where S is the surface in cm^2, L the length of the egg, and W the width of the egg. Length and width of all eggs were measured with a calliper (accuracy: 0·02 mm).

LABORATORY WORK

Eggshell samples

In the lab, samples were collected from eppendorf tubes after vigorously shaking the eppendorf in vortex (Boeco V1 Plus!) for at least three periods of 5 s. Serial decimal dilutions up to 10^{-6} were cultivated by spreading homogenously 100 μL of sample (measured with a micropipette) in plates containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar (TSA), a broadly used general medium to grow heterotrophic bacteria, and three specific 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*. Plates were incubated at 37 °C for 72 h, and afterwards the number of colonies on each plate was counted. Bacterial density was estimated as CFU (Colony Forming Units) per cm^2. Thus, we estimated bacterial density for first (soon after laying) and second (few days before hatching) samples. These counts are repeatable within the same clutch as we have shown previously using another set of nests of different species (For TSA: $R = 0.74$, d.f. $= 1,345$, $F = 2.45$, $P < 0.001$; For KF: $R = 0.79$; d.f. $= 1,345$, $F = 3.35$, $P < 0.001$;

For VJ: $R = 0.66$; d.f. $= 1,345$, $F = 1.59$, $P < 0.001$; For HK $R = 0.80$, d.f. $= 1,345$, $F = 3.60$, $P < 0.001$).

STATISTICAL METHODS

The numbers of white and black feathers and the total number of feathers approximately followed a normal distribution (Kolmogorov–Smirnov tests for continuous variables, $P > 0.15$). Frequencies of bacterial loads in media for heterotrophic bacteria (TSA) were approximately normally distributed after log_{10}-log_{10}transformation (Kolmogorov–Smirnov tests for continuous variables, $P > 0.2$). Counts of bacterial colonies in specific medium for *Enterobacteriaceae* (HK) and for *Staphylococcus* (VJ) differed significantly from normality, mainly due to bimodal distributions or because bacterial growth was only detected for approximately half of the samples, respectively. We failed to transform these bacterial counts (mainly those from the second sampling (i.e. nests close to hatching)) to a normally distributed variable, and in our analyses we thus used ranked values. In specific media for *Enterococcus* (KF), we only obtained colonies of bacteria from a single sample, and, consequently, we did not use this variable in subsequent analyses.

Estimates of eggshell bacterial loads at the end of the incubation period might depend on bacterial loads at the beginning of incubation, and, consequently, the effect of experimental treatment on eggshell bacterial density should in that case be corrected for estimates of eggshell bacterial load before the experiment. However, only bacterial counts in TSA ($R^2 = 0.16$, $N = 32$, $P = 0.023$), but not in VJ ($R^2 = 0.09$, $N = 32$, $P = 0.09$) or HK culture media ($R^2 = 0.07$, $N = 32$, $P = 0.14$) at the beginning and at the end of the incubation period were significantly positively related. In any case, we have performed analyses using between-sampling differences in bacterial counts to control for the effect of the experiment for eggshell bacterial density in nests before experimental treatment.

The effects of experimental treatment and covariates on normalized or ranked dependent variables were tested by using General Lineal Models. Sample sizes differed slightly for different analyses because eggs of five nests hatched before the second sampling, one nest was abandoned and two additional nests fell down from the wall before we collected the second samples. All statistical tests were two-tailed and performed with the software STATISTICA 8·0 (Stat Soft, Inc., Tulsa, Ok, USA).

Results

BACTERIAL LOAD AT THE START OF INCUBATION

Cultures in heterotrophic medium (TSA) of collected samples at the start of the incubation revealed the existence of bacteria in all swallow nests at the beginning of the incubation ($N = 40$). Estimated bacterial densities greatly varied among nests (colonies per cm^2: minimum $= 0.36$; maximum $= 616187.1$) with a mean value (SE) of 41975·2 (23336·8) colonies per cm^2 (median $= 5.4$ colonies per cm^2). Bacterial growth in specific cultures showed that *Staphylococcus* grew in 22 of the 40 samples, while *Enterococcus* did not grow in 1 of the 40 samples. Estimated bacterial density of *Enterobacteriaceae* was quite high and variable [mean (SE) 49680·0 (27952·8); median $= 0.4$ colonies per cm^2], while those of *Staphylococcus* was quite low [mean (SE) 0·06 (0·01); median $= 0.03$ colonies per cm^2].

Nests of barn swallows selected for different treatments did not differ significantly in the estimates of eggshell bacterial load, in general (MANOVA, Wilks = 0·99, F = 0·07, d.f. = 3,36, P = 0·97), or for any of the used culture media (TSA: F = 0·14, d.f. = 1,38, P = 0·71; VJ: F = 0·10, d.f. = 1,38, P = 0·76; HK: F = 0·01, d.f. = 1,38, P = 0·90). These bacterial counts were negatively related to the number of feathers in the nest (MANOVA, Wilks = 0·81, F = 2·90, d.f. = 3,36, P = 0·048; Fig. 1). When separately considering feathers of different colours in a multiple regression approach, we found that the number of white feathers (MANOVA, Wilks = 0·79, F = 3·18, d.f. = 3,35, P = 0·036), but not that of black feathers (MANOVA, Wilks = 0·96, F = 0·50, d.f. = 3,35, P = 0·68) significantly explained bacterial load of eggshells at the start of incubation independently of culture media used for estimations (Table 1). Finally, the percentage of white feather in nests before treatment did not significantly explain bacterial load neither in
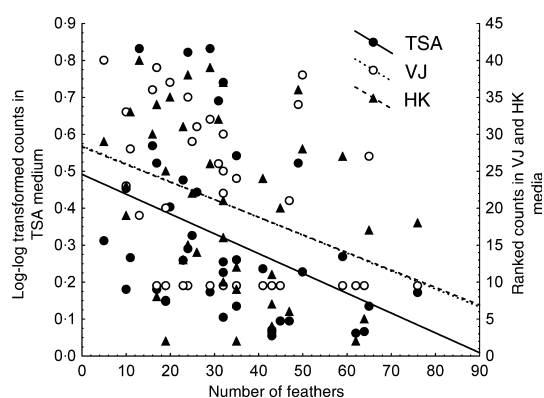
general (MANOVA, Wilks = 0·96, F = 0·52, d.f. = 3,36, P = 0·67) nor for any of the used culture media (F < 1·44, d.f. = 1,38, P > 0·23).

BACTERIAL LOAD AT THE END OF THE INCUBATION

Cultures in TSA of collected samples a few days before hatching revealed the existence of bacteria in all swallow nests at the beginning of the incubation (N = 32). Estimated bacterial densities greatly varied among nests (colonies per cm^2: minimum = 0·11; maximum = 610067·9) with a mean value (SE) of 33216·1 (20252·3) colonies per cm^2) (median = 6·8 colonies per cm^2). Bacterial growth in specific cultures revealed that *Staphylococcus* were present in 12 of the 32 samples, while *Enterococcus* did not grow in 4 of the 32 samples. Estimated bacterial density of *Enterobacteriaceae* was quite high and variable [mean (SE) = 21804·7 (15785·4); median = 0·1 colonies per cm^2], while those of *Staphylococcus* was quite low [mean (SE) 1·7 (1·4); median = 1·13 colonies per cm^2].

A few days before hatching, experimental treatment explained a significant proportion of variance in bacterial counts in general (MANOVA, Wilks = 0·57, F = 6·82, d.f. = 3,27, P = 0·001), or for estimates in all culture media, even after controlling for the number of feathers in the nest at the time of hatching (Table 1). While eggshells of the experimental white nests tend to have lower estimates of bacterial density (Fig. 2), the number of feathers and bacterial density estimated for some of the culture media were negatively related (Table 1). The effect of number of feathers on eggshell bacterial load at the end of the incubation was however lower than that detected at the beginning of the incubation since, when the effect of the experimental treatment was removed from the model, the number of feathers failed to explain any of the dependent variables (bacterial counts) (MANOVA, Wilks = 0·87, F = 1·42, d.f. = 3,28, P = 0·26; ANOVA tests:

Fig. 1. Relationships between number of feathers in nests of barn swallows at the start of incubation and eggshell density of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK). Lines are regression lines.

Table 1. Effects of changing colour composition treatment and number of total feathers (model 1) and number of white and black feathers (model 2) on load of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) on eggshells, before and after treatment (i.e. at the end of incubation)

|	TSA			VJ			HK		
	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P
Before treatment									
Number of white feathers	−0·35 (0·16)	4·85	0·034	−0·39 (0·16)	6·03	0·019	−0·46 (0·16)	8·58	0·006
Number of black feathers	−0·15 (0·16)	0·92	0·343	−0·09 (0·16)	0·35	0·558	−0·02 (0·16)	0·01	0·910
After treatment									
Model 1	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P
Number of feathers	−0·34 (0·17)	4·22	0·049	−0·40 (0·18)	4·86	0·036	−0·28 (0·15)	3·46	0·07
Treatment	0·53 (0·17)	9·88	0·004	0·23 (0·18)	1·57	0·22	0·67 (0·15)	19·37	0·0001
Model 2	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P
Number of white feathers	−0·14 (0·18)	0·58	0·453	−0·17 (0·19)	0·71	0·41	−0·10 (0·17)	0·38	0·544
Number of black feathers	−0·38 (0·18)	4·28	0·048	−0·44 (0·20)	4·92	0·035	−0·32 (0·17)	3·56	0·069
Treatment	0·51 (0·17)	9·02	0·006	0·21 (0·19)	1·34	0·26	0·66 (0·16)	17·88	0·0002
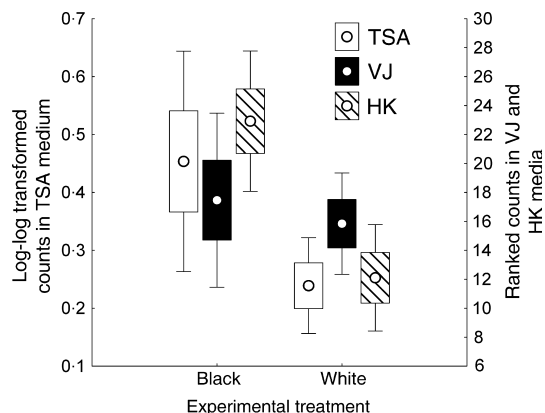
Fig. 2. Density (means, SE, and 95% confidence intervals) of load of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) on eggshells at the end of the incubation period in experimental white and black nests of barn swallows.

TSA: $F = 0.91$, d.f. $= 1,30$, $P = 0.35$; VJ: $F = 3.54$, d.f. $= 1,30$, $P = 0.07$; HK: $F = 0.13$, d.f. $= 1,30$, $P = 0.73$).

Our experimental approach resulted in experimental black nests harbouring almost twice as many feathers as experimental white nests, and, therefore, the effect of feather-colour treatment could be confounded by the effect of experimental feather number. However, several pieces of information suggest that this is not the case. First, in the model that included the experimental treatment (fixed factor), number of experimental feathers (those included in the nests at the time of performing the experiment), and number of feathers in nests at the end of the incubation (covariates), only the fixed factor (MANOVA, Wilks $= 0.65$, $F = 4.60$, d.f. $= 3,26$, $P = 0.01$), but none of the feather counts (MANOVA, Wilks > 0.90, $F < 1.00$, d.f. $= 3,26$, $P > 0.40$), explained a significant proportion of variance in eggshell bacterial density. Second, when removing the effect of experimental treatment from the previous model, none of the feather counts explained bacterial counts in general (MANOVA, Wilks > 0.86, $F < 1.48$, d.f. $= 3,27$, $P > 0.24$). Third, when we separately analyzed the effect of treatment (white vs. black nests) and that of experimental number of feathers on eggshell bacterial loads at the end of the incubation period, the former (MANOVA, Wilks $= 0.59$, $F = 6.36$, d.f. $= 3,28$, $P = 0.002$), but not the latter (MANOVA, Wilks $= 0.85$, $F = 1.61$, d.f. $= 3,28$, $P = 0.21$) factor explained a significant proportion of variance. Furthermore, when the model exclusively included these two associated factors, the results did not change (Treatment: MANOVA, Wilks $= 0.63$, $F = 5.12$, d.f. $= 3,27$, $P = 0.006$; Feather number: MANOVA, Wilks $= 0.91$, $F = 0.84$, d.f. $= 3,27$, $P = 0.48$). Finally, we tested for robustness of results related to experimental treatment by sequentially excluding from the analyses pairs of cases with the most extreme (i.e. positive and negative) values, and estimating effect sizes (partial-eta squared) associated with the treatment effect in a model that included number of feathers at hatching. Since effect sizes do not depend on sample sizes, a negative relationship between effect sizes and degrees of

freedom should appear if differences in number of feathers between experimental white and black nests were important explaining detected effect size of experimental treatment. However, estimates of effect sizes of the 10 first models (i.e. reducing degrees of freedom from 28 to 8) were not associated with degrees of freedom ($R = -0.02$, $N = 10$, $P = 0.953$). Therefore, all these analyses suggest that the detected experimental treatment effect on bacterial density was independent of the larger number of feathers in experimental black compared to white nests.

The effect of experimental treatment was still significant in explaining bacterial load on eggshells, even when separately considering the number of white and black feathers in the close-to-hatching nests of barn swallows (Table 1). In addition, the number of black (MANOVA, Wilks $= 0.82$, $F = 1.86$, d.f. $= 3,26$, $P = 0.16$), but not that of white (MANOVA, Wilks $= 0.97$, $F = 0.26$, d.f. $= 3,26$, $P = 0.85$) feathers explained an additional proportion of variance in bacterial counts (Table 1). The numbers of white and black feathers in nests at the time of the second sampling were significantly negatively related [Beta (SE) $= -0.37$ (0.16), $F = 5.68$, d.f. $= 1,35$, $P = 0.023$]. When removing the number of black feathers in the nest from the model, the effect of number of white feathers on bacterial counts were still far from significant ($F < 0.08$, d.f. $= 1,29$, $P > 0.78$). Therefore, the detected negative effects of the number of black feathers on bacterial density were not due to its association with the number of white feathers. Finally, the percentage of white feathers in close-to-hatching nests did not explain bacterial load neither in general (MANOVA, Wilks $= 0.95$, $F = 0.47$, d.f. $= 3,27$, $P = 0.70$) nor for any of the used culture media ($F < 1.40$, d.f. $= 1,29$, $P > 0.24$).

DIFFERENCES IN EGGSHELL BACTERIAL LOADS BETWEEN THE TWO SAMPLING PERIODS

In accordance with the detected effects of the experimental treatment on eggshell bacterial load, we found that after controlling for the effect of differences between the two sampling periods in number of feathers present in the nest, detected differences in heterotrophic (TSA; $F = 5.56$, d.f. $= 1,29$, $P = 0.025$), but not *Enterobacteriaceae* (HK; $F = 3.41$, d.f. $= 1,29$, $P = 0.075$) or *Staphylococcus* (VJ; $F = 1.50$, d.f. $= 1,29$, $P = 0.23$) was explained by the effect of experimental treatment.

BACTERIAL LOAD AND HATCHING SUCCESS

None of the variables describing eggshell bacterial loads at the beginning or at the end of incubation, or differences in eggshell bacterial loads between the two sampling periods, explained the probability of hatching failures in nests of barn swallows [Generalized Lineal Models, Binomial distribution and log-link function, univariate analyses; Wald Statistic (minimum – maximum) $= 0.007–0.76$, d.f. $= 1$, $P > 0.38$]. However, when including all three kinds of bacterial counts in the same multivariate models, heterotrophic (TSA: Wald

Statistic = 3·19, $P = 0.074$) and *Enterobacteriaceae* (HK: Wald Statistic = 3·00, $P = 0.083$) counts, but not that of *Staphylococcus* VJ: Wald Statistic = 0·02, $P = 0.88$) tended to explain the probability of hatching failures. Swallow nests with higher density of heterotrophic bacteria [TSA; partial regression coefficient (SE) = −4·01 (2·47)] but lower density of *Enterobacteriaceae* [HK; partial regression coefficient (SE) = 0·09 (0·05)] tended to experience higher risk of hatching failures. Multiple regression analyses including bacterial loads at the end of incubation, or differences in eggshell bacterial loads between the two sampling period, did not show any tendency explaining the probability of hatching failure [Wald Statistic (minimum – maximum) = 0·03–0·75, d.f. = 1, $P > 0.41$].

Discussion

We have shown an important role of feathers explaining bacterial density on the eggshells of barn swallows. The main results suggesting such an association are (i) a negative relationship between number of feathers and eggshell bacterial load at the start of the incubation period that was mainly explained by the number of white feathers; (ii) a significant effect of experimental modification of feather colour composition of nests of barn swallows at the beginning of incubation on eggshell bacterial loads estimated at the end of incubation; and (iii) the effect of number of feathers on eggshell bacterial load was weaker at the end of incubation and, contrary to that detected at the beginning of incubation, was mainly related to number of black feathers in the nests. Below we discuss these results in relation to the hypothetical function of feathers in controlling bacterial infection of eggshells.

Several possible scenarios predict a relationship between feather nest lining and bacterial density of eggshells of birds (see Introduction). Feathers could be a source of bacteria transferred to eggshells and, therefore, increase the probability of eggshell bacterial colonization. Furthermore, some bacteria growing on feathers (i.e. keratinolitic bacteria) are antibiotic producing microorganisms that when in contact with eggs could transfer chemicals to eggshells thereby preventing establishment of other bacteria. These two possible effects (colonization, or chemical transfer to eggshells) would result in an increased probability of successful hatching if it affects probability of eggshell colonization by pathogenic bacteria for embryos (see Soler *et al.* 2009). We found that the number of nest lining feathers at the beginning or the end of incubation was negatively related to bacterial loads of eggs at these stages of the breeding cycle, which therefore could suggest that antibiotics produced by bacteria or other microorganisms living in nest lining feathers could explain these negative relationships.

Another possible explanation for the negative relationship is that nest lining feathers are nest material with a reduced density of microorganisms capable of growing on the eggshells. Therefore, a larger number of feathers would contribute to a more sterile nest environment. However, this explanation is unlikely because keratinolitic bacteria, but also

other sometimes pathogenic bacteria, are commonly found growing on feathers (Shawkey, Pillai & Hill 2003; Gunderson 2008). Moreover, birds prevent nest feather contamination of microorganisms by preening (Shawkey, Pillai & Hill 2003; Soler *et al.* 2008), but nest lining feathers are unprotected, which predicts an even larger bacterial load of nest lining feathers in comparison with active feathers on birds. Finally, it is also possible that the negative relationship between number of feathers and eggshell bacterial load was not due directly to feathers or microorganisms living on them, but to the antimicrobial chemicals of the uropygial secretion that nest lining feathers probably included. However, birds preen feathers several times per day and, thus, a long-term effect of uropygial secretions preventing bacterial growth is unlikely. Consequently, preen secretions on nest lining feathers would in any case have a limited effect on eggshell bacterial density. Therefore, we believe that the more likely hypothesis explaining the association between number of feathers and eggshell bacterial load is related to the beneficial effect of bacteria living in nest lining feathers. However, detailed studies of the bacterial community of nest lining feathers and their antimicrobial properties in relation to bacterial load detected in the eggshell are necessary before reaching firm conclusions.

There is evidence suggesting that bacterial growth differs for feathers of different colours (see Introduction). To test the hypothesis that nest lining feathers of different colours affected eggshell bacterial load differentially, we first related the number of nest lining feathers of different colour to eggshell bacterial load. Furthermore, we experimentally modified feather colour composition of nests of barn swallows and explored the effect on eggshell bacterial load at the end of incubation. In accordance with the hypothesis, we found that the number of white, but not of black feathers explained eggshell bacterial load at the beginning of incubation. Furthermore, we found a significant treatment effect that was in accordance with the negative relationship between number of white feathers and bacterial load previously mentioned because at the end of incubation eggshells of experimental white nests harboured lower bacterial density than that of experimental black nests. Nests having the black treatment also received a larger number of feathers than experimental white nests (see Material and methods) and, thus, the detected treatment effect could be due to between nest differences in number of feathers rather that to experimental modification of feather colour composition. However, when statistically correcting for the number of experimental feathers each nest received, as well as the number of nest lining feathers found in nests of barn swallows at the end of incubation (see Results), these variables did not significantly explain bacterial load while the effect of experimental treatment did have such an effect (see Results). Therefore, experimental feather colour composition, but not experimental number of feathers was the cause of the detected experimental treatment.

In addition to the detected treatment effect explaining eggshell bacterial load, we found that the number of black, rather than the number of white feathers explained a significant proportion of variance in bacterial load at the end of the

incubation. This result, together with previous ones, could suggest a beneficial effect of feathers of black colour when present in nests at the end of incubation, while feathers of white colour would be more beneficial during laying and at the start of incubation. We lack a robust hypothesis that could explain these differences, but because shells of unincubated eggs are more prone to infection than incubated ones (Cook *et al.* 2005a; Shawkey *et al.* 2009), the hypothetical protection of nest lining feathers should be more important for early than for late incubation. In accordance with this possibility, the significant effect of feathers on eggshells bacterial density at the end of incubation disappeared when treatment was not included in the model (see Results). Consequently, it is likely that the effect of feathers preventing eggshell infections was more important for white than for black feathers.

White feathers from live chickens were more rapidly degraded *in vitro* by *B. licheniformis* than black feathers and they supported higher bacteria growth (Goldstein *et al.* 2004; Gunderson *et al.* 2008). It has been suggested that this variation could be explained by the effect of melanin that could blind keratin and make feathers more difficult to attack by bacterial keratinases (Goldstein *et al.* 2004; Gunderson *et al.* 2008), or by the direct negative effect of melanin on bacterial growth (Suh & Lee 2001; Goldstein *et al.* 2004). However, Grande, Negro & Torres (2004) found the opposite pattern with black feathers being more degraded by *B. licheniformis* than white ones. Different *Bacillus* strains could therefore be adapted to grow better on feathers of different colours (Grande, Negro & Torres 2004). Black and white feathers may then harbour different bacterial loads and/or communities that could be transmitted to the eggshell directly, or just the antimicrobial chemical, therefore explaining differences in bacterial load of eggshells in nests that varied in colour composition of lining feathers. In any case, further work is necessary to explore antimicrobial properties of the bacterial community of white and black feathers to know the underlying causes of the detected effect of the experiment of colour composition of nest lining feathers.

The results presented here may have important consequences. We estimated bacterial density of heterotrophic bacterial in general, but also of *Staphylococcus* and *Enterobacteriaceae*; two groups of bacteria that are mainly considered egg pathogens (Bruce & Drysdale 1994) (but see Introduction). Bacterial load on eggshells increase the risk of trans-shell infection and egg viability (Cook *et al.* 2003, 2005a,b). *Staphylococcus* sp. and *Enterobacteriaceae*, as the commonest bacteria found in unhatched eggs are saprophytic and opportunistic bacteria (Houston, Saunders & Crawford 1997; Singleton & Harper 1998; Cook *et al.* 2005a) that live in skin, hair and feathers of mammals and birds (Krieg & Holt 1984), and they are known to be pathogenic for avian embryos (Bruce & Drysdale 1994). Our results showed a negative association between density of these opportunistic and/or pathogenic bacteria and the number of feathers in the nest as well as experimental treatment. This scenario predicts a relationship between nest lining feathers and hatching success that should be mediated by differential bacterial load of eggshells.

In a previous paper (Peralta *et al.* unpublished data) we analyzed the effect of our experimental treatment and number of feathers of different colours on hatching success and found support for the predicted relationship. Experimental white nests with larger number of added white feathers experienced a lowest probability of hatching failures. In that article we did not explore the effect of eggshell bacterial load on egg hatchability, but directly the effect of the experiment, together with information on the number of white feathers experimentally removed (or added) from swallow nests. Here, however, we failed to find a close relationship between probability of hatching failure and eggshell bacterial load, and, consequently, we cannot conclude that the previously detected effect of feathers on hatching success is mediated by the relationship between nest-lining feathers and eggshell bacterial load. In any case, the detected negative effect of feathers on bacterial load of eggshells of swallows suggests that it should be the case. We estimated bacterial density for only four groups of bacteria, but nest lining feathers could be associated with other groups of bacteria more closely related to the probability of hatching failure. Therefore, information from a concise study of bacterial load on feathers used as lining material, as well as the antimicrobial properties of these bacteria, is necessary before discussing the expected association between nest lining feathers, nest bacterial environment, and hatching success.

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