IBIS The International Journal of Avian Science

Ibis (2012), **154**, 725–737

Avian life history traits influence eggshell bacterial loads: a comparative analysis

JUAN MANUEL PERALTA-SÁNCHEZ,[1,2]* MANUEL MARTÍN-VIVALDI,[2,3] ANTONIO MANUEL MARTÍN-PLATERO,[1,2,4] MANUEL MARTÍNEZ-BUENO,[1,2] MARTA OÑATE,[5] MAGDALENA RUIZ-RODRÍGUEZ[2,6] & JUAN JOSÉ SOLER[2,6]

[1]*Departamento de Microbiología, Universidad de Granada, Granada, Spain*

[2]*Grupo Coevolución, Unidad Asociada al CSIC, Universidad Granada, Granada, Spain*

[3]*Departamento de Zoologia, Universidad de Granada, Granada, Spain*

[4]*Polz Lab, Parsons Laboratory, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA*

[5]*Departamento de Biología Vegetal, Universidad de Barcelona, Barcelona, Spain*

[6]*Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (EEZA- CSIC), Almería, Spain*

Selection pressures due to parasitism play an important role in driving the evolution of life history traits of birds in general and of behaviour at the nest in particular. Eggshell bacterial load has been shown to predict hatching failure (i.e. the probability of embryo infection) but the relationships between the bacterial environment of the nest and life history characteristics of birds remain poorly investigated. We explored interspecific variation in eggshell bacterial load of mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae* groups across 24 bird species and assessed whether bacterial load is associated with breeding traits. Interspecific variation was much higher than intraspecific variation for all measures of bacterial load even after controlling for annual variation. Thus, we were able to assess the correlation between bacterial community characteristics and life history traits. After correcting for phylogenetic effects, we found that nest type, the use of feathers or plants as lining material, and incubation behaviour explained a significant proportion of the variance in bacterial communities on eggshells. The strength of these associations depended on study year, suggesting an important role of environmental conditions for eggshell bacterial load or community. Overall, these results suggest that bacteria on eggshells are associated with bird species traits, probably because birds are mediating the deleterious effect of eggshell microbes through behavioural traits that modify bacterial load.

Keywords: bacterial community, comparative approach, incubation behaviour, nest breeding behaviour.

Selection pressures due to parasitism play an important role in driving the evolution of life history traits of birds in general (Stearns 1992) and of behaviour at the nest in particular (Deeming 2002). Accordingly, some reproductive and behavioural features of birds have been interpreted as minimizing the probability of offspring infection by opportunistic pathogens (Clark & Mason 1985, Christe *et al.* 1994, Merilä & Allander 1995, Singleton & Harper 1998). However, empirical tests exploring the effects of microorganisms such as bacteria and viruses on the evolution of avian life history traits are scarce (Hansell & Deeming 2002, Archie & Theis 2011). Bacteria are ubiquitous in avian nests and on eggshells (Singleton & Harper 1998), and opportunistic pathogens cross eggshells and membranes and cause embryo mortality (Pinowski *et al.* 1994, Houston *et al.* 1997, Stewart & Rambo 2000). The probability of trans-shell infection

*Corresponding author.
Email: jmps@ugr.es

increases with the abundance of microorganisms on the eggshell (Cook *et al.* 2003, 2005b, Shawkey *et al.* 2009) and thus should select for traits that diminish bacterial load, and therefore the probability of trans-shell infection with pathogenic bacteria (Wellman-Labadie *et al.* 2008a,b, Shawkey *et al.* 2009).

Several environmental factors, such as nest type and moisture levels, have been shown to influence eggshell bacterial load and are potential targets of selection to reduce the probability of trans-shell embryo infection. For instance, it is known that nest type and moisture may affect bacterial colonization and load on eggshells, as experimental eggs in open nests had higher trans-shell infection rates than those in hole nests (Godard *et al.* 2007). Nest material may also affect the bacterial environment of nests, given that different nest materials may harbour different bacterial communities, as Goodenough and Stallwood (2010) have shown for two common and related hole-nesting bird species, Blue Tit *Cyanistes caeruleus* and Great Tit *Parus major*. Material used for lining the nest cup may indirectly influence the bacterial environment of nests through its effects on thermal conditions (Mertens 1977). In addition, nest material may directly affect the bacterial environment of nests through antibacterial properties. For instance, some plants that European Starlings *Sturnus vulgaris* select for nest building have antimicrobial properties and therefore may suppress bacterial proliferation (Clark & Mason 1985). Feathers are commonly used by birds for lining their nests (Cramp 1998) and it has recently been shown that the abundance of unpigmented feathers was negatively related to bacterial density on the eggshells of Barn Swallows *Hirundo rustica* (Peralta-Sánchez *et al.* 2010). Such effects may be mediated by the antimicrobial properties of uropygial oil on these feathers (Shawkey *et al.* 2003). Moreover, it is known that bacterial communities that inhabit nest-lining feathers produce antimicrobial substances themselves (Soler *et al.* 2010), which might also prevent the establishment of pathogenic bacteria on the eggshells of birds (Peralta-Sánchez *et al.* 2010, Soler *et al.* 2010) and increase hatching success (Peralta-Sánchez *et al.* 2011). Thus, there are good reasons to suggest that nest building behaviour of birds modulates and controls bacterial loads and communities on eggshells and, hence, reduces the probability of embryo infection.

Incubation behaviour is also known to reduce bacterial density and diversity, and modulate the growth of bacteria on the eggshells of birds (Cook *et al.* 2003, Shawkey *et al.* 2009). This could be mediated by the effect of incubation reducing eggshell humidity (D'Alba *et al.* 2010) or increasing temperature (Ruiz-de-Castañeda *et al.* 2011) and, consequently, early onset of incubation can be seen as an adaptive behaviour reducing the time that the first laid eggs remain unprotected in the nest from bacterial colonization (Cook *et al.* 2003, 2005a, Wang *et al.* 2011). Bird species show interspecific variation in the onset of incubation (Cramp 1998) and this variation may be related to interspecific differences in bacterial load and to changes in bacterial loads on eggshells during the incubation period (Ruiz-de-Castañeda *et al.* 2011, Wang *et al.* 2011).

Species-specific nesting behaviour may influence the bacterial load on eggs. Thus, comparison of the eggshell bacterial community across species should enable the detection of those life history traits that evolved to mediate the bacterial environment of nests. We assessed such relationships between life history traits (associated with behaviours at the nest) and eggshell bacterial load of 24 species of birds in two different study years (19 species in both years). We first assessed whether variation in bacterial density on the eggshell of different species of birds was larger than variations within bird species (species effect), which is a requisite for performing comparative analyses. We then assessed year effects on estimates of bacterial load on eggshells as evidence of environmental conditions affecting the bacterial community of eggshells. Finally, we assessed interspecific associations between eggshell bacterial load and its change during incubation as response variables, and nesting habits (hole- vs. open-nesting species), the use and type of lining material (plants or feathers), and the onset of incubation (before or after clutch completion) as predictors.

METHODS

The study was performed during the breeding seasons of 2006–2007 in Hoya de Guadix, Spain (37° 18'N, 3°11'W; 27 × 25 km), a high-altitude plateau 1000 m a.s.l., with a semi-arid climate. Typical vegetation in the area includes cultivated crops, olive and almond plantations, sparse Holm Oaks

Quercus ilex remaining from the original Mediterranean forest, small shrubs in abandoned fields and deciduous trees in temporary streams and villages. Variables describing climatic conditions during the study period are included in Appendix 1. About 600 nestboxes were present in the study area from previous years and these were checked twice weekly during March–June (for more details see Martín-Vivaldi *et al.* 2006). Nests of birds that did not breed in boxes were detected by intensively searching suitable habitats in the study area.

Information on nest-site (open vs. hole nests), use of plants or feathers as lining material and onset of incubation (before or after clutch completion) of different bird species was collected from Cramp (1998) and is given in Appendix 2.

Bacterial sampling protocol

Bacteria on the eggshells were sampled twice, once at the beginning of the incubation period (2–3 days after clutch completion, which ensured that all the eggs were incubated), and again a few days before hatching. Once a new nest was found, we visited it every 2–3 days, which allowed us to estimate laying date and clutch size. We obtained data from 281 nests in 2006 and 275 nests in 2007, with at least three nests sampled per species. Some nests, however, were predated before the second sampling visit and thus sample sizes of eggshell bacterial loads at the beginning of incubation and those of change in bacterial load of the eggshells during incubation differed. From these pools of nests and species, 19 species of birds were sampled in both years at the beginning of incubation and 15 species were sampled in both years for change in bacterial density between the first and second sample. Appendix 2 summarizes data collected over the two study years.

Samples were taken in the field whilst trying to maintain sterile conditions and prevent inter-nest contamination using new latex gloves washed with 96% ethanol for each nest. When gloves were dry, we gently handled the eggs and cleaned the complete eggshell with a sterile swab, slightly dampened with sterile sodium phosphate buffer (0.2 M; pH 7.2). All the eggs in the clutch were sampled with a single swab that afterwards was put in a microcentrifuge tube containing 1.2 mL of sterile phosphate solution and stored in a portable cooler at 4–6 °C (the detailed protocol is published in Peralta-Sánchez *et al.* 2010).

Estimates of bacterial load were standardized to the number of colonies grown from inoculations (colony-forming units, CFUs) in growing media (see below) per cm^2 by taking into account the number and area of the sampled eggs in the nests. Eggshell area was estimated according to the formula:

$$S = (3.155 - 0.0136 * L + 0.0115 * W) * L * W$$

from Narushin (2005), where S is the area 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).

Eggshell bacterial change (%) was calculated as the difference in bacterial density estimated at the end and at the beginning of the incubation period divided by bacterial load at the beginning of incubation. A decrease in bacterial density from the beginning to the end of the incubation period therefore results in a negative value for bacterial change during incubation.

Laboratory protocol

Samples were collected from microcentrifuge tubes after vortexing for at least three periods of 5 s. Serial dilutions (by 10-fold in each dilution), carried out until a dilution factor of 6 was achieved, were necessary to be able to count bacteria colonies in the Petri dishes. Microorganism cultivation was performed by spread-plating 100 μL of the series into four Petri dishes, each containing a different, sterile solid growth medium (Scharlau Chemie S.A., Barcelona, Spain). We used trypticase soy agar (TSA), a broadly used general medium, to grow mesophilic bacteria, and three selective media: Kenner fecal agar (KF) for growing bacteria in the genus *Enterococcus*; Vogel–Johnsson agar (VJ) for bacteria in the genus *Staphylococcus*; and Hecktoen enteric agar (HK) for Gram-negative bacteria in the family *Enterobacteriaceae*. These media should adequately characterize the relative load of bacterial groups living on the avian eggshell that are known to produce pathogenic infection of embryos (Peralta-Sánchez *et al.* 2010, Soler *et al.* 2011). Dishes were incubated at 32 °C for 72 h, and afterwards the CFUs on the dish were counted.

Mainly because of logistical problems and accumulation of samples during the breeding season, not all samples were stored at 4 °C for the same

period. Although sample storage has traditionally been considered as a major problem in environmental microbiology, Lauber *et al.* (2010) have shown recently that the relative abundances of most bacterial taxa from soil samples were largely unaffected by temperature (i.e. at 20.4 °C, −20 °C and −80 °C) even after 14 days of storage. Our samples were stored for 8 days on average (mean ± se = 7.94 ± 0.27) and neither sampling date, storage time nor its interaction significantly explained bacterial counts (GLM, mesophilic bacteria at the beginning of incubation as dependent variable; avian species identity as random factor, $F_{18,502} = 4.93$, $P < 0.001$; year as factor, $F_{1,502} = 0.90$, $P = 0.344$; sampling date as covariate, $F_{1,502} = 1.78$, $P = 0.182$; storage time as covariate, $F_{1,502} = 0.11$, $P = 0.740$; interaction, $F_{1,502} = 0.12$, $P = 0.733$). Moreover, the duration of the storage period did not affect rank position of different bird species, as shown by a comparison of ranked values of mesophilic bacterial loads of 21 bird species from which we collected samples in 2006 (the same 19 species sampled in both years plus two additional species sampled only in 2006) that were stored for less than 3 days ($n = 120$) and others that were stored for up to 1 month ($n = 156$) (Kendall coefficient of concordance = 0.95; average Spearman rank correlation = 0.91, Friedmann ANOVA, $\chi^2 = 38.13$, $P = 0.009$). Consequently, we are confident that variation in storage time of samples did not affect our results.

In 2011, we performed a series of field and laboratory controls to detect possible environmental and/or laboratory contamination. In the field, coincident with visits to 42 nests of Magpies *Pica pica*, Hoopoes *Upupa epops* and Spotless Starlings *Sturnus unicolor* at the egg-laying stage, we aired the swab for 10 s and stored it in a microcentrifuge tube. Within the next 24 h, samples were cultured in the lab. No bacteria were detected in 32 of the samples, whereas one and two colonies were detected in six and four samples, respectively. In the lab, we also performed two different negative controls at the time of culturing field-collected samples. The first control consisted of spreading phosphate buffer directly onto TSA plates (phosphate control). As a second negative control, we opened a TSA plate in our flow chamber and then incubated it (chamber control). No bacteria grew in any TSA control plates. Thus, our estimates of eggshell bacterial loads were not affected by external contamination.

Culture-based techniques do not characterize microbial communities as do molecular techniques, as only around 1% of microorganisms are cultivable (Amann *et al.* 1995), but culture-independent methods may also have limitations, bias and errors (Qiu *et al.* 2001, Speksnijder *et al.* 2001, Shawkey *et al.* 2005). Interestingly, the use of both methodologies has resulted in very similar conclusions when exploring the effects of incubation on eggshell bacterial communities (Cook *et al.* 2005a, Shawkey *et al.* 2009), which support the use of culture-based methods in our study.

Data analysis

To test for the effects of avian species identity and the random effect of study year, bacterial loads at the beginning of incubation, as well as the estimates of change in bacterial load during incubation (hereafter bacterial change) were rank-transformed within each type of medium.

For comparative analyses, we calculated the geometric mean of eggshell bacterial loads estimated per avian species at the beginning of incubation and their bacterial changes. The use of geometric mean values per species in comparative analyses is justified because variation among species is much greater than variation within species in bacterial counts, even after controlling for year effects (see Results and Table 1). The distribution of standardized values (i.e. mean = zero, variance = 1) of log-transformed geometric means did not differ from normality. Because of the detected effect of year on the response variables, we repeated the analyses for 2006 and 2007 separately.

Values of life history traits assigned to different species of birds, or estimates of eggshell bacterial densities and bacterial changes, cannot be considered statistically independent data points in the analyses because, due to common ancestry, species that are phylogenetically closely related are also those with more similar life histories (Harvey & Pagel 1991). We therefore took into account the phylogenetic relationships among avian species (see the phylogeny used in Appendix 3), based on Sibley and Ahlquist (1990) and Jonsson and Fjeldsa (2006) for our comparative analyses. To control for possible effects of common ancestry, we performed phylogenetic generalized least-squares regression (PGLS) analyses (Pagel 1997, 1999) as implemented in the R statistical

Table 1. Results of GLMs testing the effects of year (random factor) and avian species identity (fixed factor) on rank-transformed eggshell bacterial density estimated at the beginning of incubation (1) and change in bacterial load (BC) during incubation. We show univariate results for mesophilic bacteria (TSA 1 and BC TSA), *Enterococcus* spp. (KF 1 and BC KF), *Staphylococcus* spp. (VJ 1 and BC VJ) and *Enterobacteriaceae* (HK 1 and BC HK). Number of sampled nests is given in parentheses as a subscript to the dependent variable names.

	Factor	df	*F*	*P*		Factor	df	*F*	*P*
TSA $1_{(556)}$	Avian Species	18	8.63	0.001	BC $TSA_{(330)}$	Avian Species	14	2.15	0.010
	Year	1	132.51	0.001		Year	1	5.33	0.022
	Avian Species*Year	18	3.42	0.001		Avian Species*Year	14	1.56	0.091
KF $1_{(556)}$	Avian Species	18	14.68	0.001	BC $KF_{(330)}$	Avian Species	14	2.48	0.002
	Year	1	61.97	0.001		Year	1	1.34	0.248
	Avian Species*Year	18	2.21	0.003		Avian Species*Year	14	2.48	0.002
VJ $1_{(556)}$	Avian Species	18	6.54	0.001	BC $VJ_{(330)}$	Avian Species	14	1.26	0.232
	Year	1	2.44	0.119		Year	1	1.96	0.162
	Avian Species*Year	18	1.04	0.415		Avian Species*Year	14	1.32	0.191
HK $1_{(556)}$	Avian Species	18	9.47	0.001	BC $HK_{(330)}$	Avian Species	14	2.82	0.001
	Year	1	9.77	0.002		Year	1	4.91	0.027
	Avian Species*Year	18	3.74	0.001		Avian Species*Year	14	1.22	0.263

computing environment (using the libraries MASS, ape and mvtnorm) with an additional unpublished function by R. Freckleton (University of Sheffield) as implemented in the package 'CAIC' (London, UK). The PGLS model is a linear regression model in which phylogenetic information is incorporated into the error term and thus controls for the shared evolutionary history among species (Harvey & Pagel 1991, Martins & Hansen 1997). This method tests evolutionary hypotheses with likelihood ratio statistics (Pagel 1997) and enabled us to estimate the importance of the phylogenetic correlation signal (λ), which can vary between 0 (phylogenetic independence) and 1 (species' traits covary in direct proportion to their shared evolutionary history). We then conducted all the analyses by setting the degree of phylogenetic correlation to the most appropriate degree evaluated for each model. In addition, we corrected our comparative analyses for heterogeneity in data quality due to the large variation in sample size among bird species by using weights in the comparative analyses (Garamszegi & Møller 2010). Following Garamszegi and Møller (2007), we combined variance factors due to phylogenetic and weight effects as error terms in a form of a matrix using the $\mathbf{Q} = \boldsymbol{V} + \boldsymbol{cW}$ equation, where $\boldsymbol{V}$ is the phylogeny matrix, $\boldsymbol{W}$ is the diagonal matrix of 1/weights and $\boldsymbol{c}$ is a constant (Martins & Hansen 1997). By varying $\boldsymbol{c}$, we estimated the maximum likelihood of different combinations of the phylogeny and weight matrices. For the combination that resulted in the highest maximum likelihood, we determined the statistics (slope, standard error of the slope,
t-statistic and *P*-value) of the effect in question. This additional PGLS exercise was also performed in the R statistical computing environment, setting the degree of phylogenetic dependence (λ) to the most appropriate degree evaluated for each unweighted model.

We estimated statistical significance in PGLS models using the function 'summary' as implemented in R (pglm3.3.r), which performed a type III decomposition of errors and, consequently, the order of the categorical factors does not affect results. First, we explored the effects of nest building and incubation behaviour on each bacterial type at the beginning of incubation and its bacterial change. Secondly, we explored general effects of life history traits on bacterial load and change by combination of *P*-values ($\chi^2_{\alpha,k} = -2 * \mathrm{SUM}(\ln P)$, where k is twice the number of analyses performed) from models for each bacterial type (Sokal & Rohlf 1995).

All the statistical tests were two-tailed and conducted using the R statistical computing environment or STATISTICA 8.1 software (Tulsa, OK, USA).

RESULTS

Avian species identity explained a significant proportion of variance in eggshell bacterial density at the beginning of incubation, for mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae* (Table 1). There was also statistically sig-

nificant evidence of interspecific differences in changes in mesophilic bacteria, *Enterococcus* spp. and *Enterobactericeae*. Consequently, eggshell bacterial densities and bacterial changes were significantly more variable among than within species, validating the use of average values for interspecific comparative analyses. Interspecific *post hoc* comparisons (least significant difference (LSD) tests) of bacterial density and change during incubation (for mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae*) are given in the Supporting Information online.

Species, study year and their interaction explained significant variation in estimated eggshell bacterial loads at the beginning of incubation (MANOVA; mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp., and *Enterobacteriaceae* as dependent variables, Wilks > 0.43, all $P < 0.001$), and bacterial changes (MANOVA; mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae* as dependent variables, Wilks > 0.69; all $P < 0.001$). When considering the univariate results, study year explained a significant proportion of variance in eggshell bacterial density of mesophilic bacteria, *Enterococcus* spp. and *Enterobacteriaceae* at the beginning of incubation, as well as in bacterial changes of mesophilic bacteria and *Enterobacteriaceae* (Table 1). In general, estimates of bacterial densities and changes in them were larger in 2007 than in 2006, independent of bird species identity. Finally, the interaction between species and year explained significant variation in eggshell bacterial densities at the beginning of incubation of most analysed groups of bacteria (except for the density of *Staphylococcus* spp.; Table 1), as well as variance of bacterial changes for *Enterococcus* spp., which suggests that changes in environmental conditions differentially affect bacterial load and bacterial change on eggshells of different bird species.

Overall, life history traits (by combination of *P*-values from the models for each bacterial type) explained significant variation in eggshell bacterial loads at the beginning of incubation ($\chi^2_8 > 16.32$, $P < 0.038$). For change in bacterial loads, there was no evidence of an overall association with life history traits in 2006 ($\chi^2_8 = 3.87$, $P > 0.869$) but there was in 2007 ($\chi^2_8 = 18.71$, $P = 0.016$). Whole model tests from univariate analyses showed a significant interspecific covariation between nest building and incubation behaviours and eggshell bacterial load estimated for *Enterococcus* spp. independent of study year, and for *Enterobacteriaceae* in 2006 (Table 2). Univariate results

Table 2. Results of phylogenetic generalized least square models (PGLS) testing the relationship between standardized log-transformed geometric means of initial bacterial densities on the eggshell as dependent variables (mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae*) and nest type (i.e. hole (0) vs. non-hole nests (1)), feathers (i.e. nest cups without (0) vs. with feathers as lining material (1)), plants (i.e. nest cups without (0) vs. with plants as lining material (1)) and incubation behaviour (i.e. onset of incubation before (0) vs. after clutch completion (1)) as independent factors. *P*-values in bold, and Beta and its standard error (se) are presented for each predictor. We show in parentheses the phylogenetic signal value (λ), and percentage of variance explained by the whole model (r^2) together with the associated *P*-values.

Bacteria group	Year PGLS results	Nest type	Plants	Feathers	Incubation
Mesophilic bacteria	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.003**; $r^2 = 0.150$; $P = 0.163$)	**0.035** −1.37 (0.59)	ns	ns	ns
	2007 ($\lambda = 0.241$; $P(0) = 1$; $P(1) =$ **0.006**; $r^2 = 0.104$; $P = 0.217$)	ns	ns	ns	ns
Enterococcus spp.	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) <$ **0.001**; $r^2 = 0.413$; $P =$ **0.012**)	ns	ns	**0.035** −0.96 (0.41)	ns
	2007 ($\lambda = 0.009$; $P(0) = 1$; $P(1) =$ **0.010**; $r^2 = 0.585$; $P <$ **0.001**)	**< 0.001** −1.85 (0.39)	ns	ns	**0.029** −0.71 (0.30)
Staphylococcus spp.	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) <$ **0.001**; $r^2 = -0.029$; $P = 0.508$)	ns	ns	ns	ns
	2007 ($\lambda = 0.214$; $P(0) = 0.609$; $P(1) <$ **0.001**; $r^2 = -0.053$; $P = 0.582$)	ns	ns	ns	ns
Enterobacteriaceae	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) <$ **0.001**; $r^2 = 0.319$; $P =$ **0.036**)	**0.004** −1.76 (0.53)	ns	**0.027** −1.09 (0.45)	ns
	2007 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.005**; $r^2 = 0.123$; $P = 0.189$)	ns	ns	ns	ns

showed that nest type was associated with the density of mesophilic bacteria and *Enterobacteriaceae* in 2006, but only with the density of *Enterococcus* spp. in 2007 (Table 2). The use of feathers in nest lining explained variation in the density of *Enterococcus* spp. and *Enterobacteriaceae* in 2006. Incubation behaviour had a significant effect on *Enterococcus* spp. load in 2007 (Table 2).

Eggshells of open-nesting avian species therefore harboured lower bacterial loads at the beginning of incubation than hole-nesting species. Bird species that use feathers as nest-lining material had lower bacterial density on eggshells than those that do not. Finally, bird species with an onset of incubation before clutch completion experienced higher initial bacterial density than species that start incubation once the clutch is complete.

With respect to the estimates of change in bacterial load during the incubation period, use of feathers as nest-lining material was significantly associated with estimates for *Enterococcus* spp., whereas nest type and the use of plants were related to *Staphylococcus* spp. (Table 3). Hole-nesting bird species and those that use plants or feathers as nest-lining material had lower rates of bacterial change. These associations were detected only for one of the study years (2006), which was the year with the lowest bacterial density (Table 3).

The phylogenetic signal (λ) of most statistical models was very low and, except for models explaining interspecific variation in bacterial change during incubation in eggshell loads of *Enterobacteriaceae* estimated in 2006, λ did not differ significantly from 0, but differed from 1 (Tables 2 and 3).

DISCUSSION

The aim of this study was to highlight interspecific differences in the abundance of several groups of bacteria on the eggshells of wild birds and to explore associations with life history traits, specifically nest location, nest building and incubation behaviour. The first step was therefore to confirm interspecific differences in eggshell bacterial loads and bacterial changes that would allow interspecific analyses to be performed. We found support for such interspecific variation, even after controlling for significant interannual variation.

Differences in bacterial loads and bacterial changes between years are probably caused by differences in environmental factors. Temperature and humidity related to the nest environment are known to affect eggshell bacterial loads (Beissinger *et al.* 2005), with bacteria being more abundant in wetter and cooler nests (Cook *et al.* 2005b). In our study area, 2007 was wet compared with the drier and hotter 2006 (AEMET 2006, 2007), and accordingly our estimates of eggshell bacterial density were higher in 2007. Interestingly, except for *Enterococcus* spp., estimates of bacterial changes during incubation did not differ between species and study years, suggesting that, independently of bacterial density, change in bacterial load on eggshells during incubation remains relatively constant

Table 3. Results of phylogenetic generalized least square models (PGLS) testing the relationship between standardized log-transformed geometric means of changes of eggshell bacterial densities as dependent variables (mesophilic bacteria, *Enterococcus* spp., *Staphylococcus* spp. and *Enterobacteriaceae*) and nest type, presence of feathers in the nest lining, presence of plants in the nest lining, and timing of incubation onset. Other details are as in Table 2.

Bacteria group	Year PGLS results	Nest type	Plants	Feathers	Incubation
Mesophilic bacteria	2006 ($\lambda < 0.001$; $P(0) = 0.991$; $P(1) = 0.549$; $r^2 = -0.247$; $P = 0.867$)	ns	ns	ns	ns
	2007 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.036**; $r^2 = -0.067$; $P = 0.577$)	ns	ns	ns	ns
Enterococcus spp.	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.003**; $r^2 = 0.025$; $P = 0.413$)	ns	ns	ns	ns
	2007 ($\lambda = 0.301$; $P(0)$=0.417; $P(1) =$ **0.003**; $r^2 = 0.433$; $P <$ **0.026**)	ns	ns	**0.017** −1.24 (0.44)	ns
Staphylococcus spp.	2006 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.001**; $r^2 = -0.078$; $P = 0.582$)	ns	ns	ns	ns
	2007 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.005**; $r^2 = 0.443$; $P =$ **0.024**)	**0.008** 1.88 (0.59)	**0.004** −1.75 (0.50)	ns	ns
Enterobacteriaceae	2006 ($\lambda = 0.999$; $P(0) =$ **0.015**; $P(1) = 1$; $r^2 = -0.142$; $P = 0.694$)	ns	ns	ns	ns
	2007 ($\lambda < 0.001$; $P(0) = 1$; $P(1) =$ **0.023**; $r^2 = 0.128$; $P = 0.240$)	ns	ns	ns	ns

across years and species. Consequently, the density of bacteria on the eggshell at the beginning of incubation and change in eggshell bacterial loads during incubation may illustrate two different aspects of microbial ecology. We speculate that environmental factors related to temperature and humidity may be responsible for variation in eggshell bacterial loads. Further research exploring the effects of latitude, altitude or lay date may help to evaluate the importance of these environmental factors in determining eggshell bacterial loads in natural populations.

We found support for the predicted association between species-specific life history traits of birds and both among-species variation in eggshell bacterial loads and changes in bacterial density during incubation. At the intraspecific level, the results suggested that by selecting appropriate nest-sites (Godard *et al.* 2007), adjusting the onset of incubation (Cook *et al.* 2005a), and using nest material with thermal or antimicrobial properties (Clark & Mason 1985, Peralta-Sánchez *et al.* 2010), birds are able to reduce the probability of bacterial colonization of the eggshell. Statistical models trying to explain interspecific differences in estimates of bacterial loads and bacterial change during incubation in relation to life history traits have a small phylogenetic component (except for during-incubation change in density of *Enterobacteriaceae*). Moreover, the effect of different life history traits varies between years (see univariate results in Tables 2 and 3), highlighting the importance of environmental conditions in determining bacterial colonization and bacterial change on eggshells of birds. These results could be interpreted as effectiveness of antibacterial strategies (e.g. the use of feathers and plants as nest-lining material) depending on environmental conditions.

Eggshell bacterial loads of mesophilic bacteria, *Enterococcus* spp. and *Enterobacteriaceae* were higher in hole-nesting than in open-nesting species. Previous experiments performed by Godard *et al.* (2007) with chicken eggs demonstrated that shells of eggs in open nests harboured a higher density of bacteria than those in hole nests, probably because open nests were more exposed to environmental conditions. It is worth mentioning here that the experimental nests used by Godard *et al.* (2007) were sterilized and moistened and had no previous breeding activity. Thus, it is possible that the higher bacterial density associated with hole-nesting habits that we found indicates the influence of previous breeding activity because of the frequent re-use of holes compared with open nests (Møller & Erritzøe 1996).

The use of material for nest building was an important factor explaining eggshell bacterial loads of *Enterococcus* spp. and *Enterobacteriaceae*, as well as change during incubation in the density of *Staphylococcus* spp. and *Enterococcus* spp. (which are known to be opportunistic pathogens of embryos; see Houston *et al.* 1997, Stewart & Rambo 2000). Detecting these effects, however, depended on study year, which again suggests that environmental conditions determine the efficacy of these antiparasitic behaviours. It is known that some plants that birds use as lining material have antimicrobial properties (Clark & Mason 1985, Petit *et al.* 2002, Mennerat *et al.* 2009a) and that Blue Tits (Mennerat *et al.* 2009a,b) and European Starlings (Clark & Mason 1985) preferentially use aromatic plants with antibacterial properties in their nests, which may increase reproductive success.

Feathers are another commonly used nest-lining material (Møller 1987, Cramp 1998) and it has recently been shown that experimental modification of colour and number of lining feathers in nests of Barn Swallows affected eggshell bacterial load and hatching success (Peralta-Sánchez *et al.* 2010, 2011). Our results suggest a role in reducing (in the case of *Enterobacteriaceae*) and modulating change (*Enterococcus* spp.) in bacterial density. We predicted such a relationship because of the thermoregulatory properties of nest-lining feathers that enhance incubation activity (Lombardo *et al.* 1995), but also because preen oils active against fungi and bacteria might be present in such feathers (Shawkey *et al.* 2003) or because of the antimicrobial properties of feather-degrading bacteria (Soler *et al.* 2010). Our results emphasize the importance of feathers as antimicrobial material, although underlying causes of such a relationship should be explored further. However, detecting these relationships depended on study year, which may suggest that beneficial effects of the use of feathers as nest-lining material depend on environmental conditions.

While early incubation reduced eggshell bacterial load in tropical species (Cook *et al.* 2003, 2005a, Shawkey *et al.* 2009), this effect does not always appear in Mediterranean environments (Ruiz-de-Castañeda *et al.* 2011, Wang *et al.* 2011). We sampled the eggs of all studied species soon

after incubation started and thus our estimates are independent of a general effect of incubation. Therefore, our estimates cannot be considered to be the result of selection pressures due to eggshell bacterial loads before incubation, but just after incubation started. In our study area, bacterial density usually increases during incubation (Soler *et al.* 2011; see Appendix 2) and thus it is still possible to predict that species that start to incubate before clutch completion (i.e. under higher selection pressure due to risk of trans-shell bacterial infection) should harbour higher bacterial density on their eggs than species that start to incubate after finishing the clutch. In accordance with this hypothesis, we found that asynchronously hatching species harboured higher densities of *Enterococcus* spp. than synchronously hatching species. This effect was found for only one of the two study years, which again suggests that the beneficial effects of early incubation may depend on environmental conditions.

In summary, we demonstrate interspecific differences in life history traits of wild birds that covary with eggshell bacterial loads and changes in these loads during incubation. Given that bacterial density could be a proxy for probability of trans-shell embryo infection, this result implies differences in the risk of infection among bird species. Our results emphasize the possibility that bacteria colonizing eggshells may be important selective agents driving the evolution of bird traits. Expected relationships did not appear for all types of bacteria, suggesting that the life history traits explored may have different effects for different groups of bacteria. However, non-significant relationships should be considered cautiously because of the relatively small sample sizes obtained for some species. We also found an important between-year component of variation in the hypothesized effects of life history traits, suggesting that the expected benefits may depend on environmental conditions. We hope that the results presented here will encourage future investigation of possible functional explanations of these patterns.

We thank Rosario Millán for technical assistance, and Liesbeth de Neve, David Martín-Galvez, Maria Roldán, Juan Rodríguez, Deseada Parejo, Carlos Navarro and Juan Diego Ibañez for sampling some nests of some bird species. Anders Pape Møller, Laura W. Parfrey and Javier Balbontín provided useful comments and helpful discussions on the English text, statistics and phylogeny. Steve R. Beissinger and three anonymous reviewers made useful comments that greatly improved this manuscript. Funding was provided by Junta de Andalucía (Proyecto de Excelencia RNM 345) and the Spanish Ministerio de Educacion y Ciencia and European funds (FEDER) (CGL2010-19233-C03-01). J.M.P.-S. was funded from Junta de Andalucía fellowship (RNM 345). Permits to conduct fieldwork were issued by the Junta de Andalucía (Andalusia Regional Government) and Dirección General de Gestión del Medio Ambiente (General Directorate of Nature Management).

REFERENCES

AEMET, A.E.d.M. 2006. *Resumen Climatológico Anual de 2006*. http://www.aemet.es/es/serviciosclimaticos/vigilancia_clima/resumenes?w=0&datos=2 (accessed 5 March 2012).

AEMET, A.E.d.M. 2007. *Resumen Climatológico Anual de 2007*. http://www.aemet.es/es/serviciosclimaticos/vigilancia_clima/resumenes?w=0&datos=2 (accessed 5 March 2012).

Amann, R.I., Ludwig, W. & Schleifer, K.H. 1995. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiol. Rev.* **59**: 143–169.

Archie, E.A. & Theis, K.R. 2011. Animal behaviour meets microbial ecology. *Anim. Behav.* **82**: 425–436.

Beissinger, S.R., Cook, M.I. & Arendt, W.J. 2005. The shelf life of bird eggs: testing egg viability using a tropical climate gradient. *Ecology* **86**: 2164–2175.

Christe, P., Oppliger, A. & Richner, H. 1994. Ectoparasite affects choice and use of roost sites in the Great Tit, *Parus major*. *Anim. Behav.* **47**: 895–898.

Clark, L. & Mason, J.R. 1985. Use of nest material as insecticidal and anti-pathogenic agents by the European starling. *Oecologia* **67**: 169–176.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R. A. & Arendt, W.J. 2003. Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation? *Proc. R. Soc. Lond. B* **270**: 2233–2240.

Cook, M.I., Beissinger, S.R., Toranzos, G.A. & Arendt, W.J. 2005a. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. *Ecol. Letters* **8**: 532–537.

Cook, M.I., Beissinger, S.R., Toranzos, G.A., Rodriguez, R. A. & Arendt, W.J. 2005b. Microbial infection affects egg viability and incubation behavior in a tropical passerine. *Behav. Ecol.* **16**: 30–36.

Cramp, S. 1998. *The Complete Birds of the Western Palearctic on CD-ROM*. Oxford: Software Optimedia, Oxford University Press.

D'Alba, L., Oborn, A. & Shawkey, M.D. 2010. Experimental evidence that keeping eggs dry is a mechanism for the antimicrobial effects of avian incubation. *Naturwissenschaften* **97**: 1089–1095.

Deeming, D.C. 2002. *Avian Incubation*. New York: Oxford University Press.

Garamszegi, L.Z. & Møller, A.P. 2007. Prevalence of avian influenza and host ecology. *Proc. R. Soc. Lond. B* **274**: 2003–2012.

Garamszegi, L.Z. & Møller, A.P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. *Biol. Rev.* **85**: 795–805.

Godard, R.D., Wilson, M., Frick, J.W., Siegel, P.B. & Bowers, B.B. 2007. The effects of exposure and microbes on hatchability of eggs in open-cup and cavity nests. *J. Avian Biol.* **38**: 709–716.

Goodenough, A.E. & Stallwood, B. 2010. Intraspecific variation and interspecific differences in the bacterial and fungal assemblages of Blue Tit (*Cyanistes caeruleus*) and Great Tit (*Parus major*) nests. *Microbial Ecol.* **59**: 221–232.

Hansell, M.H. & Deeming, D.C. 2002. Location, structure and function of incubate sites. In: Deeming, D.C. (ed.) *Avian Incubation. Behaviour, Environment, and Evolution*: 8–27. New York: Oxford University Press.

Harvey, P.H. & Pagel, M.D. 1991. *The Comparative Method in Evolutionary Biology*. Oxford: Oxford University Press.

Houston, C.S., Saunders, J.R. & Crawford, R.D. 1997. Aerobic bacterial flora of addled raptor eggs in Saskatchewan. *J. Wildl. Dis.* **33**: 328–331.

Jonsson, K.A. & Fjeldsa, J. 2006. A phylogenetic supertree of oscine passerine birds (Aves: Passeri). *Zool. Scripta* **35**: 149–186.

Lauber, C.L., Zhou, N., Gordon, J.I., Knight, R. & Fierer, N. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS Microbiol. Lett.* **307**: 80–86.

Lombardo, M.P., Bosman, R.M., Faro, C.A., Houtteman, S. G. & Kluisza, T.S. 1995. Effect of feathers as nest insulation on incubation behavior and reproductive performance of Tree Swallows (*Tachycineta bicolor*). *Auk* **112**: 973–981.

Martins, E.P. & Hansen, T.F. 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. *Am. Nat.* **149**: 646–667.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Méndez, M. & Soler, J.J. 2006. Relative importance of factors affecting nestling immune response differs between junior and senior nestlings within broods of hoopoes *Upupa epops*. *J. Avian Biol.* **37**: 467–476.

Mennerat, A., Mirleau, P., Blondel, J., Perret, P., Lambrechts, M.M. & Heeb, P. 2009a. Aromatic plants in nests of the Blue Tit *Cyanistes caeruleus* protect chicks from bacteria. *Oecologia* **161**: 849–855.

Mennerat, A., Perret, P., Bourgault, P., Blondel, J., Gimenez, O., Thomas, D.W., Heeb, P. & Lambrechts, M. M. 2009b. Aromatic plants in nests of Blue Tits: positive effects on nestlings. *Anim. Behav.* **77**: 569–574.

Merilä, J. & Allander, K. 1995. Do great tits (*Parus major*) prefer ectoparasite-free roost sites – an experiment. *Ethology* **99**: 53–60.

Mertens, J.A.L. 1977. Thermal conditions for successful breeding in Great Tits (*Parus major* L) 2. Thermal properties of nests and nestboxes and their implications for range of temperature tolerance of Great Tit broods. *Oecologia* **28**: 31–56.

Møller, A.P. 1987. Nest lining in relation to the nesting cycle in the swallow *Hirundo rustica*. *Ornis Scand.* **18**: 148–149.

Møller, A.P. & Erritzøe, J. 1996. Parasite virulence and host immune defense: host immune response is related to nest reuse in birds. *Evolution* **50**: 2066–2072.

Narushin, V.G. 2005. Production, modeling, and education. Egg geometry calculation using the measurements of length and breadth. *Poultry Sci.* **84**: 482–484.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. *Zool. Scripta* **26**: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. *Nature* **401**: 877–884.

Peralta-Sánchez, J.M., Møller, A.P., Martín-Platero, A.M. & Soler, J.J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in Barn Swallow nests: an experimental study. *Funct. Ecol.* **24**: 426–433.

Peralta-Sánchez, J.M., Møller, A.P. & Soler, J.J. 2011. Colour composition of nest lining feathers affects hatching success of Barn Swallows, *Hirundo rustica* (Passeriformes: Hirundinidae). *Biol. J. Linn. Soc.* **102**: 67–74.

Petit, C., Hossaert-McKey, M., Perret, P., Blondel, J. & Lambrechts, M.M. 2002. Blue Tits use selected plants and olfaction to maintain an aromatic environment for nestlings. *Ecol. Letters* **5**: 585–589.

Pinowski, J., Barkowska, M., Kruszewicz, A.H. & Kruszewicz, A.G. 1994. The causes of the mortality of eggs and nestlings of *Passer* spp. *J. Biosci.* **19**: 441–451.

Qiu, X.Y., Wu, L.Y., Huang, H.S., McDonel, P.E., Palumbo, A.V., Tiedje, J.M. & Zhou, J.Z. 2001. Evaluation of PCR-generated chimeras: mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Appl. Environ. Microbiol.* **67**: 880–887.

Ruiz-de-Castañeda, R., Vela, A.I., Lobato, E., Briones, V. & Moreno, J. 2011. Bacterial loads on eggshells of the Pied Flycatcher: environmental and maternal factors. *Condor* **113**: 200–208.

Shawkey, M.D., Pillai, S.R. & Hill, G.E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *J. Avian Biol.* **34**: 345–349.

Shawkey, M.D., Mills, K.L., Dale, C. & Hill, G.E. 2005. Microbial diversity of wild bird feathers revealed through culture-based and culture-independent techniques. *Microbial Ecol.* **50**: 40–47.

Shawkey, M.D., Firestone, M.K., Brodie, E.L. & Beissinger, S.R. 2009. Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. *PLoS ONE* **4**: e4522.

Sibley, C.G. & Ahlquist, J.E. 1990. *Phylogeny and Classification of Birds: A Study in Molecular Evolution*. New Haven: Yale University Press.

Singleton, D.R. & Harper, R.G. 1998. Bacteria in old House Wren nests. *J. Field Ornithol.* **69**: 71–74.

Sokal, R.R. & Rohlf, F.J. 1995. *Biometry*, 3rd edn. San Francisco: W. H. Freeman.

Soler, J.J., Martín-Vivaldi, M., Peralta-Sánchez, J.M. & Ruiz-Rodríguez, M. 2010. Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. *Open Ornithol.* **2**: 29–36.

Soler, J.J., Peralta-Sánchez, J.M., Martínez-Bueno, M., Martín-Vivaldi, M., Martín-Gálvez, D., Vela, A.I., Briones, V. & Pérez-Contreras, T. 2011. Brood parasitism is associated with increased bacterial contamination of host eggs: bacterial loads of host and parasitic eggs. *Biol. J. Linn. Soc.* **103**: 836–848.

Speksnijder, A.G.C.L., Kowalchuk, G.A., De Jong, S., Kline, E., Stephen, J.R. & Laanbroek, H.J. 2001. Microvariation artifacts introduced by PCR and cloning of closely related

16S rRNA gene sequences. *Appl. Environ. Microbiol.* **67**: 469–472.

Stearns, S.C. 1992. *The Evolution of Life Histories*. New York: Oxford University Press.

Stewart, R. & Rambo, T.B. 2000. Cloacal microbes in House Sparrows. *Condor* **102**: 679–684.

Wang, J.M., Firestone, M.K. & Beissinger, S.R. 2011. Microbial and environmental effects on avian egg viability: do tropical mechanisms act in a temperate environment? *Ecology* **92**: 1137–1145.

Wellman-Labadie, O., Picman, J. & Hincke, M.T. 2008a. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. *Br. Poultry Sci.* **49**: 133–143.

Wellman-Labadie, O., Picman, J. & Hincke, M.T. 2008b. Comparative antibacterial activity of avian egg white protein extracts. *Br. Poultry Sci.* **49**: 125–132.

Received 9 November 2011;
revision accepted 24 June 2012.
Associate Editor: Alistair Dawson.

APPENDIX 1

Monthly climate variables: mean temperature (T_m; in °C), mean maximum temperature (T_{max}; in °C), mean minimum temperature (T_{min}; in °C), mean precipitation (P; in mm), temperature deviation from historical serial mean (DT; in °C) and precipitation deviation from historical serial mean (DP, mm) at Guadix (climate station location (37°19′21.34″N; 003°07′10.25″W). Data available from Junta de Andalucía, Regional Government (Spain) at http://www.juntadeandalucia.es/medioambiente/site/web/menuitem.a5664a214f73c3df81d8899661525ea0/?vgnextoid=5bfd66ad0c378010VgnVCM1000000624e50aRCRD&vgnextchannel=a1d9e2df6aaad110VgnVCM1000001325e50aRCRD&lr=lang_es.

Year	Month	T_m	T_{max}	T_{min}	P	DT	DP
2006	April	15.50	19.89	6.56	56.20	2.31	42.57
	May	20.20	23.76	10.70	31.90	2.99	−8.03
	June	23.60	27.03	12.88	24.20	0.53	−16.46
	July	27.90	32.84	16.09	0.20	1.26	−1.32
2007	April	8.70	13.59	4.52	71.90	−1.90	10.70
	May	14.80	21.02	8.17	55.10	0.30	37.40
	June	19.50	27.12	11.58	1.70	−0.50	12.60
	July	24.00	31.98	14.83	0.10	0.50	−21.00

APPENDIX 2

Standardized log-transformed values of the geometric mean of variables used as estimates of bacterial density on the eggshells of 19 avian species for the performed regression analyses. Mesophilic (TSA), *Enterococci* (KF), *Staphylococci* (VJ), and *Enterobacteriaceae* (HK) are number of colonies (per cm^2 of eggshell), at the beginning of incubation (1) and their bacterial change (BC) period.

Avian Species	N 1 2006	TSA 1 2006	KF 1 2006	VJ 1 2006	HK 1 2006	N 1 2007	TSA 1 2007	KF 1 2007	VJ 1 2007	HK 1 2007	N BC 2006	BC TSA 2006	BC KF 2006	BC VJ 2006	BC HK 2006	N BC 2007	BC TSA 2007	BC KF 2007	BC VJ 2007	BC HK 2007	Nest type	Plants	Feathers	Incubation
Athene noctua	9	0.69	1.59	−0.36	0.74	11	0.47	0.99	−0.66	0.39	5	−0.47	−2.20	−0.69	−2.05	8	1.11	1.77	−0.09	2.41	Hole	No	No	Asyn
Otus scops	15	−0.27	0.54	−0.57	0.33	14	0.08	1.07	−0.84	0.61	9	−1.29	−0.91	0.24	−1.66	14	−0.17	−0.57	−0.02	−0.18	Hole	No	No	Asyn
Falco tinnunculus	12	0.80	1.79	0.47	1.27	9	0.98	0.92	−0.38	1.04	8	−0.32	0.81	−0.10	0.01	5	−0.48	1.31	−0.20	0.32	Hole	No	No	Asyn
Columba palumbus	5	0.19	−0.88	−0.48	−0.01	6	−0.09	−0.84	−0.76	0.37											Open	Yes	No	Syn
Columba livia	25	−0.32	0.87	1.15	−0.14	16	−1.75	−0.93	1.04	−1.88	6	1.26	0.23	0.71	2.44	4	4.96	0.17	0.01	5.19	Hole	No	No	Asyn
Coracias garrulus	13	−1.02	0.71	−0.67	0.06	10	0.40	1.38	−0.09	0.67	8	−0.09	−0.66	3.24	−0.15	8	1.13	−2.01	−0.28	0.27	Hole	No	No	Asyn
Upupa epops	32	0.92	2.19	3.04	1.33	28	0.75	1.75	2.26	1.14	21	0.86	−0.04	−1.04	0.12	10	0.51	0.20	−0.22	−0.12	Hole	No	No	Asyn
Pyrrhocorax pyrrhocorax						4	−0.22	−0.81	2.85	−1.28						3	−1.00	0.82	−1.42	1.89	Hole	Yes	No	Asyn
Pica pica	52	−0.44	−0.44	0.06	−0.12	54	−2.04	−1.01	−0.80	−1.75	30	0.75	0.30	−0.37	0.38	40	1.22	0.46	0.15	2.20	Open	Yes	No	Asyn
Corvus corone	16	−0.03	0.28	−0.64	−1.20	4	−2.75	−0.37	−0.86	−2.26	14	1.04	−0.84	0.14	0.87	4	6.33	−1.17	0.08	5.69	Open	Yes	Yes	Asyn
Corvus monedula	7	0.96	−0.34	−0.32	2.14	8	0.16	0.09	−0.38	0.15	6	0.59	2.70	−0.58	0.27	7	0.50	−0.35	−0.04	1.67	Hole	Yes	Yes	Asyn
Lanius meridionalis	3	0.93	−0.95	−0.43	−1.51	6	−0.02	−1.01	−0.83	0.12	3	−2.61	−0.01	0.02	0.62	6	1.71	−0.22	−0.02	1.10	Open	Yes	Yes	Asyn
Petronia petronia	3	1.26	0.92	2.18	0.99	5	1.23	0.83	−0.67	0.13											Hole	Yes	Yes	Asyn
Passer montanus	23	0.46	−0.68	−0.27	−0.08	12	0.56	−0.77	−0.12	0.68	10	0.46	0.13	0.62	−0.50	5	0.25	−0.72	−0.29	−0.72	Hole	Yes	Yes	Syn
Passer domesticus	3	0.24	−0.81	−0.72	−0.53	6	0.94	1.69	0.71	1.36											Hole	Yes	Yes	Asyn
Serinus serinus						10	−0.56	−0.91	−0.64	−0.42											Open	Yes	Yes	Syn
Carduelis chloris	4	−1.86	−0.81	−0.32	−0.66																Open	Yes	Yes	Syn
Sturnus unicolor	15	0.83	−0.12	−0.20	0.93	19	0.00	0.30	−0.02	0.26	6	−0.07	0.21	−0.17	−0.56	13	1.55	−1.30	−0.01	0.34	Hole	Yes	Yes	Syn
Oenanthe leucura	9	−1.14	−0.92	−0.54	−1.29	7	0.98	−0.54	−0.83	−0.44	6	−1.11	−0.01	−0.51	−0.05	7	0.17	−0.92	−0.03	3.69	Hole	No	Yes	Asyn
Turdus merula	6	−2.17	−0.94	−0.54	−0.83	35	0.19	−0.83	0.40	−0.35											Open	Yes	No	Asyn
Galerida cristata						3	0.09	−1.34	−0.15	0.08						19	0.11	−0.55	−0.08	0.35	Open	Yes	No	Asyn
Hirundo rustica	18	−1.38	−0.95	−0.59	−1.47	12	0.26	−0.44	0.19	0.32	16	0.15	0.21	−0.37	−0.10	7	−2.05	−2.07	0.47	−3.08	Hole	No	Yes	Syn
Parus major	15	0.74	−0.38	0.53	0.55	13	0.32	0.78	0.57	1.06	8	0.58	0.05	−1.02	0.29	9	0.74	−1.63	−0.20	−0.24	Hole	No	Yes	Asyn
Cyanistes caeruleus	4	0.60	−0.70	−0.80	−0.50																Hole	Yes	Yes	Asyn

APPENDIX 3

Phylogenetic relationships among avian species included in the analyses based on Sibley and Ahlquist (1990) and Jonsson and Fjeldsa (2006).