BACTERIAL LOADS ON EGGSHELLS OF THE PIED FLYCATCHER: ENVIRONMENTAL AND MATERNAL FACTORS

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Abstract. We studied environmental and maternal factors explaining bacterial loads on eggshells in a population of a hole-nesting passerine, the Pied Flycatcher (*Ficedula hypoleuca*) and the possible effects of bacteria on hatching success. We collected bacteria from eggshells at early and late stages of incubation and from females' feces, as a surrogate for cloacal samples, at intermediate stages of incubation. Higher environmental temperatures during laying promoted bacterial loads on the eggshells during early incubation, supporting the fact that bacterial growth is generally favored by increasing ambient temperature. Bacterial loads on the eggshells at late stages of incubation were correlated positively with bacterial loads in the female's cloaca, suggesting possible vertical transmission of bacteria. We detected no significant differences between bacterial loads of the eggshells at early and late stages of incubation. Possible bacteriostatic effects of incubation and/or other alternative mechanisms not associated with incubation may be responsible for this pattern. Bacterial loads seemed not to affect hatching success, raising the question whether bacterial species composition rather than bacterial load affects embryo survival.

Key words: **eggshell bacterial loads**, Ficedula hypoleuca, *hatching success*, *incubation*, *Pied Flycatcher*, *temperature*, *vertical transmission*.

Carga Bacteriana en la Cáscara de los Huevos de *Ficedula hypoleuca*: Factores Ambientales y Maternos

Resumen. Estudiamos los factores ambientales y maternos que podrían explicar la carga bacteriana de la cáscara de los huevos en una población de *Ficedula hypoleuca* y examinamos los efectos de esta carga sobre el éxito de eclosión. Se muestrearon las bacterias de las cáscaras de los huevos de las puestas en fases iniciales y finales de incubación, así como de las heces maternas como un correlato de las bacterias cloacales en fases intermedias de la incubación. Mayores valores de temperatura media ambiental durante el período de puesta promovieron la carga bacteriana sobre la cáscara de los huevos a inicios de incubación, apoyando la idea de que las bacterias proliferan ante aumentos de temperatura. La carga bacteriana sobre la cáscara de los huevos en fases finales de incubación se correlacionó significativamente con la carga bacteriana cloacal materna, lo que sugiere una posible verticalidad en la transmisión de bacterias. No se detectaron diferencias significativas entre las cargas bacterianas sobre las cáscaras de los huevos desde inicios y finales de la incubación. Posibles efectos bacteriostáticos de la incubación u otros mecanismos alternativos independientes de la incubación aún no descritos podrían explicar este patrón. La carga bacteriana no pareció afectar al éxito de eclosión, destacando la necesidad de evaluar los posibles efectos de la composición bacteriana sobre la supervivencia de los embriones en estudios futuros.

INTRODUCTION

Parasites play a major role in the evolution of host life histories (Møller 1997) and may affect the expression of sexually selected characters in birds (Hamilton and Zuk 1982). However, the most diverse group of potential parasites, the bacteria (Mlot 2004), has only received scant attention in field ornithology (but see Hubálek 1994, 2004, Cook et al. 2003, 2005a,b, Benskin et al. 2009, Burtt 2009).

Bacterial growth on the eggshell surface is rapid under suitable environmental conditions and can lead to a high probability of trans-shell infection (Cook et al. 2003). Cook et al. (2003, 2005a) observed that in tropical areas microbial growth was more rapid on eggs exposed to cool humid conditions than on those experiencing warmer and drier conditions. Therefore, water may be crucial for survival and growth of bacteria on the eggshells (Cook et al. 2003). Bacteria generally maximize their metabolic activity and growth within

precise thermal ranges at which enzymatic catalysis is optimized (Zwietering et al. 1991). Temperature may be a key factor governing microbial contamination of eggs. First, bacterial growth on eggshells may be enhanced by increasing temperatures. Clay and Board (1991) found that *Salmonella enteriditis* grew on eggshells when exposed to temperatures of 25 °C but was unable to grow at 4 or 10 °C. Haines and Moran (1940) demonstrated that the difference between the high temperature within the cloaca and the lower temperature in the outer environment produces a contraction of the egg at the moment it is laid that favors bacterial penetration. Also, Kim et al. (1989) demonstrated that the chances of detection of *Salmonella enteriditis* after experimental inoculation into the egg were positively associated with higher values of storage temperatures.

Bacteria on eggshells can be vertically transmitted from the cloaca of the female at laying (Barrow 1994, Kobayashi et al. 1997). Feces from the intestine and reproductive fluids from the oviduct mix in the cloaca, creating an environment rich in bacteria (Lombardo et al. 1996, Steward and Rambo 2000, Hupton et al. 2003). Accordingly, factors affecting variation in maternal cloacal bacteria, for instance, in terms of bacterial diversity (e.g., Lombardo et al. 1999, Klomp et al. 2008), could ultimately lead to transmission of bacteria onto eggshells.

Incubation has been suggested as an adaptive mechanism for inhibiting bacterial growth on eggshells. Cook et al. (2005b), using traditional culture methods, and more recently Shawkey et al. (2009), using culture-independent methods, have experimentally demonstrated that incubation inhibits bacterial growth on eggshells. Cook et al. (2005b) have also suggested that incubation may selectively promote certain nonpathogenic bacteria that could competitively exclude potential pathogens from the embryo.

Negative effects of bacteria on the reproductive success of poultry are suggested by numerous associations between bacterial contamination of eggs and their failure to hatch. Bacteria may be able to penetrate through the eggshell's pores and proliferate within the egg, ultimately killing the developing embryo (McCabe 1964, Board and Tranter 1986, Bruce and Drysdale 1994, Board et al. 1994). Although some authors have emphasized these negative effects of bacteria on hatching success in wild bird populations (Kozlowski et al. 1989, Pinowski et al. 1994, Beissinger et al. 2005, Cook et al. 2005a,b), descriptive information about bacterial loads on and inside eggs, and on the environmental and vertical (maternal) factors explaining bacterial growth, is scant. In addition, the real effects of bacteria on avian fitness in the wild are still poorly understood. However, an increasing number of recent studies have suggested that a variety of antibacterial adaptations have evolved in birds and that these may operate at different levels. At the immunological level, Matson et al. (2006) showed that the bacteria-killing ability associated with innate immunity varies significantly by species, and Morrison et al.

(2009) suggested that this ability may depend on heritable and early maternal effects. At the behavioral level, certain birds add plants with antibacterial properties to their nests (Baggot and Graeme-Cook 2002), and certain patterns of incubation (Cook et al. 2005b, Shawkey et al. 2009) may contribute to inhibiting bacterial growth on eggshells. Moreover, certain bacterial strains may represent an indirect defense mechanism for their avian hosts since they may outcompete other potentially pathogenic bacteria, favoring positive effects on nestlings' growth (Moreno et al. 2003) or on reproductive success (Soler et al. 2008, 2010).

In the present study we were interested primarily in identifying the environmental and vertical factors that may explain bacterial loads on eggshells in a population of a hole-nesting passerine, the Pied Flycatcher (*Ficedula hypoleuca*), breeding in a montane forest in central Spain. The Pied Flycatcher is a small (12–13 g) summer visitor that readily accepts artificial nest boxes for breeding and is consequently much used as a model species in studies of avian reproductive ecology (e.g., Lundberg and Alatalo 1992, Moreno et al. 2005). We obtained quantitative estimates of viable cultivable bacteria. We considered temperature and rainfall during laying and incubation as environmental factors. We hypothesized that in this cool montane habitat, increased environmental temperatures and increased rainfall may be important variables and could promote bacterial growth on eggshells. To detect a possible vertical transmission from the laying female to her eggs, we analyzed the association between bacterial loads within the female and on the eggshell. If transmission is taking place, we hypothesized that bacterial loads on the eggshells should be positively correlated with bacterial loads in the female's cloaca. Considering the recent evidence of Cook et al. (2005b) and Shawkey et al. (2009), we hypothesized that incubation has an inhibitory effect, leading to no increase in bacterial loads during the incubation period. Alternatively, we cannot exclude that bacterial loads may remain stable as a result of other mechanisms not associated with incubation. Finally, we analyzed the possible explanatory importance of bacterial loads of the eggshell on success of hatching.

METHODS

STUDY SPECIES AND STUDY AREA

Egg laying in the Pied Flycatcher population we studied typically begins in late May, and clutch size ranges from two to eight eggs with a mode of six eggs (Sanz et al. 2003). The incubation period is about 12–15 days, and the female alone incubates. For estimated hatching success we excluded cases where eggs were damaged while being handled or were extracted from the nest by the female (Lobato et al. 2006).

During the spring of 2008, we studied a population of Pied Flycatchers breeding in artificial nest boxes in a deciduous

forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m above sea level in the vicinity of Valsaín, central Spain (40° 54′ N, 04° 01′ W). The National Institute of Meteorology (INM-AEMET) provided daily meteorological information, temperature (maximum and minimum), and rainfall for the meteorological station of Segovia, approximately 9 km from our study area. Environmental humidity data for this area were not available. These temperate montane habitats can vary significantly in their humidity from year to year, but they generally tend to become drier during the nesting period toward the end of spring and beginning of summer. In comparison to other Mediterranean plant communities of central Spain, however, these montane oak forests grow in relatively humid areas. Although our environmental data were indirect, we assumed that rainfall could provide information about humidity because soil evaporation is highly dependent on the availability of moisture in the upper layers of the soil (about 30 cm), to which rainfall makes the major contribution (Strahler and Strahler 2006).

We first estimated daily mean temperature by calculating the arithmetic mean of daily maximum and minimum temperatures. We then averaged daily mean temperatures values and daily rainfall values (from INM-AEMET) for the study site during laying and incubation at each nest to calculate the mean temperatures and mean daily rainfall to which each clutch was exposed during the each period. We used these meteorological variables of temperature and rainfall for each nest in the statistical analyses. Finally, we averaged the values of mean temperature and mean rainfall during laying and incubation to obtain a general average value of temperature and rainfall for all the nests in the study population over the season (Table 1). We defined the laying period as the time elapsed between laying of the first egg in the clutch and 0–3 days after clutch completion (first microbiological sampling of the clutch, see below for details) and the incubation period as the time elapsed between 0–3 days after clutch completion and the second microbiological sampling at late stages of incubation (see details below). Each period is are critical in avian reproduction and very different in terms of the interactions taking place between the female and her eggs. Therefore, meteorological effects on bacteria on eggshells may vary during laying and incubation.

TABLE 1. Averaged mean temperatures (°C) and mean rainfall (mm) (±SE) during the periods of laying and incubation of the Pied Flycatchers studied, with these periods' average duration.

Period	Duration (days)	Average temperature (°C)	Average rainfall (mm)	n^a
Laying	6.4 ± 0.1	12.1 ± 0.5	4.63 ± 0.18	80
Incubation	10.2 ± 0.1	12.0 ± 0.5	5.09 ± 0.18	62

[a]Number of breeding pairs.

MICROBIOLOGICAL SAMPLING

We visited nest boxes daily, beginning with early stages of the breeding season, and recorded the exact date of clutch initiation (laying date). We first sampled bacteria from eggshells 0–3 days after clutch completion (mean number of days ± SE = 0.56 ± 0.09, $n = 79$). Daily checking allowed us to confirm that clutch was completed since Pied Flycatchers can occasionally interrupt laying for 1 or 2 days (e.g., adverse weather). In addition, we could not always sample clutches on the possible day of clutch completion because of logistic reasons. We tested for possible effects of this range of sampling (0–3 days after clutch completion) on the variation of bacterial loads on the eggshells at early stages of incubation, but we detected no significant effects ($F_{1,78} = 1.47$, $P = 0.23$). We sampled eggshell bacteria for a second time at late stages of incubation, 8–11 days after the taking the first sample (mean number of days ± SE = 10.24 ± 0.10, $n = 62$). We tested for possible effects of the differences in the interval between the first and second sampling on the variation in the eggshells' bacterial loads at late stages of incubation, but we detected no significant effects ($F_{1,61} = 0.10$, $P = 0.75$). To minimize possible contamination with external bacteria not directly associated with eggshells, we used sterile sampling techniques. We always handled eggs with latex gloves previously disinfected with 97% ethanol. We held our breath while handling the eggs or wore face masks. We swabbed the entire surfaces of all the eggs of the clutch only once, taking approximately 8 sec per egg and using only one sterile cotton swab (Sterile R, Meus S.R.L., Piove di Sacco, Italy) previously moistened with sterile phosphate-buffered saline (4 g NaCl; 0.1 g KCl; 0.72 g Na_2HPO_4; 0.12 g K_2HPO_4). Moisture transferred to the eggshells being sampled could serve as an indicator of areas already sampled, which prevented us from sampling eggshells more than once. We obtained only one sample for the whole clutch. Since bacteria could be transferred from one eggshell to another by contact in the nest and since we do not know the possible fluctuations in the quantity of bacteria on each individual eggshell during laying and incubation, we assumed that the clutch may be the most appropriate statistical unit for studying bacterial loads on the eggs. This approach also prevented possible pseudoreplication in our data. Bacterial loads were not associated with clutch size during either early (general linear model [GLM], $F_{1,79} = 0.37$, $P = 0.54$) or late ($F_{1,60} = 1.00$, $P = 0.32$) incubation. Immediately after sampling, we transferred swabs into Transport Media Amies (Sterile R, Meus S.R.L.) and then conserved them in a portable cooler.

At intermediate stages of incubation (mean ± SE, 6.5 ± 0.19, $n = 65$, after the first microbiological sampling of the clutch), we captured females while they were incubating in the nest boxes. Previous experience in this population revealed no risk of clutch desertion when females are captured during intermediate or late stages of incubation. We sampled

feces produced by the female during handling and/or the feces present on the immediate outer membrane of cloacal orifice with a flexible sterile cotton swab (Sterile R, Nuova Aptaca S.R.L.). Swabs were uniformly impregnated with feces for approximately 1–3 sec. We used only swabs that appeared to be effectively impregnated with fecal material and then immediately transferred them into Amies transport medium (Amies W/0 CH Sterile R, Nuova Aptaca S.R.L). Swabs could not be inserted in the cloacae because of the birds' small size. We assumed that the microbiota in feces is directly related to the microbiota in cloacae. We transported all microbiological samples in portable coolers until transferring them to refrigerators at 4 °C at the end of the day, then kept them at this temperature until further analyses.

ESTIMATION OF BACTERIAL LOADS

We transferred swabs from Amies transport medium into 3.5 mL of phosphate-buffered saline and manually agitated them to dislodge bacteria from the cotton. We made serial dilutions (10^{-1}–10^{-5}) with sterile physiological saline (0.85% NaCl) to obtain the optimal bacterial concentration for later quantification. The remaining swab supernatant was used for other analytic purposes not considered in this study. We immediately cultured 100 μL of the original swab supernatant and 100 μL of alternate levels of dilution in plates of tryptic soy agar (bioMérieux). We cultured each sample twice in independent plates of tryptic soy agar, thus providing a more precise value of the quantity of viable bacteria. Tryptic soy agar is a general-purpose nutrient medium that promotes the growth of a wide range of aerobic bacteria of various environmental origins. It is typically used for quantification of general viable bacteria growing in a sample. Although some of these viable bacteria may not be able to grow on tryptic soy agar under laboratory conditions, we presume that the bacteria most prevalent and biologically more relevant, such as pathogens for the embryo, may be effectively detected on it. These analytical procedures are used broadly in microbiology and have recently been applied to studies in avian reproductive ecology (Cook et al. 2005b). Basically, quantification of viable bacteria is made by counting all the CFU (colony-forming units) present in the plate after the specific incubation period. We incubated plates for 48 ± 2 hr at 37 ± 1 °C and counted the number of CFU in each of the cultured replicates with the naked eye. The same observer (RR-d-C) made all counts. We sampled a total of 82 and 62 clutches at the early and late stages of incubation, respectively. We obtained feces from 35 of 65 females but used only 26 of them because the delay between collection in the field and analysis in the laboratory was too long or because of analytical problems (possible errors in serial dilutions). Duplicate CFU counts for eggshells at early (Spearman $r_{82} = 0.96$, $P < 0.001$) and late (Spearman $r_{62} = 0.97$, $P < 0.001$) stages of incubation were strongly correlated. Duplicate CFU counts for fecal samples from a single female

were also strongly correlated (Spearman $r_{26} = 0.95$, $P < 0.001$). Therefore, we averaged the CFU of the two replicates of each sample. The dilution factor allowed us to calculate the number of viable bacteria present in the original sample. In this study, we considered only samples analyzed within 7 days of collection. Within this period, we detected no effects of the delay on bacterial loads for samples from females' cloacae or from eggshells at late incubation (both $P > 0.06$). In the case of samples from eggshells at early incubation, however, we found that delay of analysis had a significant and positive effect on bacterial loads (GLM, $F_{1,80} = 76.03$, $P < 0.001$). To avoid the possible confounding effect of delay of analysis of these samples, we controlled statistically for this effect by including the delay as a covariate in all the models in which bacterial loads of eggshells early in incubation were considered.

STATISTICAL ANALYSES

Using Statistica 7.0 (StatSoft), we developed two major types of generalized linear models (GLZ), environmental and vertical, one of each type for eggshell bacterial loads early incubation and late in incubation.

The environmental model for early incubation included mean environmental temperature and mean rainfall during laying, date of laying, and delay in analysis as continuous predictors of bacterial loads on eggshells. That for late incubation included mean environmental temperature and mean rainfall during the incubation period, date of laying, the bacterial load in early incubation, and delay in analysis as continuous predictors. In both early and late incubation the eggshells' bacterial loads were normally distributed after being log-transformed.

To understand the possible vertical transmission of bacteria from the laying female to her clutch, we designed the vertical-transmission models. These models included the eggshells' bacterial load in early and late incubation as dependent variables and the female's cloacal bacterial load as a continuous predictor. For late incubation, we also included the eggshells' early bacterial loads as an explanatory variable. Cloacal bacterial loads were normally distributed after being log-transformed. The sample of cloacal bacterial loads was too small for us to consider a single model including environmental and vertical factors simultaneously.

We designed a model to explain bacterial loads late in incubation in relation to bacterial loads early in incubation. Such a model could imply effects of incubation on eggshell bacteria.

We tested the possible explanatory importance of eggshells' bacterial loads early and late in incubation for hatching success in one single model. This model included the bacterial loads both early and late in incubation as independent variables. In addition, we also included date of laying as a covariate. We defined hatching success as a binomial dependent variable with a value of 1 if all eggs hatched or 0 if at least one egg remained unhatched.

TABLE 2.   Logistic-regression models for environmental effects (mean temperature and mean rainfall during laying) on bacterial growth on eggshells early in incubation. Models are ranked according to their corrected AIC score (AIC_c). K refers to the number of parameters in each model.

Rank	Model	K	ΔAIC_c	Weight
1[a]	Mean temperature, analysis delay	3	0.00	0.46
2	Mean temperature, average daily rainfall, analysis delay	4	1.89	0.18
3	Mean temperature, analysis delay, date of laying	4	2.21	0.15
4	Analysis delay	2	3.50	0.08
5	Mean temperature, average daily rainfall, analysis delay, date of laying	5	4.11	0.06
6	Analysis delay, date of laying	3	5.19	0.03
7	Average daily rainfall, analysis delay	3	5.66	0.03
8	Average daily rainfall, analysis delay, date of laying	4	7.15	0.01
9	Average daily rainfall, date of laying	3	20.65	0.00
10	Mean temperature, average daily rainfall, date of laying	4	21.87	0.00
11	Date of laying	2	24.61	0.00
12	Mean temperature, date of laying	3	26.52	0.00
13	Mean temperature	2	54.99	0.00
14	Average daily rainfall	2	56.46	0.00
15	Mean temperature, average daily rainfall	3	57.14	0.00

[a] $AIC_c = 282.57$.

We used Akaike's information criterion (AIC) to select the models with the highest parsimony and plausibility and to identify dependent variables with greatest explanatory power. We first obtained the values of AIC and the AIC_c for each model, then calculated the differences (Δ_i) between the AIC_c of each model and the AIC_c of the most plausible model (lowest AIC_c). In addition, we calculated Akaike weights for each model from Δ_i. These weights can be interpreted as approximate probabilities that a certain model is the most plausible model in a set. Following Burnham and Anderson (2002), we estimated the relative importance of each explanatory variable by summing the Akaike weights over all the models with $\Delta_i < 2$ (more plausible models) in which the variable appeared.

Among the most plausible models ($\Delta_i < 2$) with values of $P < 0.05$, we further analyzed the effects and the sign of the effects (positive or negative) of the identified best explanatory variables on the dependent variables by a traditional contrast-of-hypotheses approach with GLM.

RESULTS

First, the most plausible environmental model (lowest ΔAIC_c) for bacterial loads on eggshells early in incubation included the mean temperature during laying as the best explanatory environmental variable. In contrast, mean rainfall had a lower explanatory potential and was first included in a model whose weight was 2.55 times lower than the most plausible model (Table 2). Specifically, considering the most plausible model, we found that mean temperature during laying was positively associated with the eggshells' bacterial loads early in incubation when the effects of analysis delay were controlled for (Fig. 1).

The most plausible environmental model for the eggshells' bacterial loads late in incubation did not include mean environmental temperature and mean rainfall during incubation. In fact, none of the six best models ($\Delta_i < 2$) incorporating five different predictors had a significant P-value (all $P > 0.09$).

The most plausible vertical-transmission model for eggshells' bacterial loads in early incubation did not include cloacal bacterial load, but this variable appeared in the second most plausible model. There was, however, no significant association between bacterial loads on eggshells and in the female's
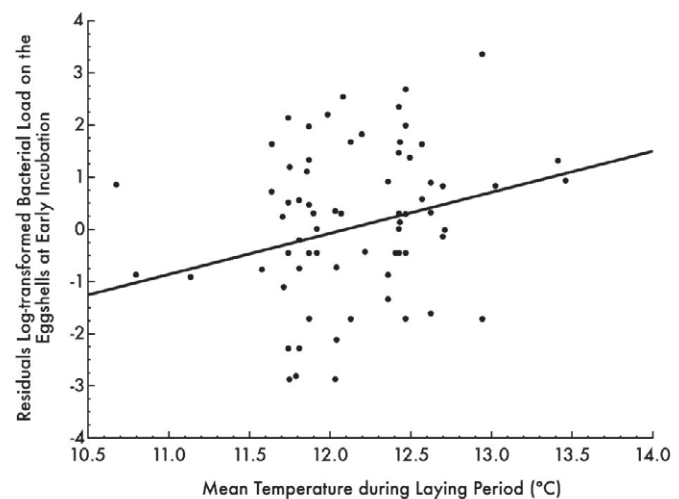

FIGURE 1.   Association between mean temperature during laying and bacterial loads on the eggshells early in incubation ($F_{1,79} = 5.64$, $r = 0.26$, $P = 0.02$). Bacterial load was statistically corrected for the effects of delay in analysis of the samples with residuals of the regression between bacterial loads and delay in analysis.

TABLE 3.   Logistic-regression models for the vertical transmission of maternal bacteria on eggshell bacteria at late stages of incubation. Models are ranked according to their corrected AIC score (AIC_c). K refers to the number of parameters of each model.

Rank	Model	K	ΔAIC_c	Weight
1[a]	Bacterial load in female's cloaca	2	0.00	0.33
2	Analysis delay, bacterial load in female's cloaca	3	0.18	0.30
3	Bacterial load in female's cloaca, eggshells' bacterial load early in incubation	3	0.50	0.26
4	Analysis delay, bacterial load in female's cloaca, eggshells' bacterial load early in incubation	4	2.59	0.09
5	Analysis delay	2	9.09	0.00
6	Eggshells' bacterial load early in incubation	2	9.39	0.00
7	Analysis delay, eggshells' bacterial load early in incubation	3	11.86	0.00

[a]$AIC_c = 84.88$.

cloaca when the effects of analysis delay were controlled for (GLM, $F_{1,26} = 0.94$, $P = 0.34$). The explanatory importance of cloacal bacterial load was more clear in the vertical-transmission model for the eggshells' bacterial loads late in incubation. Here, the three most plausible models ($\Delta_i < 2$) included cloacal bacterial load, and it was the variable with highest relative explanatory importance (Table 3). Specifically, the eggshells' bacterial load late in incubation was correlated positively with the female's cloacal bacterial load (Fig. 2).

The eggshells' bacterial load early in incubation had a low explanatory importance for their bacterial load late in incubation since this variable was not included in the more plausible model. Specifically, eggshells' bacterial loads did not change significantly through incubation when the effects of analysis delay were controlled for (GLM, $F_{1,60} = 0.02$, $P = 0.90$). In fact, none of the three models incorporating two different predictors had significant P-values (all $P > 0.09$).

During this season, hatching success in our population was high (mean $\pm$ SE = 0.94 ± 0.02, $n = 76$). Bacterial loads on
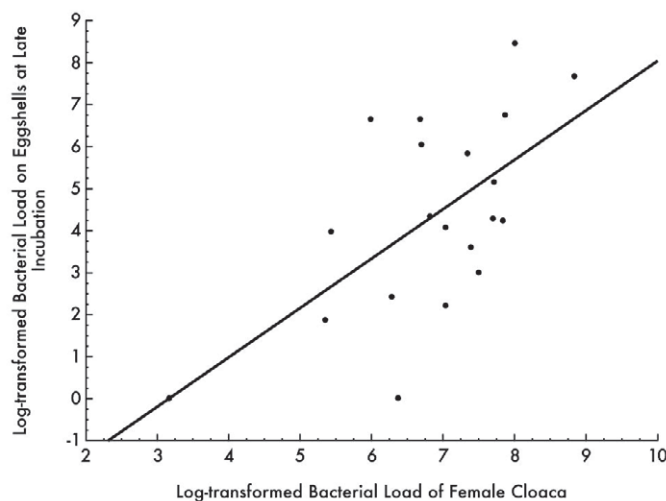

FIGURE 2.   Association between bacterial loads in the female's cloaca and those on the eggshells late in incubation ($F_{1,20} = 11.19$, $r = 0.62$, $P = 0.004$).

eggshells early in incubation were included in the best model when we considered hatching success as a dependent variable. However, when testing for possible effects of the eggshells' bacterial loads early in incubation on hatching success and controlling for analysis delay, we detected no significant effects (GLZ binomial, Wald statistic = 0.78, $n = 72$, $P = 0.38$). Bacterial load late in incubation did not appear in the best model. In general, none of the 15 models incorporating four different predictors had significant P-values (all $P > 0.07$).

DISCUSSION

Our data suggest that the ambient temperature during laying may influence eggshells' bacterial loads early in incubation, that bacterial loads in the female's cloaca could partly explain the eggshells' bacterial loads late in incubation, and that absolute bacterial loads do not increase through the incubation period. Bacterial loads on eggshells did not seem to explain hatching success. Some of these results have not been previously reported for wild populations of birds. We discuss them in turn.

Experimental and correlational evidence may support the positive effect of mean environmental temperature on eggshells' bacterial load. It is well known that temperatures around the optimum growth temperature, 15–40°C for most bacteria pathogenic to birds, have direct positive effects on metabolic activity and therefore on bacterial growth (Schaechter et al. 2006). Theron et al. (2003) experimentally demonstrated on chicken eggs that bacterial counts are much higher on eggs exposed to high temperature than on those exposed to to low temperature. Clay and Board (1991) found that eggs artificially inoculated with *Salmonella enteriditis* harbor more viable bacteria at higher temperatures. In natural environments, Cook et al. (2003, 2005a) showed that eggs in cool humid habitats harbor more bacteria on their shells than do eggs in warmer drier habitats. Therefore, in natural environments, environmental temperature may operate in close association with environmental humidity to determine bacterial growth. We found, however, in comparison to mean temperature, that mean rainfall was not a good explanatory predictor for bacterial loads on the eggshells. As Pied Flycatcher eggs are

always laid within a cavity, they are isolated from direct rainfall. In addition, the possible effects of rainfall as an indicator of environmental humidity may be too indirect to be detectable on bacterial growth on the eggshells. In future studies, relative humidity within the nest box should be considered instead. This variable would be more accurate and could have a higher explanatory value in association with temperature for the variance of bacterial loads on the eggshells.

Despite the significant effect of mean environmental temperature on eggshells' bacterial loads early in incubation, mean environmental temperature only accounted for 7% of the variation of eggshells' bacterial loads at this stage. Therefore, we presume that, in general, environmental variables explained little of the variation of bacterial loads on eggshells both early and late in incubation. Late in incubation, we presume that a possible homeostatic effect of incubation itself on environmental conditions in the nest may be partly responsible of the absence of a significant effect of environmental conditions on eggshells. We believe that more direct measures of environmental conditions within the nest boxes are required to confirm the effects of temperature and humidity on eggshells' bacterial loads and the intensity of these effects.

Females with larger bacterial loads also laid eggs with larger bacterial loads. This result seems logical, as vertical transmission of bacteria from the laying female to her eggs is well known (Barrow 1994, Kobayashi et al. 1997). However, cloacal bacterial loads partly explained eggshells' bacterial loads at the final stages of incubation but unexpectedly not at the early stages of incubation. The interpretation of these results is not straightforward and is limited by the correlative approach of this study.

One possibility is that bacteria vertically transmitted at laying may have become dormant because of stress due to limited nutrient availability and suboptimal environmental conditions on the surface of the eggs (Balaban et al. 2007), these being dramatically different from those in the intestine or cloaca of the host (e.g., temperature constant between 38 and 42 °C; Prinzinger et al. 1991). Vertically transmitted bacteria could be initially misrepresented in the counts of viable bacteria from eggshells early in incubation, but certain vertically transmitted bacteria may become gradually acclimatized to the environmental conditions and/or be selectively promoted by incubation (Cook et al. 2005b) and move from a slow-growing to an active state. If so, the detection of a significant positive association with the female's cloacal load is favored only late in incubation. This possibility may contrast with the detection of no significant variation in eggshells' bacterial loads from early to late in incubation. However, since we obtained quantitative general information only about the viable aerobic bacteria (on the basis of growth in culture media of typtic soy agar) growing on eggshells, we cannot exclude the possibility that, despite a general equilibrium in the abundance of bacteria, at least certain vertically transmitted bacteria may proliferate

through incubation. Only further studies including more diverse quantitative information about different bacterial species could provide a more solid conclusion.

The absence of significant variation in eggshells' bacterial loads through incubation could be interpreted according to the demonstrated inhibitory effects of incubation (Cook et al. 2005b, Shawkey et al. 2009). By incubating, parents may minimize the buildup of harmful bacteria (Cook et al. 2005b), by raising the temperature beyond the optimum for growth of most bacteria (Board and Tranter 1986) or by producing antibacterial secretions (e.g., in the preen gland; Shawkey et al. 2003). Because our study yielded only correlational information, we can only speculate about the possible effects of incubation; alternative mechanisms independent of incubation may also contribute to this pattern. Further experimental studies are required to confirm the effects of incubation and explore other mechanisms involved in controlling growth of eggshell bacteria in temperate habitats.

In addition to bacteria vertically transmitted onto eggshells at laying, eggshells may become colonized by air-borne bacteria, bacteria growing on nest material, bacteria present on the feathers and skin of the female during incubation, or bacteria associated with direct defecation of the female onto the eggs. This last source of bacteria seems improbable given the noticeable cleanliness of the eggs at the end of incubation (pers. obs.). The possible contribution of these environmental bacteria on the detected association between eggshell bacteria late in incubation and the female's cloacal bacteria remains unknown, so further studies are required to measure the bacterial loads of these potential sources of environmental bacteria that could be transmitted onto eggshells.

The bacterial loads of eggshells did not explain hatching success. Infection of the embryo and possible hatching failure may not depend on the general quantity of bacteria but rather on the presence of particular pathogenic strains that have developed infective strategies that may overcome the egg's defensive barriers (Baggott and Graeme-Cook 2002). Further conclusions about the possible effects of bacteria on hatching success in our study population will necessarily depend on identification of bacteria growing on eggshells and in females' feces. Alternatively, it is possible that birds breeding in natural cavities and artificial nest boxes may have selected for high resistance to bacterial contamination of eggs because they reuse cavities (Singleton and Harper 1998, Tomás et al. 2007) and the cavities are occupied during the winter by resident birds and small mammals that could be vectors for pathogens.

In conclusion, further studies are needed to clarify the patterns we found, but we can already identify some possible potential key predictors of eggshells' bacterial loads from our results. First, the implications of ambient temperature on eggshells' bacterial loads may be relevant in the context of captive breeding since bacteria growing on eggshells may ultimately have the potential to infect the embryo. Similarly, intensified

selective pressures of bacteria on eggs in the wild could result from global warming possibly promoting bacterial growth. Also, females may contribute maternal effects in the form of bacterial loads on the eggs. Finally, incubation could have a general controlling effect on eggshells' bacterial loads.

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