

EARLY ONSET OF INCUBATION AND EGGSHELL BACTERIAL LOADS IN A TEMPERATE-ZONE CAVITY-NESTING PASSERINE

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Abstract. It has been hypothesized that incubation begins before laying is completed because it controls bacterial growth on eggshells and infection of eggs. If so, early incubation should be associated with decreased abundance of bacteria on eggshells. There is no detailed information on incubation during daytime or nighttime during laying and its association with bird eggshells' bacterial loads. We used temperature sensors placed in contact with eggs and connected to data-loggers to measure early incubation by females of the Pied Flycatcher, *Ficedula hypoleuca*, a hole-nesting passerine, beginning with laying of the third egg (day 3). Incubation usually began with laying of the fourth egg (day 4), the modal clutch size in our population being six. We sampled heterotrophic bacteria on eggshells from the whole clutch soon after it was completed. We used colony-forming units counted on trypticase soy agar to estimate bacterial loads. Clutches incubated more intensively during daytime on day 4 and also during nighttime on days 3–5 had fewer bacteria on eggshells than clutches incubated less. The temperature of the air around the eggs seemed not the key explanatory variable for the eggshell's bacterial loads, suggesting that mechanisms other than thermal changes associated with incubation may be more relevant to control of bacterial proliferation. Ours is the first study showing a correlation between increased incubation on the day incubation begins during laying and a reduction in bacteria on the eggshells of wild birds. It supports the hypothesis that early incubation is an antimicrobial defense of the clutch.

Key words: behavioral antimicrobial defenses, culturable heterotrophic bacteria, eggshells, *Ficedula hypoleuca*, onset of incubation.

Inicio Temprano de la Incubación y Carga Bacteriana sobre los Huevos

Resumen. Se ha hipotetizado que la incubación comienza antes de completar la puesta porque podría controlar la proliferación bacteriana sobre los huevos así como la infección de los mismos. Si es así, la incubación temprana debería estar asociada a una reducción de la carga bacteriana sobre los huevos. No existe información detallada de la actividad de incubación durante los días o las noches del periodo de puesta así como de su asociación con la carga bacteriana sobre los huevos en aves. Usamos sensores de temperatura colocados en contacto con los huevos y conectados a dispositivos de memoria para medir la incubación temprana de hembras de *Ficedula hypoleuca*, un paseriforme que anida en oquedades, iniciando en el día de puesta del tercer huevo (día 3). La incubación generalmente se inició en el día de puesta del cuarto huevo (día 4), siendo el tamaño modal de puesta en nuestra población de seis huevos. Muestreamos las bacterias heterotróficas de toda la puesta después de que se completara. Usamos los conteos de unidades formadoras de colonias en el medio TSA como estimas de la carga bacteriana presente sobre las puestas. Aquellas puestas que fueron incubadas más intensamente en el día 4 así como durante las noches 3–5, tenían menos bacterias que aquellas que fueron incubadas en menor medida. La temperatura alrededor de los huevos no fue la variable explicativa más importante, sugiriendo la existencia de mecanismos alternativos a los cambios de temperatura asociados a la incubación, que serían más relevantes para el control de la proliferación bacteriana. Este es el primer estudio que muestra una correlación entre valores altos de intensidad de incubación durante el día de inicio de incubación durante la puesta, y una reducción de la carga bacteriana sobre huevos de aves silvestres, apoyando por tanto que la incubación temprana es una defensa antimicrobiana para las puestas.

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INTRODUCTION

Avian parents can influence the initiation of development of eggs and the resulting synchrony of hatching by varying the initiation of incubation (O'Connor 1984). In many groups of birds, most especially the Passeriformes, incubation begins before completion of the clutch (Hébert 2002, Wang and Beissinger 2009). Most hypotheses regarding the significance of patterns of hatching in birds assume that these patterns reflect patterns of incubation during laying (Hébert 2002, Wang and Beissinger 2009). However, despite much study, the evolution of patterns of hatching remains controversial (Magrath 1990, Stoleson and Bessinger 1995, Stenning 1996). Far less attention has been paid to factors relating to survival of the embryo, not the nestling, that may select for an early onset of incubation (Cook et al. 2003, Massaro et al. 2007, Wang and Bessinger 2009). In this study, we address only hypotheses related to factors promoting early incubation onset independently of hatching patterns.

Cook et al. (2003, 2005a) suggested that the onset of incubation may have evolved to reduce bacterial growth on eggshells and so reduce mortality from trans-shell infection during laying. Experiments have shown that incubation inhibits bacteria on eggshells in a cavity-nesting passerine of the tropics (Cook et al. 2005b, Shawkey et al. 2009) and another of the temperate zone (D'Alba et al. 2010), though the underlying mechanisms of such inhibition are poorly understood. Incubation may limit bacterial growth on eggshells by keeping them dry by increasing the temperature around them greatly (Cook et al. 2003, 2005a,b, D'Alba et al. 2010), above the optimum for bacterial growth (Cook et al. 2003, 2005a,b). Alternatively chemicals in secretions of the uropygial gland (Shawkey et al. 2003) or these secretions' viscosity (Reneerkens et al. 2008) may inhibit bacterial proliferation on eggshells. Also, competition with bacteria in the uropygial gland (Soler et al. 2008) or on feathers in the nest material (Peralta-Sánchez et al. 2010) may limit growth of potentially pathogenic bacteria on eggshells. One unexplored possibility is that bacteria on eggshells may be affected by possible antibacterial secretions produced in the skin of the brood patch (Menon and Menon 2000). Finally, the increased temperature of incubation could favor the release of volatile antibacterial compounds from certain plants in the nest material (Clark and Mason 1988).

The ecological and evolutionary implications of the studies by Cook et al. (2003, 2005a,b), Shawkey et al. (2009), and D'Alba et al. (2010) have raised new questions concerning bird–bacteria interactions and have contributed an important perspective to our understanding of the evolution of incubation. Their results on a passerine in a tropical habitat (Cook et al. 2003, 2005a,b, Shawkey et al. 2009), where humidity and temperature are particularly suitable for bacterial growth and subsequent trans-shell infection, have been subsequently confirmed in a species of the temperate zone (D'Alba et al. 2010). However, to our knowledge, no study has specifically

addressed the variation of early incubation during daytime and night during laying and its association with eggshells' bacterial loads in a cavity-nesting passerine in the wild.

The hypothesis of Cook et al. (2003) predicts that an early onset of incubation with respect to clutch completion should be associated with a reduction of bacterial loads on eggshells. We have tested this prediction in the cavity-nesting Pied Flycatcher (*Ficedula hypoleuca*) in a temperate montane habitat in central Spain. In a study based on other analyses of partly the same data set on bacterial loads (Ruiz-de-Castañeda et al. 2011), we found, that in this habitat, increasing ambient temperatures (mean 12.1 °C) during laying were associated with larger culturable bacterial loads on eggshells of the Pied Flycatcher. Because in that study we detected no quantitative changes in bacterial loads on eggshells from early to late stages of incubation, we hypothesized that full incubation could also operate as an adaptation to inhibit bacteria in this species. In that study, however, we did not analyze incubation activity prior to clutch completion and its possible effects on eggshells' bacterial loads soon after laying. As we know from more than two decades of study of this population that females generally start incubation during laying, it seems a good system to test the hypothesis of Cook et al. (2003).

METHODS

STUDY AREA AND SPECIES

In spring 2008 we studied Pied Flycatchers breeding in a montane forest of Pyrenean oak (*Quercus pyrenaica*) in central Spain (40° 54' N, 04° 01' W). The area covers approximately 100 ha where 300 wooden nest boxes were erected in the early 1990s. The birds normally lay clutches of 5–7 eggs (modal clutch size 6) within a well defined nest cup composed mainly of strips of bark (Moreno et al. 2009). Only the female incubates. Incubation begins during laying, and eggs hatch asynchronously within 1.25 days (Lundberg and Alatalo 1992). See Moreno et al. (2005) and Ruiz-de-Castañeda et al. (2011) for further details about the study site and species.

MEASURING INCUBATION ACTIVITY

Early in the breeding season, we visited the 300 nest-boxes every 3 days to detect initiation of nest construction. We visited 89 nests daily once they were fully lined, indicating that birds were about to lay. We noted the day on which the first egg was laid. On the day the third egg was laid (day 3), we placed Hobo temperature loggers (Onset Computer Corporation, Bourne, MA) inside 67 randomly selected nest boxes occupied by Pied Flycatchers. We unwound the sensor wire from the circuit board and extended it through the nest material until the small temperature sensor emerged in the middle of the nest cup between the eggs. This setup allowed accurate measurement of the air temperature in close contact with the eggs. We programmed the data loggers for a 40-sec measuring interval. In all nests, we measured the temperature from

14:45 on the day of laying of the third egg to 09:45 three days later, representing 6031 measurements covering 2 days, 19 hr, 40 sec per nest. We selected the daytime hours of day 3 to initiate measurements as in this population we have found no female incubating prior to that day over more than two decades of nest checks during laying. On the other hand, the great majority of females are incubating fully after laying the sixth egg. We are interested here only in incubation before clutch completion. We thus assume that days 3–5 of laying are the most critical for measuring the effects of early incubation onset.

We did not visit nest boxes while data loggers were measuring. We retrieved our data after the loggers were removed from the boxes. We divided each continuous set of temperature data into segments by daytime and nighttime according to the times of sunset and sunrise provided by the Observatorio Astronómico Nacional (Spain) for each day of the measuring period. We considered the beginning of nighttime and daytime to be 30 min after sunset and sunrise, respectively.

“Physiological zero” refers to the temperature above which embryonic development takes place and for wild birds is generally considered to be 24 °C (Webb 1987). This threshold may be critical for females’ incubation activity and for the activation of defenses against bacteria potentially able to affect early embryonic development. From the continuous temperature record, we estimated the proportion of time above 24 °C and the mean temperature for each daytime and nighttime period of days 3, 4, and 5 and for the whole pooled daytime and nighttime periods of days 3–5 (total daytime and nighttime periods). The Agencia Estatal de Meteorología (Spain) provided daily information on temperatures for the meteorological station at Segovia, 9 km from our study area. Because ambient temperatures during the period of measurement never exceeded 20 °C (mean 16.14 °C \pm SE 0.42), we assume that temperatures above 24 °C indicate incubation. To check the accuracy of thermal sensors we placed video cameras 10–15 m from 11 nest boxes and recorded females’ visits by periods of 1 hr. In three nest boxes our film showed no visits and the temperature of the eggs never exceeded 16 °C. Another film showed that the female visited the nest for only 20 sec, which, as expected, had no detectable effect on the air temperature close to the eggs. In the seven remaining nest boxes, the female’s presence in the nest coincided with egg temperatures between 20 and 32 °C, confirming that temperatures in nests above 24 °C indicate incubation and that this threshold may be somewhat conservative.

MICROBIOLOGICAL SAMPLING AND ESTIMATION OF BACTERIAL LOADS

Our samples of eggshells’ bacteria and the estimates of their heterotrophic bacterial loads are the same as the early subset of Ruiz-de-Castañeda et al. (2011). Full details of microbiological analyses are explained in that study. Briefly, using aseptic techniques, we obtained one microbiological sample

from each whole clutch after its completion. We used standard microbiological techniques to culture and quantify heterotrophic bacteria in our samples. These techniques have also been previously used in other studies of wild birds’ microbiology (Cook et al. 2003, 2005a,b, D’Alba et al. 2010). We transformed heterotrophic bacterial loads logarithmically in order to adjust this variable to a normal distribution. Samples were analyzed in the laboratory within 1–7 days of collection in the field. Given this variation in delay of analysis, we controlled statistically for its effect by including this variable as a covariate in all analyses (see Ruiz-de-Castañeda et al. 2011 for details). We finally used 61 bacterial loads for which we also had an effective record of incubation temperature.

STATISTICAL ANALYSES

We used Statistica 7.0 for statistical analyses. We first analyzed the distribution of frequencies of proportion of incubation times during daytime on day 3 ($T \geq 24$ °C) and established a conservative definition of incubation onset based on the 90th percentile of the distribution. The 90th percentile of the distribution of this variable was 5%, which means that only in 10% of the nests were eggs exposed to temperatures over 24 °C during at least 5% of the daytime or nighttime. Accordingly, we analyzed distribution of frequencies of proportion of incubation times during daytime on days 4 and 5 and during nighttime on days 3, 4, and 5 and established the day or night in which incubation began in a nest by considering proportions of incubation time that were above this 5% limit. In this way we determined the proportions of females that started incubation by day or by night of days 3, 4, or 5.

We analyzed variation in eggshells’ bacterial loads in relation to the proportion of incubation time and mean temperature of air around the eggs for daytime and nighttime independently. We included the eggshells’ log-transformed bacterial loads as the normal dependent variable and the proportion of incubation time and mean temperature around the eggs as independent continuous predictors. We also included clutch size and analysis delay in the analysis as independent predictors. Specifically, we first simultaneously included the proportions of incubation times and mean temperatures around eggs for all daytime periods on days 3, 4, and 5 and nighttime periods on days 3, 4, and 5 for the daytime and nighttime models, respectively. These models show which variables from which specific daytime or nighttime period are more important in explaining variation on eggshells’ bacterial loads during daytime and nighttime. We also constructed two additional models for the total daytime period (pooled daytime of days 3–5) and the total nighttime period (pooled nighttimes of days 3–5), including the total proportion of incubation time and mean temperature around the eggs for each category.

We used Akaike’s information criterion (AIC) to select the most plausible models. We specifically selected those

models that had the lowest AIC_c (best model). We also calculated ΔAIC_c and Akaike weights (w_i) for each model (Anderson et al. 2001). We calculated parameters for the best possible regression models with the GLM module of Statistica 7.0.

RESULTS

EARLY INCUBATION ONSET

Early incubation occurred mostly during daytime (Fig. 1a–d), and the majority of females did not incubate at all during nighttime (Fig. 2a–d). Figure 3 shows a representative continuous temperature record of a female with a clutch of six eggs that initiated incubation during daytime on day 4. According to our definition of onset (90th percentile, see above), early incubation began mainly during the daytime of day 4 and was very rare during nighttime. Specifically, 37 of 67 (55%) females started incubation during daytime of day 4, while only 7 and 11 females began incubation during daytime on days 3 and 5, respectively (Fig. 4). Onset of incubation at night was

very rare (2 females) (Fig. 4). Therefore, variation in incubation time during the day on day 4 appears most relevant for exploring the implications of early incubation.

DAYTIME INCUBATION

Incubation activity during daytime on day 4 was the key predictor of eggshells' bacterial loads during daytime. Thus, in a simultaneous analysis of incubation time and temperature during daytime on days 3, 4, and 5 in relation to eggshell bacterial loads, incubation during daytime on day 4 and not days 3 and 5 was of key importance for explaining eggshells' bacterial loads (Table 1). The best model among 200 possible models included only proportion of incubation time during daytime on day 4 and analysis delay and accounted for 44% of the variance in eggshells' bacterial loads (Table 1). During daytime on day 4, incubation time was significantly and negatively associated with eggshells' bacterial loads when analysis delay was controlled for (GLM, $F_{1,61} = 6.74$, $P = 0.01$, Fig. 5). Thus the key predictor of eggshells' bacterial loads is incubation time and not temperature.

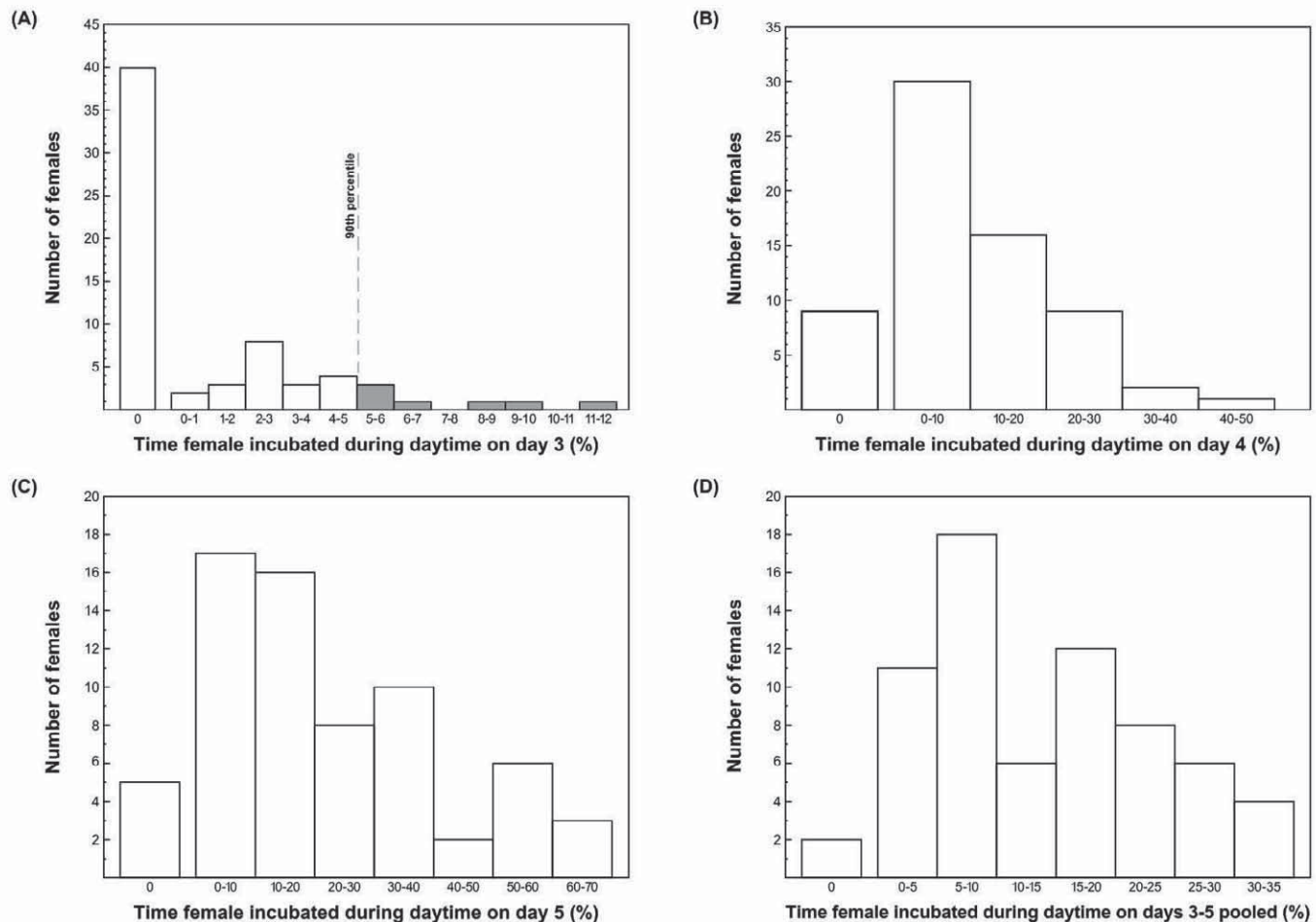


FIGURE 1. Proportion of time spent incubating during daytime by 67 female Pied Flycatchers during laying of eggs 3–5 of the clutch. A, B, C, daytime on days 3, 4, and 5, respectively; D, daytime for these three days pooled. The 90th percentile of the distribution is presented on panel 1a (see text for rationale).

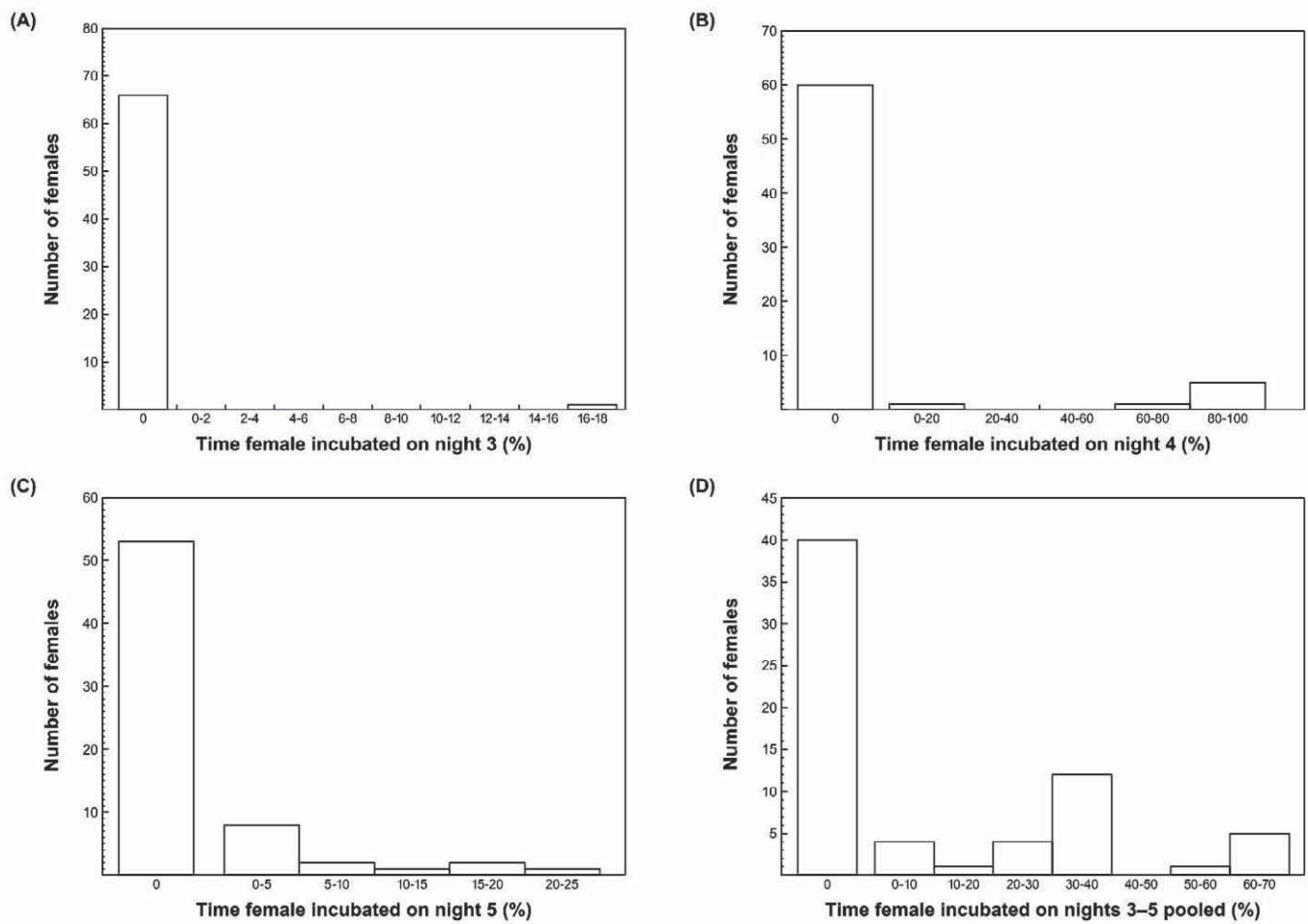


FIGURE 2. Proportion of time spent incubating during nighttime by 67 female Pied Flycatchers during laying of eggs 3–5 of the clutch. A, B, C, nighttimes on days 3, 4, and 5, respectively; D, nighttime for these three days pooled.

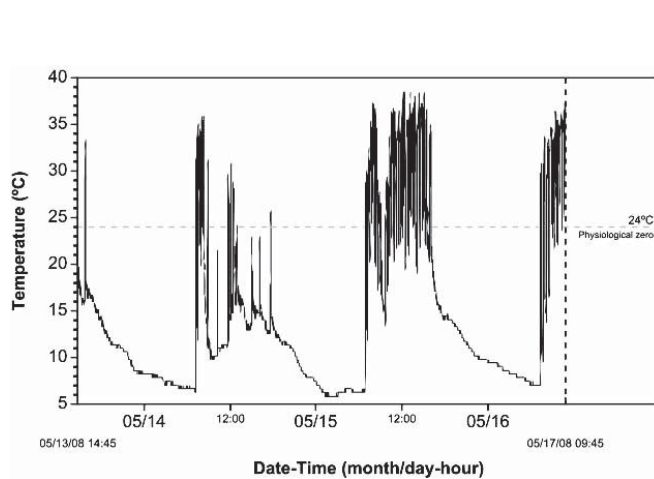


FIGURE 3. Representative record of air temperature in contact with the eggs from 14:45 on the day of laying of egg 3 to 09:45 on the day of clutch completion (6 eggs). The discontinuous horizontal line indicates the physiological zero (24 °C), the discontinuous vertical line the end of the record.

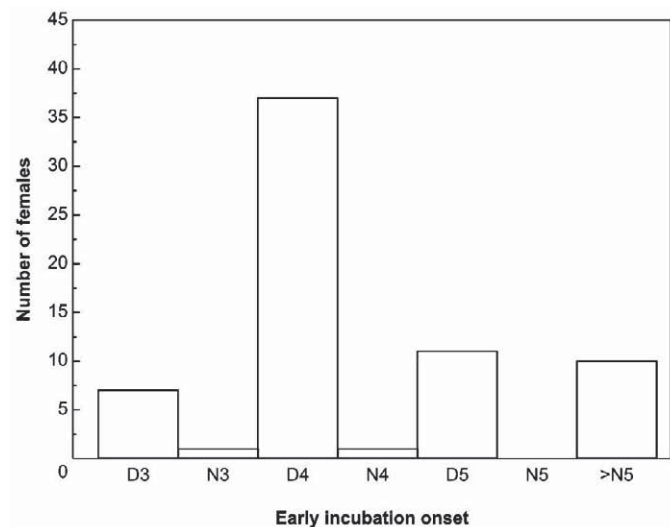


FIGURE 4. Onset of incubation of 67 female Pied Flycatchers during daytime (D) or nighttime (N) of days on which eggs 3, 4, and 5 were laid.

TABLE 1. Set of logistic-regression models with $\Delta AIC_c \leq 2$ and K parameters among 200 and 15 different possible models for 61 values of loads of culturable heterotrophic bacteria on eggshells of the Pied Flycatcher in relation to the female's daytime incubation activity (mean daytime temperature and proportion of daytime incubation) during daytime on days 3, 4, and 5 and during the total of daytime on days 3–5 (pooled). Delay in analysis of the samples and clutch size were included as covariates. Models are ranked by ΔAIC_c , which indicates the difference between each model and the best model with the lowest AIC_c , and the AIC weight (the relative likelihood of a model within a set of candidate models).

| Model | K | ΔAIC_c | Weight |
|--|-----|-------------------|--------|
| By day | | | |
| Daytime incubation day 4 (%), analysis delay | 3 | 0.00 ^a | 0.08 |
| Daytime incubation day 3 (%), daytime incubation day 4 (%), analysis delay | 4 | 0.43 | 0.06 |
| Daytime incubation day 3 (%), mean daytime temperature day 3, daytime incubation day 4 (%), analysis delay | 5 | 1.23 | 0.04 |
| Daytime incubation day 4 (%), daytime incubation day 5 (%), analysis delay | 4 | 1.00 | 0.05 |
| Daytime incubation day 3 (%), daytime incubation day 4 (%), daytime incubation day 5 (%), analysis delay | 5 | 1.43 | 0.04 |
| Daytime incubation day 4 (%), mean daytime temperature day 5, analysis delay | 4 | 1.44 | 0.04 |
| Daytime incubation day 4 (%), mean daytime temperature day 4, analysis delay | 4 | 1.55 | 0.04 |
| Days 3–5 pooled | | | |
| Analysis delay | 2 | 0.00 ^b | 0.33 |
| Mean daytime temperature days 3–5, analysis delay | 3 | 1.32 | 0.17 |
| Clutch size, analysis delay | 3 | 1.42 | 0.16 |
| Total daytime incubation days 3–5 (%), analysis delay | 3 | 1.77 | 0.14 |

^aLowest $AIC_c = 224.19$.

^bLowest $AIC_c = 228.68$.

Total incubation time and mean temperature for daytimes on days 3–5 pooled were poor predictors of eggshell bacterial loads. None of these predictors were included in the best model among the 15 possible models obtained (Table 1).

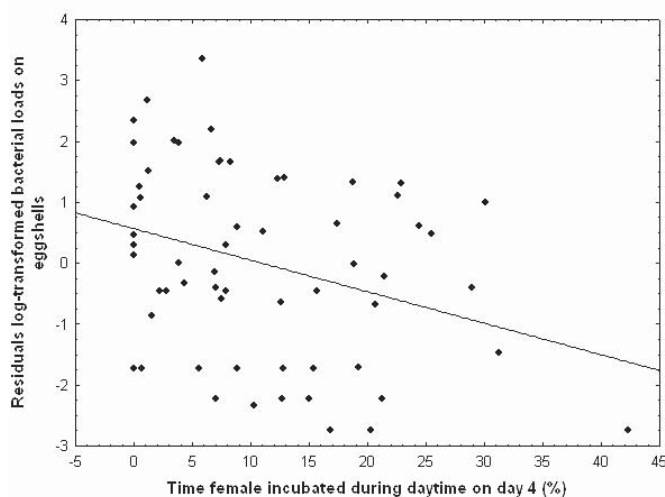


FIGURE 5. Association between proportion of incubation time during daytime on day 4 and load of culturable heterotrophic bacteria on eggshells, presented as residuals of the correlation between log-transformed bacterial loads, and delay in analysis of the samples ($n = 61$, $r = -0.32$, $P = 0.01$, $y = 0.57 - 0.05x$).

NIGHTTIME INCUBATION

Incubation time and temperature during nighttime on days 3, 4, and 5 were poor predictors of eggshells' bacterial loads. The best model among 200 possible models included only incubation time during nighttime on day 5 and analysis delay (Table 2). However, incubation time during nighttime on day 5 was not significantly associated with bacterial load when analysis delay was controlled for (GLM, $F_{1,61} = 3.32$, $P = 0.07$).

However, total incubation time but not mean temperature for nighttime on days 3–5 pooled was a good predictor of eggshells' bacterial loads. The best model among 15 possible models included total incubation time for the whole nighttime period (days 3–5) and analysis delay (Table 2). This model accounted for 42% of the variance, and total incubation time for the whole nighttime period was significantly and negatively associated with bacterial load when analysis delay was controlled for (GLM, $F_{1,61} = 4.50$, $P = 0.04$, Fig. 6)

DISCUSSION

The majority of females started incubation during daytime on day 4, and clutches incubated more in this period harbored lower loads of bacteria on their eggshells than did clutches incubated less. As there was little variation in females' incubation activity before daytime of day 4 and only a minority of females started incubation during daytime of day 5, we found

TABLE 2. Set of logistic-regression models with $\Delta AIC_c \leq 2$ and K parameters among 200 and 15 different possible models for 61 values of loads of culturable heterotrophic bacteria on eggshells of the Pied Flycatcher in relation to the female's nighttime incubation activity (mean nighttime temperature and proportion of nighttime incubation) during nighttime on days 3, 4, and 5 and during the total of nighttime on days 3–5 (pooled). Delay in analysis of the samples and clutch size were included as covariates. Models are ranked by ΔAIC_c , which indicates the difference between each model and the best model with the lowest AIC_c , and the AIC weight (the relative likelihood of a model within a set of candidate models).

| Model | K | ΔAIC_c | Weight |
|--|-----|-------------------|--------|
| By day | | | |
| Nighttime incubation day 5 (%), analysis delay | 3 | 0.00 ^a | 0.05 |
| Mean nighttime temperature day 4, analysis delay | 3 | 0.09 | 0.04 |
| Nighttime incubation day 4 (%), analysis delay | 3 | 0.14 | 0.04 |
| Mean nighttime temperature day 4, nighttime incubation day 5 (%), analysis delay | 4 | 0.74 | 0.03 |
| Nighttime incubation day 4 (%), nighttime incubation day 5 (%), analysis delay | 4 | 0.99 | 0.03 |
| Mean nighttime temperature day 5, analysis delay | 3 | 1.05 | 0.03 |
| Analysis delay | 2 | 1.18 | 0.03 |
| Mean nighttime temperature day 3, nighttime incubation day 5 (%), analysis delay | 4 | 1.24 | 0.02 |
| Mean nighttime temperature day 4, mean nighttime temperature day 5, analysis delay | 4 | 1.48 | 0.02 |
| Nighttime incubation day 4 (%), mean nighttime temperature day 5, analysis delay | 4 | 1.57 | 0.02 |
| Mean nighttime temperature day 3, nighttime incubation day 4 (%), analysis delay | 4 | 1.72 | 0.02 |
| Days 3–5 pooled | | | |
| Total nighttime incubation days 3–5 (%), analysis delay | 3 | 0.00 ^b | 0.29 |
| Mean nighttime temperature days 3–5, analysis delay | 3 | 0.24 | 0.26 |

^aLowest $AIC_c = 227.50$.

^bLowest $AIC_c = 226.33$.

no effects of incubation during daytime on days 3 or 5 on eggshells' bacterial load. Total daytime incubation (daytime of days 3–5) was neither significantly associated with bacterial load, presumably because the range of variation of this

cumulative variable is reduced in comparison with daytime incubation on day 4. On the day incubation begins, conditions around the eggs may change dramatically from those on previous days without incubation, explaining why early incubation at this time may inhibit bacteria strongly. Incubation was almost none during nighttime on days 3, 4, and 5, explaining why nighttime incubation during laying was not associated with eggshells' bacterial loads. However, when total nighttime incubation over all three days is considered, clutches attended more had lower bacterial loads.

The effects of increasing ambient temperature had a positive effect on eggshell bacteria within the moderate range of temperatures in the montane habitat we studied (mean temperature $12.1^\circ\text{C} \pm \text{SE } 0.5$, Ruiz-de-Castañeda et al. 2011). Because environmental temperatures are higher during daytime, the fact that incubation during laying takes place mainly during daytime could result from the necessity to control proliferation of bacteria on eggshells at a temperature favorable for their growth. Temperatures lower during nighttime than during daytime may limit the metabolic activity of most bacteria and their growth on eggshells, explaining why during laying incubation during nighttime is minimal. This also contradicts the alternative hypothesis that onset of partial incubation during laying is determined by thermoregulatory needs. The onset of nighttime incubation may be more related to the thermoregulatory needs of the first eggs laid (Arnold et al. 1987, Veiga et al. 1992) than to antibacterial defenses. However, the

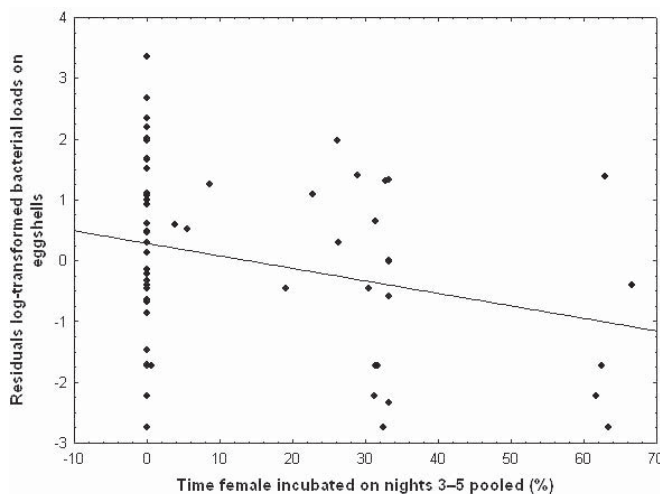


FIGURE 6. Association between proportion of incubation time during nighttime on days 3–5 pooled and load of culturable heterotrophic bacteria on eggshells, presented as residuals of the correlation between log-transformed bacterial loads, and delay in analysis of the samples, presented as the dependent variable ($n = 61$, $r = -0.27$, $P = 0.04$, $y = 0.28 - 0.02x$).

association of total nighttime incubation during the whole period of laying with bacterial loads suggests that nighttime incubation may also affect bacterial growth.

Our results provide support for the experimentally demonstrated role of early incubation in inhibiting bacterial growth on eggshells of passerines in the tropics (Cook et al. 2005b, Shawkey et al. 2009) and temperate zone (D'Alba et al. 2010). These studies found that bacterial loads (Cook et al. 2005b, D'Alba et al. 2010) and taxon richness of bacteria (Shawkey et al. 2009) were significantly lower on eggs incubated naturally than on eggs experimentally prevented from being incubated. To date, however, no published study has, to our knowledge, related a direct measure of increased incubation during the critical stage of the onset of incubation during laying to lower bacterial loads on eggshells in natural nests of a cavity-nesting species.

D'Alba et al. (2010) first demonstrated experimentally that removal of water from egg surfaces is one proximate mechanism for the antibacterial effects of incubation. This possibility had been suggested, but not tested, by Cook et al. (2003, 2005a,b). The dramatic increases we detected in temperature of the eggs (over 35 °C; see, for instance, Fig. 3) associated with the direct contact with the highly vascularized brood patch of the female may cause water to evaporate faster and may prevent condensation on eggshells (D'Alba et al. 2010). Also, it has been hypothesized that these increases of temperature may act on bacteria directly by exceeding the optimum for the growth of certain species (Cook et al. 2003, 2005a). Our data suggest that the time above a certain critical temperature is more relevant for bacterial inhibition than temperature itself because this latter variable did not appear in any of the selected best models for daytime or nighttime. Temperature may directly affect only certain bacteria not specifically quantified with the general microbiological approach we used. Temperature may have implications for the drying effect associated with incubation (D'Alba et al. 2010). Additional defensive mechanisms unrelated to thermal effects may also be relevant, though no specific information is available for the Pied Flycatcher. Possibly, Pied Flycatchers also produce antibacterial uropygial oil (Shawkey et al. 2003) and could transfer it onto eggshells directly or indirectly by contact with the plumage (Soler et al. 2008). Although Pied Flycatchers do not include feathers in their nests, the contact of the plumage of the incubating female with the eggs could also favor the transfer of benign competitive bacteria that could prevent the establishment of pathogenic bacteria (Peralta-Sánchez et al. 2010). Similarly, Cook et al. (2005b) (but see Shawkey et al. 2009) also suggested that incubation could favor the proliferation of certain bacteria on eggshells that could competitively exclude other bacteria present on eggshells that are potentially pathogenic for the embryo. Only experimental studies may unravel the mechanism behind the association we found.

To conclude, our results with a cavity-nesting passerine support the concept that the onset of daytime incubation during laying

may be necessary to control the initial growth of bacteria on eggshells, which could ultimately affect the embryo's viability. Bacteria must therefore be considered as a potential selective pressure involved in the evolution of the early onset of avian incubation.

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LITERATURE CITED

- ARNOLD, T. H., F. C. ROWHER, AND T. ARMSTRONG. 1987. Egg viability, nest predation and the adaptive significance of clutch size in prairie ducks. *American Naturalist* 130:643–653.
- CLARK, L., AND J. R. MASON. 1988. Effect of biologically active plants used as nest material and the derived benefit to starling nestlings. *Oecologia* 77:174–180.
- COOK, M. I., S. R. BEISSINGER, G. A. TORANZOS, R. A. RODRIGUEZ, AND W. J. ARENDT. 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? *Proceedings of the Royal Society of London B* 270:2233–2240.
- COOK, M. I., S. R. BEISSINGER, G. A. TORANZOS, AND R. A. RODRIGUEZ. 2005a. Microbial infection affects egg viability and incubation behaviour in a tropical passerine. *Behavioral Ecology* 16:30–36.
- COOK, M. I., S. R. BEISSINGER, G. A. TORANZOS, AND W. J. ARENDT. 2005b. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. *Ecology Letters* 8:532–537.
- D'ALBA, L., A. OBORN, AND M. D. SHAWKEY. 2010. Experimental evidence that keeping eggs dry is a mechanism for the antimicrobial effects of avian incubation. *Naturwissenschaften* 97:1089–1095.
- HÉBERT, P. N. 2002. Ecological factors affecting initiation of incubation behaviour, p. 270–279. *In* D.C. Deeming [ED.], *Avian incubation behaviour, environment and evolution*. Oxford University Press, Oxford, England.
- LUNDBERG, A., AND R. ALATALO. 1992. *The Pied Flycatcher*. Poyser, London.
- MAGRATH, R. D. 1990. Hatching asynchrony in altricial birds. *Biological Reviews* 65:587–622.
- MASSARO, M., A. N. SETIAWAN, AND L. S. DAVIS. 2007. Effects of artificial eggs on prolactin secretion, steroid levels, brood patch development, incubation onset and clutch size in the Yellow-eyed Penguin (*Megadyptes antipodes*). *General and Comparative Endocrinology* 151:220–229.
- MENON, C. K., AND J. MENON. 2000. Avian epidermal lipids: functional considerations and relationship to feathering. *American Zoologist* 40:540–552.
- MORENO, J., S. MERINO, J. J. SANZ, E. ARRIERO, J. MORALES, AND G. TOMÁS. 2005. Nestling cell-mediated immune response, body mass and hatching date as predictors of local recruitment in the Pied Flycatcher *Ficedula hypoleuca*. *Journal of Avian Biology* 36:251–260.

- MORENO, J., S. MERINO, E. LOBATO, R. RUIZ-DE-CASTAÑEDA, J. MARTÍNEZ DE LA PUENTE, S. DEL CERRO, AND J. RIVERO-DE AGUILAR. 2009. Nest-dwelling ectoparasites of two sympatric hole-nesting passerines in relation to nest composition: an experimental study. *Écoscience* 16:418–427.
- O'CONNOR, R. J. 1984. The growth and development of birds. Wiley, New York.
- PERALTA-SÁNCHEZ, J. M., A. P. MØLLER, A. M. MARTÍN-PLATERO, AND J. J. SOLER. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in swallow nests: an experimental study. *Functional Ecology* 2:426–433.
- RENEERKENS, J., M. A. VERSTEEGH, A. M. SCHNEIDER, T. PIERSMA, AND E. H. BURTT. 2008. Seasonally changing preen-wax composition: Red Knots' (*Calidris canutus*) flexible defense against feather-degrading bacteria? *Auk* 125:285–290.
- RUIZ-DE-CASTAÑEDA, R., A. I. VELA, E. LOBATO, V. BRIONES, AND J. MORENO. 2011. Bacterial loads on eggshells of the Pied Flycatcher: environmental and maternal factors. *Condor* 113:200–208.
- SHAWKEY, M. D., S. R. PILLAI, AND G. E. HILL. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *Journal of Avian Biology* 34:345–349.
- SHAWKEY, M. D., M. K. FIRESTONE, E. L. BRODIE, AND S. R. BEISSINGER. 2009. Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. *PLoS One* 4:e4522.
- SOLER, J. J., M. MARTÍN-VIVALDI, M. RUIZ-RODRÍGUEZ, E. VALDIVIA, M. MARTÍN-PLATERO, M. MARTÍNEZ-BUENO, J. M. PERALTA-SÁNCHEZ, AND M. MÉNDEZ. 2008. Symbiotic association between Hoopoes and antibiotic-producing bacteria that live in their uropygial gland. *Functional Ecology* 22:864–871.
- STENNING, M. J. 1996. Hatching asynchrony, brood reduction and other rapidly reproducing hypotheses. *Trends in Ecology and Evolution* 11:243–246.
- STOLESON, S. H., AND S. R. BESSINGER. 1995. Hatching asynchrony and the onset of incubation in birds, revisited: when is the critical period? *Current Ornithology* 12:191–254.
- VEIGA, J. P. 1992. Hatching asynchrony in the House Sparrow: a test of the egg-viability hypothesis. *American Naturalist* 139:669–675.
- WANG, J. M., AND S. R. BEISSINGER. 2009. Variation in the onset of incubation and its influence on avian hatching success and asynchrony. *Animal Behaviour* 78:601–613.
- WEBB, D. R. 1987. Thermal tolerance of avian embryos: a review. *Condor* 89:874–879.