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Drying eggs to inhibit bacteria: Incubation during laying in a cavity nesting passerine

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ABSTRACT

Early incubation has been suggested as a defensive adaptation against potentially pathogenic bacteria colonizing avian eggshells in the wild. The inhibitory mechanisms underlying this adaptation are poorly understood and only recent experimental evidence demonstrates that keeping eggs dry is a proximate mechanism for the antimicrobial effects of avian incubation. We estimated partial incubation (the bouts of incubation that some birds perform during the egg-laying period, days of lay 3–5 in our population) intensity of female pied flycatchers breeding in nest-boxes using data loggers that allowed a precise measurement of temperature just between the eggs in the nest-cup. We also measured relative humidity within the nest-boxes and related it to incubation intensity, showing that more intense incubation during laying contributes to drying the air near the eggs. We analyzed separately the effects of incubation and of relative humidity on loads of three types of culturable bacteria known to be present on eggshells, heterotrophic bacteria, Gram-negative enterics and pseudomonads. Our results show an association of early incubation with an inhibition of bacterial proliferation through a drying effect on eggshells, as we found that incubation intensity was negatively and relative humidity positively associated with eggshell bacterial loads for heterotrophic bacteria, Gram-negative bacteria and pseudomonads, although the significance of these associations varied between bacterial groups. These results point to microclimatically driven effects of incubation on bacterial proliferation on eggshells during laying in a temperate cavity nesting passerine.

1. Introduction

The study of behavioural defences against bacterial infection of eggs and embryos may contribute to our understanding of some important aspects of avian breeding biology. During laying and incubation eggs are exposed during variable periods of time (Wang and Beissinger, 2009) to different sources of environmental and maternal bacteria such as air, nest-material, cloacae and plumage (Burtt and Ichida, 1999; Cook et al., 2003, 2005a; Shawkey et al., 2009; Peralta-Sánchez et al., 2010; Ruiz-de-Castañeda et al., 2011a). Some of these bacteria could ultimately infect egg contents and kill the developing embryo (Cook et al., 2005a; Soler et al., 2008 but see Peralta-Sánchez et al., 2010; Ruiz-de-Castañeda et al., 2011a,b; D'Alba et al., 2010). Both Cook et al. (2005b) and Shawkey et al. (2009), based on culture dependent and independent methods, respectively, have demonstrated experimentally that partial incubation, the bouts of incubation that some birds perform during the egg-laying period (D'Alba et al., 2010), inhibits bacterial proliferation and diversification on eggshells in a tropical hole-nesting passerine. This was also found in temperate passerine in a correlative study by Ruiz-de-Castañeda et al. (in press). Several possible inhibitory mechanisms operating during early incubation have been proposed. For instance, antibacterial secretions produced in the uropygial gland of some species (Shawkey et al., 2003; Soler et al., 2008; Martin-Vivaldi et al., 2010), but also other secretions produced on the brood patch (Menon and Menon, 2000) may be transferred onto eggshells through contact with the incubating adult. Other species may have evolved the capacity to include in their nests certain types of biologically active plants that could release volatile antibacterial compounds during incubation (Clark and Mason, 1985; Mennerat et al., 2009).

Besides these specialized adaptations, the most obvious effect of incubation itself is a strong modification of microclimatic conditions within the nest, and this may be particularly true for cavity nesters. Thus, the presence of the incubating bird in the nest together with the nest structure may create a local environment that differs from conditions outside the cavity (Ar and Sidis, 2002). It is well known that humidity is among the most limiting abiotic factors affecting bacterial proliferation under laboratory and natural conditions (Maier et al., 2000; Madigan et al.,

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2005). Thus, behavioural strategies of incubating adults that have the potential to control humidity around the eggs may have been selected, at least in part, to prevent proliferation of harmful bacteria on eggshells. Cook et al. (2003, 2005a,b) first hypothesized about the possibility that by incubating, parents may potentially minimize the build up of harmful bacteria by reducing moisture on eggshells. Interestingly, D'Alba et al. (2010) recently demonstrated experimentally for a temperate western hole-nesting passerine that keeping eggs dry is a proximate mechanism for the antimicrobial effects of avian incubation. Removal of water from eggshells may itself occur through a number of mechanisms. For instance, increasing the temperature of eggs may cause water to evaporate faster and prevent condensation (Cook et al., 2003, 2005a,b; D'Alba et al., 2010) and/or egg turning (Deeming, 2002) may cause water to become absorbed by nest materials.

In this study we measured nest microclimatic relative humidity (RH) and temperature around the eggs during daytime periods of the critical part of the laying period for incubation onset in a population of pied flycatchers *Ficedula hypoleuca* breeding in nest-boxes in a temperate montane habitat in Central Spain (Ruiz-de-Castañeda et al., in press). We used the temperature record to detect partial incubation activity and related this activity to microclimatic variation of RH within the nest-box. We analyzed the association of incubation intensity and RH with loads of three prevalent types of culturable bacteria on eggshells, namely heterotrophic bacteria, Gram-negative enterics and pseudomonads. We specifically selected these types of culturable bacteria based on a previous descriptive study of culturable eggshell microbiota in this population (Ruiz-de-Castañeda et al., 2011b). We have tested the following predictions derived from the fact that incubation during laying serves to reduce eggshell bacterial loads through a drying effect (D'Alba et al., 2010):

(1) Incubation activity during laying produces a reduction of microclimatic RH within the nest-box.
(2) Eggshell bacterial loads are negatively affected by the reduction of RH effected by incubation activity, although the intensity of these effects may differ between bacterial groups.

To our knowledge, this is the first study specifically testing effects of incubation driven microclimatic variation of RH within a cavity nest in a wild European passerine in a temperate habitat.

2. Methods

2.1. Study area and species

We conducted this study during the spring of 2009 on a population of pied flycatchers breeding in artificial nest-boxes in a montane forest of pyrenean oak, *Quercus pyrenaica*, at 1200 m above the sea level in central Spain (40°54′N, 04°01′W). The pied flycatcher is a small hole-nesting passerine of European woodlands that readily accepts artificial nest-boxes for breeding and is frequently used as a model species in behavioural ecology (Lundberg and Alatalo, 1992). Egg laying in this population typically begins in late May, and modal clutch size is six. Eggs are laid within a well defined semi-spherical nest-cup mainly composed of strips of bark (Moreno et al., 2009) and are incubated solely by the female.

2.2. Nest microclimatic relative humidity and temperature measurements

We visited nest-boxes daily at early morning once nests were fully lined indicating that females were about to lay. Thus, we exactly recorded the day of clutch initiation. On the day of lay of the third egg (day 3, hereafter), we placed HOBO® Data Loggers (Onset Computer Corporation, MA) inside 36 nest-boxes occupied by pied flycatchers. We unwound the temperature sensor wire from the circuit board and extended it through the nest material until the small sensor (1.5 mm) showed up in the middle of the nest-cup just between the eggs. This setup allowed the measurement of temperature just between the eggs. The humidity sensor could not be used outside the logger box according to technical indications of use of the logger. Thus, we measured relative humidity of the air within the nest-box just below the position of the eggs in the nest-cup. We started measurements at 13:00 h on day 3 and programmed the data-loggers with a 1 min measuring interval covering part of the daytime period for day 3 and complete daytime periods for days 4 and 5. A previous study in this population showed that most females start nocturnal incubation after completion of the clutch (Ruiz-de-Castañeda et al., in press), so we will only consider daytime incubation in this study. Observatorio Astronómico Nacional provided us with information about sunrise and sunset in our study site during the study period (May 12 to June 2). We specifically selected day 3 to initiate measurements as no female has been found on eggs prior to that day during more than two decades of nest checks in our study population.

To control for the effects of nest material on microclimate inside nests, we also measured microclimatic humidity and temperature within 28 nest-boxes containing unoccupied nests randomly distributed in the study area. The entrance of these nest-boxes was blocked using a metallic mesh only allowing the circulation of air but preventing the entrance of birds. Within 16 of these nest-boxes we placed pied flycatchers nests collected during previous breeding seasons in nearby study areas, while in the remaining 12 nest-boxes we introduced similar amount of material and constructed artificial nests with equivalent composition and structure as in natural nests. Data loggers were then placed in these control nest-boxes in the same way as we did in nests occupied by pied flycatchers. We programmed data-loggers with a 24 s measuring interval covering part of two daytime periods from 13:00 h on a randomly selected day during the laying period of flycatchers in the study area to 10:15 h on the day after. We specifically considered the daytime period of the first day of measurements because it covered the central daytime hours when ambient temperature is expected to reach its maximum (13:00–16:00 h).

We did not visit nest-boxes while data-loggers were measuring. The program BoxCar® Pro 4 (Onset Computer Corporation, MA) allowed a read-out of the loggers after removal from nest-boxes. From this read-out we calculated the mean RH in % during incubation activity for the total period covering days 3–5.

2.3. Field microbiological sampling

We marked eggs 1–3 with a small dot on the morning they were laid using a permanent marker pen. We could not mark the remaining eggs of the clutch since data-loggers were measuring while they were laid and we did not visit nest-boxes during this period. We identified the last laid egg among the 2 (clutch size 5) or 3 (clutch size 6) non-marked eggs by the size of the air bubble (Pitts, 1995). We sampled eggshell bacteria once on the same morning of removal of the data-loggers from the nest. We sampled all eggs in the clutch excepting the last laid egg, as it was not exposed to incubation during the laying period. There were no significant differences in eggshell loads of each of the three types of bacteria due to differences in the number of eggs sampled (4 and 5 in clutches of 5 and 6 eggs, respectively) (all $F \leq 1.02$, $P \geq 0.32$).

To minimize the possible contamination of the sample with external bacteria not directly associated with the sampling surface we always handled eggs with latex gloves previously disinfected