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LETTER

Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection

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Abstract

Avian eggshells harbour microbes shortly after laying, and under appropriate ambient conditions they can multiply rapidly, penetrate through shell pores, infect egg contents and cause embryo mortality. We experimentally examined how incubation affects bacterial processes on the eggshells of pearl-eyed thrashers *Margarops fuscatus* nesting in tropical montane and lowland forests in Puerto Rico. Bacteria and fungi grew rapidly on shells of newly laid, unincubated eggs exposed to ambient conditions, but declined to low levels on shells of eggs incubated by thrashers. Divergence in bacterial growth between incubated and exposed eggs was more marked at the montane forest than at the lowland site. Pathogenic microorganisms became increasingly dominant on shells of exposed eggs, but these groups were relatively rare on incubated eggs, where more benign, less invasive groups prevailed. Some incubation during laying may be necessary to decrease the probability of trans-shell infection by reducing the growth of harmful bacteria and fungi on eggshells, although it may increase hatching asynchrony and the likelihood of brood reduction.

Keywords

Bird, egg laying, egg viability, eggs, hatching asynchrony, incubation, life history, microbial ecology.

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INTRODUCTION

Birds lay a maximum of one egg daily and many species begin incubation prior to clutch completion, which causes hatching asynchrony and can lead to mortality of latter-hatched chicks (Clark & Wilson 1981; Stoleson & Beissinger 1995). Newly laid avian eggs exhibit a rapid loss of viability if left unincubated and exposed to ambient conditions for prolonged periods, and parents may be forced to initiate incubation before laying the last egg to protect early laid eggs from environmental conditions (Arnold *et al.* 1987; Viega 1992; Stoleson & Beissinger 1999). While temperature has traditionally been considered the most critical condition affecting egg viability (Webb 1987; Stoleson 1999, Beissinger *et al.* in press), another cause of viability declines is trans-shell infection by pathogenic microorganisms (Cook *et al.* 2003, 2005). Eggshells of domestic fowl, wild ducks, thrashers, bluebirds, swallows and other avian species harbour bacteria and fungi at or shortly after laying (Baggott & Graeme-Cook 2002; Cook *et al.* 2003, 2005; J. Wang and S.R. Beissinger, unpublished data). Under appropriate ambient conditions, bacteria and fungi can multiply rapidly, penetrate the eggshell through shell pores, infect egg contents and reduce hatchability (Board & Tranter 1986; Bruce & Drysdale 1991, 1994). Recent experiments with pearl-eyed thrasher *Margarops fuscatus* and domestic fowl eggs demonstrated that infection of egg contents was positively related to shell microbial densities, occurred rapidly (between 3–5 days) and well within the period required to lay a clutch (hereafter 'laying period'), and caused a dramatic decline in hatching success (Cook *et al.* 2003, 2005).

The eggshell microbiota is comprised of a diverse array of organisms, but only certain groups appear important to the infection process. Pseudomonads and fungi can digest the cuticle layer, destroy the egg's water resistant properties, and increase the number of unplugged pores available for

trans-shell transmission (Board & Halls 1973; Board *et al.* 1979; Baggott & Graeme-Cook 2002). Microbial groups also differ in their ability to penetrate the shell and infect egg contents. Whereas desiccation-tolerant Gram-positive rods dominate the eggshell microbiota, species that infect egg contents appear to be a varied assortment of opportunistic saprophytes including certain fungi, Gram-positive cocci, Gram-negative enterics and Gram-negative fermenters (Board & Tranter 1986; Kozłowski *et al.* 1989; Bruce & Drysdale 1991, 1994; Houston *et al.* 1997; Cook *et al.* 2003, 2005). By incubating, parents may potentially minimize the build up of harmful microorganisms by reducing moisture on the eggshell, or by the antibacterial properties of certain waxes and fatty acids found on feathers, in preen gland secretions, and perhaps on the epidermal layer on the brood patch of many birds (Jacob 1978; Menon & Menon 2000; Shawkey *et al.* 2003; Sweeney *et al.* 2004). Thus, shells of incubated eggs should harbour lower densities of pathogenic microorganisms and risk a lower probability of trans-shell infection than eggs exposed to ambient conditions.

Here we present results of an experiment to test how incubation during laying affects bacterial (and some fungal) processes on the eggshell surface. We do not characterize the entire microbial community on the eggshell, but instead focus on those bacterial groups known to infect egg contents. We compared the daily growth of these bacteria on naturally incubated eggshells with that of eggshells exposed to ambient conditions using pearl-eyed thrasher eggs in Puerto Rican forests. We predicted that growth of pathogenic bacterial groups on eggshells would be greater and the proportion of eggs harbouring these bacteria would be higher for exposed eggs than for incubated eggs.

MATERIALS AND METHODS

The pearl-eyed thrasher (hereafter thrasher) is a medium-sized, omnivorous, Neotropical passerine that nests in forests from the lowlands to the cloud forest throughout Puerto Rico (Raffaele 1989). Thrashers build nests in cavities, and lay one egg daily or every second day until a typical clutch of three to four eggs is completed over 3–6 days (Arendt 1993). Only females incubate; they exhibit partial incubation (< 20% of daylight hours) on the day of or the day after clutch initiation, and the duration of incubation increases daily until a maximum is reached at or just after clutch completion (M.I. Cook and S.R. Beissinger, unpublished data). Clutches typically hatch over a 1–3-day interval.

We conducted studies in a very humid, tropical montane forest (600–810 m above sea level) in the Luquillo Experimental Forest and at a nearby warmer, less humid tropical lowland forest, Las Paulinas (10–20 m above sea level), in north-eastern Puerto Rico. The montane forest consisted of cloud forest and mid-elevation forest sites described in Cook *et al.* (2003, 2005) and Beissinger *et al.* (in press). At all sites thrashers nested in 134 wooden nest boxes that we erected in trees from 2–15 m above the ground about 0.1 km apart. During the experiment, ambient temperature and relative humidity in the montane forest averaged (± 1 SE) 20.1 ± 0.03 °C (range: 13.7–28.0) and 98.2 ± 0.1% (range: 53–100), respectively, compared with 25.8 ± 0.04 °C (range: 18.8–30.3) and 79.0 ± 0.1% (range: 33–98) in the lowland forest. See Cook *et al.* (2005) and Beissinger *et al.* (in press) for site details and nest checking methods.

We compared the daily change in shell bacteria over 3 days (the average laying period) of 24 parentally incubated eggs to that of 45 unincubated eggs exposed to ambient conditions between 3 April 3 and 30 June 2002. To replicate delayed incubation and examine the response of eggshell bacteria to prolonged exposure to ambient conditions, we removed unincubated thrasher eggs on the morning they were laid and placed them in thrasher nests situated in shaded holding-boxes identical to those used by breeding birds. We placed holding boxes in the forests within 10 m of active thrasher nest boxes to ensure climatic conditions were similar to those experienced by nesting birds, and positioned all boxes so they were sheltered from prevailing weather. This setup provided exposed eggs with climatic conditions and a nest microbiota similar to those experienced by naturally incubated eggs.

Clutches were randomly assigned to an incubation or exposure treatment. Only one clutch per nesting female and no more than two eggs per clutch were used, with each egg randomly assigned to a different holding site. We used only first-laid eggs for the parental incubation treatment because thrashers slowly increase diurnal incubation over the laying sequence, which may confound differences in microbial growth if later-laid eggs received more incubation. Exposed eggs received no incubation and were taken from throughout the laying sequence because the number of colony forming units (CFU) (see below) at laying was unaffected by laying order ($F_{2,66} = 2.07$, $P = 0.134$).

We used sterile techniques throughout for egg handling and transport (see Cook *et al.* 2003, 2005). Approximately one-fifth (3 cm^2) of the shell surface of each egg was swabbed from round to blunt end within 2 h of laying to collect microbiota. Eggs were then marked, and either returned to natal nests or transported to a holding site. At each holding site, eggs were placed in the nest at the base of the wooden nest box so they did not touch one another and were turned twice daily along their longitudinal axis. Eggs were swabbed daily during the three-day treatment period between 09:00 hours and 11:00 hours to record changes in growth and phylogenetic composition of the eggshell bacteria.

To prevent bacterial growth during transportation, swabs were immediately placed into 5 mL sterile physiological

(0.85% NaCl) saline and transported to the laboratory in an air-conditioned vehicle within 3 h of collection. We immediately cultured 0.1 mL of the swab supernatant into each of two liquefied growth media: MacConkey agar to grow Gram-negative enteric bacteria, and tryptose soya agar for heterotrophic bacteria. After 48 h, resultant microbial CFU (CFUs/0.1 mL) were counted and identified to major group level. See Cook *et al.* (2003, 2005 for additional details of microbiological techniques. Culture-based techniques do not characterize microbial communities as comprehensively as culture-independent methods (Amann *et al.* 1995), although the latter methods also have limitations of bias and error (Farrelly *et al.* 1995; Qiu *et al.* 2001; Speksnijder *et al.* 2001; Shawkey *et al.* in press). While our methods will not characterize the entire microbial community inhabiting bird eggs, the media were selected to detect the most common groups of bacteria known to live on avian eggshells and known to reduce embryo viability based on extensive studies of bacteria on domestic fowl eggs and limited studies of bacteria on wild bird eggs (Board & Tranter 1986; Kozłowski *et al.* 1989; Bruce & Drysdale 1991, 1994; Houston *et al.* 1997; Cook *et al.* 2003, 2005). Tryptose soya agar is one of the best general media for aerobic and anaerobic soil organisms, including Gram-positive bacteria. MacConkey agar is widely used to differentiate Gram-negative enteric and fermenter bacteria, which are known to be fast-growing yolk pathogens. Used together, these media should adequately characterize the relative load of bacterial groups known to produce pathogenic infections of avian eggs on the eggshells of incubated and unincubated eggs.

Comparisons between exposed and incubated eggs at each site establish the effect of incubation on microbial growth and phylogenetic composition under the two climatic regimes. We used repeated measures ANOVA to analyse microbial growth. Microbial growth data were square root transformed for all analyses. Examination of both the Huynh–Feldt and Greenhouse–Geisser adjusted *P*-values indicated that assumptions of compound symmetry (within group variances are equal) and sphericity (within groups covariances are equal) were met (SYSTAT 2004), so we report only standard ANOVA *P*-values. Fisher's exact tests were used to analyse changes in microbial composition. Values are expressed as mean ± 1 SE and analyses were carried out in SYSTAT (2004).

RESULTS

Microbial growth

Target bacterial groups were evident on the shells of 78.3% of freshly laid eggs ($n = 69$) and on all clutches ($n = 52$). Mean number of CFUs per egg was relatively low at laying, but varied greatly among eggs (32.0 ± 9.2, range 0–500).

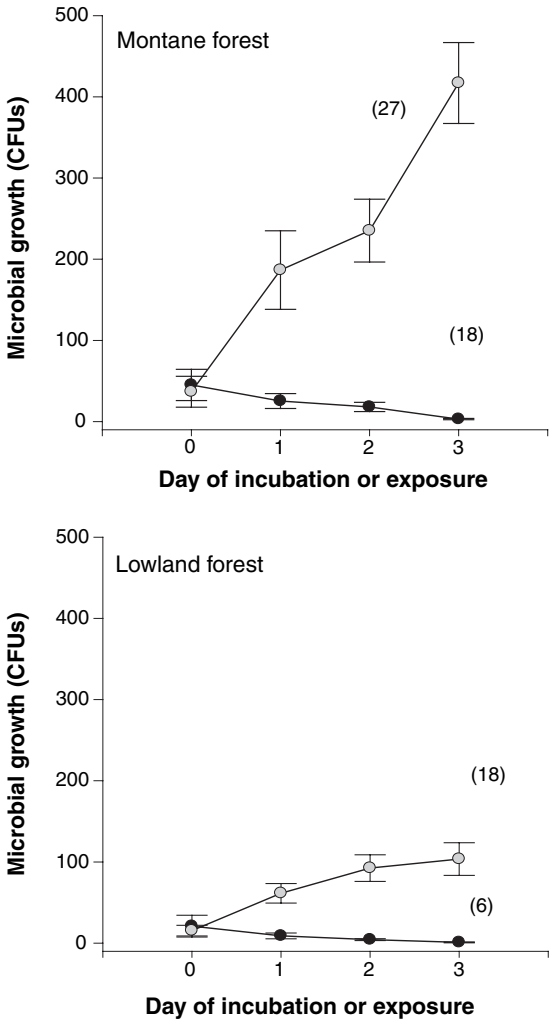

Figure 1 Microbial growth (mean ± 1 SE CFU/0.1 mL) on the shells of pearl-eyed thrasher eggs naturally incubated (black circles) or exposed to ambient conditions (grey circles) throughout the laying period at the montane forest and lowland forest sites (sample size in parentheses). Day 0 represents laying of the first egg and day 3 the last egg.

Bacterial growth differed greatly between incubated and exposed eggs (Fig. 1). CFU on the shells of incubated eggs decreased slowly throughout laying, but increased rapidly on eggs exposed to ambient conditions, as indicated by the large effects of exposure treatment and its interaction with time exposed in a repeated measures ANOVA (Table 1). Divergence in bacterial growth between incubated and exposed eggs was more marked in the cooler, very humid montane forest than in the warmer, less humid lowland site (Fig. 1; Table 1). Bacterial growth was significantly greater on the shells of montane forest eggs than on lowland forest eggs.

Table 1 Repeated measures ANOVA of the effects of site (montane forest or lowland forest) and exposure treatment (incubated or exposed) on the number of colony forming units (CFU/0.1 mL) on the shells of 69 thrasher eggs sampled daily from laying (day 0) through 3 days of exposure (time). Values in bold indicate terms accounting for the greatest amount of variation

Effects	Source	d.f.	MS	*F*	*P*-value
Among eggs	Site	1	493.6	8.9	0.004
	Exposure	1	**2232.0**	**40.2**	**0.001**
	Site * exposure	1	196.7	3.5	0.064
	Error	65	55.5		
Within eggs	Time	3	160.2	14.0	0.001
	Time * site	3	38.1	3.3	0.021
	Time * exposure	3	**436.0**	**38.1**	**0.001**
	Time * site * exposure	3	50.1	4.4	0.005
	Error	195	11.4		

Phylogenetic composition of eggshell microbiota

The eggshell microbiota was relatively heterogeneous at laying. Although none of the target groups dominated, fungi and Gram-negative enterics occurred most frequently (Fig. 2). Microbial composition at laying did not differ significantly between sites for either incubated or exposed eggs, so their biota were combined to examine differences between incubated and exposed eggs. No significant difference was evident at laying (day 0) between the proportion of incubated and exposed eggs harbouring Gram-positive rods, Gram-positive cocci, Gram-negative fermenters, Gram-negative enterics, and fungi (groups tested individually: Fisher's exact test: $n = 54$, $0.50 < P < 0.99$).

The proportion of eggs with shells harbouring pathogenic bacteria declined for naturally incubated eggs, but increased or remained the same for eggs exposed to ambient conditions (Fig. 2). After 3 days, exposed eggs were more likely to be contaminated with potentially pathogenic Gram-negative fermenters ($n = 69$, $P = 0.04$), Gram-negative enterics ($n = 69$, $P = 0.04$), and Gram-positive cocci ($n = 69$, $P = 0.08$) than incubated eggs. In contrast, growth of Gram-positive rods, which rarely invaded eggs (Cook *et al.* 2003, 2005), tended to increase more on incubated than on exposed eggs ($n = 69$, $P = 0.10$). Although exposure did not significantly reduce the proportion of eggs contaminated with fungi ($n = 69$, $P = 0.58$), the composition of fungi differed between the two treatment groups. A greater proportion of exposed eggs was contaminated with known invasive species, such as *Fusarium* and *Chrysosporium* (see Cook *et al.* 2003, 2005), whereas incubated eggs were characterized by non-invasive feather degrading species such as *Arthroderma*.

DISCUSSION

This is the first study, to our knowledge, to show that parental incubation behaviour affects bacterial processes on shells of newly laid wild bird eggs. Bacteria on thrasher eggs exposed to ambient conditions multiplied rapidly over the 3-day

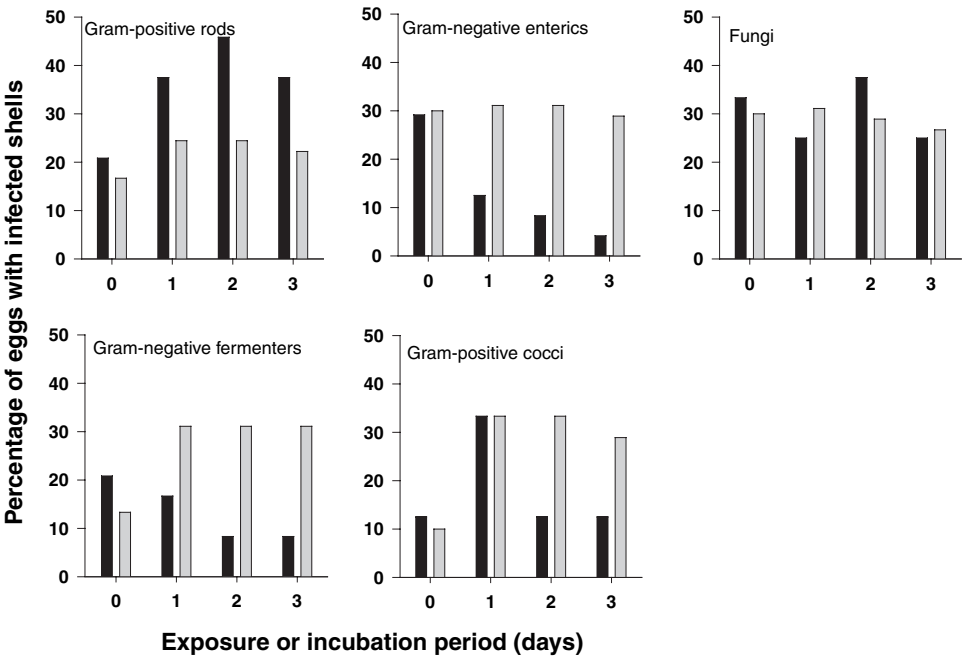

Figure 2 Percentage of thrasher eggs infected by each microbial group for eggs naturally incubated (black bars) or exposed to ambient conditions (grey bars) throughout the laying period. Day 0 represents laying of the first egg and day 3 the last egg.

treatment period (Fig. 1), and the proportion of eggs harbouring microbial groups known to be invasive or to promote trans-shell infection increased, such as Gram-negative enterics and Gram-negative fermenters (Fig. 2). In contrast, incubation by thrasher females reduced bacteria on eggshells (Fig. 1) and shifted the bacterial community composition towards a more benign shell microbiota. Pathogenic microbes became relatively less common with each day of incubation, and non-invasive Gram-positive rods and non-invasive fungi increased or remained the same (Fig. 2).

Incubation may shape microbial processes on eggshells through two mechanisms. Reduced humidity and protection from precipitation likely decrease microbial growth because most pathogenic microorganisms require damp conditions for survival and transport through shell pores into egg contents (Board & Halls 1973). Microbial growth on exposed eggs was more rapid in the cool, very humid montane forest than in the warmer, less humid lowland site (Fig. 1). Female parents may also use their feathers or the epidermal layer of their brood patches to inoculate shells with antibiotic agents, such as preen gland or fatty acids (Jacob 1978; Menon & Menon 2000; Shawkey *et al.* 2003), or with certain protective microbial species. Baggott & Graeme-Cook (2002) found *Bacillus lichenformis*, an aerobic spore-forming Gram-positive rod, dominated naturally incubated eggshells of the mandarin duck (*Aix galericulata*). This bacterium, which has been isolated from eggs and feathers of at least 40 avian species including thrashers (M.I. Cook, unpublished data), inhibits growth of Gram-positive and Gram-negative bacteria as well as certain fungi (Baggott & Graeme-Cook 2002).

Microbes on eggshells of newly laid eggs can multiply rapidly when exposed to appropriate ambient conditions, can penetrate the eggshell through pores, and can lead to a dramatic reduction in hatching success (Cook *et al.* 2003, 2005). In contrast, eggs that were parentally incubated did not become infected and exhibited high hatching success, suggesting that among its many functions incubation minimizes infection (Cook *et al.* 2005). Here we show that an important benefit of early incubation is to prevent the build up of potential pathogenic microorganisms on early laid eggs. This may lower the risk of infection and reduced hatching success, because the likelihood of trans-shell infection is positively related to microbe densities on eggshells (Cook *et al.* 2003, 2005). A cost, however, is asynchronous hatching and the possible decline in fitness because of brood reduction of last-hatched offspring. Thus, the onset of incubation may be viewed as a trade-off between the benefits of maintaining the viability of first-laid eggs and the disadvantages of an asynchronously hatching clutch (Beissinger 1999; Beissinger *et al.* in press).

High rates of microbial growth and trans-shell infection are unlikely to be limited to eggs laid in humid tropical climates. Microbes have been found on the eggs of a variety of temperate and tropical wild bird species (Baggott & Graeme-Cook 2002; Cook *et al.* 2003, 2005; J. Wang and S.R. Beissinger, unpublished data), and here we have shown they can grow rapidly in the absence of incubation in warm and cool environments. Trans-shell microbial infection has been demonstrated in domestic and wild birds (Board & Tranter 1986; Bruce & Drysdale 1991, 1994; Cook *et al.* 2003, 2005). Infection is enhanced by moisture on the eggshell surface, and a dew or rainfall event is probably sufficient to induce infection. However, infection can occur under relatively dry conditions (Cook *et al.* 2005), so birds in a variety of habitats and climatic regimes may experience a moderate to high risk of infection. Indeed, preliminary studies have shown moderate rates of microbial penetration in western bluebird *Sialia mexicanus* eggs in a Mediterranean climate in California (J. Wang and S.R. Beissinger, unpublished data). Thus, selective pressure to protect eggs from microbial infection may be widespread among birds. Nevertheless, the decision of when to initiate incubation is complicated by the interaction of many factors (Stoleson & Beissinger 1995). Although risk of microbial infection has been overlooked as a force shaping the onset of incubation and hatching patterns in birds, it will likely apply to most bird species and should be considered an important candidate factor in addition to predation, food and ambient temperatures.

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