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Microbial infection affects egg viability and incubation behavior in a tropical passerine

Mark I. Cook,[a] Steven R. Beissinger,[a] Gary A. Toranzos,[b] Roberto A. Rodriguez,[b] and Wayne J. Arendt[c]
[a]Ecosystem Sciences Division, Department of Environmental Science, Policy and Management, 151 Hilgard Hall 3110, University of California, Berkeley, CA 94720, USA, [b]Department of Biology, Box 23360, University of Puerto Rico, San Juan, PR 00931, Puerto Rico, and [c]International Institute of Tropical Forestry, USDA Forest Service, Sabana Research Station, HC 2, Box 6205, Luqillo, PR 00773, Puerto Rico

Many avian species initiate incubation before clutch completion, which causes eggs to hatch asynchronously. This influences brood competitive dynamics and often results in nestling mortality. The prevailing hypotheses contend that parents incubate early because asynchronous hatching provides fitness benefits to parents or surviving offspring. An alternative idea is that early incubation is the best of a bad job because of the costs of delaying incubation to the viability of first-laid eggs. To explore this, we examined the potential for microbial infection, and the relative effects of infection and suboptimal development temperatures on the viability of pearly-eyed thrasher (*Margarops fuscatus*) eggs. We exposed newly laid eggs for 5 days at either end of a tropical altitudinal gradient and cleaned shells of half the eggs to reduce microbial growth. Uncleaned eggs were infected more than were cleaned eggs, and infection was greater for eggs exposed at the cool, humid site than at the hot, less humid site. Parentally incubated eggs, however, were not infected, suggesting that incubation limits infection. The consequence of exposure to infection and high ambient temperatures was a dramatic reduction in viability; cleaned eggs held at the cool site had the highest hatching success, which was significantly greater than for uncleaned eggs at this site and for cleaned eggs held at the hot site. This provides the first evidence that microbes can infect unincubated eggs of a wild bird, and that infection and ambient temperature act independently to reduce hatching success. These factors could affect avian life-history strategies in diverse habitats. *Key words:* ambient temperature, egg viability, hatching asynchrony, onset of incubation, saprophytic microorganism, trans-shell transmission. *[Behav Ecol]*

Many species of birds initiate incubation before clutch completion. This gives first-laid eggs a developmental head-start, results in asynchronous hatching, and in turn establishes a within-brood size and competitive hierarchy which often contributes directly to the death of last-hatched nestlings (Clark and Wilson, 1981; Mock, 1984; Stoleson and Beissinger, 1995). Most studies have focused on the idea that early incubation is adaptive and the resulting staggered hatching pattern provides a fitness benefit to parents or first-hatched offspring (Amundsen and Slagsvold 1991; Clark and Wilson, 1981; Stoleson and Beissinger, 1995). Empirical evidence to support this, however, remains equivocal despite considerable research effort. An alternative idea posits that early incubation may be the result of a trade-off between the benefits of a large clutch size and the costs of delaying incubation to the viability of early laid eggs. Hatching success has been shown to decline rapidly after 3 days of exposure to ambient conditions, and early incubation may be necessary to protect early-stage embryos from environmental hazards (Arnold et al., 1987; Stoleson and Beissinger, 1999; Viega, 1992).

Temperature is considered the most critical condition affecting egg viability (Stoleson, 1999; Webb, 1987). Avian embryos are intolerant of chronic exposure to temperatures between physiological zero (or the threshold at which embryo development is initiated, 24°C–27°C) and optimal incubation temperature (36°C–38°C), because embryonic development continues but lethal teratogenic abnormalities can occur (Ewert, 1992; Meijerhof, 1992; Webb, 1987). However, by using domestic chicken (*Gallus gallus domesticus*) eggs exposed along a tropical altitudinal gradient, Cook et al. (2003) determined that trans-shell microbial infection of egg contents occurred within 3 days of exposure, greatly reduced egg viability, and its effect on hatchability can potentially confound that of ambient temperature.

Microbial infection of egg contents and its effect on embryo mortality is unknown for wild birds but has been investigated extensively for commercial poultry and captive waterfowl (Baggott and Graeme-Cook, 2002; Bruce and Drysdale, 1991, 1994). A high prevalence of infection observed in addled eggs of various free-living species (Houston et al., 1997; Pinowski et al., 1994) suggests that trans-shell infection is prevalent in the wild, but the source of contamination and whether microbes are the cause or consequence of egg failure remain unknown. Trans-shell infection of the egg is dependent on a suitable shell microbiota and appropriate climatic conditions. The presence of water is important because it promotes microbial growth on eggshells and is the medium of transport by which bacteria and fungi pass through shell pores (Board and Halls, 1973; Board et al., 1979). By incubating, the parent keeps the eggshell surface dry, thus minimizing the probability of infection. If bacteria infect egg contents, incubation provides additional protection by increasing the temperature of the albumen to levels at which its antimicrobial enzymes work optimally (Board and Tranter, 1986) and that exceed the optimum for growth of most microbiota. Therefore, by initiating incubation before clutch completion, first-laid eggs

Address correspondence to M Cook, who is now at the Everglades Division, South Florida Water Management District, 3301 Gun Club Road, West Palm Beach, FL 33406. E-mail: mcook@sfwmd.gov.
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should have a lower probability of microbial infection than if left exposed to the elements.

Here we present results of an experiment designed to examine the incidence of trans-shell microbial infection, its effect on egg viability, and the role of incubation in preventing infection in a box-nesting population of pearly-eyed thrashers (hereafter "thrasher"). We exposed freshly laid eggs for a duration that encompasses the laying period of thrashers at two sites at the extremes of a local environmental gradient in Puerto Rico (Beissinger SR, Cook MI, and Arendt WJ, in preparation; Cook et al., 2003): a hot, humid lowland site and a cool, very humid cloud-forest site. High humidity and cool temperatures in the cloud forest provide optimal conditions for growth and trans-shell penetration of microbes (Bruce and Drysdale, 1994; Kollowa and Kollowa, 1989), whereas less humid, hotter conditions at the lowland site may limit microbial growth (Cook et al., 2003). To distinguish between effects of ambient temperature and microbial infection on egg viability, shells of half the eggs at each site were sterilized twice daily to prevent trans-shell infection, whereas the remaining eggs were left untreated. After exposure, eggs from each treatment were either opened to examine contents for the presence of microorganisms or returned to natal nests and monitored until hatching to determine the effects on viability. To determine the effect of natural incubation on trans-shell infection, we examined the contents of untreated eggs incubated by parents for a period equivalent to the exposure of experimental eggs. We predicted (1) the probability and magnitude of microbial infection of exposed eggs would be higher at the very humid cloud forest site than at the lowland site; (2) uncleaned cloud forest eggs would exhibit lower hatchability than would cleaned cloud forest eggs owing to pathogenic effects of trans-shell microbial infection; (3) cleaned cloud forest eggs would exhibit higher hatchability than would cleaned lowland eggs, because temperatures exceeded physiological zero (24°C) 82.9% of the time at the lowland site, but 0% at the cloud forest site; and (4) the probability and magnitude of microbial infection would be lower for naturally incubated eggs than for exposed eggs.

METHODS

Study species and field site

Pearly-eyed thrashers are Neotropical passerines that typically lay a 3–4-egg clutch over 3–6 days. Partial incubation by the female is usually started soon after clutch initiation and increases daily until maximum incubation duration is attained at clutch completion (Cook MI, Beissinger SR, unpublished data). Eggs hatch over a 2–4-day period at approximately 15 days after clutch initiation. Thrashers are cavity nesters with a widespread distribution in the eastern Caribbean, and are common in most forested habitats in Puerto Rico (Raffaele et al., 1998; Snyder et al., 1987).

We conducted studies in the Luquillo Experimental Forest and at a nearby lowland coastal site, Las Paulinas, in Puerto Rico (18°19′ N, 65°45′ W). Between 1998 and 2001, we placed 20, 90, and 24 nest-boxes, respectively, within three distinct forest habitats (Ewel and Whitmore, 1973): a lowland dry forest (10–100 masl), a mid-elevation palo colorado forest (600–900 masl), and an upland cloud-forest (750+ masl). Thrashers lay multiple clutches from January–August in the palo colorado forest, where they reach their highest density, and in cloud forests (Arendt, 2000), but lay a maximum of two clutches from mid May–late June in the lowland forest (Cook MI, personal observation). Most eggs used in this study (84.7%) were laid in the palo colorado forest.

We situated holding sites at the altitudinal extremes of the gradient, and recorded temperature and humidity every 5 min to calculate hourly climatic conditions throughout the study using thermistors and data loggers (Onset Computer Corp.). Between April and June 2002, the lowland holding site (100 masl) had a mean daily temperature of 25.6 ± 1.9°C (range = 18.6°C–30.3°C) and a mean relative humidity of 83.1 ± 7.8% (range = 54.7 − 98.2%), whereas the cloud forest holding site (810 masl) experienced daily mean temperatures of 19.4 ± 1.2°C (range = 13.7°C–22.86°C) and extremely high relative humidity averaging 97.8 ± 0.3% (range = 93.4–100%).

Field experiment

To replicate delayed incubation and examine effects of exposure to microbial infection and ambient temperature, we exposed 190 unincubated thrasher eggs to ambient conditions for 5 days from 3 April–30 June 2002. Eggs were divided between two experimental groups to examine the (1) incidence and magnitude of microbial trans-shell infection ($n = 110$), and (2) effect of exposing eggs to microbial infection and ambient temperature on egg viability ($n = 80$). Following the methods of Cook et al. (2003), eggs were exposed at either the cloud forest or lowland site where, within each experimental group, they received one of two cleaning treatments: (1) wiped twice daily with 70% alcohol to kill microbes on the shell surface and to prevent trans-shell infection or (2) not cleaned and thus exposed to potential infection. The 5-day exposure period represents the maximum potential preincubation delay for this species and allowed us to examine how strongly the effects of microbial infection and ambient temperature would act if thrashers delayed the onset of incubation until clutch completion. The cleaning treatment is unlikely to affect adversely the shell cuticle or embryo viability (Cook et al., 2003).

Before laying, nest-boxes were checked every 1–7 days, depending on stage of nest construction during the previous visit. Nests with a completed lining were checked daily until clutch completion from 0700–1300 h, the typical thrasher laying time. To minimize contamination of the eggshell microbiota, we washed our hands in 70% alcohol immediately before manipulating eggs, and all eggs were individually transported between sites in sterilized zip lock bags. Because this procedure was likely to dramatically reduce transfer of microorganisms from hands to eggs, and if microorganisms were transferred they were likely to be common to the local environment, it is unlikely that contamination during handling affected results. All eggs of a clutch were removed from the nest at laying, swabbed to obtain a standardized pretreatment sample of the shell microbiota (20% of shell surface), and then labeled and replaced with a plaster dummy egg to ensure natural incubation behavior. We randomly assigned eggs among experimental groups and treatments but ensured that eggs from a clutch were allocated to a different holding site and cleaning treatment within an experimental group.

The holding environment attempted to provide treatment eggs with climatic conditions and a nest microbiota similar to that experienced by first-laid clutches of thrashers. At each holding site, eggs were placed into a single thrasher nest situated at the base of a secure, shaded, wooden nest-box (for dimensions, see Cook et al., 2003). The two nests were collected shortly after completion from nest-boxes in the palo colorado forest and had not been used for breeding. We placed a maximum of five eggs into a box so they did not touch one another, and eggs were turned twice daily along their longitudinal axis. Data loggers positioned on the inside lid of holding boxes simultaneously recorded temperature and humidity every 5 min.

After 5 days of exposure, we swabbed eggs (as above) to obtain a postexposure sample of the eggshell microbiota. All pre- and posttreatment swabs were placed into 5 ml sterile physiological (.85% w/v) saline. Eggs were either returned to natal nests to be parentally incubated and later examined for hatchability, or transported to the laboratory where we analyzed egg contents (albumen and yolk) for the presence of microorganisms. Eggs to be examined for hatching success were candled by using a portable candler at both the mid-stage (day 4–6) and late-stage (day 9–12) of parental incubation to determine embryo developmental stage and viability. Because embryo development can occur during experimental exposure if ambient temperatures exceed physiological zero, embryos were assigned to one of four stages of development based on a reference set of known development stages from unmanipulated parentally incubated eggs: stage 0 had no obvious development (equivalent to less than 1 day of parental incubation), stage 1 had limited development (equivalent to 1–4 days of parental incubation), stage 2 had intermediate development (equivalent to 5–8 days of parental incubation), and stage 3 had advanced development (equivalent to 9–13 days of parental incubation). After day 12 of parental incubation, we checked eggs daily for hatching. We also candled and examined the hatching success of an additional 54 unmanipulated eggs to determine the natural level of hatching success in this species.

Eggs to be examined for trans-shell infection were opened in a sterilized laminar-flow hood, and 0.01 ml samples of albumen and yolk were removed and placed in 5 ml physiological saline. We cultured 0.1 ml of the supernatant obtained from swab and egg-content samples, and counted and identified resultant microbial colony forming units (CFUs). See Cook et al. (2003) for details of microbiological techniques. In addition to treatment eggs, we also cultured the contents of 13 newly laid control eggs to establish the prevalence of microbial infection of egg contents prior to oviposition (trans-ovarian infection). To determine the effect of parental incubation on trans-shell transmission, we examined the contents of unmanipulated eggs that were dead and had little or no development at first candle (i.e., after 5–6 days of parental incubation). We chose dead rather than living eggs because the later contain advanced embryos with relatively small volumes of yolk and albumen, which are often difficult to extract without contamination. We feel this is a conservative test of the effects of incubation on microbial infection because it is unlikely that dead eggs provide better protection from infection than living eggs.

Data analysis

We used generalized linear models with Poisson error (GLIMMIX macro of SAS, Littell et al., 1996) to examine the roles of site and cleaning treatments on microbial colony counts. To examine effects of nesting site on pretreatment eggshell CFUs at laying, we used a mixed model analysis procedure (PROC MIXED) applied to a one-way ANOVA with nest identity included as a random term to control for parental and nest effects. For all other ANOVA analyses, we used the PROC GLM procedure because potential effects of nest were accounted for in the experimental design. We used two separate two-way ANOVAs to examine if CFUs on the eggshell at laying and microbial growth on the eggshell during exposure were related to holding site and cleaning treatments. For post hoc tests of microbial growth on the eggshell, we examined the difference in least-squares means between individual treatments using t tests. We examined the effect of holding site on the number of CFUs in egg contents of uncleaned eggs using one-way ANOVA. Means are presented with standard errors.

All other statistical analyses were performed by SYSTAT version 10 (SPSS Inc.). We compared prevalence of infection between control and treatment eggs, between experimental sites and cleaning treatments, and between unmanipulated and exposed eggs with Fisher's Exact test. We used logistic regression and Akaike's Information Criterion corrected for small sample size (AICc) to analyze factors affecting the probability of microbial trans-shell transmission following the method of Burnham and Anderson (2002) and Cook et al. (2003). Because climatic variables were highly correlated with treatment site, we did not include them in the set of candidate models. Predictive variables in models included treatment site, microbial growth during exposure, and the presence of fungi and Gram-negative fermenters on the shell during exposure, as these groups have been previously shown to break down the eggshell cuticle and promote microbial trans-shell infection (Board and Halls, 1973; Board et al., 1979). Composition of microbial communities on and in eggs was analyzed by using Pearson's chi-square statistic.

In analyses of hatching success, we excluded all eggs lost by causes other than hatching failure (e.g., predation, egg damage, desertion). We used logistic regression to examine the effects of treatment site and cleaning treatment on hatching success, and Fisher's Exact test to compare hatching success and stage of embryo mortality between treatment groups.

RESULTS

Microbial contamination of shell surface

An essential prerequisite for microbial trans-shell transmission is the presence of bacteria or fungi on the shell surface (Board and Tranter, 1986). We examined the eggshell surface at laying for the presence of microorganisms on 171 treatment eggs from 73 clutches, and microbes were evident on 80% of eggs from 93% of clutches. Mean number of CFUs at laying varied considerably by egg (103 ± 43, range = 0–5223) but did not vary among the three nesting areas when nesting box ($n = 51$) was used as a random term (one-way ANOVA: $F_{2,121} = 0.41$, $p = .665$). The average number of CFUs on shells at laying did not differ between eggs assigned to cleaned and uncleaned treatments ($F_{1,167} = 0.10$, $p = .746$) or between eggs allocated to cloud forest and lowland treatments ($F_{1,167} = 0.14$, $p = .710$); the interaction was nonsignificant ($F_{1,167} = 0.44$, $p = .509$).

Microbial growth on eggshells (Figure 1) was significantly affected by holding site (two-way ANOVA: $F_{1,164} = 21.10$, $p < .0001$) and cleaning treatment ($F_{1,164} = 9.07$, $p = .003$), but not the interaction ($F_{1,164} = 1.04$, $p = .309$). Microbes on eggshells exhibited a marked increase in CFUs relative to preexposure levels for all treatments and occurred despite cleaning, but the magnitude of growth on cleaned eggs was reduced to between one-third and one-half that of uncleaned eggs. The greatest increase in shell microbiota occurred on uncleaned eggs held at the very humid cloud forest site, which exhibited significantly greater microbial growth than did eggshells from each of the other treatment groups (difference in least-squares means: all comparisons $p < .0001$). In addition, shells of cleaned cloud forest eggs exhibited greater microbial growth than did shells of cleaned lowland eggs ($p = .036$). No difference in eggshell microbial growth was evident among cleaned and uncleaned lowland eggs ($p = .283$) or between cleaned cloud forest and uncleaned lowland forest eggs ($p = .185$).

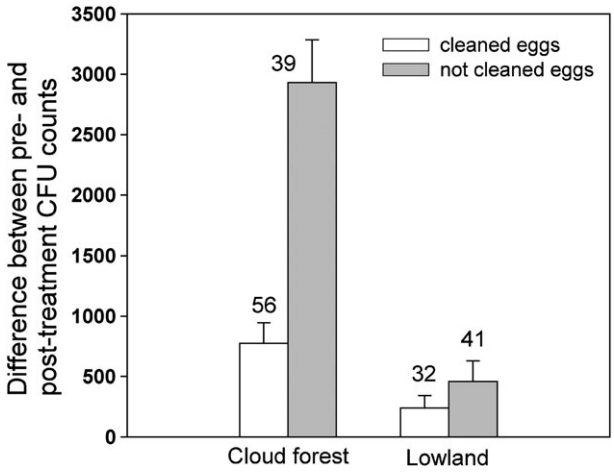

Figure 1
Effects of holding site and cleaning treatment on microbial growth on the eggshell. Mean (±1 SE) difference between post- and pre-treatment eggshell counts of CFUs is shown with sample size above each bar. Comparisons between treatments are significantly different (difference in least-squares means: $p < .0001$), except those between uncleaned lowland and cleaned lowland eggs and between uncleaned lowland and cleaned cloud forest eggs.

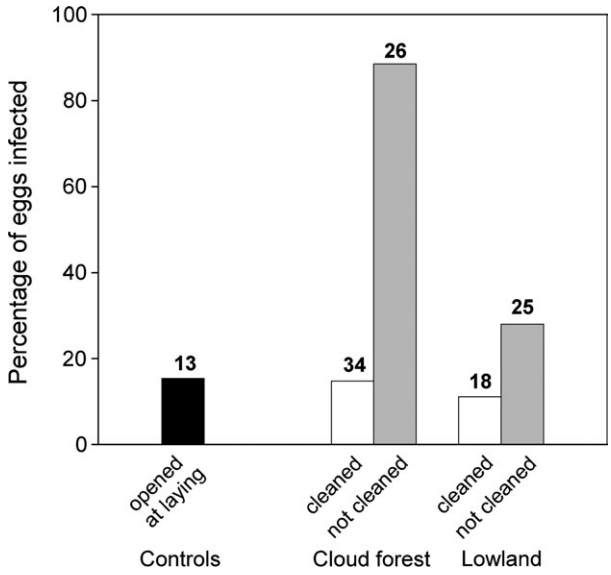

Figure 2
Microbial infection of the contents (either albumen or yolk) of unexposed control eggs and treatment eggs exposed for 5 days at two sites in Puerto Rico (sample size above each bar).

Microbial infection of egg contents

Shell microbiota penetrated egg contents of unincubated eggs within the time required to lay a clutch for this species. After 5 days of exposure, microbes infected a significantly greater proportion of uncleaned eggs (58.8%, $n = 51$) than cleaned eggs (13.5%, $n = 52$; Fisher's Exact test $p < .001$) and than eggs with contents that were examined at laying (i.e., control eggs; 15.4%, $n = 13$; $p = .011$). No difference was evident between cleaned eggs and control eggs ($p = 1.0$), suggesting that cleaning treatment prevented most microbial trans-shell transmission. Microbes did not, however, penetrate parentally incubated eggs. None of the seven unmanipulated eggs found dead after 5 or 6 days of parental incubation (all first-laid eggs from either the cloud or palo colorado forest) contained infected albumen or yolk, whereas 11 (81%) of first-laid uncleaned eggs exposed at the cloud forest were infected ($p = .0023$). Two of 13 (15.4%) control eggs harbored Gram-negative enteric colonies in the yolk but not in the albumen. This suggests infection of the yolk is occurring before laying in a small but potentially important proportion of eggs. The infectious agents of exposed cleaned eggs were Gram-negative enterics and Gram-positive cocci, the microbial groups most commonly transmitted vertically (Mayes and Takeballi, 1983).

As predicted, the probability (Figure 2) and magnitude of infection were higher at the more humid cloud forest site than at the lowland site. A logistic regression analysis (model: $\chi^2 = 45.31$, $n = 103$, df = 3, $p < .0001$; McFadden's $\rho^2 = .34$) revealed that probability of infection was strongly influence by holding site (t ratio: $p = .0001$), was not affected by cleaning treatment (t ratio: $p = .193$), but the interaction was significant (t ratio: $p = .023$). This suggests that cleaning reduced infection, but its effectiveness depended on holding site. Climatic conditions at the cloud forest site were highly conducive to trans-shell transmission, and uncleaned cloud forest eggs were almost three times more likely to contain infected contents than uncleaned lowland eggs. Nevertheless, almost one-third of uncleaned eggs were infected at the lowland site, which could have negative consequences for embryo viability. At the cloud forest site, significantly more uncleaned eggs were infected than were cleaned eggs (Fisher's Exact test: $n = 60$, $p < .0001$), but this was not true for the lowland site ($n = 43$, $p = .263$). Number (mean ± SE) of CFUs within eggs (albumen and yolk combined) tended to be higher for uncleaned cloud forest eggs (628 ± 461) than for uncleaned lowland eggs (404 ± 284) but the difference was not significant (one-way ANOVA: $F_{1,45} = 0.19$, $p = .669$).

We examined how exposure site, microbial growth, and the presence of either fungi or Gram-negative fermenters on the shell during exposure affected likelihood of infection (albumen and yolk) of 51 uncleaned eggs by examining a suite of a priori models (Table 1). Likelihood of infection was best predicted by a logistic regression model that included treatment site, microbial growth on the shell, the presence of either fungi or Gram-negative fermenters on the shell during treatment, and the interaction between the presence of these microbes and treatment site. This model had relatively strong support compared with others, with an Akaike weight of 0.61. The two next-best models were very similar and included site, microbial growth, and the presence of fungi/Gram-negative fermenters, or treatment site, presence of microbes, and the interaction term, but they received about one-fourth as much support as the best model.

Phylogenetic composition of microbes

Microorganisms differed in their ability to infect egg contents. Shells of exposed eggs were dominated by Gram-negative enterics, Gram-negative fermenters, Gram-positive rods, and Gram-positive cocci. Microbial composition did not differ significantly between albumen and yolk ($\chi^2 = .01$, df = 5, $p = 1.0$), so their microbiota were combined and tested against posttreatment shell microbiota. There was a significant difference between the composition of microbiota on shells and inside eggs ($\chi^2 = 23.9$, df = 5, $p = .0002$). Gram-negative fermenters (35.7 versus 27.1%) and fungi (26.9 versus 9.4%) were found more often inside than on the outside of eggs, whereas Gram-positive rods (4.3 versus 15.1%), Gram-positive cocci (4.3 versus 12.5%), and Actinomycetes (0 versus 6.3%) showed the reverse pattern. Gram-negative enterics were found equally in both parts of the egg.

Table 1

Logistic regression models for the likelihood of trans-shell infection of 51 thrasher eggs exposed for 5 days and their corresponding number of parameters (k), rank, and AICc score corrected for small sample size

Rank	Model	k	Loglikelihood	AICc	ΔAICc	Weight
1	**Site, growth, microbes on shell, microbes on shell × site**	**5**	**−12.605**	**36.67**	**0.00000**	**0.60629**
2	Site, growth, microbes on shell	4	−15.1986	39.35	2.67617	0.15906
3	Site, microbes on shell, microbes on shell × site	4	−15.2153	39.36	2.68742	0.15817
4	Site, microbes on shell	3	−17.4304	41.41	4.73284	0.05688
5	Growth, microbe	3	−18.8152	44.19	7.51512	0.01415
6	Site, growth	3	−19.9125	46.35	9.67332	0.00481
7	Growth	2	−23.591	51.44	14.76390	0.00038
8	Site	2	−24.1221	52.49	15.82079	0.00022
9	Microbes on shell	2	−25.7427	55.75	19.07865	0.00004

Models are ranked by ΔAICc, which indicates the difference between each model and the best model with the lowest AICc (in bold), and the AIC weight (the relative likelihood of a model given a set of candidate models, normalized to sum to one). Predictive variables in the model include treatment site (cloud forest and lowland), microbial growth (difference between pre- and posttreatment CFU counts), and microbes on shell (presence or absence of either fungi or Gram-negative fermenters on shell during treatment). All nine candidate models are presented.

Egg viability

Unincubated thrasher eggs exposed to ambient conditions for 5 days exhibited a marked reduction in viability relative to those left unmanipulated (87.3% hatched, $n = 54$), with the greatest declines found in eggs exposed to microbial infection or to temperatures above physiological zero (Figure 3). As predicted, cleaned cloud-forest eggs had the highest hatching success (42.9% hatched), and this was significantly greater than uncleaned cloud-forest eggs (0% hatched; $n = 40$, $p = .001$) and cleaned lowland eggs (6.3% hatched; $n = 37$, $p = .023$). Hatching success of cleaned cloud forest eggs, however, was significantly lower than for unmanipulated eggs ($n = 75$, $p < .001$), suggesting that mechanisms in addition to microorganisms and ambient temperature affected viability. Hatching success did not differ between cleaned and uncleaned lowland eggs ($n = 34$, $p = 1.0$), or between uncleaned cloud forest and uncleaned lowland eggs ($n = 37$, $p = .23$). A logistic regression model with the interaction term omitted (model: $\chi^2 = 8.45$, $n = 103$, df = 3, $p = .007$; McFadden's $\rho^2 = 0.15$) showed that cleaning treatment had a significant effect on egg hatching success (t ratio: $p = .020$), but there was no effect of treatment site (t ratio: $p = .118$).

Most embryos (90.2%) died before development was evident (stage 0), so for subsequent analyses we divided stage of development into those eggs with (stage 1–3) and without visible development (stage 0). Proportion of embryos dying at stage 0 was similar between sites (cloud forest 89.3%, lowland 91.3%; Fisher's Exact test: $n = 51$, $p = 1.0$) and between cleaned (86.7%) and uncleaned eggs (92.3%) at the cloud forest (Fisher's Exact test: $n = 28$, $p = 1.0$) and lowland sites (cleaned 91.7%, uncleaned 90.9%; Fisher's Exact test: $n = 23$, $p = 1.0$). Thus, the decrease in viability and timing of mortality at the two sites was similar, but the mechanisms reducing hatchability differed.

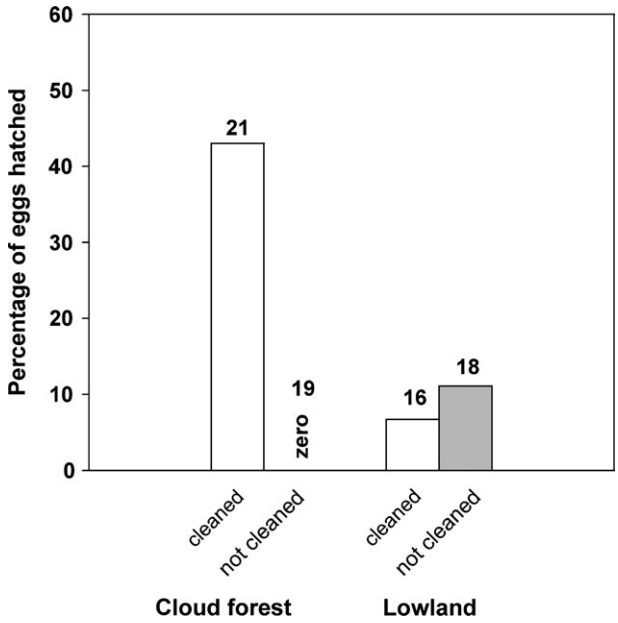

Figure 3
Hatching success of control eggs and cleaned and not cleaned treatment eggs exposed for 5 days at the cloud forest and lowland site and then parentally incubated (sample size above each bar).

DISCUSSION

Newly laid pearly-eyed thrasher eggs exposed to ambient conditions can suffer a high incidence of trans-shell microbial penetration, and the consequence of infection and exposure to suboptimal development temperatures is a dramatic reduction in viability. Our results provide the first evidence that microbial infection can reduce the hatching success of wild bird eggs and suggest that avian incubation patterns may be constrained by the need to protect the viability of eggs from microbes as well as from ambient temperatures, as previously shown in domesticated fowl eggs (Cook et al., 2003).

Microbial infection as a factor affecting the onset of incubation

Microbial growth on the eggshell surface is rapid under suitable ambient conditions and can lead to a high probability of trans-shell microbial infection (Cook et al., 2003). Unincubated thrasher eggs exhibited high rates of microbial growth on the shell surface (Figure 1) and were extremely susceptible to infection of the albumen and yolk (Figure 2). After 5 days of exposure, eggs incurred infection levels of 90% at the cool, humid cloud forest and 28% at the hotter, less humid lowland holding site. By contrast, contents of eggs that were parentally incubated for an equivalent duration were free of microbes. Although the sample size of opened incubated eggs was small, the stark contrast in infection levels between incubated and unincubated eggs, in addition to evidence that incubation considerably decreases the microbial load on the shell surface (Cook MI, Beissinger SR, unpublished data), strongly suggest that incubation helps to prevent microbial infection of egg contents by pathogenic microbes.

The primary source of infection of egg contents was likely trans-shell penetration by the shell surface microbiota rather than in situ growth of populations deposited within the egg during yolk formation. Vertical transmission of bacteria from maternal ovary to yolk has been repeatedly demonstrated in

chicken eggs (see Thiagrajian et al., 1993), and these microbes, although initially present in small numbers, can multiply rapidly (Board et al., 1989). In the present study, the proportion of control eggs with infected contents at laying was low (15.4%), indicating that vertical transmission is unlikely to account for the high proportion of infected eggs in the uncleaned treatments. Although it is possible that our sampling technique failed to detect low numbers of microbes transmitted vertically at laying, the low proportion of cleaned eggs that became infected relative to uncleaned eggs suggests otherwise and implies that the principal source of infection was the shell surface microbiota.

Trans-shell infection of thrasher eggs was largely a function of the growth and phylogenetic composition of the shell microbiota, which in turn was influenced by climatic conditions. Densities of eggshell microorganisms at laying were low but increased considerably during exposure, particularly at the cloud-forest site where cool, humid conditions are especially conducive to microbial growth (Figure 1). Water is essential for trans-shell infection because it promotes microbial growth on the eggshell surface and assists transport of bacteria and fungi pass through shell pores (Board and Halls, 1973; Board et al., 1979). The type of microbes present also appears to be an important factor affecting transmission. The likelihood of infection in thrasher eggs was influenced by the presence of either fungi or Gram-negative fermenters on eggshells during exposure (Table 1). These microorganisms can digest the protective shell cuticle and facilitate microbial infection by destroying the water resistant properties of the shell, and dramatically increase the number of open shell pores available for access (Board and Halls 1973, Board et al., 1979). Growth and phylogenetic composition of the shell microbiota and climatic conditions determined infection of domesticated fowl eggs exposed to similar treatment conditions (Cook et al., 2003), which suggests that the mechanisms governing infection may be similar among avian species.

Incubation is likely to serve an essential role in protecting eggs from microbial infection. By decreasing contact of the egg with atmospheric water, incubation may minimize growth of pathogenic and cuticle digesting microorganisms, thereby limiting the probability of trans-shell transmission. Heat transfer to the egg may also provide an important "cleaning" function by raising the egg's temperature to levels at which antimicrobial enzymes in the albumen function optimally (Board and Tranter, 1986) and beyond the optima for most environmental microbiota (Cook et al., 2003). Furthermore, early incubation may be necessary to reduce the growth and pathogenic effect of vertically transmitted microbes, regardless of the risk of trans-shell transmission. Nevertheless, the temperature and duration of warming required to protect eggs from microbial infection are not well known, and further studies are needed to determine whether incubating birds can warm eggs sufficiently to sterilize them without promoting the process of asynchronous hatching.

Egg viability and avian life histories

Our results concur with previous studies on egg viability (Arnold et al., 1987; Stoleson, 1999; Stoleson and Beissinger, 1999; Viega, 1992) and show that continuous exposure of unincubated wild-bird eggs to ambient conditions results in reduced hatching success after 3–5 days. A decline in viability of thrasher eggs was clearly owing to a lack of incubation rather than effects of handling and transportation, because these procedures did not reduce hatching success of experimental eggs compared to control eggs (Beissinger et al. in preparation). If thrashers were to delay incubation by 5 days, the maximum laying period in this species, the probability of hatching for first-laid eggs would be greatly reduced, perhaps to as low as 0–20% viability (Beissinger et al. in preparation) (Figure 3). Therefore, starting incubation before clutch completion may be very important for maintaining the viability of early laid eggs in this species, and the resulting asynchronous hatching pattern may be a consequence of this.

A conspicuous difference between these results with thrasher eggs and our earlier similar experiments using chicken eggs (Cook et al., 2003) was the extreme loss of viability for the passerine species, which had 50–66% lower hatching success for all treatments. Although earlier studies suggest the decline in viability of waterfowl eggs is slower than in other species (Arnold, 1993; Arnold et al. 1987; Stoleson, 1999), the comparison of thrasher and chicken eggs provides the first direct evidence that viability differs between eggs of birds with altricial and precocial modes of development exposed to the same environmental conditions. Thrasher eggs may have been more sensitive to microbial infection and suboptimal temperatures, perhaps because of their relatively small size or thin shells. Another factor that might differ between the two species is eggshell pore number and size, which could affect the likelihood of trans-shell infection. Alternatively, the large clutch size and constraint to hatch eggs synchronously may have selected for domestic fowl to endow albumen with higher concentrations of antimicrobial properties than are in thrasher eggs, which receive some incubation soon after laying.

The very powerful effect of microbial infection and suboptimal temperatures on unattended eggs before the onset of incubation in both climatic regimes tested here suggests these environmental factors will be pervasive among species nesting in a diversity of habitats and climates. Initiating full or partial incubation before clutch completion may be essential for maintaining egg viability, and the consequence is hatching asynchrony, in which late-hatched nestlings are often disadvantaged when competing with siblings (Mock et al., 1990; O'Connor, 1978). Egg viability may also have a selective influence on other reproductive traits such as clutch size. If viability constraints result in a hatching pattern in which the fitness costs of sibling competition exceed the benefits of increased viability, then selection will likely opt for a downward adjustment in egg number (Beissinger, 1999). This hypothesis has yet to be investigated but is supported by declines in clutch size and increases in hatching asynchrony with increasing temperature, both seasonally (Perrins and McCleery, 1989; Stoleson and Beissinger, 1995; Stutchberry and Robinson, 1988) and with latitude (Lack, 1968; Skutch, 1985, Viñuela and Carrascal, 1999).

Although we have shown dramatic declines in the viability of newly laid eggs owing to prolonged exposure to environmental factors, a fundamental question remains unanswered: Is the loss of viability through exposure to ambient conditions the selective force promoting early incubation, or is this loss a consequence of initiating incubation early because eggs incubated shortly after laying have lost those mechanisms that protect against ambient conditions (Ankney et al., 1991)? Stoleson and Beissinger's (1999) discussion of comparative and experimental evidence suggest the improvement in egg viability owing to incubation is the selective cause of initiating incubation early. Experiments with domestic fowl provide strong evidence that embryo viability is a conservative trait that cannot be increased without adverse pleiotropic effects (Crittenden and Bohren 1961, Nordskag and Hassan 1971). However, similar studies on wild birds are lacking. More work is needed to determine embryo tolerance to environmental

conditions and how it varies among species with differing life-history strategies and evolutionary history. Nonetheless, future studies of avian life history need to consider loss of embryo viability of newly laid eggs as a potential cause of early incubation before assigning an adaptive function to hatching patterns and other traits.

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