THE ROYAL SOCIETY

Received 21 February 2003
Accepted 2 July 2003
Published online 28 August 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?

Mark I. Cook[1*], Steven R. Beissinger[1], Gary A. Toranzos[2], Roberto A. Rodriguez[2] and Wayne J. Arendt[3]

[1]*Ecosystem Sciences Division, Department of Environmental Science, Policy and Management, 151 Hilgard Hall 3110, University of California, Berkeley, CA 94720, USA*
[2]*Department of Biology, Box 23360, University of Puerto Rico, San Juan, PR 00931, Puerto Rico*
[3]*International Institute of Tropical Forestry, United States Department of Agriculture Forest Service, Palmer, PR 00721, Puerto Rico*

Many birds initiate incubation before clutch completion, which results in asynchronous hatching. The ensuing within-brood size disparity often places later-hatched nestlings at a developmental disadvantage, but the functional significance of the timing of the onset of incubation is poorly understood. Early incubation may serve to maintain the viability of early-laid eggs, which declines over time owing to the putative effects of ambient temperature. An unexplored risk to egg viability is trans-shell infection by micro-organisms. We experimentally investigated the rate and magnitude of microbial trans-shell infection of the egg, and the relative effects of ambient temperature and micro-organisms on hatching success. We show that infection of egg contents is prevalent and occurs within the time required to lay a clutch. The probability of infection depends on the climatic conditions, the exposure period and the phylogenetic composition of the eggshell microbiota. We also demonstrate that microbial infection and ambient temperature act independently to reduce egg viability considerably. Our results suggest that these two factors could affect the onset of avian incubation in a wide range of environments.

Keywords: saprophytic micro-organism; trans-shell transmission; egg viability; incubation behaviour; ambient temperature

1. INTRODUCTION

Unlike most animals, avian parents influence the initiation of development, and the resultant hatching synchrony of a brood, through the onset of incubation. Birds lay no more than one egg daily and most species initiate incubation prior to laying a full clutch (Clark & Wilson 1981). This behaviour, which causes first-laid eggs to receive a developmental advantage and the clutch to hatch asynchronously, often leads to the mortality of last-hatched young. Studies of avian hatching patterns have focused on identifying adaptive functions for nestling-size disparities, with equivocal success (Clark & Wilson 1981; Amundsen & Slagsvold 1991; Stoleson & Beissinger 1995), while assuming that embryos are maintained in a protected stasis, waiting for parents to initiate incubation in a manner that creates optimal hatching patterns. Incubation before clutch completion may be necessary to maintain the viability of first-laid eggs, which diminishes over time owing to the putative effects of ambient temperature (Arnold *et al.* 1987; Viega 1992; Stoleson & Beissinger 1999). An unconsidered factor that may also reduce the viability of eggs is trans-shell infection of egg contents by saprophytic micro-organisms. Initiation of incubation prior to clutch completion may be necessary because non-incubated eggs incur a higher risk of infection by micro-organisms than do incubated eggs.

The prevalence of trans-shell infection and its effect on embryo mortality are unknown in wild birds (Baggott & Graeme-Cook 2002) but are better understood for commercial species (Board & Tranter 1986; Bruce & Drysdale 1994). Eggs possess physical and chemical mechanisms to protect against microbial invasion (Board 1966; Board & Fuller 1974) and the effectiveness of defence depends on the environmental conditions and the attributes of invading organisms (Board & Tranter 1986). Micro-organisms enter the egg through the small proportion of eggshell pores that are not covered by the shell cuticle (Board & Board 1967). Water appears essential for trans-shell transmission, because it promotes the conditions required for microbial growth on the eggshell and transports micro-organisms through the shell pores. When sufficient water is present, certain microbial species can digest the cuticle layer and dramatically increase the number of unplugged pores available for penetration (Board & Halls 1973; Board *et al.* 1979). By incubating, the parent physically protects the egg surfaces from atmospheric water, thereby reducing the chance of trans-shell transmission. Should micro-organisms breach the shell, incubation further protects the egg by raising its temperature to levels at which antimicrobial proteins in the albumen function optimally (Board & Tranter 1986) and that exceed the optimum for the growth of most microbiota. Thus, incubated eggs should receive greater protection from microbial infection than exposed eggs.

*Author for correspondence (mcook@nature.berkeley.edu).

We examined the potential for trans-shell infection by exposing freshly laid eggs for durations that encompass the laying period of many avian species at two sites at the extremes of a local tropical environmental gradient in Puerto Rico: a hot humid tropical lowland site and a cool very humid cloud forest site. High humidity at the cloud forest site provides optimal conditions for penetration of microbes, while warmer temperatures at the lowland site reduce bacterial growth (Bruce & Drysdale 1994). After exposure, we tested egg contents and shells for the presence of micro-organisms. We predicted that the probability and magnitude of microbial infection of eggs would be: (i) positively related to exposure period; and (ii) higher at the very humid cloud forest site than at the lowland site. In addition, because microbial infection may not necessarily cause subsequent mortality of the embryo, we conducted a second experiment to determine the fitness consequences of infection on non-incubated eggs and to disentangle the effects of micro-organisms and ambient temperatures on egg viability. Extended exposure of eggs to temperatures above the threshold at which embryo development is initiated, physiological zero (24–27 °C), and below the optimal incubation temperature (36–38 °C) can cause abnormal development and embryo mortality (Webb 1987; Ewert 1992; Meijerhof 1992; Stoleson 1999). We exposed freshly laid eggs at both sites, and sterilized the shells of half the eggs at each site twice daily to prevent trans-shell infection, while the remaining eggs were untreated. After exposure, we artificially incubated the eggs and compared hatching success between treatment groups. We predicted that: (i) uncleaned cloud forest eggs would exhibit lower hatchability than cleaned cloud forest eggs owing to the effects of pathogenic micro-organisms; and (ii) cleaned cloud forest eggs would exhibit higher hatchability than cleaned lowland eggs, because temperatures exceeded physiological zero 72.8% of the time at the lowland site, but less than 0.5% of the time at the cloud forest site.

2. MATERIAL AND METHODS

(a) *Study sites and source of eggs*

Studies were conducted at a cloud forest in the Luquillo Experimental Forest (810 m above sea level) and a nearby lowland forest, Las Paulinas (20 m above sea level), Puerto Rico. The two sites differ dramatically in ambient temperature and relative humidity, as recorded hourly throughout the study using thermisters and data loggers (Onset Computer Corp., Pocasset, MA, USA). The lowland site had a mean daily temperature of 27.3 ± 0.1 (s.d.) °C (range of 22.9–31.2 °C) and a mean relative humidity of $81.2 \pm 0.6\%$ (range of 65–97%), while the cloud forest site experienced daily mean temperatures of 21.1 ± 0.1 °C (range of 17.5–25.6 °C) and extremely high relative humidity averaging $97.5 \pm 0.5\%$ (range of 77–100%).

We used domestic chicken (*Gallus gallus domesticus*) eggs because their microbiology is familiar (Board & Fuller 1974) and they hatch synchronously, so may have evolved greater resistance to infection than asynchronously hatching species, yet their susceptibility (i.e. number of open pores and water-vapour conductance) is similar to that of other birds (Bruce & Drysdale 1994). We used freshly laid free-range eggs from a local supplier to ensure that laying conditions and contamination of the eggshell were relatively natural and embryo development was minimal. We used brown eggs because their shells are thicker and the protective cuticle more developed than white eggs (Board & Halls 1973). Breeds included Brown Leghorn, Plymouth Rock and Turken.

(b) *Experiment 1*

We exposed a total of 164 eggs at the two sites for periods of 1, 3, 5 or 7 days. Sterile handling procedures were used throughout. Each egg was randomly assigned a holding site and exposure period, swabbed to obtain a pre-treatment sample of the microbiota on the eggshell and labelled. The number of colony-forming units (CFU) on eggshells did not vary with holding site (analysis of variance (ANOVA): $F_{1,156} = 0.82$, $p = 0.366$), exposure period ($F_{3,156} = 2.18$, $p = 0.093$) or their interaction ($F_{3,156} = 1.35$, $p = 0.262$). At the holding site, eggs were set in an open cotton-lined tray at the base of a secure shaded wooden nest-box (24 cm × 22 cm × 73 cm with a 16 cm diameter hole located 6 cm from the top). The lining was replaced weekly.

An experimental replicate consisted of 16 eggs (two per exposure period per site) placed on the same day into boxes at each site. This procedure limited the variation in holding conditions that eggs of different treatment periods experienced. A maximum of eight eggs per box were positioned with their long axis horizontal and did not touch one another. We ran 11 replications over 11 weeks between June and August 2001. After exposure, we swabbed eggs to obtain post-treatment samples of the eggshell microbiota. Eggs were subsequently broken open under aseptic conditions and the contents (membrane, albumen and yolk) were analysed for the presence of micro-organisms (see § 2c).

Two additional eggs per replicate (20 in total) were opened at laying and served to control for vertical transmission of micro-organisms. Only one control egg harboured a single bacterial colony in the yolk (a Gram-negative enteric), which suggests that transovarial infection may occur in a very limited proportion of eggs. Evidence of sperm in the blastodisc of all 20 eggs indicated 100% fertility (Kosin 1944).

(c) *Microbiology*

To obtain pre- and post-treatment samples of microbiota from the shell surface, we swabbed one-fifth of the eggshell surface. After the post-treatment swab, the shell surface was sterilized with 95% alcohol. Using sterile techniques, each egg was cracked open at the blunt end, the eggshell peeled away with forceps and a 1 cm^2 sample of membrane removed from the air-sac region. We then enlarged the opening and poured the egg contents into a Petri dish. After removing a 0.01 ml aliquot of albumen with a calibrated 10 µl loop, the yolk surface (perivitelline membrane) was sterilized with 70% alcohol and opened aseptically using a spatula, and a 0.01 ml aliquot of yolk was taken. We placed all shell swabs and egg-content samples in 5 ml of sterile physiological (0.85% w/v) saline.

We cultured micro-organisms by plating 0.1 ml of saline supernatant into each of two liquefied growth media (Difco Laboratories, Detroit, MI, USA): MacConkey agar to grow Gram-negative enteric bacteria, and Tryptose Soy agar for heterotrophic bacteria. Fungi (unicellular and multicellular) grow on both media. All samples were plated within 1 h of collection. MacConkey and Tryptose plates were incubated aerobically for 48 h at 35 °C and 23 °C, respectively. We repeated the above culturing procedure without using egg components for each group of experimental eggs examined and found no false positives caused by contamination.

Following incubation, representative bacterial colonies from each plate were isolated and identified to a major group using morphological characteristics and the Gram method (Carter & Cole 1990), and to genus level using BIOLOG (Hayward, CA, USA) bacterial-identification kits. Fungi were identified following Barnett & Hunter (1972) and Malloch (1981).

(d) *Experiment 2*

Experiment 2 followed a similar procedure to Experiment 1, except that all eggs ($n = 144$) were exposed for 5 days, and eggs were not swabbed for the presence of micro-organisms. In addition, eggs underwent one of two treatments: half were wiped twice daily with 70% alcohol to kill microbes on the shell surface and prevent trans-shell infection; the other half were not cleaned and were exposed to potential infection. An experimental replicate consisted of 48 eggs (12 per treatment per site), and three replicates were run between April and August 2002. After exposure, eggs were incubated (Lyon Incubators, Chula Vista, CA, USA) with an automatic turner at 38 °C and 55–65% relative humidity for 18 days, and for the 3 days before hatching at 37.5 °C and 75% relative humidity in a brooding incubator (Brower, Houghton, IA, USA). Eggs were candled at 5 day intervals to assess embryo development.

To test that the cleaning procedure prevented microbial infection, we exposed an additional 18 eggs at the cloud forest site and wiped them with 70% alcohol twice daily. After 5 days of exposure, only one yolk out of the 18 eggs was infected.

(e) *Data analysis*

Two-way ANOVA with Tukey's post-hoc test was used with colony-count data, sometimes log transformed to achieve normality, to test for pre- and post-treatment effects, and for the roles of experimental site and exposure duration. Mann–Whitney *U*-tests were used when data could not be normalized. Means are presented with standard errors. The group compositions of microbial communities on and in eggs were analysed using Pearson's chi-square statistic (χ^2), excluding actinomycetes because they were rarely recorded.

Levels of infection were compared between control and experimental eggs with Fisher's exact test, and between experimental sites and among exposure periods using likelihood ratio tests (LRT). We used logistic regression and Akaike's Information Criterion corrected for small sample size (AICc) to analyse factors affecting the probability of microbial trans-shell transmission, following the methods of Burnham & Anderson (1998). Rather than testing statistical hypotheses, AICc discriminates among a suite of *a priori* models by evaluating how well each model in a set of candidate models fits a particular set of data (measured by the log-likelihood estimate) and penalizes models as they become more complex (measured by their number of parameters). Models were ranked using ΔAICc (the difference in AICc between a model and the model with the lowest AICc value, or best model) and AIC weights (the relative likelihood of a model given a set of models, normalized to sum to 1). Climatic variables were not considered in the set of candidate models because they were highly correlated with treatment site. We included treatment site, exposure period and both pre- and post-treatment loads of both fungi and *Pseudomonas* spp. on the shell, as these groups have been previously shown to break down the eggshell cuticle and facilitate microbial invasion (Board & Halls 1973; Board *et al.* 1979).

We used Fisher's exact test to compare hatching success and stage of embryo mortality between treatment groups.

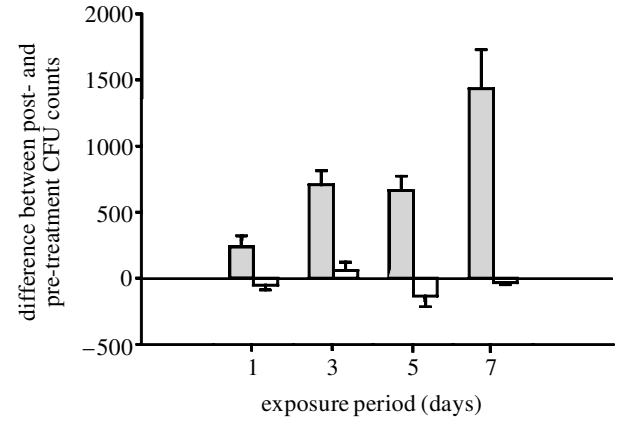

Figure 1. Effects of holding site and duration of exposure on microbial growth on the eggshell. Mean (± 1 s.e.m.) difference between post- and pre-treatment eggshell counts of CFUs is shown. Growth was affected by site (ANOVA: $F_{1,156} = 80.54$, $p < 0.001$), period ($F_{3,156} = 8.06$, $p < 0.001$) and their interaction ($F_{3,156} = 7.42$, $p < 0.001$). Cloud forest (grey bars) treatment periods of 3, 5 and 7 days had larger increases in CFUs than all four lowland (open bars) treatments (Tukey's HSD, all comparisons: $p < 0.015$). The number of CFUs was greater at the 7 day than at the 1, 3 and 5 day cloud forest treatments (all comparisons: $p < 0.01$).

3. RESULTS

(a) *Shell microbiota*

The presence of micro-organisms on the eggshell is essential for trans-shell infection (Board & Tranter 1986), and most experimental eggs (96%) possessed a shell microbiota prior to manipulation. The number of CFUs on eggshells increased rapidly and significantly with exposure duration at the cloud forest site, but not at the lowland site (figure 1). High humidity appears crucial for the survival and growth of micro-organisms on eggshells.

(b) *Microbial trans-shell transmission*

Shell microbiota penetrated the egg contents rapidly (figure 2), well within the time required to lay a clutch for many bird species (Viñuela & Carrascal 1999). Microbes appeared on the inner membrane after 1 day of exposure, by day 3 had invaded the albumen and by day 5 reached the yolk. As predicted, the probability and magnitude of infection were positively related to exposure period, and were higher at the very humid cloud forest site than at the lowland site. Bacteria and fungi occurred in a significantly greater proportion of treatment than control eggs after 5 days (Fisher's exact test: $n = 40$, $p = 0.003$) and 7 days ($n = 40$, $p = 0.001$), although not after 1 day ($n = 40$, $p = 1.0$) or 3 days ($n = 44$, $p = 0.66$). At the cloud forest site, microbes invaded 60% of eggs after 5 days and 70% after 7 days (figure 2*a*), whereas the chance of infection for lowland eggs was lower, the rate of infection was slower and the number of micro-organisms was fewer. Nevertheless, invasion occurred in a sizeable proportion of lowland eggs (25% and 33% after 5 and 7 days, respectively). The number of CFUs per sample also tended to be greater for cloud forest eggs than for lowland eggs (cloud forest versus lowland for: membrane 231.1 ± 174.8 versus 1.5 ± 0.5; albumen 47.0 ± 21.6 versus 4.4 ± 1.7; yolk 122.4 ± 87.0 versus 4.0 ± 1.4), but

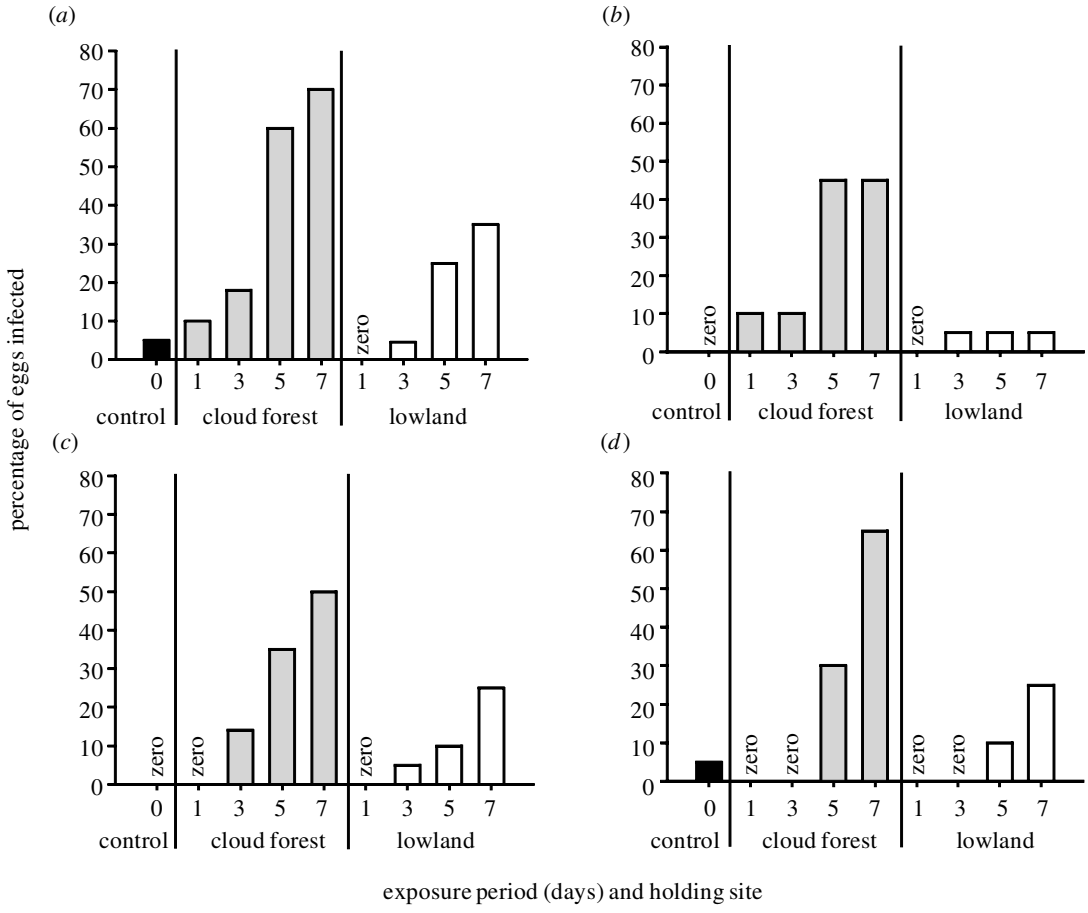

Figure 2. Microbial infection of the contents of unexposed control eggs and treatment eggs exposed for 1–7 days at two sites in Puerto Rico for (*a*) any egg content, (*b*) inner shell membrane, (*c*) albumen and (*d*) yolk.

varied so greatly between eggs that the difference was not significant for any component (Mann–Whitney *U*-test: $z = 83$, $p > 0.13$) with our sample size.

Likelihood of infection of each component of the experimental eggs (figure 2*b–d*) was best predicted by a logistic regression model that included treatment site, exposure and presence of fungi on the shell after exposure (table 1). This model had strong support compared with the others, with Akaike weights ranging from 0.71 to 0.90. The second-best model had only site and period terms, or site and post-treatment fungi terms, depending on the egg component analysed, but received about one-twelfth to one-third as much support as the best model (table 1).

(c) *Phylogenetic composition of microbes on the eggshell surface and in the contents*

The major contaminants on the eggshell were Gram-positive rods (table 2), comprising up to 40% of the colony types. Gram-positive cocci, Enterobacteriacea, Pseudomonadaceae and fungi were also prevalent. Microbial composition on the shell did not differ between holding sites prior to treatment ($\chi^2 = 6.5$, d.f. = 4, $p = 0.163$). After treatment, there was a marginal difference ($\chi^2 = 8.3$, d.f. = 4, $p = 0.079$), as shells held at the cloud forest site had relatively more Enterobacteriacea and fewer Gram-positive rods than shells held at the lowland site. There was no difference between the pre- and post-treatment microbiotas on shells held at either the cloud forest site ($\chi^2 = 0.53$, d.f. = 4, $p = 0.971$) or the lowland site ($\chi^2 = 3.52$, d.f. = 4, $p = 0.475$).

By contrast, Gram-negative enterics and fermenters, Gram-positive cocci and fungi invaded egg contents. Microbial composition did not differ significantly between egg components ($\chi^2 = 5.40$, d.f. = 8, $p = 0.700$), so their faunas were combined and tested against the post-treatment shell microbiota. There was a significant difference between the microbial composition on shells and that inside eggs ($\chi^2 = 32.6$, d.f. = 4, $p = 0.001$). Groups found in a higher percentage inside than outside the eggs were fungi (22.6% versus 10.3%) and Enterobacteriacea (29.2% versus 16.0%), while Gram-positive rods formed a higher proportion outside (14.2% versus 40.7%). Small sample size prohibited comparison between sites.

(d) *Egg viability*

Non-incubated eggs exposed to microbial infection and to temperatures above physiological zero exhibited a marked reduction in viability (figure 3). As predicted, cleaned cloud forest eggs had the highest hatching success (76.5%), which did not differ from that of unexposed controls (83.3% hatched; Fisher exact test: $n = 52$, $p = 0.73$), but was significantly greater than those of uncleaned cloud forest eggs (22.7% hatched; $n = 69$, $p < 0.001$) and cleaned lowland eggs (40% hatched; $n = 69$, $p = 0.007$). The reduction in hatchability of uncleaned cloud forest eggs was similar to the level of microbial infection at this

Table 1. Logistic regression models for infection of 164 treatment eggs, and their corresponding number of parameters (k), rank and AICc score corrected for small sample size. (Models are ranked by ΔAICc, which indicates the difference between each model and the best model with the lowest AICc (top line), and the AIC weight (the relative likelihood of a model given a set of candidate models, normalized to sum to 1). Predictive variables in the model are: treatment site (cloud forest or lowland); treatment period (1, 3, 5 or 7 days); pre-treatment presence of fungi or pseudomonads on the eggshell (presence or absence); and post-treatment presence of fungi or pseudomonads on the eggshell (presence or absence). *Interaction.)

		membrane				albumen				yolk			
model	k	rank	AICc	ΔAICc	weight	rank	AICc	ΔAICc	weight	rank	AICc	ΔAICc	weight
site, period, post-fungi	**6**	**1**	**84.52**	**0.00**	**0.711**	**1**	**120.06**	**0.00**	**0.899**	**1**	**95.80**	**0.00**	**0.741**
site, post-fungi*	4	2	86.73	2.21	0.236	7	136.19	16.14	0.000	7	134.49	38.69	0.000
site, post-fungi	3	3	89.72	5.20	0.053	6	134.79	14.73	0.001	6	132.68	36.88	0.000
site, period	5	4	102.28	17.76	0.000	2	125.34	5.28	0.064	2	98.70	2.90	0.174
site, period, pre-fungi	6	5	103.40	18.87	0.000	3	126.95	6.89	0.029	3	100.32	4.51	0.078
post-fungi	2	6	103.97	19.45	0.000	8	136.85	16.80	0.000	8	136.85	41.05	0.000
site, period*	8	7	108.39	23.87	0.000	5	131.71	11.65	0.003	4	105.25	9.44	0.007
site	2	8	113.99	29.47	0.000	9	147.61	27.56	0.000	9	145.21	49.41	0.000
period	4	9	121.32	36.79	0.000	4	130.63	10.58	0.005	5	108.29	12.49	0.001
pre-fungi	2	10	130.79	46.27	0.000	11	153.59	33.54	0.000	10	153.66	57.86	0.000
pre-pseudomonads	2	11	130.97	46.45	0.000	10	152.49	32.44	0.000	12	153.93	58.13	0.000
post-pseudomonads	2	12	133.31	48.79	0.000	12	153.63	33.58	0.000	11	153.81	58.01	0.000

site (60%; figure 2*a*), suggesting that micro-organisms accounted for most of the loss in egg viability at the cloud forest site. Hatching success did not differ between cleaned and uncleaned lowland eggs ($n = 70$, $p = 0.808$), suggesting that ambient temperature exerts a strong effect on egg viability under warmer conditions and that microbial infection has a small additive effect. The impact of microbial infection at the lowland site may have been reduced owing to the lower infection rates (figure 2) or because warmer temperatures increased the efficiency of the antimicrobial properties of the egg, thereby overcoming microbial invasion. The hatchabilities of uncleaned cloud forest and uncleaned lowland eggs did not differ significantly ($n = 70$, $p = 0.279$).

Most embryos (61%) died very early in development (less than 5 days), with a smaller peak in mortality occurring between day 15 and hatching (26%). There was no difference in the timing of mortality between sites (Fisher's exact test: $n = 77$, $p = 0.35$), or between cleaned and uncleaned eggs at either the cloud forest site ($n = 34$, $p = 1.0$) or the lowland site ($n = 43$, $p = 0.76$). Thus, the decrease in viability and the timing of mortality at the two sites were similar, but the mechanisms reducing hatchability differed.

4. DISCUSSION

Our results present strong evidence that birds' eggs have a limited shelf life under natural conditions. A decline in the viability of wild birds' eggs occurs after 3–5 days of exposure to ambient conditions and thereafter can accelerate rapidly (Stoleson 1999; Stoleson & Beissinger 1999). Temperature is assumed to be the critical factor causing non-incubated eggs to lose viability (Webb 1987; Ewert 1992; Meijerhof 1992), but here we demonstrate that microbial infection of egg contents can occur equally quickly, and that the two factors act independently to reduce hatching success considerably (figures 2 and 3). It is difficult to distinguish between the two causes of embryo mortality and previous work has not attempted to do so. Our results also suggest that the relative effects of microbial infection and ambient temperatures on hatching success are difficult to disentangle.

The occurrence of trans-shell infection depends on both climatic conditions and the phylogenetic composition of the eggshell microbiota. Studies conducted under laboratory conditions have shown that water is essential for trans-shell transmission because it promotes bacterial growth on eggshells and is the medium of transport by which microbes pass through shell pores (Board & Halls 1973; Board *et al.* 1979). Our results concur, showing that infection is most prevalent under conditions of high relative humidity. Previous studies also suggest that bacteria are more important than fungi in the addling of eggs (e.g. Board & Tranter 1986). By exposing eggs to natural conditions, however, we found that the probability of infection was highly dependent on the presence of fungal growth on eggshells (table 1). Eggs have limited chemical defence against fungi (e.g. no chitinases), which can break down the shell cuticle and facilitate microbial invasion by radically increasing the number of open pores available for access and by transporting bacteria through the pores on hyphae (Board *et al.* 1964; Board & Halls 1973; Board *et*

Table 2. Relative frequencies of microbial groups and genera on exposed eggshells and in egg contents.
(Asterisks represent the percentage of experimental eggs harbouring the identified genus: * < 1%, ** 1–5%, *** 6–10%, **** > 10%. Totals represent the percentage of a microbial group with respect to all microbes identified.)

		frequency of occurrence				
group	genus	pre-treatment shell	post-treatment shell	membrane	albumen	yolk
Gram-negative enterics	*Escherichia*	***	****	—	**	**
	Proteus	***	**	—	*	*
	Serratia	*	**	—	—	—
	Hafnia	—	—	—	—	—
	Citrobacter	**	*	—	—	—
	Enterobacter	**	**	—	*	*
	Klebsiella	—	**	—	—	—
	Salmonella	—	—	*	—	*
	Yersinia	**	**	*	—	—
	unknown	**	*	*	***	**
	total (%)	16	16	22	30	31
Gram-negative fermenters	*Pseudomonas*	**	***	**	**	**
	unknown	****	****	**	**	**
	total (%)	15	15	19	19	22
Gram-positive rods	*Bacillus*	****	****	**	**	**
	unknown	****	****	**	**	*
	total (%)	39	40	21	9	9
Gram-positive cocci	*Streptococcus*	*	*	*	*	*
	Staphylococcus	***	**	*	—	—
	Micrococcus	**	*	—	—	—
	unknown	****	****	*	**	**
	total (%)	20	18	8	16	13
actinomycetes	actinomycete	*	**	*	**	**
	unknown	—	—	—	—	—
	total (%)	1	1	3	7	6
fungi	*Aspergillus*	**	**	**	*	*
	Blastomyces	—	—	*	—	—
	Candida	*	**	*	*	—
	Fusarium	—	**	**	**	**
	Ovulariopsis	—	*	—	—	—
	Trichoderma	—	*	—	—	—
	yeast	—	—	—	*	*
	unknown	***	**	**	**	**
	total (%)	9	10	27	19	19

al. 1979). In addition, the growth of fungal mycelia in egg contents was frequently rapid and extensive, and often associated with the breakdown (gelling) of the albumen and the perivitelline membrane. This may account for the relatively fast infection rates observed in this study compared with those in studies conducted under artificial conditions where fungi were less pervasive (e.g. Board & Tranter 1986). We also found that microbial groups differed significantly in their ability to penetrate eggs, perhaps owing to intrinsic features of the passive defence mechanisms of eggs. The phylogenetic composition of the eggshell microbiota (table 2) was similar to that found in the eggs of captive fowl (Board *et al.* 1964) and was dominated largely by Gram-positive rods. This group is relatively tolerant of the dry conditions encountered on the shell owing to an ability to form desiccant-tolerant spores. By contrast, fungi, Gram-negative enterics, Gram-negative fermenters and Gram-positive cocci characterized the microbiota of egg albumen and yolk. Similar groups have been found in the eggs of domestic fowl (Bruce & Drysdale 1994) and wild birds (Houston *et al.* 1997; Stewart & Rambo 2000), often with pathogenic consequences in the former.

Despite the importance of a high-humidity environment to microbial penetration in this study, the infection of egg contents is likely to be an important constraint on the onset of incubation for birds nesting in a wide range of climates. Infection at the hotter less humid lowland site was frequent and occurred without condensation occurring on eggshells. Moreover, eggs in less humid environments are likely to suffer a high risk of infection if exposed to precipitation, condensation or damp nesting material, all of which may commonly occur during natural incubation. It is also unlikely that the rate of infection reported here is an artefact of using hens' eggs. The high levels of microbial infection found in addled eggs of wild temperate species (Kozłowski *et al.* 1989; Houston *et al.* 1997; Stewart & Rambo 2000) and pearly-eyed thrashers' eggs (*Margarops fuscatus*) in Puerto Rico (M. I. Cook & S. R. Beissinger, unpublished data) suggest that the infection

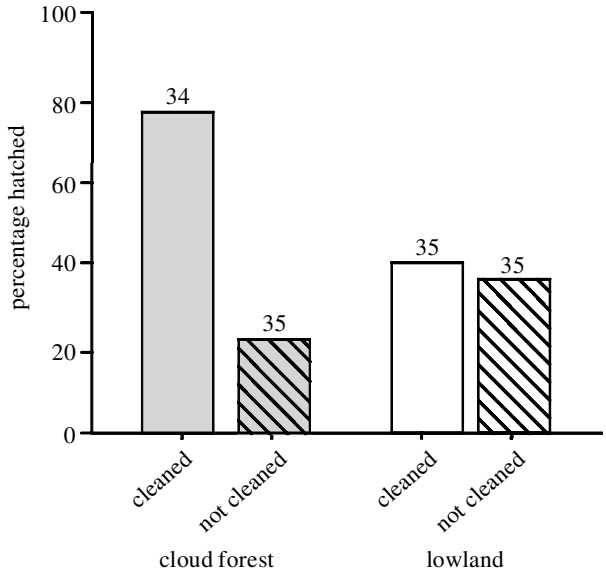

Figure 3. Hatching success of cleaned and uncleaned eggs exposed for 5 days at the cloud forest site or the lowland site and then artificially incubated (sample size above each bar). Logistic regression analysis indicates that the probability of hatching is strongly influenced by site, sterilizing treatment and their interaction (LRT = 22.4, $n = 139$, d.f. = 3, $p < 0.001$).

rate reported here is reasonable, and may be generally applicable to birds. Loss of egg viability appears to be a conservative trait in birds (Crittenden & Bohren 1961), so it is unlikely that the microbial resistance of domestic hens' eggs differs greatly from that of wild birds' eggs.

An early onset of incubation has the potential to maintain the viability of early-laid eggs by reducing the probability and magnitude of microbial infection and the impacts of ambient temperature. The strong effects of these two environmental factors on embryo mortality suggest that declines in egg viability will affect birds residing in a broad range of habitat and climatic types, and may select for the early onset of incubation even though the resulting hatching asynchrony may lead to the mortality of later-hatched chicks (Beissinger 1999). Risk of microbial infection may also shed light on the phenomenon of intermittent (partial) incubation prior to clutch completion (e.g. Afton 1979), a strategy employed by many species that could raise egg temperatures to levels sufficient to initiate chemical-defence mechanisms in the albumen (Williams *et al.* 1968). Finally, preventing trans-shell microbial infection may explain why some birds apply chemical control during nesting. The green-plant component of the nest lining effectively inhibits bacterial growth (Wimberger 1984; Clark & Mason 1985; Petit *et al.* 2002), and is employed primarily by species whose eggs are probably exposed to high levels of micro-organisms (i.e. those that reuse nests for multiple breeding attempts or nest in enclosed sites or cavities). Certain waxes and fatty acids found in preen-gland secretions also have antibacterial and antimycotic properties, which may function in nest hygiene (Jacob 1978) and may protect against infection by increasing the water repellence of the eggshell. Preen wax exhibits a sharp shift in chemical composition during incubation (Reneerkens *et al.* 2002), but whether these changes reflect an increase in antimicrobial efficiency is unknown. Further studies are required to catalogue the microbiota present and the prevalence of microbial invasion in wild birds' eggs, how incubation and climatic variables affect invasion and how invading species affect embryo fitness.

The authors thank A. Kong for help in the laboratory, M. Ford, K. Janaes and M. Anderson for help in the field, and F. Calderon for assistance with bacterial identification, which was done at the University of Puerto Rico R. C. M. I. Center for Fingerprinting of Microorganisms. We are grateful to J. Lodge for providing laboratory space and help with fungal identification, J. Wunderle for logistic support, A. Budden, C. Nordby, L. Grenier, Z. Peery, E. Punkay, J. Wang and two anonymous referees for reviewing this manuscript, and U. Abbot and J. Pisenti for helpful discussions. This study was financially supported by National Science Foundation grant 99-04754 to S.R.B. and by the USDA Forest Service.

REFERENCES

Afton, A. D. 1979 Incubation temperatures of the northern shoveler. *Can. J. Zool.* **57**, 1052–1056.

Amundsen, T. & Slagsvold, T. 1991 Asynchronous hatching in the pied flycatcher: an experiment. *Ecology* **72**, 797–804.

Arnold, T. W., Rohwer, F. C. & Armstrong, T. 1987 Egg viability, nest predation, and the adaptive significance of clutch size in prairie ducks. *Am. Nat.* **130**, 643–653.

Baggott, G. K. & Graeme-Cook, K. 2002 Microbiology of natural incubation. In *Avian incubation behaviour, environment, and evolution* (ed. D. C. Deeming), pp. 179–191. Oxford University Press.

Barnett, H. L. & Hunter, B. B. 1972 *Illustrated genera of imperfect fungi*, 3rd edn. Minneapolis, MN: Burgess Publishing Co.

Beissinger, S. R. 1999 Interaction of egg viability, brood reduction and nest failure on the onset of incubation. In *Proc. 22 Int. Ornithol. Congr. Durban* (ed. N. J. Adams & R. H. Slotow), pp. 638–646. Johannesburg: BirdLife South Africa.

Board, P. A. & Board, R. G. 1967 A method of studying bacterial penetration of the shell of the hen's egg. *Lab. Pract.* **16**, 471–482.

Board, R. G. 1966 The course of microbial infection of the hen's egg. *J. Appl. Bacteriol.* **29**, 319–341.

Board, R. G. & Fuller, R. 1974 Non-specific antimicrobial defenses of the avian egg embryo and neonates. *Biol. Rev. Camb. Phil. Soc.* **49**, 15–49.

Board, R. G. & Halls, N. A. 1973 The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poultry Sci.* **14**, 69–97.

Board, R. G. & Tranter, H. S. 1986 The microbiology of eggs. In *Egg science & technology*, 3rd edn (ed. W. J. Stadelman & O. J. Cotterill), pp. 75–96. Westport, CT: AVI Publishing Co.

Board, R. G., Aynes, J. C., Kraft, A. A. & Forsythe, R. G. 1964 The microbiological contamination of egg shells and egg packaging material. *Poultry Sci.* **43**, 584–595.

Board, R. G., Loseby, S. & Miles, V. R. 1979 A note on microbial growth on eggshells. *Br. Poultry Sci.* **20**, 413–420.

Bruce, J. & Drysdale, E. M. 1994 Trans-shell transmission. In *Microbiology of the avian egg* (ed. R. G. Board & R. Fuller), pp. 63–91. London: Chapman & Hall.

Burnham, K. P. & Anderson, D. R. 1998 *Model selection and inference: a practical theoretic approach*. New York: Springer.

Carter, G. R. & Cole Jr, J. R. 1990 *Diagnostic procedures in veterinary bacteriology and mycology*, 5th edn. San Diego, CA: Academic Press.

Clark, A. B. & Wilson, D. S. 1981 Avian breeding adaptations: hatching asynchrony, brood reduction, and nest failure. *Q. Rev. Biol.* **56**, 253–277.

Clark, L. & Mason, J. R. 1985 The use of nest material as insecticidal and antipathogenic agents by the European starling. *Oecologia* **67**, 169–176.

Crittenden, L. B. & Bohren, B. B. 1961 The genetic and environmental effects of hatching time, egg weight and holding time on hatchability. *Poultry Sci.* **40**, 1736–1750.

Ewert, M. A. 1992 Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In *Egg incubation: its effects on embryonic development in birds and reptiles* (ed. D. C. Deeming & M. W. J. Ferguson), pp. 173–191. Cambridge University Press.

Houston, S., Saunders, J. R. & Crawford, R. D. 1997 Aerobic bacterial flora of addled raptor eggs in Saskatchewan. *J. Wildl. Dis.* **33**, 328–331.

Jacob, J. 1978 Uropygial gland secretions and feather waxes. In *Chemical zoology X, Aves* (ed. M. Florkin, B. T. Sheer & A. H. Brush), pp. 165–211. New York: Academic Press.

Kosin, L. L. 1944 Macro- and microscopic methods of detecting fertility in unincubated hen's eggs. *Poultry Sci.* **23**, 266–269.

Kozłowski, S., Małysko, E., Pinkowski, J. & Kruszewicz, A. 1989 The effect of microorganisms on the mortality of house sparrow (*Passer domesticus*) and tree sparrow (*Passer montanus*) embryos. In *Proc. Int. Symp. of the Working Group of Granivorous Birds* (ed. J. Pinowski, B. P. Kavanagh & W. Górski Warszawa), pp. 121–128. Słupsk, Poland: Intecol.

Malloch, D. 1981 *Moulds: their isolation, cultivation and identification*. University of Toronto Press.

Meijerhof, R. 1992 Pre-incubation holding of hatching eggs. *World's Poultry Sci. J.* **48**, 57–81.

Petit, C., Hossaert-McKey, M., Perret, P., Blondel, J. & Lambrechts, M. M. 2002 Blue tits use selected plants and olfaction to maintain an aromatic environment for nestlings. *Ecol. Lett.* **5**, 585–589.

Reneerkens, J., Piersma, T. & Sinninghe Damsté, J. S. 2002 Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proc. R. Soc. Lond.* B **269**, 2135–2139. (DOI 10.1098/rspb.2002.2132.)

Stewart, R. & Rambo, T. B. 2000 Cloacal microbes in house sparrows. *Condor* **102**, 679–684.

Stoleson, S. H. 1999 The importance of the early onset of incubation for the maintenance of egg viability. In *Proc. 22 Int. Ornithol. Congr. Durban* (ed. N. J. Adams & R. H. Slotow), pp. 600–613. Johannesburg: BirdLife South Africa.

Stoleson, S. H. & Beissinger, S. R. 1995 Hatching asynchrony and the onset of incubation in birds, revisited: when is the critical period? *Curr. Ornithol.* **12**, 191–270.

Stoleson, S. H. & Beissinger, S. R. 1999 Egg viability as a constraint on hatching synchrony at high ambient temperatures. *J. Anim. Ecol.* **68**, 951–962.

Viega, J. P. 1992 Hatching asynchrony in the house sparrow: a test of the egg-viability hypothesis. *Am. Nat.* **139**, 669–675.

Viñuela, J. & Carrascal, L. M. 1999 Hatching patterns in non-precocial birds: a preliminary comparative analysis. In *Proc. 22 Int. Ornithol. Congr. Durban* (ed. N. J. Adams & R. H. Slotow), pp. 584–599. Johannesburg: BirdLife South Africa.

Webb, D. R. 1987 Thermal tolerance of avian embryos: a review. *Condor* **89**, 874–898.

Williams, J. R., Dilliard, L. H. & Hall, G. O. 1968 The penetration patterns of *Salmonella typhimurium* through the outer structures of chicken eggs. *Avian Dis.* **12**, 69–97.

Wimberger, P. H. 1984 The use of green plant material in bird nests to avoid ectoparasites. *Auk* **101**, 615–618.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.