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Microbial and environmental effects on avian egg viability: Do tropical mechanisms act in a temperate environment?

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Abstract. The viability of freshly laid avian eggs declines after several days of exposure to ambient temperatures above physiological zero, and declines occur faster in tropical than temperate ecosystems. Microbial infection during preincubation exposure has recently been shown as a second cause of egg viability decline in the tropics, but whether microbial processes influence the viability of wild bird eggs in temperate ecosystems is unknown. We determined the microbial load on eggshells, the incidence of microbial penetration of egg contents, and changes in the viability of wild bird eggs (*Sialia mexicana*, *Tachycineta bicolor*, *Tachycineta thalassina*) experimentally exposed to temperate-zone ambient conditions in situ in a mediterranean climate in northern California. Initial microbial loads on eggshells were generally low, although they were significantly higher on eggs laid in old boxes than in new boxes. Eggshell microbial loads did not increase with exposure to ambient conditions, were not reduced by twice-daily disinfection with alcohol, and were unaffected by parental incubation. The rate of microbial penetration into egg contents was low and unaffected by the duration of exposure. Nevertheless, egg viability declined very gradually and significantly with exposure duration, and the rate of decline differed among species. In contrast to studies performed in the tropics, we found little evidence that temperature or microbial mechanisms of egg viability decline were important at our temperate-zone site; neither temperatures above physiological zero nor alcohol disinfection was significantly related to hatching success. Delaying the onset of incubation until the penultimate or last egg of a clutch at our study site may maintain hatching synchrony without a large trade-off in egg viability. These results provide insight into the environmental mechanisms that may be responsible for large-scale latitudinal patterns in avian clutch size and hatching asynchrony.

Key words: clutch size; egg viability; environmental constraints; hatching asynchrony; life-history strategy; microbial ecology; Sialia mexicana; Tachycineta bicolor; Tachycineta thalassina.

Introduction

Life-history traits are adaptations that have evolved within environmental constraints, such as time and resource allocation limits or trade-offs (Ghalambor and Martin 2001, Ricklefs and Wikelski 2002). Food availability and predation risk are the primary environmental constraints thought to drive many avian life-history traits, such as clutch size (Monaghan and Nager 1997, Martin et al. 2000, Ricklefs 2000) and hatching asynchrony (Stoleson and Beissinger 1995). Both food and predation hypotheses emphasize the importance of processes acting as selective forces primarily after a clutch has been laid or hatched, through brood reduction or offspring mortality. However, ambient environmental conditions acting on events earlier in the reproductive cycle could also be a constraint that drives avian clutch size and hatching patterns. For example, the viability of freshly laid avian eggs declines over time when eggs are exposed to ambient conditions (Arnold et al. 1987, Veiga 1992, Stoleson and Beissinger 1999) and may necessitate early incubation of eggs (egg viability hypothesis: Arnold et al. 1987). A decline in egg viability often begins after several days of preincubation exposure, increases with duration of exposure, and occurs faster in tropical than in temperate ecosystems (Stoleson and Beissinger 1999, Beissinger et al. 2005), although few in situ experiments on wild species have been conducted.

Two independent and interacting mechanisms are known to cause declines in egg viability: ambient temperature and microbial infection (Cook et al. 2003). When ambient temperature exceeds physiological zero (24–27°C) but remains below optimal incubation temperature (34–36°C), abnormal embryonic development commences, resulting in increased embryonic mortality (Webb 1987, Meijerhof 1992). Exposure of a week or more to temperatures below physiological zero but above freezing can also erode egg viability by increasing albumen pH and decreasing albumen viscosity (Arnold 1993, Fasenko et al. 2001, Fasenko 2007). Microbial infection can cause egg viability to decline

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with increased duration of preincubation exposure (Cook et al. 2003, 2005*b*, Godard et al. 2007). Warm, moist conditions in the tropics are conducive to fungal and bacterial growth on eggshells and to microbial penetration of egg contents, but temperature and humidity can act independently (Cook et al. 2003, 2005*b*). Parental incubation reduces microbial growth on eggshells in tropical environments (Cook et al. 2005*a*, Shawkey et al. 2009). Little is known about microbial processes on eggs exposed to temperate environments. Low levels of microbial infection occurred in chicken (*Gallus gallus*) eggs exposed to temperate ambient conditions, but these eggs were moistened daily (Godard et al. 2007).

In this study we measure microbial loads on eggshells, the incidence of microbial penetration of egg contents, and changes in the viability of wild bird eggs experimentally exposed to ambient conditions in situ in a temperate, mediterranean climate in northern California. We examine these processes in three box-nesting passerines by experimentally exposing freshly laid eggs to ambient conditions for up to eight days, opening some to assess microbial penetration and returning others to nests to measure hatching success. Hypothesizing that temperate ambient conditions are conducive to microbial growth, we predicted that (1) microbial load on eggshells and the probability of egg-content infection should increase with duration of ambient exposure, (2) disinfecting eggshell surfaces should reduce shell microbial loads and the probability of egg-content infection compared to untreated eggs, and (3) egg viability (i.e., hatching success) should decline with increased length of preincubation exposure and with increased exposure to ambient temperatures above physiological zero. Moreover, we predicted that the changes in microbial load, the probability of microbial penetration, and the rate of decline in egg viability would be less than that reported from tropical environments because of lower ambient temperature and humidity at our study site compared to tropical conditions. We also sampled bacteria on the eggshell and in the egg contents, to provide the first sequence-based identification of wild-egg-associated bacteria in the temperate region. We predicted that eggshell bacteria were more likely to be gram-positive and egg-content bacteria would be gram-negative, as has been found on eggs in the tropics (Cook et al. 2003, 2005*a*). Our results provide the first quantification of microbial processes on the eggs of wild birds in a temperate environment and present an important comparison with past work in tropical ecosystems.

Methods

Study species, study site, and field monitoring

Experiments were performed using freshly laid eggs of the Western Bluebird (*Sialia mexicana*), Tree Swallow (*Tachycineta bicolor*), and Violet-green Swallow (*Tachycineta thalassina*). All three species are single-sex intermittent incubators that nest in similar habitat, overlap in breeding season and clutch size (3–7 eggs), and experience similar environmental conditions during nesting (Wang and Beissinger 2009). They are widespread species that regularly nest in wooden boxes, lay eggs daily, and exhibit a large degree of variation among individuals in the onset of incubation (Wang and Beissinger 2009).

The study was performed at the Hopland Research and Extension Center in Mendocino County, California (39°00′ N, 123°04′ W) from March to July of 2005–2007. The site is composed of sheep pastures and oak woodland and experiences a mediterranean climate with hot, dry summers and cool, wet winters. During the study, daily air temperatures averaged 15.6 ± 8.2°C (mean ± SD) and exceeded 24°C (a conservative estimate of physiological zero) 17.3% of the time. See Wang and Beissinger (2009) for details of the study site and nest boxes. Top-opening boxes were 12 years old and front-opening boxes were 1–6 years old when our study began.

Dataloggers (Hobo H8; Onset Corporation, Bourne, Massachusetts, USA) were attached halfway up the rear inside wall of holding boxes. Eggs ($N = 667$) experienced ambient temperatures >24°C and >34°C (incubation temperature) for 18.7% ± 11.6% (mean ± SD) and 2.0% ± 3.7% of their exposure duration, respectively. Daily temperature experienced by eggs inside of holding boxes averaged 16.7° ± 3.2°C (mean ± SD; minimum, 8.1° ± 3.1°C; maximum, 31.9° ± 6.0°C), and relative humidity averaged 56.7% ± 12.2%.

We checked boxes at least every three days from 07:00 to 13:00 hours. Nests near completion were checked daily for new eggs until clutch completion, which was designated when clutch size remained unchanged for three consecutive days. Daily checks for hatching success started 11 days after clutch completion and continued until all eggs had hatched or their fates were otherwise known. We performed nest box checks either without handling the eggs and nest material, or by using gloves disinfected with 70% isopropyl alcohol.

Microbial penetration experiment

To determine how microbial loads on eggshells and the probability of egg-content infection changed with duration of ambient exposure, eggs were removed on the morning of laying, sampled for eggshell microflora, transported and placed in empty nest boxes ("holding boxes") at a central location, and removed after a randomly assigned exposure of 2, 4, or 6 days for microbial sampling of eggshells and contents. At the natal nest before transport, each egg was swabbed over approximately one-half of the shell surface with a sterile Dacron swab moistened in 800 uL of sterile phosphate-buffered saline solution (0.05% Tween 80/15% glycerol). Then the egg was individually marked on its blunt end, weighed in an alcohol-disinfected container using a 5-g Pesola scale, placed in a sterile 4-oz (113 g) Whirl-Pak

(Nasco, Fort Atkinson, Wisconsin, USA), and transported to a holding box in a cooler with an ice pack to prevent warming. To prevent the interruption of laying in natal nests, each egg was replaced with a fake, painted egg made from self-hardening clay.

Holding boxes (12 new and 3 old boxes) contained recently constructed nests of the corresponding species to provide environmental microflora similar to natal nests. Eggs were placed so that they were not in direct contact with each other and were rotated 180° around their long axis twice daily to simulate egg turning that may occur in natural nests prior to incubation (Cook et al. 2003, 2005*b*). At the end of each exposure period eggs were swabbed, weighed, and transported to the laboratory to be opened aseptically. We wiped the shell surface with a 70% isopropyl alcohol swab and allowed it to air dry, cracked the shell above the air cell, and removed the shell and shell membranes with flame-sterilized forceps. We poured the egg contents into a sterile petri dish and collected 200 μL of albumen using a sterile micropipette. The vitelline membrane was punctured with a flame-sterilized loop to collect 200 μL of yolk with a sterile micropipette. Each sample was diluted in 600 μL of a 15% glycerol/phosphate-buffered saline (PBS) solution and shaken vigorously.

We employed two types of controls: a "day-0" control and an unmanipulated control for incubation (Cook et al. 2003). One egg per clutch was randomly assigned to be the day-0 control, which was handled and transported identically to other experimental eggs at the natal box, but was opened on the day of laying to determine the rate of vertical transmission (i.e., infection passing from the oviduct to the egg contents). The unmanipulated control accounted for the effects of incubation and was designated as any egg found warm on the day of laying, typically the last-laid egg of a clutch. It was swabbed on the day of laying and left in the natal nest to undergo incubation for four days, then re-swabbed and opened in the laboratory. In summary, eggs in the microbial penetration experiment were assigned to one of three conditions: exposed, day-0 control, or unmanipulated control.

Egg viability experiment

To determine how egg viability was affected by duration of exposure to ambient conditions, eggs were removed upon laying and exposed in holding boxes for periods of 2–8 days following the procedures used in the *Microbial penetration experiment*, except that one-half of the eggs were also assigned to a "cleaning" treatment to remove microflora from the shell surface. Cleaning consisted of wiping the eggs with a 70% isopropyl alcohol cotton pad during their twice-daily rotation. Cleaning treatments were alternately assigned to eggs in natal clutches for each exposure duration. After exposure, eggs were returned to active nests and monitored for hatching success. We used two types of controls per clutch following Beissinger et al. (2005): (1) transport controls were swabbed to sample microflora, transported for 104 ± 116 min (mean ± SD, $N = 170$), and returned to natal nests; and (2) unmanipulated controls were swabbed for microflora on the day of laying and left in natal nests for four days to undergo natural incubation, upon which they were re-swabbed and monitored for hatching.

Viability experiments were conducted in multiple years. In 2005 we conducted two- and four-day exposures for all three species. We assigned first and second eggs to four-day exposures, and third and fourth eggs to two-day exposures to mimic the maximum duration of exposure that each egg could have received in the wild. Fifth-laid eggs were either transport controls or unmanipulated controls. This protocol was continued in 2006–2007 for Violet-green Swallow eggs, to achieve sample size goals. We conducted six- and eight-day ambient exposures for Tree Swallow and Western Bluebird eggs in 2006–2007, randomizing the assignment of transport controls, six-day, and eight-day exposures within each natal clutch and returning exposed eggs to non-natal clutches at which full incubation had started. Some eggs were transferred a second time if the incubation period ended before the transferred eggs had hatched ($N = 39$). Eggs placed in non-natal nests were fully accepted and incubated.

Microbiology

All eggshell swabs and egg content samples were kept cold in the field or at 4°C in the laboratory until same-day inoculation onto two types of media (Difco tryptic soy agar and Difco MacConkey agar; BD, Franklin Lakes, New Jersey, USA). We vortexed each sample for 3–5 s and removed 0.1-mL aliquots for culturing on four plates: TSA at 27°C and 35°C and MAC at 27°C and 35°C. Plates were incubated for 72 hours and colony counts were performed on days 1, 2, and 3 of incubation. The maximum count during the three-day period was used for analyses. A subset of colonies was isolated and identified by 16S rDNA sequencing following the methods in Appendix A.

Data analysis

Microbial loads on eggshells were measured as the sum of colony counts from four cultures per egg swab (a total inoculation volume of 0.4 mL) and cube-root transformed to achieve normality. Changes in microbial load, measured as the cube-root transformed difference of pre- and postexposure colony counts, were examined with linear regression in SAS (versions 9.1.3 and 9.2; SAS Institute 2006, 2008), which was used for all subsequent analyses. We used logistic regression to assess the probability of microbial penetration of the albumen or yolk with the effects of species and exposure duration, and with eggshell fungal presence and microbial loads before and after exposure (Appendix B).

We used stepwise logistic regression to assess the probability of hatching success using $\alpha = 0.15$ for the

TABLE 1. GLM models of initial microbial load on eggshells ($N = 556$ eggs) and change in microbial load during exposure ($N = 370$ eggs).

Microbial flora, factor	df	F	P
Initial load			
Species	2, 529	2.41	0.091
Exposure	4, 529	0.42	0.791
Year	2, 529	3.37	0.035
Natal box age	1, 529	5.55	0.019
Cleaned	1, 529	0.09	0.761
Species × exposure	8, 529	0.69	0.704
Species × year	4, 529	1.39	0.237
Species × natal box age	2, 529	0.40	0.672
Year × natal box age	2, 529	2.63	0.073
Change in load			
Species	2, 343	3.80	0.023
Exposure	1, 343	0.96	0.329
Cleaned	1, 343	0.01	0.935
Year	2, 343	0.15	0.865
Initial load	1, 343	26.99	0.001
Species × exposure	2, 343	3.52	0.031
Species × cleaned	2, 343	0.69	0.504
Species × year	4, 343	0.69	0.601
Species × initial load	2, 343	0.25	0.780
Exposure × cleaned	1, 343	0.67	0.414
Exposure × year	2, 343	1.10	0.333
Exposure × initial load	1, 343	0.01	0.926
Cleaned × year	2, 343	1.08	0.342
Cleaned × initial load	1, 343	0.52	0.470
Initial load × year	2, 343	6.47	0.002

Note: Exposure was coded as categorical for initial microbial load and as continuous for change in microbial load during exposure.

enter-and-stay threshold. A logistic model with only the effects of species and exposure duration was then estimated to illustrate the declines in egg viability for each species. To investigate the factors influencing egg mass loss, we used a repeated-measures design with species, exposure duration, and their interaction as effects.

To facilitate cross-study comparisons, we converted eggshell microbial counts into standardized units of colony-forming units (CFU) per egg by multiplying by a dilution factor of (diluent volume [mL]/proportion of eggshell swabbed) and dividing by the total inoculant volume (mL). Thus, mean counts were multiplied by three in the present study (0.6 mL·[0.5 eggshell]$^{-1}$·0.4 mL^{-1}), by 125 for Cook et al. (2003, 2005*a*, *b*), and by 100 for Godard et al. (2007). The standardized counts were then log-transformed.

Colony counts from the albumen and yolk were converted into CFU/mL for comparison with other studies. A theoretical limit of detection, the smallest infection detectable under ideal conditions, was calculated by converting one colony detected per sample into the concentration of colonies in the source material, using $c = F(z/v)$ (Niemela 2003), where c = estimated microbial content per unit volume of sample, F = dilution factor, z = number of colonies observed (set at 1), and v = volume of the inoculant (in milliliters of final dilution). The theoretical limit of detection was 10 CFU/mL for the present study, and 2505 CFU/mL for Cook et al. (2003) and Godard et al. (2007).

RESULTS

Microbial processes on eggshells

Initial microbial loads on eggshells were small (median 6 CFU/egg) and did not differ among eggs assigned to different exposure durations or cleaning regimens (Table 1). However, initial eggshell loads differed significantly with natal-box age and year (Table 1). Initial loads were over twice as high on eggs originating from old boxes (least-squares mean 22.5 CFU/egg) as from new boxes (9.9 CFU/egg), and almost three times greater on eggs laid in 2005 (27.0 CFU/egg) than in 2006 (11.8 CFU/egg) or 2007 (10.2 CFU/egg). Neither species nor any of its interactions were significant (Table 1).

Initial microbial load and the interaction of initial load and year significantly affected changes in load with exposure (Table 1). Eggs with higher initial microbial loads experienced less microbial growth and even decreases in microbial loads (Fig. 1). The majority of eggs with initial shell loads >8 CFU decreased in microbial load over time, while most eggs with initial loads ≤8 CFU had small increases in load. This relationship was most pronounced in 2005 and weakest in 2007. The species interaction with exposure duration was significant but so small that it was not biologically meaningful (Table 1); microbial loads on eggshells did not significantly change with exposure duration in the Western Bluebird, but increased in swallow eggs (0.22 CFU/day in Violet-green Swallows and 0.06 CFU/day in Tree Swallows). Changes in eggshell loads were unaffected by cleaning (Table 1).

We isolated 30 genera and five phyla from eggshells (Appendix A; Table 2). Most isolates were gram-positive bacteria from the Actinobacteria and Firmicutes phyla. The most common genera found were *Bacillus*, *Staphylococcus*, *Arthrobacter*, and *Sporosarcina*. Only one gram-negative bacterium occurred multiple times, *Pantoea* from the family Enterobacteriaceae in phylum Proteobacteria. The composition of microflora on eggshells before and after exposure (Table 2) did not change significantly ($\chi^2 = 4.17$, df = 3, $P = 0.232$).

Eggs that were exposed for four days experienced changes in bacterial loads similar to unmanipulated control eggs that were incubated by females for four days between swab sampling (Table 3). Year, initial microbial load, and the interaction of species with incubation were significant. Microbial loads increased significantly more on eggs in 2005 (least-squares mean 18.9 CFU·egg^{-1}·d^{-1}) than in 2006 (0.6 CFU·egg^{-1}·d^{-1}) or 2007 (0.9 CFU·egg^{-1}·d^{-1}), and changed less on eggs with higher initial loads. Although the species × incubated interaction was significant, none of the pairwise differences of least-squares means were significant after adjustment for multiple comparisons.

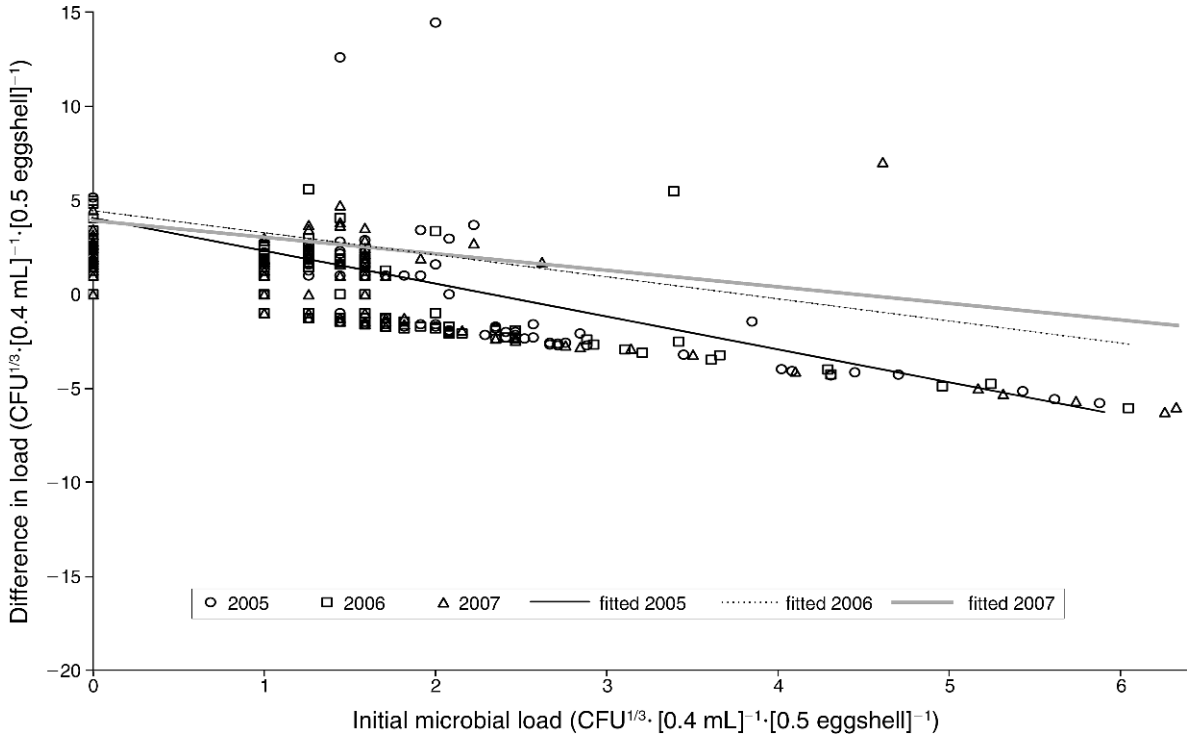

FIG. 1. Initial microbial loads on eggshells vs. change in microbial loads during ambient exposure in non-incubated eggs ($N = 370$). CFU stands for colony-forming units.

Microbial penetration into egg contents

The rate of microbial penetration into egg contents was low and unaffected by duration of exposure (Fig. 2; Appendix B). The risk of penetration in either the albumen or yolk did not differ significantly among species. Neither pre- vs. postexposure microbial loads nor the presence of fungi on eggshells significantly affected the probability of albumen or yolk infection (Appendix B). None of the eggs with albumen infection had fungi detected on their shells after exposure. *Staphylococcus*, *Bacillus*, and *Micrococcus* were the most commonly detected genera in the egg contents, in order of frequency (Appendix A).

Effects of exposure to ambient conditions on egg viability

Egg viability declined very gradually but significantly with exposure duration, and the rate of decline differed among species (Fig. 3, Table 4). Tree Swallows had significantly less loss of viability with exposure and Violet-green Swallows had significantly more viability loss with exposure, compared to Western Bluebirds. Transport time, cleaning, average temperature, proportion of time >24°C, and the interactions of species with exposure duration and cleaning were not significant. Initial egg mass and egg mass loss during exposure marginally affected hatching success. The estimated average rate of mass loss was 0.01 g/day ($F = 13.41$, df $= 1, 399$, $P < 0.001$) and did not differ by species. The average daily percentages of mass loss were 0.70% for the two species of swallows and 0.42% for the Western Bluebird.

DISCUSSION

Low microbial loads resulted in little risk of infection

Microbial loads on our eggshells were generally low ($<10^1$ CFU/egg), did not increase with exposure to ambient conditions, were not reduced by twice-daily disinfection of eggshells, and were unaffected by parental incubation. In contrast, these factors all

TABLE 2. Number and percentage of eggs with isolates by exposure, component, and phylum.

Phylum	Preexposure	Postexposure		
	Eggshell ($N = 43$)	Eggshell ($N = 43$)	Albumen ($N = 21$)	Yolk ($N = 4$)
Actinobacteria	18 (14.9%)	12 (27.9%)	8 (38.1%)	3 (60%)
Bacteroidetes	2 (4.7%)	0	0	0
Firmicutes	31 (72.1%)	41 (95.3%)	16 (76.2%)	2 (40%)
Proteobacteria	6 (14.0%)	5 (11.6%)	0	0

Note: N is sample size of eggs.

TABLE 3. Generalized linear model of change in eggshell microbial load comparing incubated vs. non-incubated eggs ($N = 126$ eggs).

Effect	df	F	P
Species	2, 106	0.82	0.445
Incubated	1, 106	0.63	0.430
Year	2, 106	4.65	0.012
Initial load	1, 106	68.73	0.001
Species × incubated	2, 106	3.65	0.029
Species × year	4, 106	1.19	0.321
Species × initial load	2, 106	0.94	0.395
Incubated × year	2, 106	2.59	0.080
Incubated × initial load	1, 106	1.99	0.161
Initial load × year	2, 106	1.62	0.204

Note: Change in microbial load was cube-root transformed, and incubation was coded as a categorical variable (yes or no).

affected microbial loads on wild and domestic bird eggs exposed in nest boxes in tropical environments (Cook et al. 2003, 2005*a*, *b*, Shawkey et al. 2009). Shell loads on eggs in the tropics were 10^3–10^4 CFU/egg and increased up to 10-fold over time (Cook et al. 2005*a*, *b*). One other study (Godard et al. 2007) exposed chicken eggs in a temperate environment in both nest boxes and open cup nests, and also found that microbes grew little on eggshells exposed in nest boxes for up to five days. However, eggs exposed in open cup nests received daily simulated rains by direct misting of eggs, which complicates interpretations and comparisons because moisture on eggshells stimulates microbial growth and aids transport of microbes through shell pores (Board et al. 1979). For consistency, we restrict comparisons of our work below to Godard et al.'s nest box treatment, but return to their results on open-cup nests in the final section of the *Discussion*.

Two environmental factors that did affect microbial loads on eggshells were box age and year of study. Eggs laid in older boxes had higher initial microbial loads than eggs laid in newer boxes, likely due to the accumulation of feces, fungi, and other sources of microbes in old boxes, as well as the breakdown of tannins in wood. Initial microbial loads were twice as high in 2005, the year with the wettest peak egg-laying month (May precipitation totaled 120 mm in 2005, 11 mm in 2006, and 12 mm in 2007), compared to other years. Differences in annual rainfall may also account for the smaller decrease in eggshell loads in 2007 (Appendix B). Not only do wetter environments promote microbial growth, but they may stimulate the activity of *Bacillus* spp., which were commonly found on our eggshells and are dominant in avian plumage assemblages (Burtt and Ichida 1999, Whitaker et al. 2005).

Eggs exposed to ambient conditions at our temperate mediterranean site experienced small rates of microbial penetration above the background levels of vertical transmission (2.9% in albumen and 7.9% in yolk), and the rate of infection did not increase significantly over time (Fig. 2, Table 4). Our study environment corresponds to lower-risk conditions; both dry ambient conditions and nest boxes reduce the chance for water to occur on eggshells, which promotes trans-shell infection (Board et al. 1979, Cook et al. 2003). Similarly, albumen and yolk of chicken eggs exposed to a temperate environment in nest boxes were uninfected (Godard et al. 2007). In tropical environments, however, infection rates of eggs of a cavity-nesting thrasher were high (60%) after five days of exposure and had increased two to six times from the background rate of infection (Cook et al. 2005*b*). Likewise, infection rates of chicken eggs exposed in nest boxes rose eightfold after five days of tropical exposure (Cook et al. 2003).

Identification and sources of microbes on wild eggs

The genera *Bacillus* and *Staphyloccocus*, which are common on chicken eggshells (Board and Tranter 1986, Bruce and Drysdale 1994, Cook et al. 2003), were the most frequent isolates from our wild bird eggshells (Appendix A). These results contrast with those of Cook et al. (2003), where the composition of eggshell bacteria in the tropics was dominated by gram-positives but the composition of bacteria in the shell membrane, albumen, and yolk was characterized as gram-negatives. None of our identified yolk colonies were pathogenic gram-negative enterics or gram-negative fermenters,

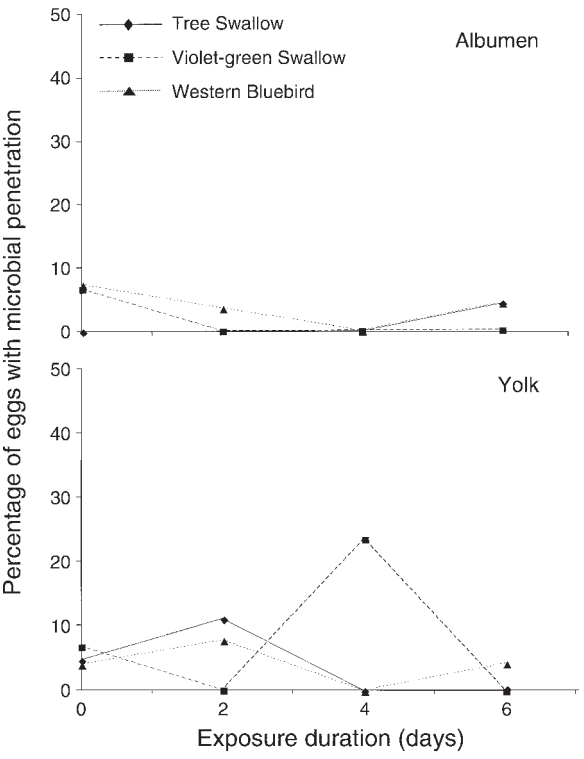

FIG. 2. Percentage of eggs with infections in the albumen or yolk greater than that detected in ~90% of control eggs (Appendix B). Eggs were opened on the day of laying or after 2, 4, or 6 days of exposure to ambient conditions.

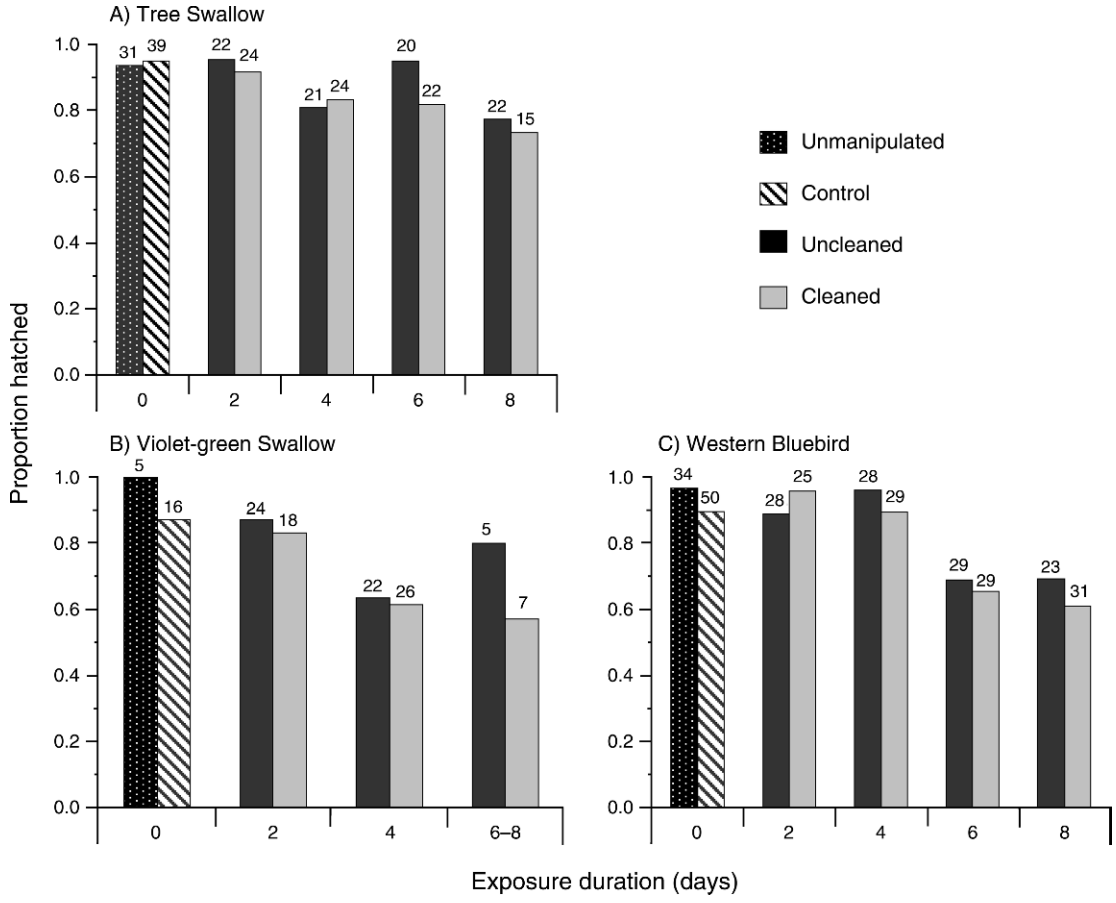

FIG. 3. Hatching success of eggs by species, exposure duration, and cleaning treatment. Two groups of eggs were not exposed or cleaned: unmanipulated eggs were left in natal nests, and control eggs were transported and returned to natal nests. Sample sizes are indicated above each bar.

groups that are commonly found in the digestive tracts of animals. Although gram-negative enterics of the Enterobacteriaceae are commonly found on eggs and nests (Bruce and Drysdale 1994, Berger et al. 2003, Peralta-Sanchez et al. 2010), we found only one genus on our eggs, *Pantoea*. Conspicuously missing from our eggs were genera common on eggshells (Cook et al. 2003), rotten eggs (Mayes and Takeballi 1983, Board and Tranter 1986), and nests (Singleton and Harper 1998, Berger et al. 2003): *Pseudomonas*, *Streptococcus*, *Alcaligenes*, and most Enterobacteriaceae, including *Escherichia*, *Proteus*, *Serratia*, and *Salmonella*.

We found microbes on eggshells that have not been documented in previous studies and that appear to have originated from several sources. Thirty of the 33 genera that we identified were not previously reported from tropical eggs using culture-based identification (Cook et al. 2003). Seventy-six percent (25/33) of the genera of our California eggs were from orders favored by dry conditions, Actinomycetales and Bacillales (Dubinsky 2008). Two genera found on our California eggshells (*Pantoea* and *Sphingobacterium*) belong to families strongly associated with the microflora of unincubated eggs in the tropics, Enterobacteriaceae and Sphingobacteriaceae (Shawkey et al. 2009), although neither genus was common. We found three nonfermenter genera (*Chryseobacterium*, *Psychrobacter*, and *Sphingobacterium*) that have not been previously isolated from eggs, nests, or feathers but are known from plant, soil, or water isolates (Bergey and Holt 1994, Tai et al. 2006,

TABLE 4. Stepwise logistic regression of factors affecting hatching success for 596 eggs.

Parameter	df	Estimate	SE	χ^2	$P <$
Intercept	1	2.85	0.32	118.7	0.001
Species: TRES	1	0.53	0.27	3.9	0.049
Species: VGSW	1	−0.66	0.28	5.4	0.021
Exposure	1	−0.22	0.05	22.7	0.001
Egg mass	1	−0.30	0.17	2.9	0.090
Egg mass loss	1	−0.06	0.04	2.7	0.102

Notes: Nonsignificant effects that did not enter into the final model included transport time, cleaning, average temperature, proportion of time >24°C, box age, percentage egg mass lost, standardized egg mass, and interactions of species with exposure duration and cleaning. Species are abbreviated as Tree Swallow (TRES) and Violet-green Swallow (VGSW). Western Bluebird is the reference species.

Heylen et al. 2007). Their presence on eggs may represent colonization from air or rainfall. Six genera we detected on eggshells have been previously isolated from feathers (*Acinetobacter*, *Arthrobacter*, *Pantoea*, *Microbacterium*, *Agrobacterium* and *Stenotrophomonas*), which may be a source of mostly nonpathogenic bacteria (Shawkey et al. 2005, Whitaker et al. 2005, Bisson et al. 2007).

Implications for latitudinal trends in avian life-history traits

Stoleson and Beissinger (1995, 1999) proposed and demonstrated that tropical birds experience a high loss of viability if their eggs are left unincubated for extended periods during laying, and they hypothesized that this could select for the smaller clutch sizes and greater hatching asynchrony exhibited by tropical species. Hatching failure for several temperate passerines examined by Cooper et al. (2005) increased with declining latitude, supporting this hypothesis. However, microbial processes that act independently of temperature also erode egg viability and may be linked with moisture availability on the eggshell, complicating simple predictions of viability declines with latitude (Cook et al. 2003, 2005*b*, Beissinger et al. 2005).

Our results from eggs exposed in cavities to a low-risk temperate environment support the underlying premise of the egg viability hypothesis for latitudinal trends in avian life-history traits. Hatchability of the eggs of three passerines declined slowly in a temperate mediterranean climate with exposure to ambient temperatures that rarely exceeded physiological zero and to low moisture conditions (Fig. 3; Appendix B). The daily loss of egg viability in this study (−2.5% per day from 0 to 8 days) was similar to the slow rate of decline in waterfowl eggs (−1.4%) exposed to high-temperate zone conditions, but was much lower than the loss of viability for two tropical species (−4.6% and −14.6%) and a passerine inhabiting a humid mediterranean climate (−4.6%) (summarized in Beissinger et al. 2005). In our dry environment, egg mass loss occurred during the exposure period. This may be another mechanism contributing to hatching failure because hatchability suffers if water balance in the albumen is not maintained (Deeming 1991, 2002). In comparison, egg mass loss was negligible for eggs exposed to moist tropical environments (Cook et al. 2003, 2005*b*).

Our results provide a glimpse of how strongly the factors affecting egg viability itself can differ between temperate and tropical ecosystems, but we caution that temperate environments and avian nesting strategies are extremely variable. Most studies, including ours, have exposed eggs in nest boxes. Unattended eggs in open-cup nests may experience warmer temperatures if exposed to direct solar radiation and may be more susceptible to wetting from rain. Although both factors may frequently be more intense in tropical than temperate ecosystems, there will be many exceptions. For example, when chicken eggs were exposed in open-cup nests to warm, temperate conditions and simulated daily rainfall for 3–5 days, microbial growth rivaled tropical ecosystems with eggshell microbial loads increasing by 10^1–10^2 CFU/egg and high infection rates of albumen and yolk, trends that were not exhibited by eggs exposed in nest boxes (Godard et al. 2007). How differences in bacterial growth between open and cavity nests affect egg viability may depend on whether the proportion of pathogenic microbes differs between nest types, the duration of preincubation exposure, and the bacteriostatic effects of partial incubation during the laying period (Cook et al. 2005*a*, Shawkey et al. 2009, Wang and Beissinger 2009). Natural cavities can be more insulated than nest boxes (Stoleson and Beissinger 1999) and possibly more humid, which could lead to a more favorable environment for bacteria.

Delaying the onset of incubation for 3–5 days until the penultimate or last egg of a clutch is laid may maintain hatching synchrony without a large trade-off in egg viability at our study site, unlike eggs exposed to warm, moist, tropical conditions (Stoleson and Beissinger 1999, Beissinger et al. 2005). This may explain why the onset of partial incubation exhibited such great intraspecific variability in our study species and why the timing of incubation onset may be influenced more by energetic constraints on adults than by environmental constraints on egg viability (Wang and Beissinger 2009). Less selective pressure on egg viability at our temperate site allows for flexibility in the onset of incubation that may not be possible in locations with stronger selective pressure from microbial or temperature mechanisms of egg viability decline (Grenier and Beissinger 1999, Stoleson and Beissinger 1999, Cook et al. 2005*b*).

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Literature Cited

Arnold, T. W. 1993. Factors affecting egg viability and incubation time in prairie dabbling ducks. Canadian Journal of Zoology 71:1146–1152.

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

Beissinger, S. R., M. I. Cook, and W. J. Arendt. 2005. The shelf life of bird eggs: testing egg viability using a tropical climate gradient. Ecology 86:2164–2175.

Berger, S., R. Disko, and H. Gwinner. 2003. Bacteria in starling nests. Journal fur Ornithologie 144:317–322.

Bergey, D. H., and J. G. Holt. 1994. Bergey's manual of determinative bacteriology. Ninth edition. Lippincott Williams and Wilkins, Baltimore, Maryland, USA.

Bisson, I. A., P. P. Marra, E. H. Burtt, M. Sikaroodi, and P. M. Gillevet. 2007. A molecular comparison of plumage and soil bacteria across biogeographic, ecological, and taxonomic scales. Microbial Ecology 54:65–81.

Board, R. G., S. Loseby, and V. R. Miles. 1979. A note on microbial growth on hen egg-shells. British Poultry Science 20:413–420.

Board, R. G., and H. S. Tranter. 1986. The microbiology of eggs. Pages 75–97 *in* W. J. Stadelman and O. J. Cotterill, editors. Egg science and technology. AVI, Westport, Connecticut, USA.

Bruce, J., and E. M. Drysdale. 1994. Trans-shell transmission. Pages 63–91 *in* R. G. Board and R. Fuller, editors. The microbiology of the avian egg. Chapman and Hall, London, UK.

Burtt, E. H., and J. M. Ichida. 1999. Occurrence of feather-degrading *Bacilli* in the plumage of birds. Auk 116:364–372.

Cook, M. I., S. R. Beissinger, G. A. Toranzos, and W. J. Arendt. 2005*a*. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. Ecology Letters 8:532–537.

Cook, M. I., S. R. Beissinger, G. A. Toranzos, R. A. Rodriguez, and W. J. Arendt. 2003. Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation? Proceedings of the Royal Society B 270:2233–2240.

Cook, M. I., S. R. Beissinger, G. A. Toranzos, R. A. Rodriguez, and W. J. Arendt. 2005*b*. Microbial infection affects egg viability and incubation behavior in a tropical passerine. Behavioral Ecology 16:30–36.

Cooper, C. B., W. M. Hochachka, G. Butcher, and A. A. Dhondt. 2005. Seasonal and latitudinal trends in clutch size: thermal constraints during laying and incubation. Ecology 86:2018–2031.

Deeming, D. C. 1991. Reasons for the dichotomy in egg turning among birds and reptiles. Pages 307–324 *in* D. C. Deeming and M. W. J. Ferguson, editors. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge, UK.

Deeming, D. C. 2002. Embryonic development and utilisation of egg components. Pages 43–53 *in* D. C. Deeming, editor. Avian incubation: behaviour, environment, and evolution. Oxford University Press, New York, New York, USA.

Dubinsky, E. A. 2008. Environmental controls on microbial community structure and iron redox dynamics in upland soils. University of California, Berkeley, California, USA.

Fasenko, G. M. 2007. Egg storage and the embryo. Poultry Science 86:1020–1024.

Fasenko, G. M., F. E. Robinson, A. O. Whelan, K. M. Kremeniuk, and J. A. Walker. 2001. Prestorage incubation of long-term stored broiler breeder eggs: 1. effects on hatchability. Poultry Science 80:1406–1411.

Ghalambor, C. K., and T. E. Martin. 2001. Fecundity-survival trade-offs and parental risk-taking in birds. Science 292:494–497.

Godard, R. D., M. Wilson, J. W. Frick, P. B. Siegel, and B. B. Bowers. 2007. The effects of exposure and microbes on hatchability of eggs in open-cup and cavity nests. Journal of Avian Biology 38:709–716.

Grenier, J. L., and S. R. Beissinger. 1999. Variation in the onset of incubation in a Neotropical parrot. Condor 101:752–761.

Heylen, K., B. Vanparys, F. Peirsegaele, L. Lebbe, and P. De Vos. 2007. *Stenotrophomonas terrae* sp. nov. and *Stenotrophomonas humi* sp. nov., two nitrate-reducing bacteria isolated from soil. International Journal of Systematic and Evolutionary Microbiology 57:2056–2061.

Martin, T. E., P. R. Martin, C. R. Olson, B. J. Heidinger, and J. J. Fontaine. 2000. Parental care and clutch sizes in North and South American birds. Science 287:1482–1485.

Mayes, F. J., and M. A. Takeballi. 1983. Microbial contamination of the hen's egg: a review. Journal of Food Protection 46:1091–1098.

Meijerhof, R. 1992. Preincubation holding of hatching eggs. World's Poultry Science Journal 48:57–68.

Monaghan, P., and R. G. Nager. 1997. Why don't birds lay more eggs? Trends in Ecology and Evolution 12:270–274.

Niemela, S. I. 2003. Measurement uncertainty of microbiological viable counts. Accreditation and Quality Assurance 8:559–563.

Peralta-Sanchez, J. M., A. P. Møller, A. M. Martin-Platero, and J. J. Soler. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. Functional Ecology 24:426–433.

Ricklefs, R. E. 2000. Lack, Skutch, and Moreau: the early development of life-history thinking. Condor 102:3–8.

Ricklefs, R. E., and M. Wikelski. 2002. The physiology/life-history nexus. Trends in Ecology and Evolution 17:462–468.

SAS Institute. 2006. Version 9.1.3 of the SAS System for Windows. SAS Institute, Cary, North Carolina, USA.

SAS Institute. 2008. Version 9.2 of the SAS System for Windows. SAS Institute, Cary, North Carolina, USA.

Shawkey, M. D., M. K. Firestone, E. L. Brodie, and S. R. Beissinger. 2009. Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. PLoS ONE 4:e4522.

Shawkey, M. D., K. L. Mills, C. Dale, and G. E. Hill. 2005. Microbial diversity of wild bird feathers revealed through culture-based and culture-independent techniques. Microbial Ecology 50:40–47.

Singleton, D. R., and R. G. Harper. 1998. Bacteria in old house wren nests. Journal of Field Ornithology 69:71–74.

Stoleson, S. H., and S. R. Beissinger. 1995. Hatching asynchrony and the onset of incubation in birds, revisited: when is the critical period? Pages 191–270 *in* D. M. Power, editor. Current Ornithology. Plenum Press, New York, New York, USA.

Stoleson, S. H., and S. R. Beissinger. 1999. Egg viability as a constraint on hatching synchrony at high ambient temperatures. Journal of Animal Ecology 68:951–962.

Tai, C. J., H. P. Kuo, F. L. Lee, H. K. Chen, A. Yokota, and C. C. Lo. 2006. *Chryseobacterium taiwanense* sp. nov., isolated from soil in Taiwan. International Journal of Systematic and Evolutionary Microbiology 56:1771–1776.

Veiga, J. P. 1992. Hatching asynchrony in the house sparrow: a test of the egg-viability hypothesis. American Naturalist 139:669–675.

Wang, J. M., and S. R. Beissinger. 2009. Variation in the onset of incubation and its influence on avian hatching success and asynchrony. Animal Behaviour 78:601–613.

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

Whitaker, J. M., D. A. Cristol, and M. H. Forsyth. 2005. Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage. Journal of Field Ornithology 76:264–270.

APPENDIX A

Identification of bacterial isolates from eggshell swabs, albumen, and yolk (*Ecological Archives* E092-092-A1).

APPENDIX B

Quantification and analyses of infection rate of experimental eggs (*Ecological Archives* E092-092-A2).