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Forum

Do birds differentially distribute antimicrobial proteins within clutches of eggs?

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Parents can considerably affect the fitness of their offspring through the additive influences of inherited traits and parental effects (Rossiter 1996; Mousseau and Fox 1998). These latter, nongenetic factors may be adaptive phenotypic responses to environmental variation that are invested in the egg at some cost and can increase offspring growth and survival (Schwabl 1993, 1996; Schwabl et al. 1997; Mousseau and Fox 1998; Eising et al. 2001). Among their many functions, eggs must protect embryos from microbial infection because marine and terrestrial environments harbor microbes capable of infecting embryos. Eggs of a variety of animal taxa contain antimicrobial agents (Brogden 2005), but antimicrobial defenses in eggs of wild animals remain poorly understood despite the obvious significance of this function (Giacomello et al. 2006).

Avian eggs contain a network of defenses against microbial infection. The shell provides physical protection that is complemented by the strong antimicrobial effects (through unknown mechanisms) of incubation (Cook et al. 2005a). Microbes multiply rapidly on the shells of unincubated eggs, such as those laid early in the sequence before the onset of incubation, and can penetrate to the embryo and cause hatching failure (Cook et al. 2003, 2005b). Further, chicken eggs contain at least 6 antimicrobial proteins in the albumen (Board and Fuller 1974, 1994). One of these antimicrobials, ovotransferrin, chelates Fe^{3+}, Cu^{2+}, and Zn^{2+}, necessary for growth of most microbes, and may also have active antimicrobial properties (Valenti et al. 1983, 1985; Ibrahim 1996). A second antimicrobial, avidin, binds biotin, a vitamin necessary for growth of most microbes (Board and Fuller 1974), and is presumed to slow the growth of a broad spectrum of bacteria. A third antimicrobial, lysozyme, catalyzes the lysis of gram-positive bacteria (Rogers and Perkins 1968) by hydrolyzing the peptidoglycan in their cell walls. Activity of these antimicrobial proteins is higher at incubation temperatures (34–36 °C) than at ambient temperatures (Tranter and Board 1984) and increases with protein concentration (Salton 1957; Yamanishi et al. 2002).

Birds could, therefore, increase protection of eggs through deposition of higher concentrations of antimicrobial proteins. In addition, indirect evidence suggests that effects of antimicrobials like lysozyme may be carried over from albumen into the nestling for a short period of time after hatching (Saino et al. 2002). Because earlier-laid eggs are at greater risk for infection because of the rapid growth of microbes on unincubated eggs (Cook et al. 2005a), earlier-laid eggs should have higher levels of these proteins than later-laid eggs. However, this should only be true if production or deposition of these proteins is costly in some way, which was suggested for lysozyme (Saino et al. 2002, 2007). A decline in the concentration of lysozyme with laying order in the eggs of European barn swallows (*Hirundo rustica*) (Saino et al. 2002) supports this prediction. Whether antimicrobials are allocated in a similar manner in the eggs of other wild birds is unknown.

Our goal in this study was to test whether birds nonrandomly allocated antimicrobial proteins to eggs within clutches. We analyzed 444 eggs from 97 complete clutches of known laying sequence for 8 species from 6 families of birds (Table 1). These species inhabit environments ranging from temperate wetlands and grasslands to tropical forests and exhibit preincubation delays ranging from 0 (green-rumped parrotlet, *Forpus passerinus*) to 8 days (blue-winged teal, *Anas discors*). We predicted that antimicrobial concentration would decline with laying order in all species except the parrotlet. Our null hypothesis was that antimicrobials would be evenly or randomly distributed across all eggs in clutches of each species.

MATERIALS AND METHODS

Field methods

Study species were chosen to represent a range of preincubation exposure periods (i.e., the number of days the first-laid egg sits in the nest prior to incubation) in birds (see Table 1) and for logistical reasons. Exposure periods ranged from 0 to 8 days, although most were between 1 and 3 days. Infection can occur within 1–3 days in unincubated eggs (Cook et al. 2003), so these exposure periods were sufficiently long to test our hypothesis. Logistically, we needed to find nests before egg laying began and to collect eggs daily. Thus, we chose species that nested conspicuously (primarily in nest boxes), were not sensitive to disturbance and were already under long-term study. We collected complete clutches of eggs from 8 species (see Table 1 and references therein for more details): pearly-eyed thrashers *Margarops fuscatus* in a tropical montane forest (El Yunque National Forest; 18°19′N, 65°47′W) in Puerto Rico; green-rumped parrotlets in tropical savannahs of Venezuela (8°34′N, 67°35′W); western bluebirds *Sialia mexicana* and tree and violet-green swallows *Tachycineta bicolor* and *Tachycineta thalassina*, respectively, in temperate grasslands in northern coastal California (Hopland Field Station; 38°99′N, 123°07′W); eastern bluebirds *Sialia sialis* in subtropical grasslands in central Alabama (32°35′N, 82°28′W); red-winged blackbirds *Agelaius phoeniceus* in temperate marshes in Kansas (Konza Prairie Biological Station; 39°05′N, 96°35′W); and blue-winged teal in temperate marshes in North Dakota (48°41′N, 99°15′W). Details of study sites and species are listed in Table 1. Eggs were collected in May and June 2006, except for parrotlets (July–November 2005). Nests were checked between 0900 and 1300 every 1–3 days until nest cups were fully formed, at which point we checked them daily. We removed eggs on the day

Table 1

Taxonomy and reproductive characteristics of the bird species used in this study whose clutches were sampled and tested for differential deposition by laying order of the antimicrobials ovotransferrin, avidin, and lysozyme

Species	Habitat type	Location	Nest	Family	Clutch size	Incubation onset	Exposure
Pearly-eyed thrasher	Tropical, terrestrial	PR, USA	Cavity	*Sturnidae*	3 (2–4)	Penultimate	1 day
Tree swallow	Temperate, terrestrial	CA, USA	Cavity	*Hirundidae*	4 (3–7)	Penultimate	2 days
Violet-green swallow	Temperate, terrestrial	CA, USA	Cavity	*Hirundidae*	4 (3–7)	Penultimate	2 days
Red-winged blackbird	Temperate, terrestrial	KS, USA	Cup	*Icteridae*	3 (2–4)	Penultimate	1 day
Eastern bluebird	Subtropical, terrestrial	AL, USA	Cavity	*Turdidae*	4 (3–7)	Penultimate	2 days
Western bluebird	Temperate, terrestrial	CA, USA	Cavity	*Turdidae*	5 (3–7)	Penultimate	3 days
Green-rumped parrotlet	Tropical, terrestrial	Venezuela	Cavity	*Psittacidae*	7 (5–12)	First	0 days
Blue-winged teal	Temperate, aquatic	ND, USA	Floating	*Anatidae*	9 (8–10)	Last	8 days

All data (except location of sampling) are from Birds of North America account (http://bna.birds.cornell.edu/BNA) for each species, except the green-rumped parrotlet (Grenier and Beissinger 1999). Common names, typical breeding habitat, location of sampling for this study, nest type, family, mean clutch size, incubation onset, and exposure are listed. Incubation onset refers to the egg upon which incubation is initiated. Exposure is the number of days the first-laid egg sits in the nest prior to the onset of full incubation.

they were laid and replaced them with painted clay dummy eggs to facilitate normal laying behavior. Collected eggs were then cracked open in a clean manner using ethanol and flame-sterilized forceps, yolk was separated from albumen using sterile pipettes, and albumen was stored at −20 °C.

We collected 13 clutches (41 eggs; clutch size range 2–4, mean 3.19 ± 0.09 standard error) from pearly-eyed thrashers, 10 clutches (44 eggs; range 2–5, mean 4.43 ± 1.13) from tree swallows, 10 clutches (41 eggs; range 3–5, mean 4.17 ± 0.08) from violet-green swallows, 15 clutches (43 eggs; clutch size range 2–5, mean 3.23 ± 0.15) from red-winged blackbirds, 18 clutches (69 eggs; range 2–5, mean 4.09 ± 0.09) from eastern bluebirds, 10 clutches (44 eggs; range 3–5, mean 4.60 ± 0.10) from western bluebirds, 11 clutches (79 eggs; range 5–10, mean 7.61 ± 0.20) from green-rumped parrotlets, and 10 clutches from blue-winged teal (83 eggs; range 2–12, mean 9.72 ± 0.26).

Protein assays

We measured concentrations of ovotransferrin, lysozyme, and avidin because they are the most abundant and well-characterized antimicrobials in egg albumen. We used the total iron-binding capacity assay of Yamanishi et al. (2002) to measure concentration of ovotransferrin in each egg. This assay measures the amount of iron necessary to saturate ovotransferrin in the sample and thus correlates well with ovotransferrin concentration (Yamanishi et al. 2002). We added 125 µl of a 1:500 dilution of an iron-standard solution (1000 mg/ml; Sigma, St Louis, MO) in buffer (pH 8.4) containing 300 mmol/l Tris (Fisher, Pittsburgh, PA), 150 mmol/l sodium hydrogen carbonate (EMD, Darmstadt, Germany), and 4.2 g/l Triton X-100 (Sigma) to 24 µl of each albumen sample in wells of a 96-well plate (Nunc MaxiSorp, Rochester, NY). After 5 min of incubation at 37 °C, we added 25 µl of a second reagent (pH 4.0) containing 10 mmol/l ferrozine (Baker, Phillipsburg, NJ) and 32.6 mmol/l L-ascorbic acid (Acros, Geel, Belgium) in 50 mmol/l Tris buffer to each well and (Yamanishi et al. 2002) incubated plates at 37 °C for 5 min before adding 100 µl of a third reagent containing 600 mmol/l citric acid (Baker) and 25.6 mmol/l thiourea (Baker). We then measured absorbance (color change) every 20 s at 570 and 660 nm for 6.2 min using a Versamax microplate reader (Molecular Devices, Sunnyvale CA). To calculate ovotransferrin concentration, we determined the difference in absorbance at 570/660 nm at the beginning and end of the 6.2-min period. We then determined absolute ovotransferrin concentration by comparing these values with those in a standard curve.

To determine avidin concentration, we used a modified version of the colorimetric method of Gan and Marquardt (1999). We diluted each albumen sample 4-fold in carbonate–bicarbonate buffer (Sigma) and added serial dilutions (100 µl) to columns 1 through 11 of each row. The bottom row contained a serial dilution of avidin (Sigma, 5–0.002 µg/ml) in carbonate–bicarbonate buffer. We incubated these plates at 4 °C overnight and then rinsed them 3 times with phosphate-buffered saline (PBS)/0.05% Tween-20 (Sigma). Nonspecific binding was prevented by adding Superblock buffer (Pierce, Rockford, IL) and incubating for 30 s at room temperature 3 times. We then added 100 µl of a 1:4000 dilution of biotin/horseradish peroxidase (Sigma) in Superblock/0.05% Tween-20 to each well and incubated plates for 25 min at room temperature. Finally, we washed the plates 5 times with PBS/0.05% Tween-20 and added 100 µl of blue peroxidase (POD) substrate (Roche, Basel, Switzerland) to each well before incubating the plates at room temperature for 30 min. We measured absorbance at 450 nm with a Versamax microplate reader. Concentration of avidin in each sample was calculated by comparison of absorbance values to those in the standard curve.

To measure lysozyme concentration, we used a version of the lysoplate method of Osserman and Lawlor (1966) modified for 96-well plates (Millet et al. 2007). We added 25 mg dried *Micrococcus lysodeikticus* (Sigma) to 50 ml 1% agar (Difco, Detroit, MI) and kept the suspension at a temperature of 50–60 °C. Then 150 µl of this suspension was added to each 10-µl albumen sample in a well of a 96-well plate. We obtained a standard curve by adding the bacterial suspension to serial dilutions of a standard lysozyme solution. Plates were incubated overnight at room temperature and absorbance was measured with a Versamax microplate reader at 850 nm. Concentration of lysozyme in each sample was calculated by comparison of absorbance values to those in the standard curve.

All assays were run in duplicate by an observer blind to the identity of the sample being tested and were highly repeatable (sensu Lessels and Boag [1987] using values taken from the duplicate measurements; repeatability = 0.86 for ovotransferrin, 0.89 for avidin, and 0.79 for lysozyme).

Statistical analyses

All statistical tests were performed in SPSS 13. Only avidin data required log transformation to obtain normality.

Concentrations of the 3 antimicrobials were not strongly correlated with one another in any of the species (Pearson correlation coefficient; all $P > 0.09$), so we ran separate analyses for each antimicrobial. To test for covariation between laying order and antimicrobial concentration, we used a separate analysis of covariance (ANCOVA) (general linear model univariate function in SPSS) for each species with ovotransferrin (milligrams per milliliters), lysozyme (micrograms per milliliters), or avidin (log scale from data measured in micrograms per milliliters) concentration as the dependent variable, nest of origin as a random factor, and clutch size and egg-laying order as covariates. We included clutch size in models because we predicted that differential distribution would be stronger in larger clutches, as first-laid eggs in larger clutches are exposed for longer periods of time than in smaller clutches. The models were first run with the interaction term clutch size × laying order included. In cases where this interaction term was not significant ($\alpha = 0.05$), the models were then run again without this interaction term. Because of the large number of comparisons made (24), a sequential Bonferroni correction could be applied to P values, which would lower the threshold for significance to $\alpha = 0.002$. However, use of Bonferroni correction in such a manner may be overly conservative (Nakagawa 2004; Garamszegi 2006), especially in cases with small sample sizes such as our study. We present results with and without Bonferroni correction (Tables 2–4) to allow readers to make their own decisions about the significance of the tests. Most of our results were not significant with or without correction.

Ethical considerations

All research was carried out with approval of the Animal Ethics Committee of the University of California at Berkeley (protocol No. R233:1004–1008); under collecting permits from the US Fish and Wildlife Service; California and North Dakota Game and Fish Departments; Alabama Department of Conservation and Natural Resources; Kansas Department of Wildlife and Parks; and in cooperation with the Instituto Venezolano de Investigaciones Científicas. Although we collected complete clutches of eggs, we minimized impact on the populations by removing all dummy eggs immediately after clutch completion. In almost all cases, the birds nested again. Furthermore, we do not expect that the collection of these eggs will have a large effect on the population dynamics of these common species. Any temporary effect on population dynamics from an increase in mortality of eggs due to clutch collection is more than offset by the increased number of nesting females producing offspring due to the large number of nest boxes constructed and placed in the wild.

RESULTS

Antimicrobial enzymes varied little by laying order (Tables 2–4, Figures 1–3). Of 24 comparisons, only 5 patterns of differential antimicrobial distribution among eggs were statistically significant before Bonferroni correction and only 2 remained significant after Bonferroni correction (Tables 2–4). Ovotransferrin significantly declined with laying order in pearly-eyed thrashers ($\beta = -0.01$; Table 2, Figure 1a) and the interaction term between laying order and clutch size for ovotransferrin in pearly-eyed thrashers was significant ($\beta = 0.01$;Table 2), suggesting that this pattern was stronger in smaller clutches. However, no significant patterns were detected when data from each clutch size were analyzed separately (ANCOVA; all $P > 0.13$). Consistent with the predictions, ovotransferrin significantly declined with laying order in violet-green swallows at the $\alpha = 0.05$ level ($\beta = -0.04$; Table 2, Figure 1c), but this pattern did not remain significant after Bonferroni correction. Contrary to predictions, avidin levels significantly increased with laying order in mountain bluebirds, violet-green swallows, and tree swallows ($\beta = 0.14$, 0.18, and 0.14, respectively; Table 3, Figure 2b,c,f), and the latter 2 patterns remained significant after Bonferroni correction. All other patterns for clutch size and laying order, and their interaction, were nonsignificant (Tables 2–4, Figures 1–3).

Variation in antimicrobial protein concentration among females was the most frequent significant effect before Bonferroni correction (8 of 24 cases; Tables 2–4). Ovotransferrin varied significantly among nests in parrotlets, eastern bluebirds, and tree swallows (Table 2). Avidin significantly varied among nests in tree swallows, violet-green swallows, and blue-winged teal (Table 3). Lysozyme varied significantly among nests in tree swallows and violet-green swallows (Table 4). However, only variation in avidin among teal remained significant after Bonferroni correction (Table 3). Removal of outliers did not substantially alter any of these results.

As an additional test for effects of laying order, we recoded eggs within a clutch as first laid, intermediate, and last laid and ran an analysis of variance with nest as a random factor and laying position as a fixed effects factor. This analysis largely confirmed the results of our previous analyses; only increasing avidin deposition in tree swallow eggs and decreasing

Table 2

Overall means and 95% confidence intervals (CIs) of means and ANCOVA of concentration of the antimicrobial enzyme ovotransferrin in the egg albumen of 8 bird species by clutch size (CS), laying order (LO), and nest of origin (Nest ID)

	Mean (95% CI)	LO			CS			CS × LO			Nest ID		
Species	mg/ml	df	F	P	df	F	P	df	F	P	df	F	P
Pearly-eyed thrasher	16.4 (15.2–17.5)	1,25	8.17	**≤0.01**	1,25	1.00	≤0.33	1,25	7.38	**≤0.01**	12,25	1.12	≤0.45
Tree swallow	13.8 (12.6–15.0)	1,32	0.74	≤0.40	1,32	3.40	≤0.08	1,32	2.17	≤0.15	9,31	2.14	**≤0.05**
Violet-green swallow	13.9 (12.4–15.3)	1,29	4.56	**≤0.04**	1,29	2.49	≤0.13	1,29	1.42	≤0.24	9,28	1.83	≤0.11
Red-winged blackbird	13.6 (12.4–14.7)	1,26	0.20	≤0.66	1,26	0.23	≤0.69	1,26	1.48	≤0.24	14,25	0.88	≤0.58
Eastern bluebird	15.4 (14.0–16.8)	1,49	2.23	≤0.14	1,49	0.60	≤0.44	1,49	0.21	≤0.65	17,48	1.82	**≤0.05**
Western bluebird	15.5 (14.3–16.7)	1,32	0.61	≤0.44	1,32	1.89	≤0.18	1,32	0.01	≤0.98	9,31	1.07	≤0.41
Green-rumped parrotlet	28.4 (26.3–30.6)	1,66	1.17	≤0.28	1,66	0.15	≤0.74	1,66	0.15	≤0.70	10,66	2.61	**≤0.01**
Blue-winged teal	22.6 (21.1–24.0)	1,71	0.07	≤0.80	1,71	0.05	≤0.82	1,71	0.06	≤0.80	9,70	1.70	≤0.10

The models were first run with the interaction term clutch size (CS) × LO included. In cases where this interaction term was not significant, the models were then run again without this interaction term. F and P values for CS and LO are presented from these latter models unless the interaction term was significant ($\alpha = 0.05$). F and P values for nest ID are presented from the original models. Significant ($\alpha = 0.05$) P values are listed in bold. If a sequential Bonferroni correction was applied, the threshold of significance for all analyses would be $\alpha = 0.002$. df, degrees of freedom.

Table 3

Overall means and 95% confidence intervals (CIs) of means and ANCOVA of concentration of the antimicrobial enzyme ovotransferrin in the egg albumen of 8 bird species by clutch size (CS), laying order (LO) and nest of origin (Nest ID)

	Mean (95% CI)	LO			CS			CS × LO			Nest ID		
Species	μg/ml	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Pearly-eyed thrasher	0.02 (0.01–0.03)	1,25	0.39	≤0.54	1,25	1.44	≤0.24	1,25	0.22	≤0.64	12,25	1.23	≤0.31
Tree swallow	0.33 (0.26–0.40)	1,32	14.88	**≤0.01***	1,32	0.39	≤0.54	1,32	0.10	≤0.76	9,31	2.53	**≤0.03**
Violet-green swallow	0.30 (0.24–0.37)	1,29	11.75	**≤0.01***	1,29	1.31	≤0.26	1,29	2.32	≤0.08	9,28	7.27	**≤0.01**
Red-winged blackbird	0.14 (0.12–0.15)	1,26	1.89	≤0.18	1,26	0.50	≤0.62	1,26	1.00	≤0.33	14,25	0.82	≤0.64
Eastern bluebird	0.22 (0.08–0.36)	1,49	0.76	≤0.39	1,49	0.13	≤0.72	1,49	0.01	≤0.98	17,48	1.39	≤0.19
Western bluebird	0.19 (0.09–0.29)	1,32	8.23	**≤0.01**	1,32	0.01	≤0.90	1,32	0.85	≤0.36	9,31	0.85	≤0.36
Green-rumped parrotlet	0.02 (0.00–0.05)	1,66	0.44	≤0.51	1,66	0.85	≤0.57	1,66	0.03	≤0.87	10,66	0.83	≤0.60
Blue-winged teal	0.88 (0.67–1.10)	1,71	0.52	≤0.47	1,71	0.02	≤0.88	1,71	0.29	≤0.59	9,70	5.27	**≤0.01***

The models were first run with the interaction term clutch size (CS) × LO included. In cases where this interaction term was not significant, the models were then run again without this interaction term. *F* and *P* values for CS and LO are presented from these latter models unless the interaction term was significant ($\alpha = 0.05$). *F* and *P* values for nest ID are presented from the original models. Significant *P* values are listed in bold. If a sequential Bonferroni correction was applied, the threshold of significance for all analyses would be $\alpha = 0.002$. *P* values significant at this level are indicated with an asterisk (*). df, degrees of freedom.

ovotransferrin concentration in violet-green swallow eggs were significant ($P = 0.04$ and 0.02, respectively), but neither of these patterns remained significant after correction. Variation between clutches was only significant for avidin in blue-winged teal eggs after correction ($P < 0.01$), as found in our previous analyses.

DISCUSSION

Distribution of antibiotic enzymes in avian eggs

We present the first survey of multiple antimicrobial enzymes in the eggs of a fairly widely dispersed group of wild bird species, but our results provide little support for the hypothesis that female birds allocate higher concentrations of antimicrobials to earlier- than later-laid eggs. Indeed, after correction, the only significant patterns were in the opposite direction: the concentration of avidin increased with laying order in eggs of 2 swallow species. These findings suggest 2 nonexclusive hypotheses: 1) females favor last-hatched nestlings by providing them with more antimicrobial defense or 2) females favor first-hatched nestlings by providing higher biotin availability. Biotin, the vitamin bound by avidin, is also important in chick growth (Klasing 1998) and is assimilated by the nestling when it swallows the albumen the day before hatching (White et al. 1992). Low levels of avidin could lead to high levels of unbound biotin available to early-hatching nestlings. In any case, this pattern does not appear to be widespread but warrants further investigation. Other than these 2 cases, within-clutch concentration of antimicrobials appears to be fairly constant.

Although not supporting our initial hypothesis, our data suggest that some bird species may vary in antimicrobial distribution at the individual level. Only variation among teal remained significant after Bonferroni correction, but the relative frequency of a degree of variation in antimicrobial distribution among these species suggests some plasticity in antimicrobial deposition among clutches. Differences among females in the antibiotic concentrations of egg albumen could be caused by variations in environmental conditions, age, condition, or genetic factors (Saino et al. 2004). Indeed, some evidence suggests a link between temperature and lysozyme deposition (Saino et al. 2004). Saino et al. (2007) suggested that maternal favoritism in lysozyme deposition can occur because females preferentially deposit lysozyme in eggs sired by male barn swallows with longer tails (although the evidence is unpublished [Saino N, personal communication]).

Table 4

Overall means and 95% confidence intervals (CIs) of means and ANCOVA of concentration of the antimicrobial enzyme ovotransferrin in the egg albumen of 8 bird species by clutch size (CS), laying order (LO) and nest of origin (Nest ID)

	Mean (95% CI)	LO			CS			CS × LO			Nest ID		
Species	μg/ml	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Pearly-eyed thrasher	7.2 (4.5–10.0)	1,25	2.85	≤0.10	1,25	1.27	≤0.27	1,25	0.00	≤0.96	12,25	1.37	≤0.23
Tree swallow	3.2 (2.24–4.19)	1,32	0.58	≤0.45	1,32	0.58	≤0.45	1,32	0.10	≤0.76	9,31	2.53	**≤0.03**
Violet-green swallow	3.3 (2.4–4.2)	1,29	0.35	≤0.56	1,29	0.00	≤0.97	1,29	0.04	≤0.84	9,28	2.89	**≤0.02**
Red-winged blackbird	32.9 (26.3–39.5)	1,26	0.02	≤0.89	1,26	0.98	≤0.30	1,26	1.09	≤0.31	14,25	0.97	≤0.50
Eastern bluebird	36.1 (28.3–44.0)	1,49	1.04	≤0.31	1,49	0.24	≤0.62	1,49	0.01	≤0.98	17,48	1.39	≤0.19
Western bluebird	48.5 (32.1–64.9)	1,32	0.49	≤0.49	1,32	0.00	≤0.99	1,32	0.85	≤0.36	9,31	0.85	≤0.36
Green-rumped parrotlet	5910 (5460–6360)	1,66	0.05	≤0.83	1,66	0.35	≤0.76	1,66	3.00	≤0.09	10,66	1.63	≤0.12
Blue-winged teal	4115 (3610–4620)	1,71	0.57	≤0.32	1,71	0.17	≤0.68	1,71	0.17	≤0.68	9,70	0.02	≤0.88

The models were first run with the interaction term clutch size (CS) × LO included. In cases where this interaction term was not significant, the models were then run again without this interaction term. *F* and *P* values for CS and LO are presented from these latter models unless the interaction term was significant ($\alpha = 0.05$). *F* and *P* values for nest ID are presented from the original models. Significant *P* values are listed in bold. If a sequential Bonferroni correction was applied, the threshold of significance for all analyses would be $\alpha = 0.002$. df, degrees of freedom.

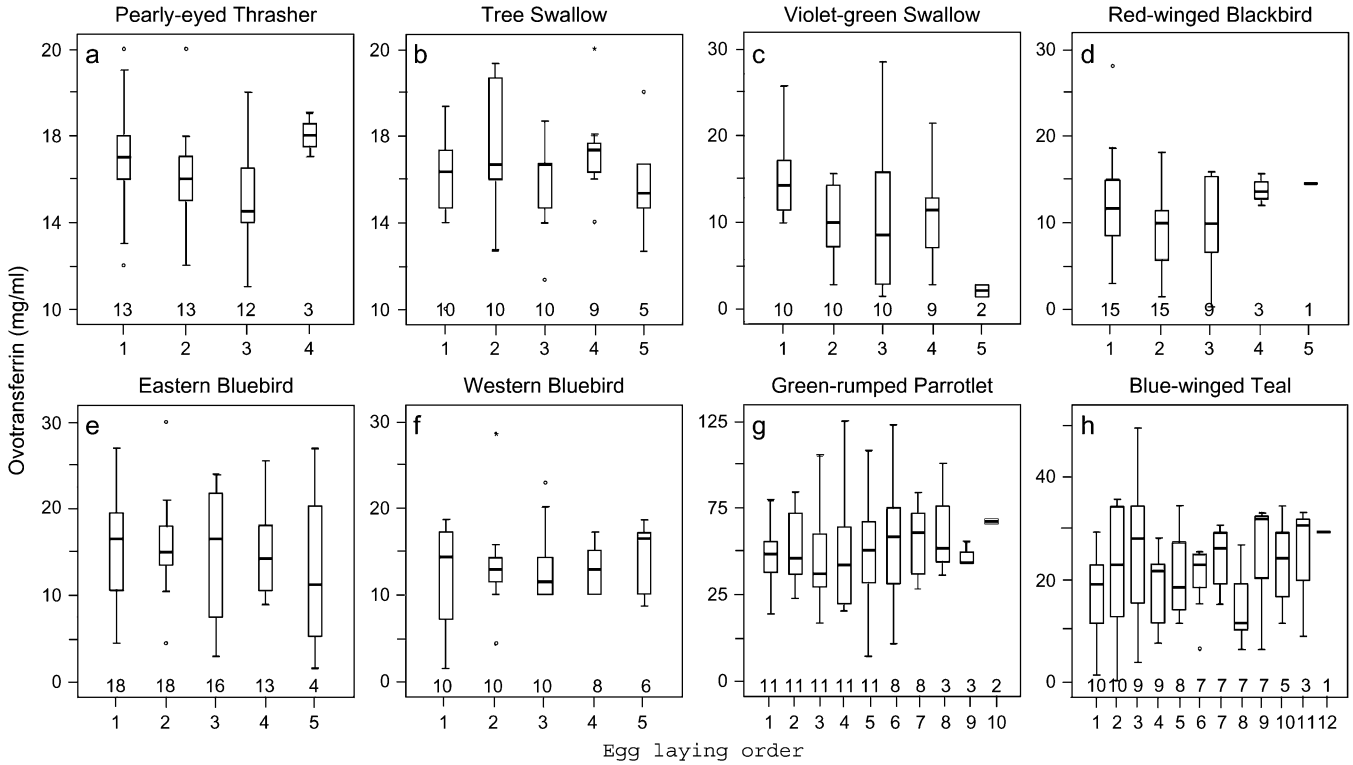

Figure 1
Boxplots of concentration of the antimicrobial enzyme ovotransferrin by laying order in eggs of 8 species of birds (labeled a–h). The line within each box represents the median concentration, the lower and upper borders of each box are the 25th and 75th percentiles, and the lower and upper bars are the 10th and 90th percentiles. Sample sizes are indicated at the bottom of each figure. Outliers are represented with circles.

Is deposition of antibiotics in avian albumen costly enough to warrant differential distribution?

Our results contrast with the only previous study of intraclutch patterns of lysozyme allocation in the albumen of wild birds (Saino et al. 2002) and with some studies of other maternally derived factors in egg yolks, such as androgens and vitamins (Sockman et al. 2006, but as a counterexample, Navara et al. [2006] found no pattern). These previously described patterns and the potentially high risk of infection for early-laid unincubated eggs (Cook et al. 2003, 2005a, 2005b) led to our prediction. However, the production cost of these antimicrobials in albumen appears to be low, and behavioral regulation of their function may make their differential distribution unnecessary.

Production and deposition of antimicrobials may be less costly than deposition of yolk factors. Steroids deposited in the yolk can entail immunosuppressive costs in chicks (Mooradian et al. 1987; Ketterson and Nolan 1999; Peters et al. 2000; Roberts et al. 2004), and antioxidants, such as vitamin E, may be energetically costly to deposit relative to other yolk components (Navara et al. 2006). However, the amount of energy needed to manufacture and deposit antimicrobial proteins is no greater than for other albumen components (calculations from Tristram 1953). Furthermore, a tradeoff between blood and egg lysozyme (and hence between embryo and adult immunity), as proposed by Saino et al. (2002, 2007), appears unlikely because egg and adult lysozyme are produced by different, specialized cells rather than originating from a common pool. Adult lysozyme is produced by mature macrophages, whereas egg lysozyme is produced by tubular gland cells in the oviduct (Mandeles and Ducay 1962; Shutz et al. 1978; Moen and Palmiter 1980).

Thus, it is not surprising that, to the best of our knowledge, no evidence exists for a relationship between blood and egg lysozyme levels. Prusinowska et al. (2000) and Saino et al. (2002) showed positive relationships between maternal and offspring plasma lysozyme levels but did not compare egg and adult plasma lysozyme levels. Similarly, only 1 paper suggests a positive relationship between plasma lysozyme and egg hatchability (Saino et al. 2002). Of the other papers examining plasma lysozyme and egg hatchability, 1 shows a negative relationship (Prusinowska and Jankowski 1996), 1 shows no relationship (Prusinowska et al. 2000), and 2 do not explicitly compare them (Melek 1977; Krykanov 1982). Thus, production of egg lysozyme does not appear to directly compromise adult immunity, and the cost of lysozyme deposition in albumen may be minimal.

Ovotransferrin expression in the blood and the oviduct is also regulated by different mechanisms (Lee et al. 1978, 1980; McKnight et al. 1980; Burley and Vadehra 1989; Xie et al. 2002). Ovotransferrin production is largely regulated by hormones in the oviduct (Lee et al. 1978), by iron deficiencies in the liver (McKnight et al. 1980), and by infection state in the blood (Xie et al. 2002). Avidin production is regulated by hormones in the goblet cells of the oviduct (Hertz et al. 1943; O'Malley 1967), but little is known of its regulation in the blood.

Energy expenditure and immunosuppression, therefore, do not appear to be the costs of antimicrobial deposition in eggs. Thus, birds may have little reason to adjust antimicrobial concentrations of eggs within a clutch, unless adding more antimicrobials decreases the amount of nutrients and protein in the albumen available to the developing embryo. Albumen supplies essential vitamins and nutrients to the embryo and chick, and its amino acid balance is optimized for embryonic

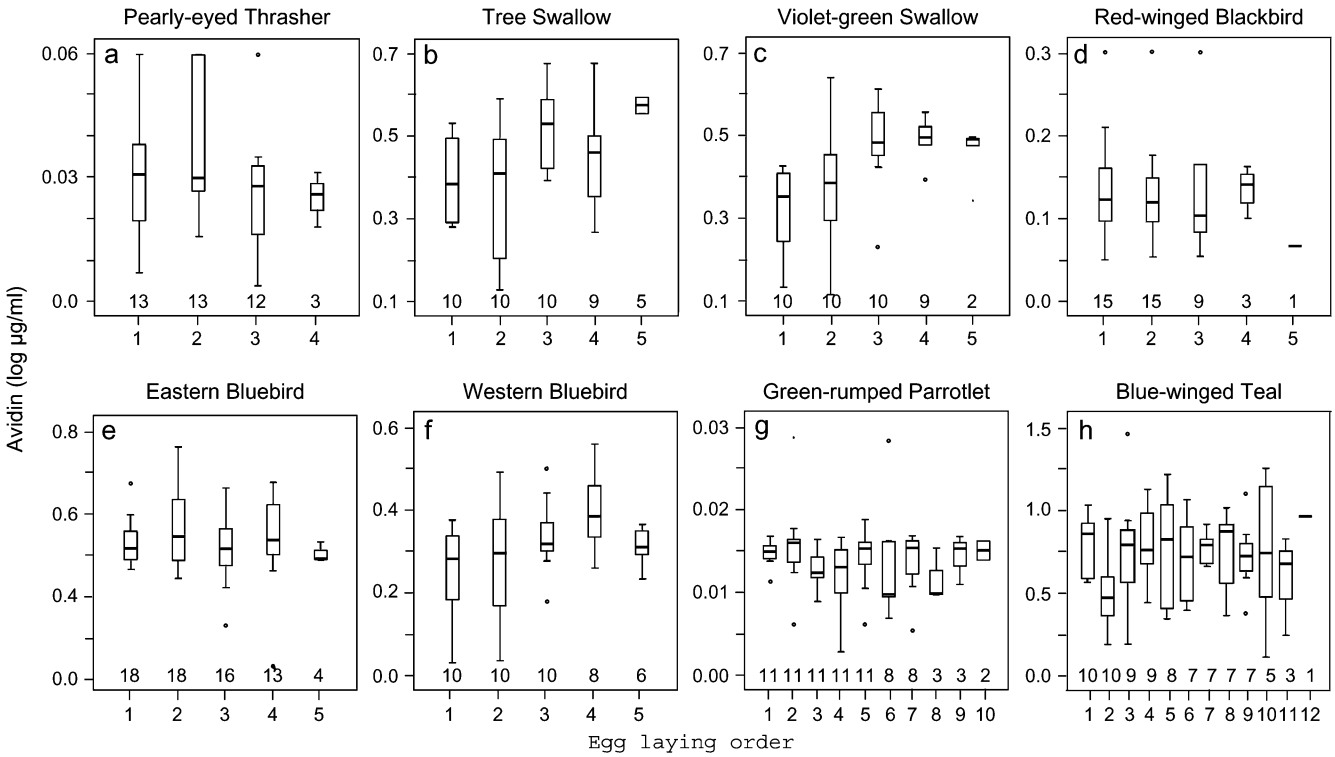

Figure 2
Boxplots of concentration of the antimicrobial enzyme avidin by laying order in eggs of 8 species of birds (labeled a–h). The line within each box represents the median concentration, the lower and upper borders of each box are the 25th and 75th percentiles, and the lower and upper bars are the 10th and 90th percentiles. Sample sizes are indicated at the bottom of each figure. Outliers are represented with circles.

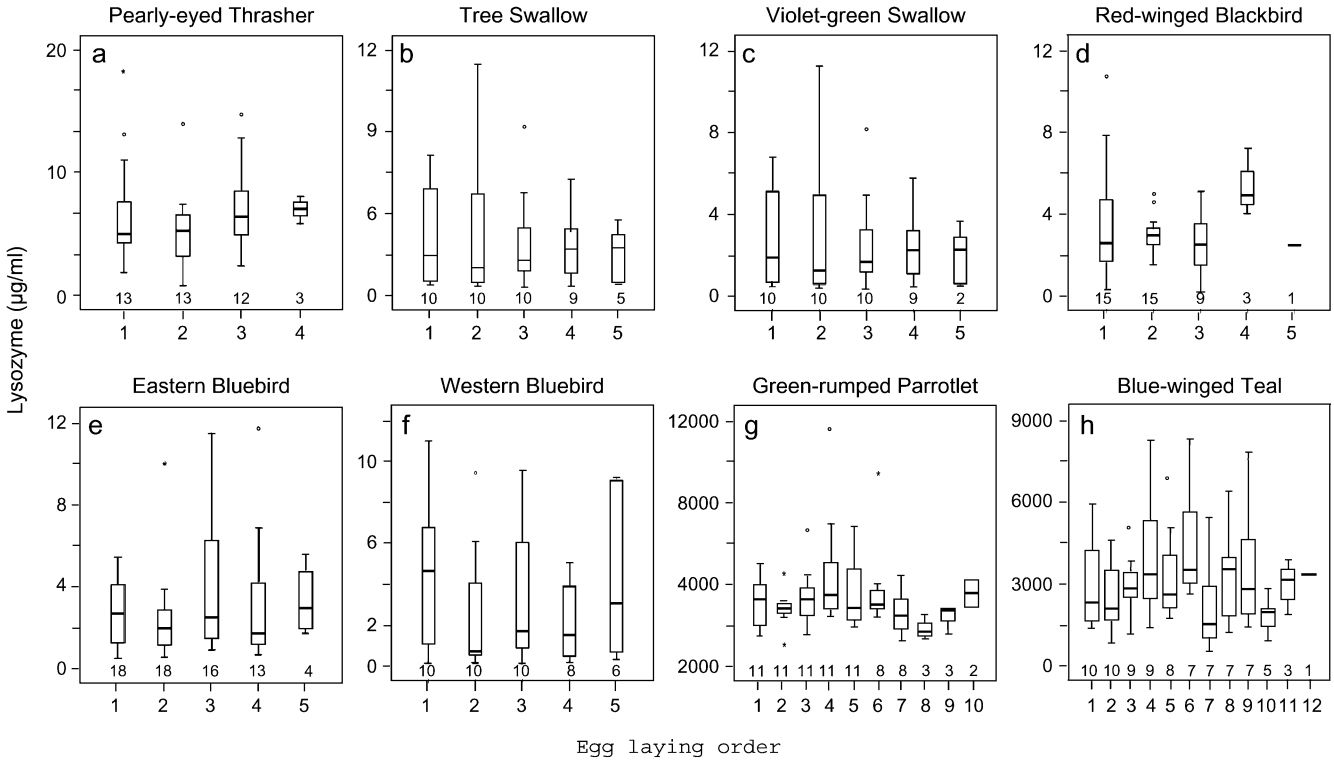

Figure 3
Boxplots of concentration of the antimicrobial enzyme lysozyme by laying order in eggs of 8 species of birds (labeled a–h). The line within each box represents the median concentration, the lower and upper borders of each box are the 25th and 75th percentiles, and the lower and upper bars are the 10th and 90th percentiles. Sample sizes are indicated at the bottom of each figure. Outliers are represented with circles.

growth (Klasing 1998). Therefore, adding more antimicrobials could entail a concomitant decrease in other aspects of albumen quality. Thorough surveys and manipulations of the nutritional and protective contents of albumen are required to determine the effects of exchanging nutrients for protective proteins on embryo and chick health. Overall amount of antibiotics in the egg may also vary because of variation in overall albumen content and/or egg size (Ferrari et al. 2006; Alquati et al. 2007).

Differential deposition of antimicrobials within clutches may be unnecessary because birds can increase the activity of antimicrobials through incubation (Board and Fuller 1974). Many birds incubate intermittently before the onset of full incubation (Haftorn 1981; Banbura and Zielinski 1995; Anderson 1997; Grenier and Beissinger 1999). Partial incubation may temporarily raise the temperature of eggs to levels at which antimicrobial enzymes are more effective. Partial incubation appears to be widespread in ducks (Loos and Rowher 2004) as well as passerines (references above) and, among other functions, may allow birds to increase the activity of antimicrobials in their eggs without altering their concentration. Future studies should examine the effects of partial incubation on microbial infection and egg hatchability.

Finally, variation in antimicrobial concentration among species was very high, ranging orders of magnitude for all 3 antimicrobials. This pattern confirms previous findings for lysozyme by Saino et al. (2007). Future studies should use phylogenetically controlled sampling and experimental approaches to identify the causes of this vast variation. Environmental conditions, nest habitat and sanitation, and microbial abundance and diversity may all drive the evolution of optimal concentrations for individual species. Antimicrobial concentrations were significantly different neither between eastern and western bluebird eggs, despite nesting in subtropical and temperate nesting habitats, respectively, nor between violet-green and tree swallows nesting in the same habitat (data not shown). However, the pressures driving high concentrations of some antimicrobials in, for example, teal and parrotlet eggs, will be a fascinating area for future research.

Antimicrobial defense of eggs is a critical function in other animal taxa, particularly those that lay their eggs in water, such as frogs and fish, where bacterial abundance is many times greater than in the air (Reinheimer 1992; Brodie et al. 2007). Examining the allocation of antimicrobial defense mechanisms at multiple scales and in multiple organisms will offer important insights into the evolution of parental care. For example, a recent study showed that male blenny fish actively apply antimicrobials to their eggs, creating a "paternal effect" (Giacomello et al. 2006). Microbes may play critical roles in the evolution of vertebrate parental care patterns, and their effects need greater consideration.

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