The role of disease risk and life history in the immune function of larks in different environments

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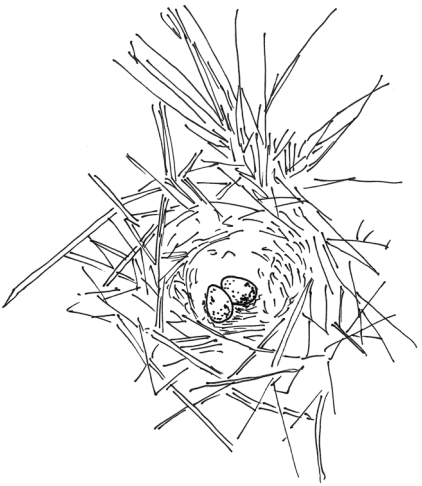

CHAPTER 4

Antimicrobial proteins in avian eggs: ovotransferrin increases but lysozyme decreases with environmental correlates of trans-shell infection

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Abstract

Understanding how immune investment relates to disease risk could be one means of explaining the variation in immune defences observed among animals in different environments. A useful way to study this is to use eggs, since they represent a simplified version of the immune system, with a reduced suite of immune defences to measure, and fewer potential sources of infection to be considered. The albumen of avian eggs contains antimicrobial proteins. These proteins protect the embryo from microbes, which can penetrate the eggshell and cause loss of egg viability. Microbial loads on eggs, microbial penetration of the eggshell and declines in egg viability increase with environmental humidity, while eggs that are kept dry experience little microbial contamination. Using humidity as a proxy for risk of trans-shell infection, we tested whether concentrations of antimicrobial proteins were higher in eggs laid in conditions that are more humid. We collected eggs of larks (Alaudidae) that live along a humidity gradient, gathered climatic data, and measured concentrations of two antimicrobial proteins, ovotransferrin and lysozyme. We also measured pH of the albumen, since this could influence the microbicidal ability of the albumen. As expected, concentrations of ovotransferrin were highest in eggs collected from environments that are more humid. Contrary to expectations, lysozyme concentrations decreased in eggs from increasingly humid environments and correlated negatively with ovotransferrin levels. Albumen pH was not significantly related to humidity. Temperature explained more of the variation in egg defences than precipitation, a result inconsistent with studies stressing the importance of water for trans-shell infection. Our study raises interesting questions about the functional role of albumen defence proteins and the potential trade-offs between them, as well as highlighting the usefulness of eggs as a simplified model for studying the evolution of immune defences in different environments.

Introduction

Viability of avian eggs exposed to ambient temperatures above physiological zero decreases over time (Beissinger, Cook and Arendt 2005). One cause of reduced viability is trans-shell microbial infection (Cook *et al.* 2003; Cook *et al.* 2005a; Cook *et al.* 2005b). To minimise infection by microbes, eggs possess physical barriers in the form of the shell, membranes and viscous albumen, and chemical barriers that include antimicrobial proteins in the albumen and the pH of the albumen (Board and Fuller 1974). Two such antimicrobial proteins are ovotransferrin, which has bactericidal properties and binds iron to make it unavailable for bacterial growth (Superti *et al.* 2007), and lysozyme, which catalyses the lysis of cell wells of gram-positive bacteria (Callewaert and Michiels 2010). In some bird species these proteins show patterns related to ambient temperature, or to lay order of eggs in a clutch, but these patterns are not universal (Saino *et al.* 2002; Saino *et al.* 2004; Shawkey *et al.* 2008; Cucco *et al.* 2009; Bonisoli-Alquati *et al.* 2010; D'Alba *et al.* 2010). The antimicrobial activity of ovotransferrin and lysozyme is influenced by pH of the albumen (Tranter and Board 1984), which changes during embryonic development (Romanoff 1944). Albumen at high pH (9-10) is bactericidal, while albumen with lower pH (6-8) displays only bacteriostatic properties (Tranter and Board 1984).

Concentrations of ovotransferrin and lysozyme, like other defensive properties of the egg, are set by the mother during egg formation. It has been shown that for antibodies in the yolk, concentrations are proportional to levels circulating in the mother (Grindstaff *et al.* 2006; Grindstaff 2008) which are likely influenced by recent or repeated exposure to pathogens. Similarly, the amount of lysozyme deposited in the albumen may relate to plasma levels in the mother (Saino *et al.* 2002), and both plasma lysozyme and ovotransferrin levels are related to infection status (Xie *et al.* 2002a; Millet *et al.* 2007). Thus, mothers can transmit their experience of the wider environment to their offspring (Gasparini *et al.* 2001; Boulinier and Staszewski 2008) thereby influencing offspring phenotype and survival. However, aside from vertical transmission of microbes from mother to egg, the risks of infection faced specifically by eggs are primarily associated with the microenvironment of the egg. Likely sources of potential contamination include the substrates that eggs contact, such as nest materials (Singleton and Harper 1998; Berger, Disko and Gwinner 2003), and the skin and feathers of incubating parents (Shawkey *et al.* 2005; Ruiz-de-Castañeda *et al.* 2011a; Ruiz-de-Castañeda *et al.* 2011b; but see below for effects of incubation). This makes eggs a simplified yet useful model for studying the evolution of immune defences in different environments.

Egg qualities differ among environments. For example, declines in egg viability are greater and occur more rapidly in the humid tropics than in temperate ecosystems, and microbial loads on eggshells and trans-shell infection rates are also

highest in the tropics (Cook *et al.* 2003; Beissinger, Cook and Arendt 2005; Cook *et al.* 2005a; Cook *et al.* 2005b; Messens, Grijspeerdt and Herman 2005; Wang, Firestone and Beissinger 2011). Rates of trans-shell infection and levels of egg viability in arid environments have not been studied. However, eggs that are kept dry, either experimentally (D'Alba, Oborn and Shawkey 2010) or through incubation (Cook *et al.* 2005a; Shawkey *et al.* 2009), have reduced microbial loads and diminished opportunities for trans-shell infection, highlighting the apparent importance of moisture in mediating this process (Bruce and Drysdale 1994). Temperature affects avian egg viability independently of microbial infection through its effect on embryonic development (Webb 1987). Temperature also influences microbial infection of eggs, either by affecting the growth of microbes on eggshells (Ayres and Taylor 1956) or through the potentiation of antimicrobial proteins, which work optimally at physiological temperatures (Tranter & Board 1984).

The activity of antimicrobial proteins increases with protein concentration (Salton 1957; Horrocks, Tieleman and Matson 2011, but see Wilcox and Daniel 1954; Friedberg and Avigad 1966 for effects in lysozyme). If antimicrobial defences have evolved to match the risk of microbial infection (Wellman-Labadie, Picman and Hincke 2008; Horrocks, Matson and Tieleman 2011), then concentrations of antimicrobial proteins in eggs should vary with environmental conditions. To test this hypothesis we collected eggs from larks (*Alaudidae*) living in different locations along a gradient of environmental humidity that ranges from humid (tropical and temperate) to hyper-arid (desert; Tieleman, Williams and Bloomer 2003; Tieleman, Williams and Visser 2004; Tieleman 2005). We focused on humidity as a proxy for the risk of trans-shell infection because microbial infection of eggs is correlated with humidity (Cook *et al.* 2003; Beissinger, Cook and Arendt 2005; Cook *et al.* 2005a; Cook *et al.* 2005b). We measured concentrations of ovotransferrin and lysozyme in the albumen of these eggs and, since it may contribute to the antimicrobial properties of the albumen (Tranter and Board 1984), we recorded the pH of the albumen.

Larks are an ideal system of comparable species for studying geographic differences in egg defences because different species inhabit a variety of environments with different macroclimates and show a range of well-documented physiological and life history traits associated with these environmental differences (Tieleman, Williams and Bloomer 2003; Tieleman, Williams and Visser 2004). Furthermore, all lark species build open-cup nests on or close to the ground in open habitats. This leaves the eggs relatively unprotected from environmental conditions and might make them more vulnerable to microbial contamination than eggs of species that nest off the ground or in cavities (Godard *et al.* 2007). We predicted that the concentrations of ovotransferrin and lysozyme and the pH of the albumen would be lowest in eggs from hot and dry environments and higher in eggs from cooler and wetter, more humid locations.

Materials and methods

Antimicrobial protein assays

We collected 125 eggs from nine lark species in 14 locations (Table 4.1). We dissected eggs into constituent parts, recorded the pH of the albumen, and if present, we weighed the mass of any embryonic material. We used the quotient of embryo mass over total egg mass as a proxy for egg age. Of the 125 eggs, 76 were collected on the day of laying and only 13 were estimated to be more than four days old based on embryo mass (maximum estimated age 7 days for one egg). The incubation period in all lark species is normally 12 days (del Hoyo, Elliott and Christie 2004). Ovotransferrin concentration (mg ml^{-1}) was measured as described in Horrocks, Tieleman and Matson (2011), using 10 μl of albumen instead of plasma. Lysozyme concentration was measured by recording the rate of optical density change (OD, 450 nm) following addition of 200 μl of a one mg ml^{-1} solution of *Micrococcus lysodeikticus* (M3770) in potassium phosphate buffer (pH 7.0, 100 mM) to microplate wells containing 50 μl sample. OD was recorded every ten seconds for 60 minutes at 25°C on a spectrophotometric microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Samples were run at two dilutions (Appendix 4.1). Standards (over the range 0.04–0.004 mg ml^{-1}) of 50 μl purified chicken egg white lysozyme (L6876) were run in duplicate. The time at which OD had decreased to 75% of the OD of a negative control (potassium phosphate buffer only) was recorded (T75). A standard curve relating T75 to lysozyme concentration of the standards was used to calculate mean lysozyme concentrations (mg ml^{-1}) of the two sample dilutions. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Climatic data and indices of environmental humidity

We obtained high-resolution (0.5 × 0.5 degree) gridded data on climatic variables for the period 1901–2009 from http://badc.nerc.ac.uk/view/badc.nerc.ac.uk_ATOM_dataent_1256223773328276. This dataset is described in more detail by Mitchell & Jones (2005). For each egg-collection location, we extracted mean annual values for precipitation (P; mm), temperature (T; °C) and potential evapotranspiration (PET; mm). PET is a derived reference measurement of how much water could be lost to the atmosphere through the combined processes of evaporation and plant transpiration. PET is dependent on a range of climatic and environmental factors including solar radiation, cloud cover and vegetation cover but tends to be low in cool and wet environments and high in arid locations such as deserts. We used the three climatic variables to calculate two alternative indices of humidity: the United Nations Environment Programme index A_{UNEP} (P / PET; UNEP 1992) and de Martonne's index A_M (P / T + 10; de Martonne 1926). Originally created as a means to define the drylands of the world, these indices may be considered to define either humidity or aridity, depending on perspective (e.g.

Table 4.1. Number of eggs (*n*), number of nests (nest n), geographic origin and climatic variables for nine species of lark. The climatic variables are mean annual values for precipitation (P), temperature (T), potential evapotranspiration (PET), and two indices of humidity: A_{UNEP} (P / PET) and A_M (P / T + 10).

species	*n*	nest *n*	latitude	longitude	P (mm)	T (°C)	PET (mm)	A_{UNEP}	A_M
hoopoe lark *Alaemon alaudipes*	18	9	22° 14' N	41° 50' E	99.57	24.72	2359.83	0.04	2.87
black-crowned finchlark *Eremopterix nigriceps*	3	3	21° 15' N	40° 41' E	201.26	21.12	2165.66	0.09	6.47
crested lark *Galerida cristata*	1	1	21° 15' N	40° 41' E	201.26	21.12	2165.66	0.09	6.47
red-capped lark *Calandrella cinerea*	12	7	0° 52' S	36° 23' E	570.48	20.25	1463.95	0.39	18.86
	2	1	0° 37' S	36° 28' E	806.77	15.39	1043.3	0.77	31.78
horned lark *Eremophila alpestris*	4	4	37° 10' N	72° 53' E	399.41	0.08	867.98	0.46	39.61
	7	7	37° 24' N	73° 30' E	369.06	-4.25	728.81	0.51	64.18
	1	1	40° 02' N	83° 09' W	1002.33	10.89	946.27	1.06	47.98
Hume's short-toed lark *Calandrella acutirostris*	3	3	37° 11' N	72° 49' E	399.41	0.08	867.98	0.46	39.61
	10	9	37° 25' N	76° 39' E	369.06	-4.25	728.81	0.51	64.18
	1	1	37° 18' N	73° 03' E	419.10	-3.75	745.90	0.56	67.06
oriental skylark *Alauda gulgula*	1	1	37° 01' N	72° 41' E	465.90	-1.44	818.66	0.57	54.43
Skylark *Alauda arvensis*	29	14	52° 55' N	6° 15' E	770.11	9.19	557.82	1.38	40.13
Woodlark *Lullula arborea*	33	21	52° 55' N	6° 15' E	770.11	9.19	557.82	1.38	40.13

Hulme, Marsh and Jones 1992) and are alternately referred to as humidity or aridity indices. For both indices, low values are associated with arid conditions and increasing values indicate increasing humidity.

Statistical analyses

During embryonic development, the pH and water content of albumen decreases (Deeming 2002). Therefore, we first explored the relationships between egg age and antimicrobial concentrations and pH, before relating these to the environmental variables. We corrected antimicrobial concentrations and pH values for egg age using two approaches: i) correction based on the differences in group means between fresh and incubated eggs (i.e. eggs with no embryonic development vs. eggs with any embryonic development); ii) correction based on a regression against amount of embryonic development. Neither correction approach qualitatively changed the outcome of any analyses, so we present all results based on analyses of uncorrected values. We took a conservative approach and calculated mean values per population (i.e. per species per location) of each dependent variable. To do this, we first calculated mean values per nest for each population, and used these values to calculate mean values per population. We treated species and geographically distinct populations as independent points and used linear models to investigate relationships between antimicrobial concentrations, pH and climate. Because sample size varied among species (Table 4.1), regression models were weighted by the square root of the number of nests sampled in each population (Sokal and Rohlf 1995). We performed all statistical analyses using R 2.13.0 (R Development Core Team 2009).

Results

Ovotransferrin, lysozyme and pH related to climatic variables and humidity indices in different ways (Fig. 4.1). Ovotransferrin correlated negatively with precipitation, temperature and PET (Figs 4.1A-C), but temperature was the only climatic variable where the relationship was significant (Table 4.2). Therefore, ovotransferrin concentrations matched predictions regarding temperature, but not regarding precipitation. However, there was a positive but non-significant trend (Table 4.2) for ovotransferrin concentrations to increase with increasing

Figure 4.1. (Right) Concentrations of ovotransferrin and lysozyme, and the pH of the albumen of eggs from nine lark species, in relation to three climatic variables and two indices of humidity, the UNEP humidity index A_{UNEP} (P/PET) and de Martonne's index A_M. (P / T + 10). Humidity indices are plotted such that humidity increases along the x-axis. The size of each data point is proportional to the number of records (number of nests) contributing to the value.

ovotransferrin conc. (mg ml-1)
lysozyme conc. (mg ml-1)
pH
mean annual precipitation (mm)
mean annual temp. (°C)
mean annual PET (mm)
UNEP humidity index
de Martonne's index
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
r2 = 0.05
r2 = 0.28
r2 = 0.06
r2 = 0.00
r2 = 0.23
r2 = 0.15
r2 = 0.58
r2 = 0.57
r2 = 0.22
r2 = 0.62
r2 = 0.07
r2 = 0.25
r2 = 0.04
r2 = 0.02
r2 = 0.13

humidity according to de Martonne's index A_M (Fig. 4.1E). The UNEP humidity index A_{UNEP} showed no relationship with ovotransferrin (Fig. 4.1D).

Lysozyme also showed a non-significant negative relationship with precipitation (Fig. 4.1F), but correlated positively and highly significantly with temperature and PET (Figs 4.1G, H; Table 4.2). This resulted in a negative correlation between lysozyme and both humidity indices (Figs 4.1I, J) that was highly significant in the case of A_M but not in the case of A_{UNEP} (Table 4.2). Thus, for all climatic variables and humidity indices, lysozyme concentrations displayed patterns contrary to our predictions.

There were no significant relationships between any climatic variables and pH (Figs 4.1K-O), although the positive relationship with temperature (Fig. 4.1l) was only marginally non-significant (Table 4.2). There was also no relationship between A_{UNEP} and the pH of the albumen (Fig. 4.1N), but a trend for pH of the albumen to be higher in eggs from less humid environments according to A_M (Fig. 4.1O).

Lysozyme and ovotransferrin concentrations were negatively correlated with each other, but this relationship was not significant either when testing at the level of individual eggs (Spearman's rank correlation $S = 3.6 \times 10^5$, $P = 0.090$, $rho = -0.15$) or at the level of populations ($S = 622$, $P = 0.1973$, $rho = -0.37$).

Table 4.2. Results (F tests and *P* values) of linear models examining relationships between concentrations of ovotransferrin and lysozyme and the pH of the albumen of eggs from nine lark species, in relation to mean annual precipitation, temperature and potential evapotranspiration (PET) and two indices of humidity, A_{UNEP} and A_M. Nominator and denominator degrees of freedom are 1 and 12 for each model. Significant *P* values are shown in bold.

response variable	explanatory variable	F	*P*
ovotransferrin	precipitation	0.67	0.430
	temperature	4.59	**0.053**
	PET	0.83	0.380
	A_{UNEP}	0.04	0.844
	A_M	3.67	0.079
lysozyme	precipitation	2.08	0.174
	temperature	16.46	**< 0.001**
	PET	15.95	**0.002**
	A_{UNEP}	3.35	0.092
	A_M	19.18	**< 0.001**
pH	precipitation	0.94	0.350
	temperature	3.91	0.072
	PET	0.54	0.476
	A_{UNEP}	0.25	0.626
	A_M	1.80	0.204

Discussion

One prediction related to the immunological variation that has been documented among individuals, populations, and species is that immune defences might match the risk of infection or disease (Horrocks, Matson and Tieleman 2011). This study provides a novel insight into this prediction by investigating the non-specific chemical defences of eggs collected along a gradient of presumed infection risk, as indexed by environmental variation in humidity. The main finding of our study is that, when applied to the antimicrobial defences of eggs, support for this prediction is mixed. In general, concentrations of ovotransferrin supported our hypothesis that chemical defences should be highest in eggs from more humid environments where the risk of trans-shell infection is also highest. In contrast, concentrations of lysozyme were lower in eggs from wetter environments, were positively correlated with environmental temperature and PET, and were significantly and negatively correlated with humidity. Thus, lysozyme showed patterns directly contrary to our predictions. pH, a third quality of the albumen, showed qualitatively similar trends to those of lysozyme in relation to climatic variables, although these trends were never significant.

Concentrations of ovotransferrin and lysozyme showed opposing patterns in relation to humidity, our proxy for risk of trans-shell infection. One explanation for contrasting patterns might be the existence of a trade-off between these proteins. In fact, concentrations of ovotransferrin and lysozyme were negatively correlated, although not significantly. Eggs containing high concentrations of ovotransferrin – and that presumably are well protected – might require less lysozyme, and vice versa. One testable possibility is that the microbial assemblages on eggshells in different environments differ in composition and not just density (Cook *et al.* 2003). In that case, one antimicrobial protein may be more effective than another. For example, on its own, lysozyme is only effective against gram-positive bacteria, while the iron-binding function of ovotransferrin makes it more broadly effective. Interestingly however, when combined, ovotransferrin and lysozyme may display synergistic effects. In the presence of lactoferrin, a protein similar to ovotransferrin, lysozyme becomes bactericidal against gram-negative bacteria (Ellison and Giehl 1991) increasing its range of antimicrobial activity. Lysozyme may also potentiate the activity of ovotransferrin (Ko *et al.* 2009). A balance between the concentrations of ovotransferrin and lysozyme might maximise antimicrobial defence of the egg while minimising the amounts of protein required. This could be important if production of antimicrobial proteins was costly (but see Shawkey *et al.* 2008), or if increasing the concentration of these proteins in the egg came at the expense of other albumen components that are required for embryonic development. Nonetheless, the existence of such a trade-off in protein levels cannot explain why the balance between ovotransferrin and lysozyme shifts with respect to predicted trans-shell infection risk and fur-

ther points to the need to evaluate egg microbial assemblages.

An explanation for why lysozyme does not correlate with predicted infection risk could be that the primary function of lysozyme in egg albumen is not as a defensive protein. This argument has been proposed previously (Board and Fuller 1974), but largely ignored in subsequent investigations of egg antimicrobial properties. Aside from antimicrobial properties, lysozyme in albumen possesses additional functions, some of which might relate to environmental factors such as temperature. For example, the viscosity of the albumen is determined by the binding of lysozyme to another albumen protein called ovomucin (Burley and Vadehra 1989), and albumen viscosity correlates strongly with lysozyme activity (Trziska and Clostermann 1993). In commercial chicken lines, albumen viscosity may relate to egg hatchability, (Hurnik, Reinhart and Hurnik 1978), and albumen viscosity degrades faster at higher temperatures (Reijrink *et al.* 2008). The physicochemical properties of lysozyme may therefore make it more valuable in eggs laid in hot and dry environments, where the risk of trans-shell infection may also be lower. In cooler and wetter environments reductions in hatchability due to temperature-induced albumen degradation may be less relevant. In these environments, if microbes are the dominant selection pressure, then the broader-spectrum antimicrobial activity of ovotransferrin may be more valuable. This scenario could further support the idea of a trade-off between concentrations of ovotransferrin and lysozyme. Nonetheless, given the ubiquitous function of lysozyme as an antimicrobial agent in other biological systems (reviewed in Callewaert and Michiels, 2010), a complete lack of any antimicrobial function of lysozyme in egg albumen seems unlikely.

We found no significant correlations between albumen pH and any climatic variables. We suggest that this is because physiological constraints related to embryonic development limit the extent to which the pH of an egg can be altered. Upon laying, eggs lose carbon dioxide, which results in an increase in pH of the albumen from about pH 7.6 to pH 9.5 (Sharp and Powell 1931), changing the albumen from bacteriostatic (pH 6-8) to bactericidal (pH 9-10; Tranter and Board 1984). This pH burst, which occurs during the first days after laying, could effectively sterilise the albumen of any microbial contamination, both by making the albumen inhospitable to microbial growth, and by potentiating the activity of antimicrobial proteins (Tranter and Board 1984; Reijrink *et al.* 2008). Yet, albumen with a pH above pH 8.2 is detrimental to embryonic development (Reijrink *et al.* 2008). This implies that beyond the initial short-term increase in pH, birds are limited in their ability to rely on high albumen pH as an antimicrobial defence.

Temperature explained the highest amount of variation in ovotransferrin and lysozyme concentrations and also in albumen pH. Our data are in line with an earlier study showing that eggs contain more lysozyme when ambient temperature during egg formation is higher (Saino *et al.* 2004), which might relate to

albumen viscosity and protein trade-offs, as discussed already. The relative importance of temperature to egg defences also provides an interesting contrast to a related study of immune function in adult larks measured along a similar gradient of climatic variation and disease risk (chapter 3). In that study, immune indices of birds declined with disease risk, as predicted by environmental humidity. Contrary to the findings of the current study however, variation in the immune indices of birds (including plasma concentrations of ovotransferrin) was explained by precipitation and not by temperature. We suggest that the importance of temperature in explaining variation in immune defences of eggs but not of birds relates to the ectothermic nature of eggs and the endothermic abilities of birds. Although the enzymatic activity of antimicrobial proteins is believed to be temperature-dependent (Tranter and Board 1984) it is interesting to note that in our study ovotransferrin showed a negative (although very weak and non-significant) trend with temperature while lysozyme showed a positive relationship.

Despite the apparent necessity of water for trans-shell infection (D'Alba, Oborn and Shawkey 2010), mean annual precipitation explained little to no variation in any albumen defence component. This result could be explained if the negative effects of water on trans-shell infection are countered by incubation (Cook *et al.* 2005a; Shawkey *et al.* 2009; D'Alba, Oborn and Shawkey 2010). With the exception of lysozyme concentration, PET was also poor at explaining variation in albumen defences. Most likely for that reason, and because of the strong correlations with temperature, albumen defences correlated much more strongly with humidity index A_M (based on precipitation and temperature) than with humidity index A_{UNEP} (calculated from precipitation and PET). Humidity indices more specifically, and environmental variation in general, may still be useful proxies for risk of microbial infection, as evidenced by the decrease in soil microbial diversity and abundance associated with decreasing annual precipitation (and presumably humidity; Bachar *et al.* 2010). However, describing and quantifying the microbial assemblages of eggs and the surrounding microenvironment, such as nest material, is essential to developing a better understanding of the risks of trans-shell infection in different environments (Cook *et al.* 2005b; Wang, Firestone and Beissinger 2011). Ultimately, studies that combine these measurements with quantification of albumen defences and assessment of egg hatchability will be informative in understanding how immune protection evolves to match the risk of infection. In the meantime, we have uncovered interesting inverse relationships between ovotransferrin and lysozyme and environmental parameters. These relationships provide new insight regarding the functional role of albumen defence proteins, the potential trade-offs between proteins, and the value of abiotic environmental variables as proxies for the variation in biotic infection risk.

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