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ORIGINAL PAPER

Egg antimicrobials, embryo sex and chick phenotype in the yellow-legged gull

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Abstract Maternal effects through albumen quality are largely unexplored, despite the fundamental role that albumen exerts as source of proteins and water, as well as for antimicrobial defence of the embryo. We analysed the variation of two major albumen antimicrobials, avidin and lysozyme, by extracting samples from freshly laid eggs of the yellow-legged gull (*Larus michahellis*) and by correlating their levels to egg features. Lysozyme concentration increased along the laying sequence, while avidin concentration decreased. Both antimicrobials declined during the season. In addition, avidin concentration declined from first- to last-laid male eggs, whereas the opposite was true among the female eggs. We also analysed chick body mass and size and immune response, in relation to albumen antimicrobial levels in their original egg while controlling for potential covariation between egg quality and rearing conditions by cross-fostering eggs between nests. Tarsus length decreased with avidin concentration, particularly early in the season. Avidin concentration negatively predicted tarsus length of chicks and the phytohaemagglutinin response of females, but not males. However, chick phenotype did not covary with lysozyme albumen concentration. This is the first study where maternal effects mediated by albumen antimicrobials are investigated in relation to both sex and egg features in any wild bird species. Whether the observed patterns of variation in antimicrobial concentration are the by-product of maternal physiological constraints, or reflect adaptive allocation strategies, cannot be ascertained. The covariation between chick cell-mediated immunity and albumen avidin concentration might be causal, according to the documented effects of albumen proteins on immunity in other species.

Keywords Albumen · Antimicrobial proteins · Avidin · Lysozyme · Maternal effects · Yellow-legged gull · Cross-fostering

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Introduction

The phenotype of the mother and the environment she experiences has the potential to profoundly modify offspring phenotype, besides the effects of maternal contribution to offspring genotype (Mousseau and Fox 1998). One major pathway of prenatal maternal effects is mediated by egg quality (Bernardo 1996). Cleidoic eggs of birds contain all the materials needed for embryo development, with limited exchange with the external environment (Romanoff and Romanoff 1949).

A number of studies have investigated maternal resource allocation strategies to the yolk, for constituents as diverse as hormones, antioxidants and immune factors (Groothuis

et al. 2005; Badyaev et al. 2006a; Rubolini et al. 2006; Saino et al. 2008). It has been suggested that these maternal strategies may be adaptively tuned to sex-specific requirements, as well as to the position in the laying sequence (Young and Badyaev 2004; Müller et al. 2005; Badyaev et al. 2006a, b). On the other hand, surprisingly, little research has been devoted to explore maternal effects mediated by the amount and composition of the albumen (Hill 1993; Finkler et al. 1998; Ferrari et al. 2006; Bonisoli-Alquati et al. 2007, 2008). The albumen represents the main source of water and proteins for the embryo (Romanoff 1967). Experimental studies have shown that the amount of albumen can affect embryo size at hatching, with potential consequences for post-natal growth, physiology and social interactions with siblings (Finkler et al. 1998; Ferrari et al. 2006; Bonisoli-Alquati et al. 2007, 2008). One important function of the albumen, largely neglected in evolutionary ecology studies, is to provide antimicrobial defence to the embryo (Mine and Kovacs-Nolan 2004; Wellman-Labadie et al. 2007; but see Cucco et al. 2006). Developing eggs are susceptible to infectious agents that can be incorporated in the egg during its formation and/or penetrate it after laying, ultimately affecting embryo or chick viability (Board and Tranter 1986; Bruce and Drysdale 1994; Pinowski et al. 1994; Cook et al. 2003, 2005). The fibrous and viscous nature of the albumen, particularly of its inner layers, can represent a mechanical barrier to the diffusion of bacteria penetrating the egg and isolate the embryo from the porous eggshell (Brooks and Hale 1959). In addition, several albumen proteins can reduce the risk of virulent bacterial infections by means of either direct bactericidal actions or by creating an environment unsuitable to bacterial growth (reviewed in Palmer and Guillette 1991; Mine and Kovacs-Nolan 2004; Wellman-Labadie et al. 2007). Lysozyme accomplishes a direct bactericidal action through the hydrolyzation of glycosidic bonds of bacterial cell walls (Pellegrini et al. 1997; Bera et al. 2005). Other albumen proteins can sequester minerals or vitamins that are essential to bacteria (Seviour and Board 1972). For example, the bacteriostatic action of avidin is mainly accomplished by strongly binding biotin, thus making it unavailable to bacterial proliferation (Green 1975). Indeed, although most of the egg biotin (up to 90% in fowl) is contained in the yolk (Romanoff and Romanoff 1949), biotin can diffuse into the albumen during embryonic development due to change in permeability of the perivitelline membrane (Bush and White 1989), thus potentially fuelling bacterial growth. Some albumen proteins (e.g. lysozyme and ovomucin) have also been assigned an antiviral action (Li-Chan et al. 1995) and can be passed onto the embryonic circulation (Marshall and Deutsch 1951), where they can prevent viral haemagglutination (Palmer and Guillette 1991).

Antimicrobial proteins are also directly involved in oviductal transfer of nutrients from the mother to the developing egg (White 1987; Palmer and Guillette 1991). For example, avidin may be involved in transferring to the embryo maternal biotin, a cofactor in the metabolism of fatty acids and amino acids and an essential nutrient for embryonic growth (White 1985; Taniguchi and Watanabe 2007). Most of these maternal antimicrobial proteins are passed directly to the embryo and are subsequently found in the embryo blood as well as in other tissues (Marshall and Deutsch 1951), where they may exert an antimicrobial role similar to the one they have in the egg. Experimental studies on domestic species suggest that egg lysozyme might have positive consequences for early post-hatching survival (Prusinowska et al. 2000). Poultry studies are still the main source for the relevance of egg antimicrobial proteins for hatching success, protection against pathogens and maternal transfer of nutrients to the embryo (Melek 1977; Prusinowska et al. 2000; see reviews in Board and Fuller 1974; Board and Tranter 1986; Palmer and Guillette 1991; Wellman-Labadie et al. 2007). Very few ecophysiological studies have investigated adaptive maternal effects via egg antimicrobials in natural bird populations. Indeed, the likely physiological cost for females of transferring these substances to the eggs (but see Shawkey et al. 2008) and the multiple crucial functions that antimicrobials perform during embryonic development both suggest that mothers are selected for tuning antimicrobial transfer to the reproductive value of their offspring.

Similarly, variation in antimicrobial concentration among eggs in a clutch may reflect adaptive maternal strategies of allocation of egg constituents (see Saino et al. 2002b, 2007; Shawkey et al. 2008). There are several reasons why concentration of antibacterial factors may vary among eggs in a clutch. First, mothers may discriminate among eggs in relation to the expected reproductive value of the offspring. A wealth of studies has shown that severe fecundity and survival costs of egg production do indeed exist in birds (Monaghan et al. 1998; Nager et al. 2001). Albumen components with antimicrobial action make up a large proportion of albumen proteins, thus meeting the assumption of their costly allocation. In facultative brood reducing species, mothers may therefore better equip the eggs carrying the offspring that will be likely privileged by post-hatching parental decisions. Since brood reduction strategies are typically mediated by the sacrifice of the less valuable, last-hatched chicks, a negative covariation of antimicrobial concentration and laying order could be expected. Second, laying order is negatively associated with the time elapsed between laying and hatching because hatch spread is shorter than laying spread even in species with pronounced hatching asynchrony. This results in a prolonged risk of bacterial egg infection and in an increased time available to bacteria to

proliferate in first- compared to last-laid eggs. Overall, the infection risk for first- vs last-laid eggs should depend on the exact incubation patterns at the onset and at the end of the incubation period, as well as on variation in ambient temperature (Cook et al. 2003, 2005). In addition, small eggs have a higher surface-to-volume ratio, the difference in the surface exposed to bacterial infection relative to egg volume between large and small eggs being in the order of 5–10% in the study species (our unpublished data). This suggests that, other things being equal, small eggs should be provisioned with larger amounts of antimicrobials than large eggs to attain the same level of defence. Moreover, allometric relationships may exist between egg and albumen volume (Deeming 2007), and albumen relative to egg volume can vary with laying order (e.g. Ferrari et al. 2006), potentially generating additional variance in antimicrobial concentration.

Finally, the sex of the embryo is a totally neglected source of variation in antimicrobial concentration in the eggs. Mothers might fine-tune the allocation of antimicrobials to the eggs depending on embryo sex because of sex-specific susceptibility to pathogens or because male and female embryos differ in their requirements of egg components bound to antimicrobial molecules. In birds, susceptibility to virulent pathogens after hatching can vary between the sexes (e.g. Badyaev et al. 2006b), and physiological differences leading to sex-related susceptibility to parasitism after hatching may be well established before hatching. Several studies have reported sex-related maternal effects in the form of differential resource allocation to the eggs (Young and Badyaev 2004; Müller et al. 2005; Badyaev et al. 2006a). Overall, due to the multiplicity of roles of albumen proteins and to the differences in selection pressures among eggs in a clutch, drawing firm predictions about variation in albumen protein content relatively to egg laying order, egg mass and sex of the embryo seems, thus, premature.

Natural variation in egg antimicrobials according to egg features (i.e. egg laying order, date and mass) is still poorly explored in wild birds. In the present study of yellow-legged gulls (*Larus michahellis*), we first focus on variation of two major antimicrobials of avian egg albumen—avidin and lysozyme—in relation to egg features and embryo sex. Second, we analyse the covariation between the phenotype (i.e. body mass and size and an index of immune response) of male and female chicks during the first 8 days post-hatching, when the effects of egg composition may be predicted to be strongest, and the concentration of avidin and lysozyme in their original egg by extracting a small bioptic sample of albumen from freshly laid eggs. In order to uncouple the potential effects of concentration of antimicrobials from those of post-hatching environment in determining chick phenotype, we performed a reciprocal cross-fostering of eggs within pairs of synchronous clutches.

Methods

Study species and general field procedures

Our model species is a large gull (800–1,500 g) inhabiting mainly coastal habitats across the Mediterranean region (Cramp 1998). Eggs are laid at 2–3-day intervals till completion of a clutch of two to three eggs (rarely one or four; modal clutch size=3 eggs in 90% of clutches; our unpublished data). Egg mass (mean 86.27 (7.39 standard deviation (SD)), range 63–110 g, n=3695; our unpublished data) decreases along the laying sequence, with change in mass being smaller between first and second eggs (a- and b-eggs, respectively) than between b- and c-eggs (see Rubolini et al. 2006, 2009). Hatching is markedly asynchronous (hatch span 1–3 days), with hatch order paralleling laying order, and results in a marked size/age brood hierarchy that can mediate a facultative brood reduction strategy. Nidifugous, altricial chicks fledge at 35–40 days of age (Cramp 1998). Sexual size dimorphism is established already at hatching (our unpublished data).

We studied yellow-legged gulls in a large colony located in the Comacchio lagoon (NE Italy, 44°20′ N–12°11′ E) during spring 2004 and 2005. The field methods have been extensively described in previous companion papers (Rubolini et al. 2006; Saino et al. 2008) and will be briefly outlined below. In those papers, we mainly investigated the covariation between chick phenotype and composition of the yolk of their original egg in terms of carotenoid concentration and antioxidant capacity (Rubolini et al. 2006; Saino et al. 2008) by extracting a bioptic yolk sample from freshly laid eggs, which were subsequently incubated by their parents. In the present study, we focus on the covariation between egg features (i.e. laying order, date and mass), embryo sex or chick phenotype and concentration of antimicrobials in the albumen, using a bioptic albumen sample that was extracted immediately after extraction of the yolk sample. The analyses of the concentration of antimicrobials could only be done after publication of the two related papers (Rubolini et al. 2006; Saino et al. 2008).

At clutch completion, we applied a reciprocal cross-fostering procedure, wherein either one or two eggs of the same laying order were swapped between dyads of clutches where the last egg was laid on the same day. For each dyad, we randomly chose one of the six possible cross-fostering schemes (i.e. three schemes for cross-fostering of two eggs plus three schemes for cross-fostering of one egg of the

same laying order; see also Rubolini et al. 2006). This cross-fostering procedure was aimed at controlling for incubation and post-hatching environmental effects (including parental effects). However, we are aware that cross-fostering protocols leave the possible covariance between direct additive genetic effects and egg quality uncontrolled, as well as part of the covariation between egg quality and offspring environment (Krist and Remeš 2004). Hatching failure of biopsized eggs was ca. 23% higher than unmanipulated eggs of the study population (overall hatching success for eggs subjected to biopsy was 55%; see Rubolini et al. 2006). Because most of the eggs that did not hatch failed to develop an embryo, the sex of unsuccessful eggs could not be determined. In addition, the causes of hatching failures are uncertain. Bacterial infections could have been responsible if the unavoidable opening of a hole to extract the biopsies caused bacterial contamination. Alternatively, insertion of the needle to extract the albumen or, more likely, the yolk, could have resulted in a mechanical injury to the embryo or other structures (e.g. the perivitelline membrane). Because the relative contribution of these and other potential factors to the observed proportion of failures could not be estimated, we decided not to investigate whether a larger concentration of antimicrobials predicted hatchability (see also Saino et al. (2008) for further details on hatchability issues).

The colony was visited regularly in order to locate and mark newly started clutches and newly laid eggs according to laying order, which could be identified for all the eggs included in this study. Once a new egg was found, it was temporarily removed from the nest and replaced with a dummy egg for the duration of the bioptic procedures. We opened a hole through the eggshell by means of a sterile pin. After the yolk biopsy (see Rubolini et al. 2006), the egg was kept with its longitudinal axis inclined to a ca. 30° angle on the horizontal plane, with the acute pole pointing downwards, for a few seconds. Albumen bioptic samples (100–200 mg, corresponding to ca. 0.2% of average albumen mass) were collected using a 2.5-mL disposable sterile syringe with a 21-gauge, 40-mm-long needle. We standardised the bioptical procedures by always inserting the needle by ca. 15 mm into the egg. After bioptical procedures, the hole was sealed by glueing a piece of eggshell upon it. The egg was then carried back to its nest of origin within a few hours after removal. For the purpose of subsequent analyses, egg laying order was scored both according to the laying sequence (i.e. eggs were classified as a-, b-, or c-eggs; 'laying order' hereafter) or to whether the egg was the last one in its clutch (i.e. the b-egg in two-egg clutches or a c-egg in three-egg clutches) or not ('position in the laying sequence' hereafter). This was decided because the patterns of allocation of maternal substances to a- and b-eggs may differ depending on whether b-eggs are followed or not by a c-egg. If this is the case, b-eggs in three-egg clutches should not be considered in the same way as b-eggs in two-egg clutches, where they represent the last-laid egg. This is confirmed by the fact that in a large sample of 669 clutches measured in the same population during 2007–2008, b-eggs were significantly smaller in two-egg than in three-egg clutches, while a-eggs did not significantly differ (Rubolini et al. 2009). All analyses were thus performed using this dual approach.

Regular visits to the nests around hatching allowed us to individually mark the chicks before hatching by inoculating a small drop of food dye (either blue or green) in the pipping egg, thus tracking individual egg of origin (Rubolini et al. 2005).

Body mass (to the nearest 1 g) and tarsus length (to the nearest 0.1 mm) of the chicks for which antimicrobial concentration in the original egg was assessed were measured at an average age of 4.44 (0.05 SE) days (hereafter age 4), and again at an average age of 8.44 (0.05 SE) days (hereafter age 8). At age 8, we also performed an in vivo immunological test (the so-called phytohaemagglutinin (PHA) wing web swelling response test), according to a standard protocol (Saino et al. 1997; see Rubolini et al. (2005, 2006) for details). The values of the wing web swelling index were highly repeatable within individuals, as determined by measuring twice the wing web of 12 individuals ($F_{11,24}=33.58$, $P<0.0001$, $R^2=0.97$). Blood samples were collected at age 4 by puncturing the ulnar vein (Rubolini et al. 2006), for the purposes of quantifying plasma lysozyme concentration and for molecular sexing of the chicks. Blood samples were then kept cool until they were centrifuged (10 min at 11,000 rpm) to separate the plasma (within 12 h from collection) and stored at −20 ºC. Molecular sexing was carried out on the red blood cells using the W chromosome-linked avian chromobox-helicase-DNA-binding gene (CHD-W; Griffiths et al. 1998; see Saino et al. (2002a) for a detailed account of the procedure).

Avidin and lysozyme assay in the albumen and plasma

The albumen samples were stored at −20 °C until analysis. The albumen was homogenised for 1 min, and then lysozyme was quantified as described by Taylor et al. (1992) and Vidal et al. (2003). ELISA plates (F96 Maxisorp Nunc-Immuno Plate, Nunc, Roskilde, Denmark) were coated overnight at 4 ºC with 100 µl of primary rabbit polyclonal anti-lysozyme antibodies diluted 1:1,000 (immunogen: lysozyme from hen egg white, cat no. NB600-851, Novus Biologicals, Littleton, CO, USA) in phosphate-buffered saline (PBS) containing 0.2% Tween (T-PBS). After washing, the remaining sites for protein binding on the microtiter plate were saturated by incubation with blocking buffer (PBS-bovine serum

albumin (BSA) 3%) at room temperature for 60 min. The wells were washed with T-PBS, and then 100 μl of specimen, diluted 1:6 with PBS, was added to each well. The assay was standardised with hen egg lysozyme (Sigma-Aldrich), diluted to known concentrations (0.5, 1, 2, 5, 10, 20 μg/mL) and standards were included in quadruplicate on all plates to calibrate absorbance measures across plates (intra-assay coefficient of variation=4.4%). The plates were incubated for an additional 120 min at room temperature after which they were washed and 100 μl of horseradish peroxidase-conjugated rabbit polyclonal anti-lysozyme conjugated to HRP (full-length native protein (purified), lysozyme (hen egg white) cat. no. NB600-850 Novus Biological) diluted 1:1,000 in T-PBS were added and incubated for another 60 min at room temperature. After washing, 100 μl of enzyme substrate OPD (*o*-phenylenediamine dihydrochloride, Sigma-Aldrich, Italy) was added to each well and incubated for 30 min at room temperature. The absorbance was read in triplicate on a plate reader (Sirio S, SEAC, Florence, Italy) at 450 nm. All samples were processed blind to their clutch of origin and randomly assigned to plates. Three albumen samples were assayed twice, and repeatability of the procedure was estimated by means of a one-way analysis of variance (ANOVA) with individual as the main effect. This analysis showed that lysozyme concentration measures were highly consistent within individuals ($F_{2,\ 3}$=101.93, P=0.002, adjusted R^2= 0.976). Concentration values for samples assayed twice were calculated as the mean of the two measures. In order to quantify lysozyme concentration in the plasma samples, we utilised the same ELISA procedure employed for egg albumen, with the exception that samples were diluted 1:15 with PBS. Lysozyme concentration was measured in microgram per millilitre and can be assumed to positively predict defence against Gram-positive bacteria (Bera et al. 2005).

The biotin-binding capacity (i.e. active avidin concentration) of albumen samples was measured using biotinylated insulin and biotinylated alkaline phosphatase (New England Biolabs, Celbio, Milan, Italy), according to the method reported in Groman et al. (1990). The 96-well plates were coated with biotinylated insulin (10 μg/mL) in sodium carbonate buffer (50 mM, pH 9.6) at 37 ºC for 2 h, followed by washing three times with T-PBS and blocking with 1% BSA in PBS (PBS-BSA) for 1 h. Albumen samples were diluted 1:6 with 0.5% BSA in PBS, vortexed briefly and incubated for 2 h at room temperature. Duplicate samples (100 μl each) were allowed to bind to biotinylated insulin at 37 ºC for 1 h, followed by washing five times with T-PBS. Biotin saturated samples (17 mg/L, Sigma-Aldrich, Milan, Italy) and BSA were used as negative controls, and the assay was standardised with chicken avidin (Sigma-Aldrich), diluted to known concentrations. Standards were included on all plates to calibrate absorbance measures across plates. Biotinylated AP was used to probe the bound biotin-binding proteins diluted 1:3,000 in PBS-BSA (1 h, 37 ºC). *Para*-nitrophenyl phosphate (1 mg/mL, Sigma-Aldrich) was used as a signal molecule, and absorbencies were measured at 405 nm with a plate reader (Sirio S, SEAC, Florence, Italy), 20 min after adding substrate buffer. Avidin concentration was expressed in microgram per millilitre. A subsample of 100 samples was assayed twice, allowing us to estimate repeatability of the procedure, by means of a one-way ANOVA with individual as the main effect, as for lysozyme concentration. The biotin-binding capacity was highly repeatable ($F_{99,100}$=7.16, P<0.001, adjusted R^2=0.754). The concentration values of samples assayed in duplicate were calculated as the mean of the two measures.

The biochemical assay we adapted to measure avidin concentration provides a quantitative estimate of the concentration of free avidin, i.e. avidin not bound to its main ligand, biotin, which is required for bacterial growth. Thus, our measure of avidin concentration may positively correlate with antimicrobial defence. This would be the case if a larger amount of free avidin indicates a larger residual capacity of binding albumen biotin or biotin diffusing from the yolk during embryo development, with negative effects on bacterial growth. However, to the best of our knowledge, the actual importance of yolk biotin transfer to the albumen to bacterial proliferation has not been quantified.

Statistical analyses

Data were analysed mainly by means of general linear mixed models (GLMM). All models were simplified through a step-down procedure, whereby terms not reaching statistical significance (α<0.05) were removed, starting from interaction terms. Factors involved in significant interactions were retained even if they did not attain statistical significance. Lysozyme and avidin concentrations were analysed using laying date, egg mass and either laying order or position in the laying sequence (see above), together with chick sex and all the two-way interactions. Clutch identity was included as a random factor. Main and interaction effects were always removed when non-significant.

The covariation between chick phenotype and antimicrobial concentration in their original eggs was analysed using chick phenotypic traits (tarsus length, body mass and PHA response) as dependent variables. Egg mass, laying date and laying order or position in the laying sequence were included in the analyses as covariates, together with sex and all the two-way interactions. Dependent variables

were log-transformed when needed to attain normality and homoscedasticity.

All analyses were performed with SAS (ver. 9.0, SAS Inst., Cary, NC, USA).

Results

Antimicrobial albumen concentration and egg features

The concentration of lysozyme in the albumen was measured in 117 eggs from 76 nests (n=42, 51, 24 for a-, b-, and c-eggs, respectively), but one extreme outlier whose value was more than 10 SDs larger than the mean was excluded from subsequent analyses. Mean lysozyme concentration was 15.7 (3.38 SE) μg/ml. The concentration of avidin was measured in 113 eggs (n=40, 50, 23 for a-, b-, and c-eggs, respectively) from the same clutches. Mean avidin concentration was 0.65 (0.021 SE) μg/ml.

In a step-down GLMM, lysozyme was found to decline with laying date of the egg and to increase with laying order (Table 1; Fig. 1a and b). The decline in lysozyme concentration with laying date across the entire laying period equals to 1.79 SDs ($\log_{10}$-scale) of the concentration recorded in the whole sample of eggs, while the increase with laying order corresponds to 0.81 SDs. In this analysis, the effects of two-way interactions between laying order and date and egg mass did not attain statistical significance, as was the case for the main and interaction effects of chick sex (P>0.05 in all cases, details not shown). An analysis where the eggs were classified according to their position in the laying sequence (i.e. whether an egg was the last in the clutch or any of the previous eggs; see "Methods") disclosed a significant position by date interaction, with a larger decline of lysozyme activity in the last eggs (see Fig. 1a).

The same dual approach was used to investigate avidin concentration in relation to laying order or, respectively,

Table 1 GLMM of lysozyme and avidin concentrations in the albumen in relation to egg features

	F	df	P	t	Coefficient (SE)	P
Lysozyme						
Model with laying order						
Laying order	16.90	1, 94.6	<0.001		0.192 (0.048)	
Laying date	28.56	1, 74.9	<0.001		−0.034 (0.006)	
Model with position in the laying sequence						
Laying date	27.51	1, 101	<0.001			
Position in laying sequence	15.67	1, 88.2	<0.001			
Laying date × position in laying sequence	4.04	1, 83.8	0.048			
First eggs				3.96	−0.027 (0.007)	<0.001
Last eggs				4.27	−0.052 (0.013)	<0.001
Avidin						
Model with laying order						
Laying order	3.94	1, 103	0.050		−0.059 (0.030)	
Laying date	5.28	1, 92.1	0.024		0.072 (0.031)	
Egg mass	0.03	1, 86.9	0.870		−0.001 (0.004)	
Laying date × egg mass	4.46	1, 93.4	0.037		−0.001 (0.000)	
Model with position in the laying sequence						
Sex	3.62	1, 102	0.060			
Laying date	8.36	1, 97.7	0.005		0.098 (0.034)	
Egg mass	1.37	1, 91.4	0.245		0.012 (0.006)	
Position in laying sequence	0.00	1, 99.7	0.958			
Sex × position in laying sequence	9.24	1, 104	0.003			
Laying date × egg mass	7.63	1, 99.3	0.007		−0.000 (0.000)	
Sex × egg mass	3.83	1, 103	0.053			
Males				0.36	−0.002 (0.004)	0.722
Females				1.82	0.012 (0.006)	0.072

Laying order was classified either according to the laying sequence (i.e. eggs were classified as a-, b-, or c-eggs; 'laying order') or classifying the eggs whether the last one or not in the clutch ('position in the laying sequence'; see "Methods" for further details)

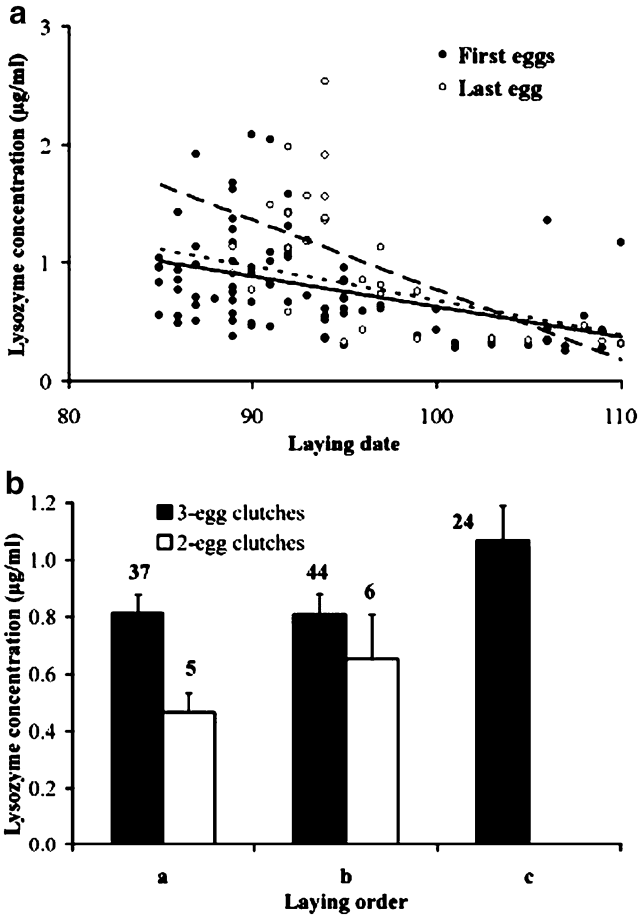

Fig. 1 Lysozyme concentration (log_{10}-transformed) in relation to laying date (**b**) and laying order (**b**). In **a**, eggs are classified according to their position in the laying sequence, i.e. whether they were the last egg or not in their clutch. Linear regression lines are fitted to first eggs only (*solid line*), last eggs only (*dashed line*) or all eggs pooled (*dotted line*). In **b**, mean lysozyme concentration is shown in relation to laying order according to size of the original clutch (two-egg clutches, *white bars*; three-egg clutches, *black bars*). Sample sizes are shown above *bars*

position in the laying sequence, and laying date, egg mass and sex. Contrary to lysozyme concentration, avidin concentration decreased with laying order (Table 1). The slope of this relationship yields a reduction in the concentration by 0.53 SDs from a- to c-eggs. Avidin concentration depended on the combined effects of laying date and egg mass (Table 1). The coefficients reported in Table 1 imply that the decline in avidin concentration with egg mass was more pronounced late in the season. No additional significant effects of egg features or sex emerged. The pattern of variation in avidin concentration was more complex when we included in the analysis the position in the laying sequence instead of laying order. The significant interaction between laying date and egg mass according to the pattern observed in the analysis with laying order was confirmed (Table 1). In addition, the effect of position in the laying sequence differed between male and female eggs (Table 1; Fig. 2). Among the male eggs, avidin concentration decreased with position in the laying sequence, whereas the opposite was true among female eggs. Thus, among first eggs, those carrying a male had larger avidin concentration than those with a female, whereas the opposite was the case among last eggs (Fig. 2). These differences between groups roughly correspond to 0.2–0.4 SDs in avidin concentration. The interaction between egg mass and sex in predicting avidin concentration, though not significant, was retained in the model (Table 1). Avidin concentration was almost invariant with egg mass in male eggs, while it showed a non-significant positive association with mass of female eggs (Table 1).

In the above analyses, lysozyme concentration was found to vary significantly among clutches ($z>2.76$, $P<0.002$ in both step-down models), while avidin concentration did not ($z<1.07$, $P>0.140$).

Albumen avidin and lysozyme concentrations were not significantly correlated ($r=-0.087$, $P=0.364$, $n=112$). In a GLMM with nest as a random factor, lysozyme concentration did not predict avidin concentration ($F_{1,100}=0.03$, $P=0.853$), and the same held true in two models where we included the terms that predicted avidin concentration (see Table 1; details not shown). In a similar set of analyses, no association between the two antimicrobials was found if avidin was used as a predictor of lysozyme concentration (details not shown).

Albumen composition and chick phenotype

Lysozyme albumen concentration and its interactions with sex and egg features did not predict tarsus length or body mass at the ages of 1, 4 and 8 days, nor the response to the

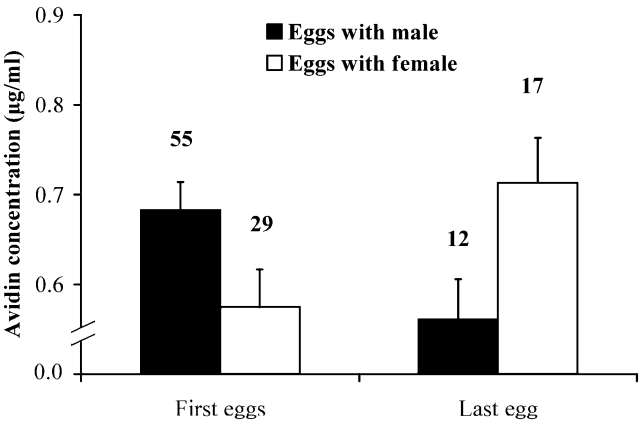

Fig. 2 Means (+SE *bar*) of avidin concentration in relation to position in the laying sequence. First eggs are a- and b-eggs in clutches of three eggs and a-eggs in clutches of two eggs; last eggs are b- or c-eggs in clutches of two or, respectively, three eggs. The four pair-wise differences between sexes within position and between positions within sex were all significant at $P<0.05$, LSD tests

PHA test (main effect of lysozyme: P values at removal always >0.350). Avidin albumen concentration per se or in combination with sex and egg features did not predict chick body mass at any age ($P>0.05$ in all cases) nor tarsus length at hatching ($P=0.36$, details not shown).

However, tarsus length at age 4 was significantly predicted by the interaction term between avidin albumen concentration and laying date ($F_{1,86}=6.69$, $P=0.011$, coefficient=0.257 (0.099 SE); avidin concentration: $F_{1,90.8}=2.56$, $P=0.113$, coefficient=−1.424 (0.891 SE); date: $F_{1,87.6}=8.04$, $P=0.006$, coefficient=−0.211 (0.074 SE)). The coefficients associated with the covariates indicate that an increase in avidin concentration had a stronger negative association with tarsus length early than late in the breeding season. In a model where we controlled for the effects of sex, laying order and original egg mass, tarsus length at age 8 was found to decline with avidin albumen concentration ($F_{1,88.3}=6.71$, $P=0.011$, coefficient=−2.967 (1.145 SE)). Finally, covariation of PHA-response with avidin albumen concentration differed in the two sexes ($F_{1,100}=7.69$, $P=0.007$), with the relation being significantly negative among females (model-derived coefficient=−99.66 (38.08 SE), $t=2.62$, $P=0.010$) and non-significantly positive among males (coefficient=39.48 (32.20 SE), $t=1.23$, $P=0.223$).

Plasma lysozyme concentration and chick phenotype

We investigated if plasma lysozyme concentration at age 4 predicted chick morphology at ages 4 and 8 and PHA response. In these analyses, we included the main effects and two-way interactions between lysozyme concentration and sex or egg features, as well as the main effect of albumen lysozyme concentration. A significant positive covariation was observed between plasma lysozyme concentration and tarsus length at age 4 ($F_{1,107}=4.90$, $P=0.029$, coefficient=3.672 (1.658 SE)). All the other predictors included in this model were not significant ($P>0.115$ in all cases). Similarly, no significant effect was disclosed on any of the other phenotypic traits ($P>0.05$ in all cases). The positive covariation between lysozyme concentration and tarsus length at age 4 was confirmed when tarsus length was considered as a covariate and plasma lysozyme concentration as the dependent variable (details not shown).

The correlation between lysozyme concentration measured in the plasma of the chicks at age 4 and in the albumen of their original eggs was weak and not significant ($r=0.147$, $P=0.123$, $n=112$).

Discussion

In the first part of the study, we analysed allocation of antimicrobials to the eggs in relation to embryo sex and egg features, including egg mass, laying date and laying order or position in the laying sequence (i.e. whether an egg was the last one laid or not), by using a correlational approach. We then analysed the covariation between antimicrobial concentration and chick phenotypic traits, by partly controlling for covariation between parental and egg quality in a cross-fostering experiment.

Lysozyme concentration increased whereas avidin concentration declined with laying order. Differential allocation of lysozyme and avidin to the eggs in relation to laying order is apparently anything but a universal phenomenon. For example, while a study of barn swallows reported a decrease of lysozyme concentration with laying order (Saino et al. 2002b), a more recent study of eight species from three avian orders showed only very limited evidence for variation in avidin, lysozyme or ovotransferrin concentrations with laying order (Shawkey et al. 2008). Such differences in allocation patterns among the species investigated so far have no straightforward explanation. Variation in concentration of antimicrobials in the eggs (e.g. lysozyme) across species spans over six orders of magnitude (White 1985; Saino et al. 2007; Shawkey et al. 2008). It should be noted that species for which information on the covariation between antimicrobial concentration and laying order is available broadly differ in developmental mode, incubation behaviour, clutch size, reproductive strategies (e.g. brood reduction vs survival), as well as habitat and geographic distribution. Thus, different covariation patterns of antimicrobial concentration with laying order could be predicted across species. In fact, a rapidly expanding number of studies have been demonstrating that the concentration of maternal substances in the eggs is not invariant with laying order, and variation patterns broadly differ among species and even conspecific populations (Groothuis et al. 2005; Badyaev et al. 2006a). This variation is expected both under the general hypothesis of maternal adaptive allocation strategies, as well as under conditions where the ability to invest in individual eggs declines along the laying sequence as a result of physiological and/or ecological constraints. Differential allocation of antimicrobials might be tuned to transfer higher defence to the eggs left for longer in the nest, a pattern that is consistent with our finding of a decline in avidin concentration along the laying sequence. However, it could also be predicted that females allocate more antimicrobials to their last-laid eggs because horizontal transmission of bacteria from the other eggs in the clutch or from the body of the incubating parent may be more intense for c-eggs than for previous eggs. This pattern is consistent with our finding of an increase in lysozyme concentration with laying order.

These opposite trends in the concentrations of the two antimicrobials along the laying sequence may reflect specific requirements of first- vs last-laid offspring immune

defence. The two antimicrobials have different functions, with the lysozyme enhancing phagocytic activity and stimulating monocytes (Wellman-Labadie et al. 2007), while the avidin mainly inhibits microbial growth by creating a biotin-free environment (Palmer and Guillette 1991). Admittedly, this interpretation is a posteriori and entirely speculative.

The observed decline of lysozyme concentration late in the season, particularly so for the last eggs in a clutch, might indicate that the ability to transfer antimicrobials to the eggs is influenced by maternal phenotype and environmental factors, suggesting some form of ecological constraint on maternal allocation of antimicrobial proteins. In particular, seasonal trends in lysozyme concentration may reflect variation in average phenotypic quality of mothers (Rubolini et al. 2006; Saino et al. 2008) although a reduction in maternal investment according to the reduced reproductive value of the offspring is also plausible. Females laying late might be in poorer condition and thus lay last eggs of inferior quality compared to last eggs laid by early breeding females. Along the same reasoning, the larger decline of avidin concentration with egg mass late in the season may suggest that late-breeding females are unable to produce eggs qualitatively similar to those laid by early-breeding females for a given egg size.

Several recent studies of birds have investigated sex allocation strategies by analysing the relationship between maternal effects via the eggs and embryo sex (Young and Badyaev 2004; Müller et al. 2005; Badyaev et al. 2006a, b; Romano et al. 2008; reviewed in Pike and Petrie 2003). However, to the best of our knowledge, no study has ever tested for a differential allocation of antimicrobials to male or female eggs. In this study, there was no evidence of sex-related allocation of lysozyme to eggs or that sex-related lysozyme allocation depended on the concomitant effects of egg features. However, variation in avidin concentration with position in the laying sequence (see above for a definition) markedly differed between male and female eggs, as position positively predicted avidin concentration in female eggs whereas the reverse was true among male eggs. Larger concentration of avidin may boost the quality of last-laid (mostly c-) eggs carrying a female and thus promote viability of females that hatch later than their siblings. In a different perspective, sex-related variation in avidin concentration with position in the laying sequence may represent a side effect of mechanisms of sex determination. Although the causal role of maternal hormonal profile at the time of meiosis and ovulation in sex determination is still debated, it has been experimentally shown that high levels of progesterone, the main follicular hormone secreted during meiosis, favour the production of female ova (Correa et al. 2005). Since avidin secretion by laying female birds is under oestrogen and progesterone control (Hertz et al. 1943; Kohler et al. 1968), higher levels of circulating progesterone when females lay their last egg may concomitantly result in a larger proportion of female eggs and a higher avidin concentration.

Importantly, evidence for a differential association between avidin concentration and laying sequence in relation to sex arose when position in the laying sequence, rather than laying order, was included in the analysis. This suggests that variation in egg composition along the laying sequence differs in relation to clutch size. More generally, present results also suggest that analyses of variation in egg quality with laying order should be complemented with analyses where the position in the laying sequence of individual eggs is considered (i.e. whether an egg was, for example, the last in its clutch), particularly in species with marked variation in clutch size.

It should be noted that the latter analysis was based on a larger number of males than females from first-laid eggs, while the converse was true in last-laid eggs (see samples sizes in Fig. 2). At present, given that our analyses are based on all chicks that survived to 4 days of age (when sex of the chicks was determined), we are unable to conclude if this uneven distribution of the sexes between first- and last-laid eggs was due to a difference between the sexes in hatching rate or in post-hatching survival or even to an association between sex and position in the laying sequence (but see Rubolini et al. 2009). Therefore, we cannot rule out the possibility that avidin had interacted with the sex of the embryo in determining hatching and survival prospect of the chicks.

We investigated the covariation of chick phenotypic traits, including body mass, tarsus length and cell-mediated immune response, in relation to concentration of antimicrobials in the original egg. This approach is novel and

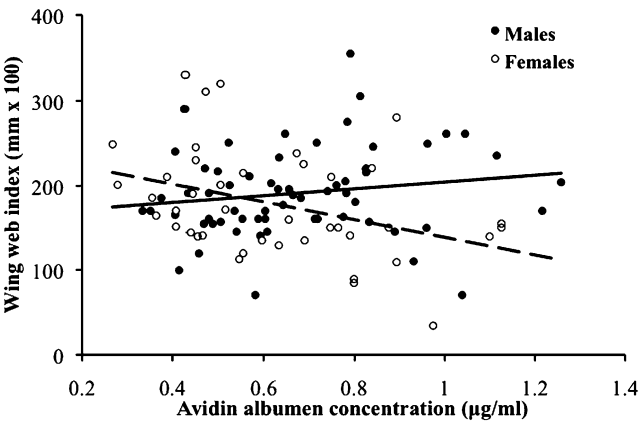

Fig. 3 Wing web swelling response (8 days of age) reflecting a component of T cell-mediated immune response in relation to avidin concentration in the albumen of the original egg in chicks of the two sexes (males, n=63; females, n=41). Linear regression lines fitted to males (*solid line*) and females (*dashed line*) are shown

powerful in that the phenotype of individual chicks is directly related to their embryonic environment. The cross-fostering experimental design allowed us to control for the confounding effects of covariation between egg and parental quality. Yet, the correlational nature of the analyses of variation of chick phenotype in relation to egg quality does not allow inferences about causation, as covariation could result from the effect of other egg components that covary with antimicrobial concentration. Lysozyme in the eggs did not predict chick phenotype. However, cell-mediated immune response of female chicks declined with concentration of avidin in the original egg whereas it was unrelated to avidin concentration in male chicks (Fig. 3). Albumen proteins that exert antimicrobial action have been found to have immunomodulatory and/or immunostimulatory effects (e.g. Sugahara et al. 2000). Although this activity has not been investigated for avidin, it has been documented for, e.g. ovotransferrin, ovomucoid, ovomucin and lysozyme (Palmer and Guillette 1991). If the same action is exerted by avidin, albumen composition and incorporation of albumen components into the embryo tissues may affect development of the immune system and contribute to modulating immune response in the post-hatching period. The differential allocation of avidin to first- or last-laid eggs in relation to their sex, together with the differential effects of avidin concentration on immunity, may suggest that avidin participates in strategic sex-related maternal allocation. However, the adaptive value of the avidin allocation pattern, if any, remains to be elucidated. The negative association between egg avidin and chick tarsus length at age 4 and 8 remains similarly obscure.

Finally, we observed a positive covariation between chick tarsus length at age 4 and concentration of lysozyme in their plasma. This positive relationship may reflect an increase in lysozyme concentration during somatic growth or a positive effect of nutritional condition on both body size, as indexed by tarsus length, and lysozyme concentration.

In conclusion, this study contributes to the scant information on allocation patterns of major antimicrobials to the eggs, showing significant variation in relation to laying order and complex associations with laying date and egg mass. In addition, it provides the first test and evidence that allocation of antimicrobials differs between eggs carrying male or female offspring. Finally, the cross-fostering experiment showed that chick phenotype is predicted by the concentration of antimicrobials in their original egg. The causal nature of these relationships remains obviously questionable. Given that natural selection by bacterial parasites is likely to be intense, though little explored, this study prompts for further investigation of egg maternal effects that mediate antibacterial defence in wild populations.

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