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ORIGINAL PAPER

Eggshell pigmentation has no evident effects on offspring viability in common kestrels

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Abstract Infectious diseases and parasitism are major environmental forces decreasing fitness, and thus individual strategies aimed at preventing pathogen infections, either in an individual or their offspring, should be favoured by natural selection. The mineral fraction and some organic compounds in the shells of bird eggs are considered physical and chemical barriers against pathogen penetration to the embryo. However, eggshell pigment deposition has only recently been considered as a mechanism to resist pathogen penetration into the egg. By wiping the eggshell surface, the amount of pigment and some cuticle proteins were experimentally manipulated for the first time in nature. The effects on egg hatchability and offspring viability measured as nestling condition, immunocompetence and probability of recruitment were investigated in the common kestrel *Falco tinnunculu*s. Protoporphyrin IX and biliverdin IXα to a lesser extent were the only identified pigments. The concentration of protoporphyrin IX and cuticle proteins were significantly reduced in the wiped with respect to the control treatment. Our study shows no evidence of a detrimental effect of the reduction of eggshell pigments on egg hatchability, mortality of the

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chicks during the nesting period, nestling body condition, nestling local immune response to PHA antigen or probability of recruitment. Further research will be necessary to elucidate the direct role of protoporphyrins and other pigments in egg bacterial infection.

Keywords Biliverdin IX · Egg colouration · Eggshell proteins · *Falco tinnunculus* · Maternal effects · Protoporphyrin IX

Introduction

Shell pigmentation in bird eggs has attracted the attention of ecologists for decades (Wallace 1889; Lack 1958). The pigments responsible for eggshell colour and patterning are mainly protoporphyrins and biliverdins (Lang and Wells 1987). Protoporphyrin IX is the most abundant pigment and produces red, brown and black colourations while biliverdin and its zinc chelate generate blue and blue-green colouration (Kennedy and Vevers 1976; Lang and Wells 1987; Mikšík et al. 1996). A number of adaptive hypotheses have been postulated to explain the ecological functionality of eggshell pigmentation and patterning. These hypotheses have focused on aposematism, thermoregulation, egg recognition, crypsis, sexual conflict, structural reinforcement of the shell and signalling (reviewed by Underwood and Sealy 2002; Moreno and Osorno 2003; Kilner 2006; Cherry and Gosler 2010).

Porphyrins are photosensitizer molecules, which in combination with visible light, are used in medicine for photodynamic therapies to inactivate microbes (Nitzan et al. 1994; Wainwright 1998; Brígido-Aparicio et al. 2008). Protoporphyrin IX is among the porphyrins with reported photosensitizing capacity (Kennedy and Pottier 1992; Nitzan et al. 1994). Although the effects of porphyrins on bacteria inactivation and the negative effects of eggshell bacteria on offspring survival and egg hatchability are known (Nitzan et al. 1994; Wainwright 1998; Brígido-Aparicio et al. 2008), none of the adaptive hypotheses proposed to explain eggshell colouration and pigment patterning consider the possibility of eggshell pigments as a mechanism to decrease trans-shell contamination by pathogens. Recently, Ishikawa et al. (2010) have demonstrated in the laboratory and under visible light conditions that bacterial growth was lower in the presence of brown and green eggshell pigmentation compared to white eggshells. These results suggest the possibility that pigments deposited by bird mothers in the eggshell may have evolved in nature as a defence system against microbial pathogens (Ishikawa et al. 2010). Consequently, eggshell pigmentation might also be considered as an adaptive maternal strategy aimed to prevent offspring pathogen exposure during early development.

Selective pressures induced by pathogens have forced the development of defense mechanisms of organisms in order to counterbalance the deleterious effects of microbial infection. Bird parents devote considerable resources to prevent the proliferation of bacteria during early stages of development either by incubating (Cook et al. 2005; Shawkey et al. 2009) or depositing bioactive compounds in the eggs, such as proteins, lipids, vitamins and minerals (Huopalahti et al. 2007). Particularly, some proteins deposited in the yolk (immunoglobulin Y) and in the egg white (e.g. lysozyme, ovotransferrin, riboflavin-binding-proteins, antiproteases) protect embryos against pathogenic microbes through antibacterial activity or by immune system enhancement (Baron and Réhault 2007; Schade and Chacana 2007). Also, eggshell membranes, the calcite layer and cuticle constitute a

first line of physical defence against external pathogens (Berrang et al. 1999; Huopalahti et al. 2007). The most external layer in the egg is the cuticle, an organic layer mainly composed of proteins clothing the shell and plugging the pores, thus preventing microbial penetration (Board and Halls 1973; Berrang et al. 1999). The cuticle also contains the majority of pigments involved in eggshell colouration (Mikšík et al. 2003, Samiullah and Roberts 2013), although in some bird species showing red-brown colouration, such as Falconiformes, pigments form a different layer deposited outside the cuticle (Tyler 1966). The eggshell mineral fraction is associated with an occluded organic matrix composed of proteins, glycoproteins and proteoglycans (Nys et al. 2004). Some of the proteins found in the uterus and constituting the organic components of the eggshell (including the cuticle), such as lysozyme, ovotransferrins, ovocalyxins, ovocleidins or ansocalcins show antimicrobial activity (Hincke et al. 1995; Mikšík et al. 2007, 2010; Wellman-Labadie et al. 2008a; Jonchère et al. 2010), revealing that the chemical action of the eggshell might also play a role in preventing pathogen infections. It is worth noting that these studies were mainly done in chicken eggs and that there is no experiment manipulating these egg compounds to study their effect on egg infection.

The common kestrel *Falco tinnunculus* lays eggs coloured by a reddish-brown pigment (Martínez-Padilla et al. 2010; Fig. 1), although there are no studies identifying the pigment responsible for this colouration in kestrel eggs. In the present study, eggshell pigments and some cuticle proteins were identified and measured. To evaluate the role of eggshell pigments and proteins on egg hatchability, and offspring viability, immunity, mortality, and recruitment we performed a within-nest experiment where pigment concentration and eggshell proteins were reduced. Regarding egg hatchability, other potential functions of the eggshell pigments, such as thermoregulation or structural eggshell strength (reviewed by Kilner 2006) might also affect egg failure. Finally, the natural relationship between pigment concentration and chick condition was analysed in un-manipulated eggs. It is predicted that if eggshell pigments have a role in providing external defences against pathogens or reinforcing eggshells, a reduction in these components will promote detrimental effects on offspring development and survival.

Materials and methods

Study species and study area

Common kestrel females lay one egg every 2 days (Aparicio 1994), and mean clutch size in the study population is five ranging from three to seven eggs (Fargallo et al. 2001). Full incubation (95–100 % of the time) begins on the day, or the day after, the penultimate egg is laid (Wiebe et al. 1998). The study was conducted in a common kestrel population in Campo Azálvaro, central Spain, where kestrels breed mainly in nest boxes (Fargallo et al. 2001). Common kestrels are not strict hole-nesters for which nest boxes designed for this species have to allow entry of direct sunlight (Online resource 1). All nest boxes installed in our population have the same design.

Experimental procedures

In the breeding season of 2009, nest boxes were monitored daily until the first egg was laid, then every two days during egg laying. It is known that protoporphyrin can be removed from newly laid eggs by wiping their surface (Tyler 1966). In every nest, each freshly laid

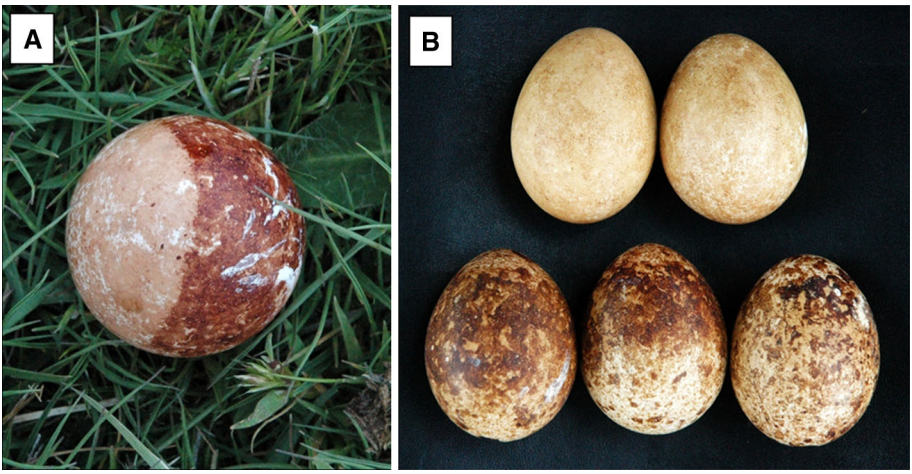

Fig. 1 Pictures showing: **a** experimental protoporphyrin reduction in a kestrel egg on the laying day and **b** a kestrel clutch 23 days after the treatment in which two eggs belong to wiped (*top*) and three to control (*bottom*) treatments

egg was assigned to wiped or control treatments as follows: in 12 nests, eggs occupying positions 1, 3 and 5 in the laying sequence were assigned to the wiped group and eggs 2, 4 and 6 to the control group. In the other 13 nests, eggs 1, 3 and 5 were assigned to the control group and eggs 2, 4 and 6 to the wiped group. Each pattern was applied to nests in an alternating manner. For this purpose, recently laid eggs from the wiped group were gently rubbed with a piece of cotton dampened with sterilized water in order to remove eggshell pigments (see Fig. 1). No between-treatment differences were observed in egg size (volume = width^2 × length × 0.51) or egg position in the laying sequence (ANOVA, both $P > 0.55$). During the year prior to our study (2008) we used the wiping procedure on 10 freshly laid eggs from kestrel nests. In those eggs we recorded the time elapsed from the moment we began wiping the egg until we finished. The longest time needed for wiping eggshells was 54 s; for this reason rubbing was stopped at one minute. Procedures for the control group were done in a similar manner, but in some cases rubbing was ceased when the piece of cotton began showing red colouration in order to avoid removing pigment. We sampled 129 eggs from 25 nests. All cotton pieces were of similar size and only one piece was used for each egg. Sixteen cotton pieces from different nests (8 wiped and 8 control) were used to analyse between-treatment differences in the amount of removed pigments and proteins. These cotton pieces were cut into halves with respect to the red pigment residue, one half to estimate cotton pigment concentration and the other one to estimate cotton protein concentration. The relationship between pigment and protein concentration was analysed within each cotton piece. Cottons were stored in plastic bags at 4 °C during field work and then frozen at −20 °C in the lab few hours later for later pigment and protein analyses. All eggs were marked with an indelible and nontoxic colour felt pen after manipulation (Staedtler® felt pen).

Some eggshell fragments (37 from wiped and 31 from control eggs) in which marks were still visible, could be collected and stored at 4 °C in the field, then frozen at −20 °C for subsequent pigment analyses. Eggshell and cotton samples were lyophilised at the National Museum of Natural Sciences (Madrid) and sent by mail to the Institute of

Physiology, Academy of Sciences in the Czech Republic (Prague), where pigments and proteins were analysed.

Nestling measurements and recruitment

At the time of hatching, nests were checked one to three times per day in order to assign the hatchling to its corresponding egg. Hatchlings were identified by marking them with the indelible pens on the hatching tooth during the hatching process, and later on wings until banding. Twenty-six days after hatching, we took nestling body measurements (wing length, tarsus length and body mass). At that time, the common phytohaemagglutinin-P (PHA) injection assay was used to evaluate in vivo local immunity. The cell-mediated immune response measured as the local inflammatory response to the PHA mitogen reflects the capacity of inflammatory response (Martin et al. 2006) in this species as well (Fargallo et al. 2002; Kim et al. 2013). PHA was injected at the age of 26 days and swelling measured 24 h later with similar procedures as described in Fargallo et al. (2002). For molecular sexing, a blood sample was extracted (Fargallo et al. 2002). Eight (6 %) out of the 129 monitored eggs did not hatch. One hundred and five chicks (87 %) from hatched eggs survived until fledging. Egg origin could be assigned to 95 surviving chicks from 23 nests.

Recruits were monitored during the 3 years following the birth of chicks, as almost all kestrels in our population recruit between 1 and 2 years of age, but some individuals have also been recruited in their third year after fledging (pers. obs.). For this reason local recruitment was based on the ring codes of breeding kestrels captured from 2010 to 2012. Breeding individuals were captured at the nest box during the chick-rearing period.

Eggshell pigments and proteins

The dimethylester form of protoporphyrin IX and biliverdin IXα were the only pigments determined in eggshells and half-cotton pieces, although we have to consider the possible existence of other unknown (and undiscovered yet) red-brown colorant not measured in our analyses. The procedure used by Mikšík et al. (1996) was followed. Methodology for pigment identification and measurement is described in Online Resource 2.

The small size of the collected eggshell fragments was insufficient for both pigment and protein analyses, for which reason proteins were examined in cotton only. The number of eggshell proteins described is relatively low and not all bands could be used to identify proteins. A special database for chicken (*Gallus gallus*) IPI chick and also a general database SwissProt were used. For more precise identification a second round of peptide/sequence searching (error tolerant search) was applied in order to elucidate possible changes in amino acid composition of peptides (compare to proteins described in databases) as well as to apply semitrypsin miscleavage. The set of determined proteins remained small and it was assumed that proteins present in the eggshell of kestrels differ significantly from chicken and have not yet been described. Methodology for protein identification and measurement is described in Online Resource 2. Only proteins present in the five most dense gel bands were considered in the analyses.

Pigment concentration measured in eggshells was expressed as nmol of pigment per gram of eggshell (nmol/g eggshell). In the case of cottons, all the pigment in the cotton piece was extracted and concentration is given in nmol/cotton piece. To quantify proteins extracted from cottons, gel bands resulting from electrophoresis were scanned with a GS-800 Calibrated Densitometer and processed by software for image analysis (Quantity

One©, Bio-Rad, Hercules, CA, USA). Protein concentration values are given in Optical Density (O. D.) units.

Statistical procedures

To test the effect of the experimental manipulation on chick body condition, body mass as response variable was analysed using a General Linear Mixed Model (LMM) in which wing length was included as a covariate to control for size, sex and treatment were used as fixed factors and nest as a random factor. Immune response of the chicks was analysed using a LMM in which body mass was included as a covariate, sex and treatment as fixed factors and nest as a random factor. Egg hatchability, nest mortality and probability of recruitment were analysed using Generalized Linear Mixed Models (GLMM; binomial error, logit function) were the value "1" was assigned to hatched chicks, dead nestlings and recruited individuals and the value "0" was assigned to unhatched eggs, live nestlings and unrecruited individuals. In GLMMs for egg hatchability and nest mortality, treatment was included as a fixed factor and nest as a random factor. In the GLMM for recruitment, the effects of sex (fixed factor) and body mass (covariate) were analysed. Nested designs were run by nesting experimental treatment within kestrel nests. In all models, except those for egg hatchability and nest mortality, two term interactions were also analysed. In these analyses, different sets of models with possible combinations of independent variables were constructed. We used Akaike's information criterion corrected for small sample size (*AICc*) for model selection. The best model was the one with the lowest *AICc* value with a difference > 2 from the second best model. Pigment and protein concentration in eggshells and cottons were analysed by using ANOVAs in which concentration was the dependent variable and the experiment was a fixed factor. Pigment concentration values were log transformed. In order to explore the natural relationships between eggshell pigment content and nestling growth-immunocompetence, pigment eggshell concentration from the control group was analysed using LMMs, in which sex was included as a fixed factor and nest as a random factor. All analyses were two-tailed and were performed in SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA). Mean $\pm$ SD are given.

Results

Pigments and proteins

Chromatography analyses confirmed the existence of protoporphyrin IX and very low concentration of biliverdin IXα in kestrel eggshells. Eggshell concentration of both pigments was lower in wiped than in control eggs (Table 1). Analyses done in cottons showed that the concentration of protoporphyrin was higher in wiped than control groups, while no differences were found in biliverdin concentration (Table 1).

Proteins found are listed in Online Resource 3. Eighteen (49 %) out of 37 proteins identified in cotton samples were keratins probably belonging to hair, scales and feathers present in kestrel nest material (Online Resource 3). Lizards, micromammals and birds are common kestrel prey whose remains are commonly found at the nests in our population (Navarro-López et al. 2014), although some keratins can also naturally occur in the eggshell. In order to know whether the wiped treatment reduced the concentration of proteins present in the cuticle, the differences in the band density were analysed. Bands of 105 kDa correspond to unidentified proteins, 69 kDa to proteins with presence of serum albumin

Table 1 Between-treatment differences in pigments found in shells of common kestrels *Falco tinnunculus* and in pigments and proteins found in cotton pieces

Term	*df*	*F*	*P*	Control	Wiped
Eggshell pigments (nmol/g eggshell)					
Protoporphyrin IX	1.21	26.3	<0.001	58.0 ± 45.3	25.1 ± 17.9
Biliverdin IXα	1.21	4.5	0.047	0.02 ± 0.01	0.00 ± 0.00
Pigments in cottons (nmol/cotton piece)					
Protoporphyrin IX	1.16	70.5	<0.001	4.3 ± 2.9	152.3 ± 110.4
Biliverdin IXα	1.16	2.6	0.133	0.00 ± 0.00	0.02 ± 0.0
Proteins in cottons(O.D.)					
105 kDa band	1.14	8.4	0.012	0.7 ± 0.2	1.1 ± 0.5
69 kDa band	1.14	6.9	0.020	1.4 ± 0.2	1.9 ± 0.5
45 kDa band	1.14	13.0	0.003	0.9 ± 0.4	1.4 ± 0.7
38 kDa band	1.14	15.4	0.002	0.9 ± 0.3	1.5 ± 0.7
20 kDa band	1.14	6.3	0.025	0.8 ± 0.4	2.4 ± 2.1

Note that contrary to eggshells, cotton used in the wiped treatments is expected to have more cuticle components (pigments and proteins) than cotton used in the control treatment. Differences in eggshells were analysed using General Linear Mixed Models (LMM) and General Linear Models (LM) were used for pigments in cottons. Mean ± SD are given. Note that concentrations found for biliverdin were below the detection error

precursor, 45 kDa with presence of ovocleidin-116, 38 kDa with presence of OMP38 and CACNA1C-calcium channel and 20 kDa with presence of ovocalyxin-32 and connective tissue growth factor. Cottons from the wiped group showed significantly higher concentrations of proteins in all analysed bands (Table 1).

Egg hatchability and chick viability

The experiment had no significant effects on egg hatchability, nestling mortality, nestling body condition, immune response to PHA or recruitment (Table 2).

Protoporphyrin IX and biliverdin IXα in un-manipulated conditions

Controlling for nest in the control group, eggshell protoporphyrin IX concentration was not significantly correlated with egg volume or egg position in the laying sequence, nestling body condition or immune response to PHA (LMM, all $P > 0.18$). No significant correlations were found for eggshell biliverdin IXα concentration (LMM, all $P > 0.36$). Concentrations of both pigments correlated positively in the eggshell (LMM, $F_{1,9} = 8.7$, $P = 0.016$, *estimate* $= 3.679$, $n = 31$).

In the case of cottons, except for proteins located in the 38 kDa band (OMP38 and CACNA1C-calcium channel), the remaining proteins showed a negative trend with protoporphyrin IX concentration (all "r" were negative) measured in cotton used for wiping eggshells (Online Resource 4); however, only the 45 kDa band (associated with the presence of ovocleidin-116) and 20 kDa band (associated with the presence of ovocalyxin-32 and connective tissue growth factor) were significantly correlated (Online Resource 4). No significant correlations were found for biliverdin IXα concentration (all $P > 0.11$). Concentrations of both pigments were not correlated in the cottons ($P = 0.240$).

Table 2 Linear mixed models for the effect of treatment on each dependent variable in common kestrels *Falco tinnunculus*

Effect	Estimate	SE	(95 % CI)	*df*	*F*	*P*
Hatchability						
Treatment	−0.5244	0.76	(−2.08, 1.04)	24	0.5	0.495
Mortality 1 (dead chicks)						
Treatment	0.2125	0.52	(−0.86, 1.28)	24	0.2	0.686
Mortality 2 (unhatched eggs + dead chicks)						
Treatment	0.0533	0.72	(−1.55, 1.44)	24	0.00	0.942
Body mass						
Treatment	−3.0572	4.01	(−11.39, 5.28)	21	0.4	0.561
Sex	7.4351	4.54	(−2.25, 17.12)	15	12.0	0.003
Wing length	0.8674	0.16	(0.53, 1.20)	29	28.0	0.000
Treatment × sex	9.5448	6.07	(−2.86, 21.96)	29	2.5	0.213
Immune response to PHA						
Treatment	−5.4870	19.7	(−46.51, 35.54)	21	0.0	0.994
Sex	461.74	177	(82.45, 841.0)	15	6.9	0.019
Body mass	2.4679	0.72	(0.98, 3.96)	28	7.8	0.010
Treatment × sex	11.1971	29.6	(−49.36, 71.75)	28	0.1	0.708
Recruitment						
Treatment	−0.0161	0.68	(−1.43, 1.40)	21	0.0	0.981
Sex	0.2134	0.70	(−1.20, 1.62)	46	0.1	0.765

Estimates, standard errors (S.E), 95 % confidence intervals (95 % CI), degrees of freedom (*df*), *F* and *P* values are shown. Hatchability (GLMM): $AICc = 710.8$, $n = 129$. Mortality-1 (GLMM, considering only dead chicks at the nest): $AICc = 633.5$, $n = 121$. Mortality-2 (GLMM, Including dead chicks at the nest and unhatched eggs): $AICc = 652.2$, $n = 129$. Nestling body mass (LMM): $AICc = 760.8$, $n = 92$ (*AICc* for the second best model = 768.9. This model included a non-significant treatment x sex interaction). Immune response to PHA (LMM): $AICc = 1043.1$, $n = 92$. (*AICc* for the second best model = 1,051.5. This model included a non-significant treatment x body mass interaction). Recruitment (GLMM): $AICc = 466.3$, $n = 92$ (*AICc* for the second best model = 468.0. This model included a non-significant effect of nestling body mass)

Discussion

Since reddish protoporphyrin IX was found in high concentrations in the shell is feasible to assume that the colouration observed in kestrel eggshells is due to this pigment. Some hours after the egg was laid, the shell pigments of kestrel eggs can be partly removed by softly rubbing the eggshell with moistened cotton. This can be done because some the mottled reddish pigments found in Falconiform species are not embedded within the shell matrix, but is deposited in the outermost layer on the cuticle (Tyler 1966). Furthermore, a negative correlation between protoporphyrin and cuticle protein concentration found in cottons was shown. This suggests two possibilities: (1) kestrel females deposited less cuticle protein on more pigmented eggshells, or (2) a high proportion of pigment covering the cuticle prevented us from removing more proteins within it. Both cases indicate a lower external exposure of cuticle proteins in more pigmented eggshells. Long and complex treatments with EDTA or N1 CLH to dissolve the shell are necessary to extract proteins forming the organic matrix of the outer mineral layer in the eggshell (see Wellman-Labadie et al. 2008b, c; Mikšík et al. 2010). Therefore, the gentle manipulation we applied

on the eggs only, or mainly, reduced the pigment layer and some cuticle compounds. Our findings show that the significant reduction of protoporphyrin IX concentration to less than half of the average amounts and the reduction of biliverdin IXα and other proteins did not significantly affect egg hatchability, mortality of the chicks during the nesting period, nestling body condition, nestling local immune response to PHA antigen or recruitment.

Results of this study do not support our initial prediction: that reducing protoporphyrin, and other essential eggshell compounds, will have detrimental effects on offspring development, condition and/or recruitment. This prediction was derived from the effects of porphyrins and other eggshell compounds (biliverdin and ovocalyxin-32) on bacteria inactivation and the negative effects of eggshell bacteria on offspring survival and egg hatchability. The only known work investigating the role of pigments in bacteria proliferation (Ishikawa et al. 2010) found that survival of gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) cultivated in the lab decreased drastically in the presence of brown protoporphyrin (isolated and in eggshell) and green biliverdin (in eggshell) pigments under artificial light conditions. This result was not observed under dark conditions or for gram-negative bacteria (*Escherichia coli* and *Salmonella enteritidis*) under either artificial light or dark conditions, indicating that eggshell pigments possess photoantimicrobial activity. Also, protoporphyrin may act chemically, by killing bacteria or inhibiting their growth, much like antibiotic substances (see Martín-Vivaldi et al. 2010), or physically, by blocking bacteria installation on the rough and porous eggshell surface (Berrang et al. 1999; Baggott and Graeme-Cook 2002). Furthermore, it has been reported that bacteria growth diminishes in the presence of outer eggshell matrix and cuticle extracts of domesticated and wild bird species (Wellman-Labadie et al. 2008b, c), while the effect of the different eggshell compounds could not be separated. Among the proteins we could identify, only ovocalyxin-32 has been suggested to have antimicrobial activity (Xing et al. 2007), however we cannot exclude that our manipulation did not reduce the presence of other unknown proteins with a similar function. Taking into account these potential beneficial effects, our study however did not detect detrimental effects on chicks when these eggshell compounds were reduced, not even in the immune response to PHA. Similarly, our findings did not perceive evidence of a weakening in the shell, such as breaks or any potential effect of this on hatching success.

There are several scenarios on which to speculate given our results. First our manipulation may not have been powerful enough to detect effects on egg hatchability or chick viability. Second, bacteria could have been removed from the eggshell in both treatments, as they are easily eliminated from eggshell surfaces by wiping with wet swabs (Cook et al. 2005; Peralta-Sánchez et al. 2012; Shawkey et al. 2009). If that is the case, the effect of the treatment would be expected to be even lower. Third, other more subtle effects on more sensitive immunological functions to microbial infection, such as humoral, interferon and interleukin responses (e.g. Dorman and Holland 1998; Okamuraa et al. 2004) were not investigated in this study. Fourth, not many highly virulent pathogen microbes exist in our kestrel population. And fifth, the particular environmental conditions experienced in a given year or location may have prevented a high proliferation of pathogen microbes (Ruiz-de-Castañeda et al. 2011; Wang et al. 2011; Peralta-Sánchez et al. 2012) that may affect the potential detrimental effect of bacteria on hatching success (Wang et al. 2011). Although eggshell pigments seem to exert some control on microbe proliferation in lab conditions (Ishikawa et al. 2010), this important result that lends a new perspective to our understanding of eggshell colouration needs to be confirmed in nature. Our experimental removal of eggshell pigments in a wild kestrel population did not find any significant effects on offspring health or recruitment. Further experiments, especially those focussed

on determining the role of pigments on the composition and proliferation of the eggshell bacteria community are required in nature.

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