Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success

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Summary

1. Animals live in a bacterial world, and detecting and exploring adaptations favouring mutualistic relationships with antibiotic-producing bacteria as a strategy to fight pathogens are of prime importance for evolutionary ecologists.

2. Uropygial secretion of European hoopoes (*Upupa epops,* Linnaeus) contains antimicrobials from mutualistic bacteria that may be used to prevent embryo infection. Here, we investigated the microscopic structure of hoopoe eggshells looking for special features favouring the adhesion of antimicrobial uropygial secretions.

3. We impeded female access to the uropygial gland and compared microscopic characteristics of eggshells, bacterial loads of eggs and of uropygial secretion, and hatching success of experimental and control females. Then, we explored the link between microbiological characteristics of uropygial secretion and these of eggs of hoopoes, as well as possible fitness benefits.

4. The microscopic study revealed special structures in hoopoes' eggshells (crypts). The experimental prevention of females' gland access demonstrated that crypts are filled with uropygial secretion and that symbiotic enterococci bacteria on the eggshells come, at least partially, from those in the female's uropygial gland. Moreover, the experiment resulted in a higher permeability of eggshells by several groups of bacteria and in elimination of the positive relationships detected for control nests between hatching success and density of symbiotic bacteria, either in the uropygial secretion of females or on the eggshell.

5. The findings of specialized crypts on the eggshells of hoopoes, and of video-recorded females smearing secretion containing symbiotic bacteria at a high density onto the eggshells strongly support a link between secretion and bacteria on eggs. Moreover, the detected associations between bacteria and hatching success suggest that crypts enhancing the adhesion of symbiont-carrying uropygial secretion likely protect embryos against infections.

Key-words: antimicrobial defences, birds, co-evolution, mutualism, symbiotic bacteria, uropygial gland

Introduction

Animals using chemicals from metabolism of symbiotic bacteria against pathogenic micro-organisms and parasites is one of the most exciting discoveries in ecological immunology. Bacteria produce an extraordinary diversity of antimicrobial compounds to inhibit other micro-organisms (Ji, Beavis & Novick 1997; Riley & Wertz 2002) and, when they are in symbiotic associations with animals, such chemicals may function for hosts as defences against pathogenic micro-organisms and parasites. For instance, chemicals produced by symbiotic bacteria are known to protect ants' gardens, wood galleries of beetles and

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embryos of shrimp, lobsters, squid, wasps and some sala-manders from pathogenic bacteria and/or competitor fungi (Gil-Turnes, Hay & Fenical 1989; Barbieri *et al.* 1997, 2001; Currie *et al.* 1999; Kaltenpoth *et al.* 2005; Cardoza, Klepzig & Raffa 2006; Banning *et al.* 2008; Scott *et al.* 2008), or aphid hosts from their parasitoids (Oliver *et al.* 2003), which illustrate the great variety of animals that use antibiotic-producing bacteria as defence against pathogenic infection.

Animals live in a bacterial world, and exploring their physiological, morphological and behavioural characteristics facilitating colonization of beneficial microbes is of prime importance for understanding animal–bacteria interactions in general and the evolution of the mutualistic relationship in particular (McFall-Ngai *et al.* 2013). Animal behaviour plays a central role in the establishment and regulation of beneficial association with micro-organisms. It is, for instance, the case of newborn bug nymphs feeding on capsules of symbionts (Hosokawa *et al.* 2008) or juvenile iguanas eating soil or faeces (Troyer 1984) (see, Ezenwa *et al.* 2012). Some animals may even have evolved specialized storage compartments on their body as those described for pine beetles (Scott *et al.* 2008), the accessory nidamental gland of squids (Barbieri *et al.* 2001) or the specialized crypts (depressions in the tegument) of fungus-growing ants where beneficial bacteria grow (Currie *et al.* 2006). As far as we know, these specialized compartments have only been described in insects and are interpreted as the result of a co-evolutionary process between micro-organisms and animal hosts, favouring the survival of the microbes specialized in protecting hosts against the attack of antagonistic ones (Currie *et al.* 2006).

The hoopoe (*Upupa epops,* Linnaeus) is the only bird species in which the use of protective bacteria-produced antibiotic substances has been suggested (Soler *et al.* 2008). Nesting hoopoe females and nestlings but not males secrete a brown and malodorous uropygial secretion that harbours bacteria with antimicrobial capabilities at a high density (Martín-Platero *et al.* 2006; Soler *et al.* 2008; Martín-Vivaldi *et al.* 2010; Ruiz-Rodríguez *et al.* 2012; Ruiz-Rodriguez *et al.* 2013) that protects feathers from bacterial degradation (Ruiz-Rodriguez *et al.* 2009) and that may also help to prevent trans-shell infection of eggs by pathogenic bacteria (Soler *et al.* 2008). Shells of avian eggs are usually quite smooth, with the outmost eggshell layer of protein-hydrophobic nature (Becking 1975; Tullett 1984; Mikhailov 1997) where uropygial secretions would hardly result adhered. However, some evidence suggests that colour changes experienced by hoopoe eggs few days after being laid may be caused by the deliberated impregnation by incubating females with their uropygial secretions (Martín-Vivaldi *et al.* 2009). This colour change may therefore suggest that eggshells of hoopoes may have special structures to maximize the amount of uropygial secretion containing bacterial symbiont added to the shell, which would therefore enhance protection of embryos against pathogenic infection. Here, we explored characteristics of eggshells of hoopoes that may enhance the adhesion of uropygial secretion containing antimicrobials from symbiotic bacteria, and its possible beneficial effects protecting embryos against trans-shell pathogenic infections.

The aims of the study were therefore (i) to explore the hypothesis that hoopoes use their uropygial gland secretion onto the eggshells likely to protect embryo from pathogenic infection and (ii) to examine hoopoe eggshells microscopically looking for special places facilitating retention of uropygial secretion. To achieve these objectives, we performed an experiment preventing female access to the uropygial gland secretion (hereafter UGS) and explored the effect of the experiment on (i) microscopic eggshell structure, (ii) microbial communities inside the egg and on the outer shell surface and (iii) hatching success. If uropygial secretion of females accumulates on the eggshell, those of experimental females at the end of the incubation period should show (i) a different microscopic structure due to the absence of UGS, (ii) less abundant symbiotic bacteria (enterococci) on eggshells, (iii) increased trans-shell bacterial contamination and (iv) reduced hatching success than those of control females. We also video-recorded incubating females and compared the microscopic structure of eggshells of hoopoes with those of some other species of birds.

Bacterial abundances were estimated for aerobic mesophilic bacteria, *Enterococcus* (which included the symbiotic bacteria of hoopoes), and two groups that included well-known pathogens of avian embryos, *Staphylococcus* and bacteria belonging to the family *Enterobacteriaceae*. We analysed bacterial abundance in the uropygial secretion, on the eggshell and inside unhatched eggs of hoopoes and looked for relationships among these variables. Moreover, we also relate hatching success and bacterial density on eggshells and uropygial secretion. We predicted that (v) abundance of mutualistic enterococci on the eggshell should be positively related to hatching success, while (vi) the effect of staphylococci and *Enterobacteriaceae* on hatching success should be negative.

Materials and methods

STUDY SITES AND STUDY POPULATIONS

Fieldwork was performed during the breeding seasons 2008–2010 in Hoya de Guadix (37°18′N, 38°11′W), southern Spain, where hoopoes, spotless starlings (*Sturnus unicolor,* Temminck), rollers (*Coracias garrulus*, Linnaeus), scops owls (*Otus scops*, Linnaeus) and house sparrows (*Passer domesticus*, Linnaeus) breed within nest boxes placed in trees or buildings, and rock doves (*Columba livia*, Gmelin) nest on shelves in abandoned house-caves. The experiment to prevent female hoopoes from having access to the uropygial gland was performed in 2009–2010 in a captive population maintained at Hoya de Guadix (Granada, University of Granada) and in Finca Experimental 'La Hoya' (Almería, Estación Experimental de Zonas Áridas, CSIC) since 2008. Breeding

pairs were housed in independent cages of at least 3 m × 2 m × 2 m placed in the open, with access to soil and provided with live food (crickets and fly larvae) and meat (beef heart) *ad libitum*. The treatments were balanced within each captive subpopulation.

EXPERIMENTAL MANIPULATION OF FEMALE ACCESS TO UROPYGIAL GLAND

Access of female hoopoes to the uropygial secretion was manipulated in 19 females by using sterile cat catheters (Buster, width 1·0 mm) inserted in the opening of the papilla of the uropygial gland connected with flexible silicone tubes with 8 mm width and 70 mm length that served as a store of the secretion, by means of small sections of two tubes of intermediate widths (Fig. 1). The flexible tube connected with the catheter was plugged to the gland, covering the entrance. It was fixed to the skin with surgical glue (3M Vetbond) and adhesive bandage (Fig. 1a). Therefore, experimental birds did not have access to uropygial gland secretion, which was retained in the plastic tube. Every second day, we removed and changed the plastic tube and checked the fixation of the apparatus on the gland opening. In order to control for possible effects in females of having a plugged tube of 7 cm on their uropygial gland, a first group of control birds (control I, $n = 21$) were provided with a similar structure of tubes, but in this case, the tube did not cover the gland entrance and thus did not prevent normal access of females to secretions (Fig. 1b). A third group of breeding females (control II, $n = 15$, Fig. 1c) were visited and handled at the same rate as those of experimental and control I groups. Most females bred more than once during the two years of study and were randomly assigned to different experimental treatments when caring for different clutches. During incubation and the first half of the nestling period, female hoopoes stay within the nest the whole day, and all food that they consume is provided by the male (Martín-Vivaldi *et al.* 1999; Krištín 2001). At this time, they only leave the cavity for defecation a few times daily. Although the experimental

manipulation may slightly affect flight capacity of females, they easily flied back to the nest. None of the experimental females died in the course of the experiment. Moreover, the experiment did not affect the body condition of females as those that did [$n = 11$; initial weight: mean (SE) = 73·7 (1·5) g; weight loss: mean (SE) = 2·5 (1·7) g] or did not wear [$n = 5$; initial weight: mean (SE) = 75·0 (1·0) g; weight loss: mean (SE) = 1·2 (2·3) g] flexible silicon tubes (repeated-measures ANOVA, $F_{1,14} = 0·18$, $P = 0·68$), or that of females with [$n = 12$; initial weight: mean (SE) = 75·3 (1·2) g; weight loss: mean (SE) = 2·5 (1·6) g] and without [$n = 4$; initial weight: mean (SE) = 70·8 (1·5) g; weight loss: mean (SE) = 0·75 (2·4) g] access to uropygial secretion (repeated-measures ANOVA, $F_{1,14} = 0·31$, $P = 0·59$) did not differ in body mass reduction experienced from first to second reproductive events (body mass measured at the beginning of each breeding attempt, when laying the first eggs). Thus, we are confident that our experimental manipulation did not negatively affect the adequate nourishment and health of breeding females in our conditions of captivity. The experiment was conducted according to relevant Spanish national guidelines (Real Decreto 1201/2005, 10th October) and under the permission of Junta de Andalucía, Environmental Management Agency, which authorized the establishment and maintenance of the captive breeding population (Resolution of 14th of April 2008) and field protocols (Resolutions of 14th of April of 2008, and 23rd of March of 2010).

MICROSCOPIC STUDY OF EGGSHELLS

During the breeding seasons 2009–2010, we collected recently laid (<24 h) eggs from nests that were partially depredated or abandoned during laying by hoopoes ($n = 10$), scops owls ($n = 3$), rollers ($n = 2$), rock doves ($n = 2$), spotless starlings ($n = 4$) and house sparrows ($n = 5$). At hatching, we also collected shells from nests within the first 12 h after hatching [scops owls ($n = 2$), rollers ($n = 4$), rock doves ($n = 3$), spotless starlings ($n = 4$), house sparrows ($n = 2$) and hoopoes ($n = 10$)]. Only a single egg was used
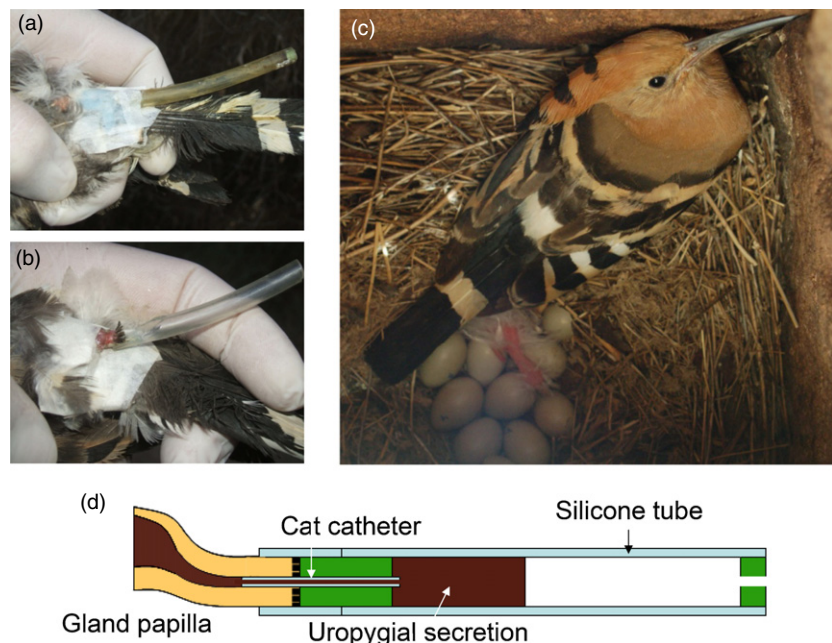

Fig. 1. The experiment consisted of impeding (experimental, a) the access by some females to uropygial secretion with flexible silicone tubes (d). Other females wore silicone tubes that did not covered gland entrance, thus they had access to the secretions (control I, b), while a third group of females did not wear silicone tubes (control II, c).

per clutch. In addition, eggshells from experimental hoopoe nests at the end of incubation (i.e. eggshells from hatchlings) were also collected for microscopic study of the effect of the experiment ($n = 80$ eggshells from 43 clutches). A small fragment (about 3 mm × 3 mm) of the equator of each egg was fixed in 2·5% glutaraldehyde in PBS for 24 h, introduced three times in PBS to wash up eggshells from glutaraldehyde and stored at 4 °C in distilled water to avoid crystalline mineral precipitation until the microscopic analyses. Afterwards, samples were air-dried overnight and coated with approximately 10 nm of gold/palladium using a Nanotech Semprep2 sputter coater. Observations were made with a Hitachi S-510 scanning electron microscope (SEM) at the Scientific Instrumental Services of the University of Granada, and images were digitalized with Scan Vision. Three randomly selected fields of each piece of eggshell were photographed at 900× magnification. In the case of hoopoe eggshells, the photographs were used to evaluate where there exists any kind of crypts, their abundance and the percentage of them filled with secretion.

The SEM images of recently laid eggs were examined looking for shell textures identifiable as cuticle, a fissured external layer with the appearance of dried mud (Becking 1975) or other shell accessory material covering pores (Tullett 1984). The first two images of eggshells of ten randomly selected hoopoe females (five wild and five captive) were processed with Adobe Photoshop 7·0 to estimate the percentage of eggshell surface occupied by crypts (i.e. in units of pixels). These estimates were repeatable within eggs ($R = 0·78$, $F_{9,10} = 7·93$, $P = 0·002$), and therefore, we used the mean values for eggs in subsequent analyses. Furthermore, eggshells of wild and captive females did not differ in the percentage of their surface occupied by crypts at laying [wild females: mean (SE) = 31·9 (3·22); captive females: mean (SE) = 33·9 (1·68); $F_{1,8} = 0·30$, $P = 0·60$], and consequently, we pooled data of these nests to describe eggshell structure.

For the estimation of percentage of eggshell crypts that were or were not filled with material, we used three different pictures of each of the 172 studied hoopoe eggs (except for one with only two available pictures). Each of the 515 pictures received one randomly assigned identification number and was evaluated independently by two observers (JMP-S & LA) who were unaware of the identity of either the eggs or their experimental treatment. The estimates of percentage of eggshell crypts that were filled with material were repeatable between observers ($R = 64·9$, $F_{514,515} = 4·69$, $P < 0·0001$), and thus, we used mean values. Moreover, within-egg estimates were also repeatable ($R = 88·5$, $F_{171,343} = 24·13$, $P < 0·0001$), suggesting that the estimates reflect the state of the entire eggshell.

ESTIMATING BACTERIAL LOADS OF UROPYGIAL SECRETIONS, EGGSHELLS AND EGG CONTENTS

While sampling eggs, females or secretions, we wore new latex gloves washed with ethanol 70% to avoid among-nest contamination.

Secretion of incubating females

At the beginning of laying (with the first or second egg) and at the end of incubation (day 15 after the first egg was laid), we collected 5 μL of uropygial gland secretion from incubating females to estimate bacterial loads. Samples were collected with a micropipette directly from within the uropygial gland after feathers around the gland were separated and washed with ethanol to avoid contamination. The secretion was introduced in a sterile Eppendorf tube and stored at 4 °C until processed in the laboratory within the following 24 h. In the laboratory, we added 45 μL of sodium phosphate buffer (0·2 M, pH 7·2) and vigorously mixed it by repeated pipetting before inoculating growth media. The total volume of secretion available in the gland was also estimated (in μL) by using a 1- to 10-μL micropipette (Finnpipette).

Eggshells

We sampled a single egg per clutch at the end of incubation (day 15 after the first egg was laid) during the 2010 breeding season by cleaning the entire eggshell with a sterile swab slightly wet with sterile sodium phosphate buffer (0·2 M, pH 7·2). The swab was preserved in an Eppendorf tube at 4 °C containing the sterile buffer until laboratory analyses during the following 24 h. Estimates of bacterial load were standardized to eggshell surface sampled, which were estimated according to the formula $S = 3L^{0·771}B^{1·229}$ following Narushin (2005), where S is the surface in cm², L the egg length and B the egg breadth.

Unhatched egg contents

To estimate the effects of experimental manipulation on transshell bacterial contamination, during the 2009–2010 breeding seasons, we collected all eggs ($n = 60$ from 23 different clutches) that failed to hatch in nests under control ($n = 16$ clutches) and experimental ($n = 7$ clutches) treatments and that did not show any sign of breakage. These eggs were collected two or three days after the expected hatching date for the last egg in the clutch. After disinfection of the eggshell surface with 70% ethanol, unhatched eggs were broken in a laminar flow cabinet and after carefully cutting egg membranes, whenever possible, 300 μL of yolk and 300 μL of egg white were collected and homogeneously mixed using a sterile single-use inoculation loop. Afterwards, 300 μL of this mix was used for the final dilution. When egg membranes were deteriorated and it was impossible to extract yolk and egg white separately, egg contents were homogeneously mixed using a sterile single-use inoculation loop and 300 μL of mixture collected (embryos were separated before mixture and in no case were longer than 2 mm; i.e. embryos that died during the first few days of incubation) (for further details see Soler *et al.* 2011). Samples were diluted in 300 μL of sterile sodium phosphate buffer (0·2 M, pH 7·2) in an Eppendorf tube from where bacteria were cultured in Petri dishes.

Culturing bacteria

In the laboratory, samples were collected from Eppendorf tubes after vigorously shaking them in vortex for at least three periods of 5 s, and afterwards, 100 μL (eggshell samples) or 5 μL of the dilution (secretions and unhatched egg contents) was surface-plated onto Petri dishes containing different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used tryptic soy agar (TSA), a broadly used general medium to grow mesophilic bacteria, and three selective media: Kenner faecal agar (KF) for growing bacteria belonging to the genus *Enterococcus*, Vogel-Johnson agar (VJ) with addition of potassium tellurite for growing bacteria of the genus *Staphylococcus*, and Hektoen enteric agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*.

Enterobacteriaceae and *Staphylococcus* sp. are saprophytic and opportunistic bacteria (Houston, Saunders & Crawford 1997; Singleton & Harper 1998; Cook *et al*. 2005) that live on skin, hair and feathers of mammals and birds (Krieg & Holt 1984). They commonly appear on avian eggshells and are known to include pathogens for avian embryos (Bruce & Drysdale 1994). Enterococci, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce & Drysdale 1994) and are opportunistic pathogens (Franz, Holzapfel & Stiles 1999), although some species might also have beneficial effects (Moreno *et al*. 2003; Soler *et al*. 2008, 2010).

Egg samples were inoculated both in the general (TSA) and the three selective media (KF, VJ and HK), while the uropygial secretions were inoculated only in TSA (we have previously shown that most aerobic cultivable bacteria in hoopoe UGS are enterococci (Soler *et al*. 2008) so we can assume growth in TSA from these samples to correspond mostly to enterococci). Cultures were incubated aerobically, in the case of uropygial secretions at 28 °C for 12 h and egg samples at 37 °C for 72 h. When the number of bacterial colonies was too dense to count, we performed serial dilutions to obtain isolated colonies allowing us to estimate the bacterial density of the sample. Bacterial density was expressed as colony-forming units (CFU) per cm^2 (for repeatability estimates of intraspecific variation of eggshell bacterial loads see Peralta-Sánchez *et al*. 2012), or as CFU per μL of sample (secretions and unhatched egg contents).

HATCHING SUCCESS

We estimated hatching success of nests as the percentage of eggs that successfully hatched among those that remained in the nests at hatching. Some nests were deserted after sampling the eggs at the end of incubation, and thus, sample sizes differ from those involving only bacterial loads. We collected information of hatching success for 40 nests (23 control and 17 experimental). Three of the control nest estimates of bacterial density of UGS of females were not available, and thus, sample size for the relationships between hatching success and bacterial density of the UGS and of the eggshell differs.

STATISTICAL ANALYSES

Statistical analyses were performed using the software STATISTICA 10 (Statsoft Inc. 2011). We did not collect all information for all reproductive attempts of experimental females, and thus, sample sizes used in different analyses differ.

We estimated the effects of early vs. late incubation on the percentage of filled crypts (structures found on eggshells, see Results), both with natural and cavity nests by means of general linear mixed models (GLMM) with incubation (laying vs. hatching), kind of nests (wild vs. captivity) and the interaction between these two variables as fixed factors. Nest identity, nested within the interaction between incubation and kind of nest, was included as a random factor to account for the non-independence of estimates within the same nests. Since the effects of incubation did not differ for eggs incubated in wild or captivity nests (see Results), we did not include this factor in subsequent models.

The effects of the experiment preventing access by females to the uropygial secretion on percentage of crypts in the eggshells filled with material (performed only with captive females) were tested with estimates at the end of incubation as the dependent variable, treatment (experimental, control I and control II) as the fixed factor and nest identity nested within treatment as the random factor to account for the non-independence of estimates within the same nest.

Bacteria counts

Except for mesophilic bacteria, bacterial loads on eggshells or in the contents of unhatched eggs were not normally distributed even after Box–Cox transformation. Thus, we conservatively used ranked values for our analyses. Date of collection did not explain significant variance in eggshell bacterial loads ($P > 0.30$) and thus was not included in the model. Moreover, we did not find any significant differences in bacterial loads of eggshells of the two types of control females [MANOVA, dependent variables: eggshell bacterial loads (mesophilic bacteria, enterococci, staphylococci and enterobacteria), treatment (control I vs. control II) as the fixed factors and sampling date as covariate; effect of treatment: *Wilks* $\lambda = 0.84$, $F = 0.20$, $P = 0.93$]. Thus, we combined data from control I and control II treatments to improve statistical power of the analyses.

The effects of experimental manipulation of the uropygial gland on eggshell bacterial load were analysed using two different approaches. First, we used GLMMs with the four different bacterial counts as dependent variables, experimental treatment as the fixed factor and female identity as a random factor to account for the repeated-measures nature of our data set. The interaction between treatment and female identity was also included in the model for testing whether the experiment resulted in a similar effect on different females. Secondly, by mean of MANCOVAS, we analysed whether the relationship between eggshell bacterial loads and bacterial density of the uropygial gland secretion differed for experimental and control females. Therefore, the models included eggshell bacterial densities as dependent variables, treatment as the fixed factor, bacterial density of uropygial gland secretion of females at the beginning of the incubation as covariate, and the interaction between treatment and the covariate.

Next, we analysed the bacterial loads of contents of unhatched eggs. Log-transformed bacterial loads of contents of unhatched eggs did not differ from normal distributions (Kolmogorov–Smirnov test, $P > 0.05$), and we therefore used parametric statistics. Moreover, among-nest variation in bacterial loads of unhatched eggs was significantly larger than the within-nest variations ($F_{25,32} > 2.14$, $P < 0.033$), and thus, we used mean values of experimental nests in subsequent analyses. In addition, we did not find evidence of between-years variation ($F_{1,10} > 0.45$, $P > 0.51$), and thus, we did not include year as an independent factor in subsequent analyses. Finally, unhatched eggs in nests of control I and control II females harboured similar amounts of bacteria (TSA: $F_{1,14} = 1.29$, $P = 0.28$; HK: $F_{1,14} = 3.11$, $P = 0.10$; KF: $F_{1,14} = 0.01$, $P = 0.94$; VJ: $F_{1,14} = 0.23$, $P = 0.64$). Thus, we combined data from control I and control II treatments to improve the statistical power of the analyses.

Hatching success

Frequencies of hatching success differed from normal distributions even after squared-root arcsine or Box–Cox transformation, and therefore, we conservatively used ranked values in our statistical analyses. The effect of experimental manipulation on hatching success was analysed using a GLMM by including female

identity (nested within study year) as the random factor and treatment (control (I and II) vs. experimental) as the fixed factor. The interaction between the experimental treatment and female identity (nested within study years) was also included to explore whether the expected experimental effect differed among females. Finally, the relationship between eggshell bacterial loads and hatching success was analysed by mean of linear regression. We analysed the relationship between bacterial counts and hatching success, and later, we excluded from the analyses samples without growth in culture media for *Enterococcus*. Because we were interested in detecting the influence on hatching success by each category of bacteria after controlling for the effect of the others, we used semipartial regression coefficients.

Results

THE USE OF SECRETION, FEMALE BEHAVIOUR AND EGGSHELL STRUCTURE

Video-recordings confirmed that incubating females collect the UGS with the bill and use it to either preen feathers, including those of the belly (Video S1, Supporting information), or to smear the eggshells by opening the bill tip holding a drop of secretion when it contacts with the egg (Video S2, Supporting information).

The microscopic study of eggshells of hoopoes revealed special structures that have never been described for birds: the external crystal layer is full of crypts of different size and depth that end at the spongy palisade layer (i.e. they do not pierce the eggshell, Fig. 2). We examined eggshells of five other bird species from four different orders, but did not find any evidence of crypts or similar structures at laying or at the end of incubation (Fig. 3). Moreover, these or similar structures do not appear in any of the SEM images of eggshells of the 31 group of birds (i.e. orders that include 90 species) analysed in Mikhailov (1997).

The appearance of hoopoe eggshell crypts changed drastically during incubation. Most crypts become filled with a doughy material at hatching (comparison of the percentage of filled crypts at the beginning and the end of the incubation period, $F_{1,185} = 180.5$, $P < 0.001$), and this change did not differ between eggs from nests in the wild and those in captivity ($F_{1,56.8} = 0.88$, $P = 0.35$, Fig. 2c,e). Close examination of crypts in eggs of un-manipulated females at the end of the incubation period (i.e. SEM images at greater magnification) allowed us detecting bacteria within the matrix of the filling substance (Fig. 2g,h).

This drastic change does not occur in clutches of female hoopoes with experimentally restricted access to the uropygial gland (Fig. 4; effect of experimental treatment: $F_{2,43.3} = 50.0$, $P < 0.0001$). Post hoc comparisons revealed statistically significant differences in the percentage of crypts filled with secretion between eggs from experimental and control females (LSD, $P < 0.0001$), while eggs of
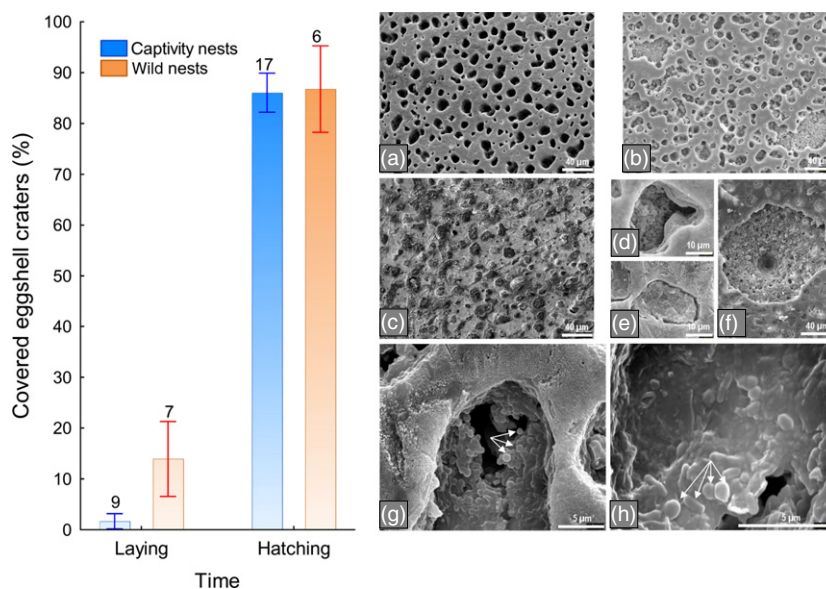

Fig. 2. Percentage (± 95% confidence intervals) of crypts of hoopoe eggshells that were filled with material at laying and hatching in captivity and in nests of wild non-manipulated females. Pictures are SEM images showing the surface of eggshells of hoopoes. SEM photographs a and b show recently laid (<24 h) eggs, while c shows the appearance of an eggshell of the same clutch as in a, but at the end of the incubation period. Image d shows a detail of a typical empty crypt that has vertical walls and ends at the palisade layer (spongy texture). Crypts occupy between 24.6% and 42.1% of the eggshell surface [for 10 females mean (SD) = 32.9 (5.5)], and they are usually rounded, with the longer diameter being up to 20–30 μm. Image e shows the typical aspect of crypts at the end of the incubation period, filled with material. The eggs of some females show much wider crypts, but of similar depth as the rounded ones (i.e. they do not penetrate the spongy palisade layer, as in b). Image f shows a detail of a conic pore that is much larger than crypts (diameter 80–120 μm) and perforates the eggshell deeper by piercing it completely. These pores occur with a frequency of 12.58 pores cm^{-2} and are not covered by material at the end of incubation. Finally, the two SEM images at the bottom (g and h) are examples of bacteria found within the material filling the crypts of hoopoe eggshells.

the two kinds of control females did not differ significantly (*LSD*, $P = 0.83$). The crypts in eggs of experimental females did not stay completely empty at the end of the incubation period (Fig. 4), probably because some crypts can be filled by the material surrounding eggs, such as soil particles in the nest bottom.

BACTERIA ON THE EGGSHELLS OF HOOPOES AND ITS RELATIONSHIP WITH FEMALE UROPYGIAL SECRETION

Eggshells of females experimentally prevented from accessing the gland had lower bacterial densities than
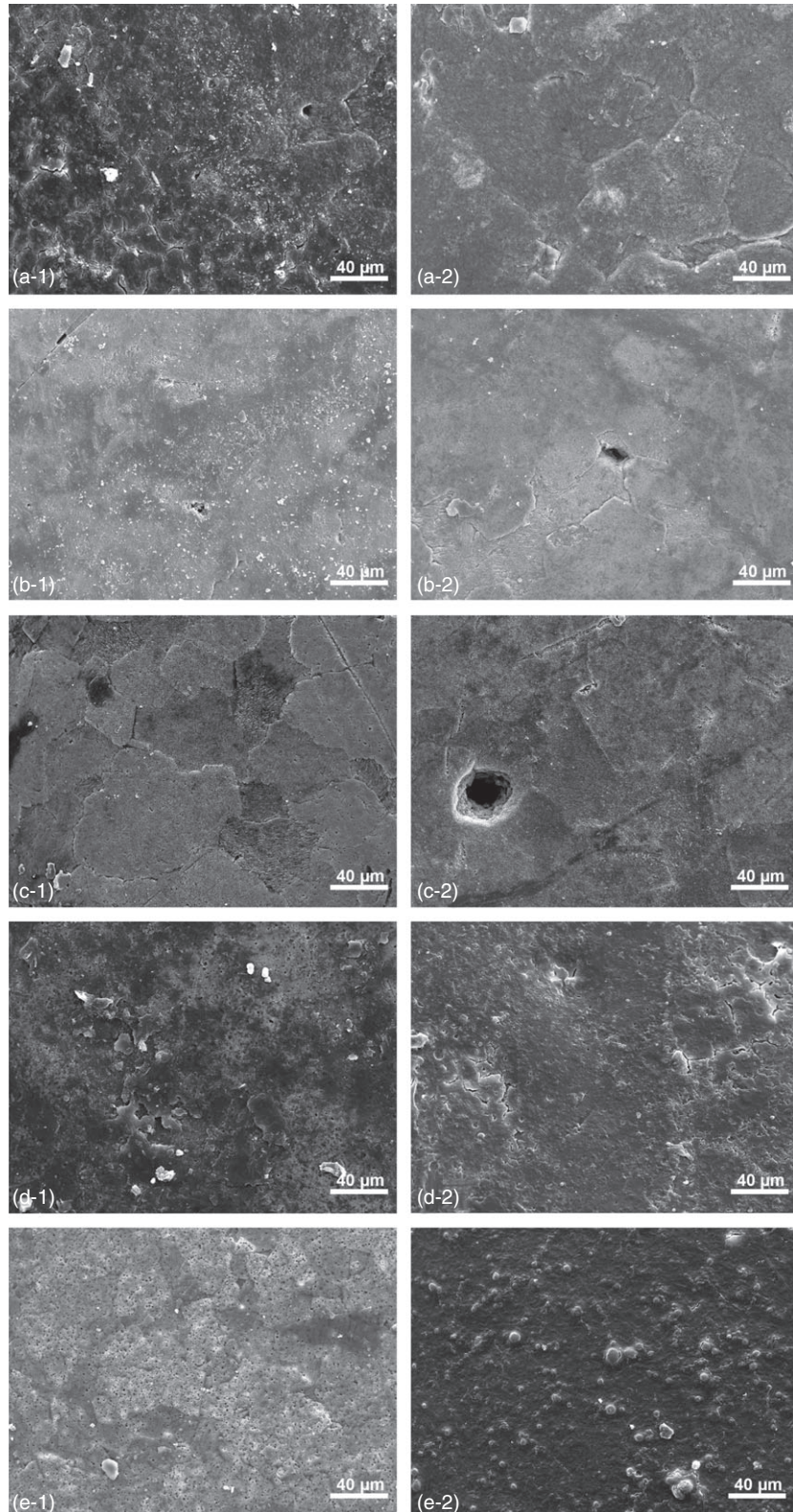

Fig. 3. SEM images of the eggshell surface of (a) scops owl, (b) roller, (c) rock dove, (d) spotless starling and (e) house sparrow, at laying (left, numbered 1) and at the end of incubation (right, numbered 2). Eggshells of these species do not have crypts, and in some (a-1, d-1, d-2, e-1, e-2), but not in other pictures (b and c) the cuticle or an organic cover can be detected as a thin layer usually fractured with the appearance of dried mud.
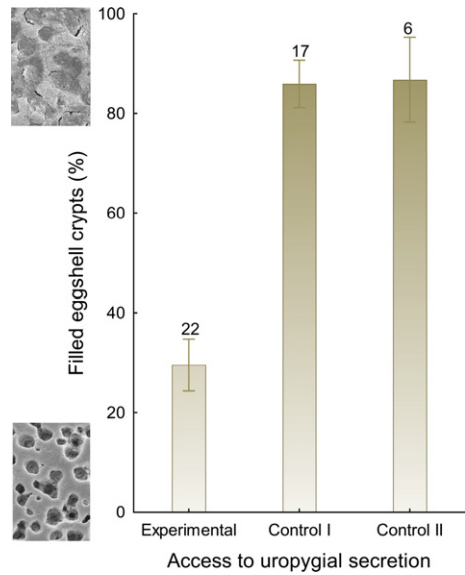
Fig. 4. Percentage (± 95% confidence intervals) of crypts of eggshells of experimental hoopoes that were filled with material at hatching. SEM pictures show empty (0%) and filled (100%) eggshell crypts.

those of control females. Interestingly, these differences were especially pronounced for total mesophilic bacteria (GLM, treatment effect: $F_{1,11\cdot4} = 12\cdot31$, $P = 0\cdot005$) and *Enterococcus* (GLM, treatment effect: $F_{1,\ 11\cdot5} = 7\cdot64$, $P = 0\cdot018$), but not for *Staphylococcus* (GLM, treatment effect: $F_{1,11\cdot4} = 0\cdot03$, $P = 0\cdot86$) and *Enterobacteriaceae* (GLM, treatment effect: $F_{1,11\cdot2} = 4\cdot16$, $P = 0\cdot072$) (Fig. 5a). These results suggest that the experiment limited the access of symbiotic enterococci from the uropygial secretion to the eggshells.

Several pieces of evidence are in accordance with that interpretation. First, abundance of total mesophilic bacteria and enterococci on the eggshell were strongly positively related in control [(beta (SE) = 0·74 (0·14), $t_{20} = 5\cdot41$, $P < 0\cdot0001$, staphylococci and *Enterobacteriaceae* did not explain significant additional variance ($P > 0\cdot2$)], but not in experimental nests (abundance of *Enterobacteriaceae* did predict abundance of mesophilic bacteria (beta (SE) = 0·82 (0·15), $t_{15} = 5\cdot65$, $P < 0\cdot0001$) with no other group of bacteria explaining additional significant variance). Secondly, density of bacteria in the UGS of females was strongly positively associated with density of mesophilic bacteria (beta (SE) = 0·57 (0·20), $F_{1,38} = 7\cdot01$, $P = 0\cdot012$) and *Enterococcus* on the eggshell [beta (SE) = 0·70 (0·20), $F_{1,38} = 8\cdot74$, $P = 0\cdot005$], but not for other kinds of bacteria (*Staphylococcus* ($F_{1,38} = 1\cdot42$, $P = 0\cdot24$) and *Enterobacteriaceae* ($F_{1,38} = 3\cdot81$, $P = 0\cdot058$). Finally, slopes of such relationships for control and experimental females differed significantly for *Enterococcus* (interaction between experimental treatment and bacterial density of secretion, $F_{1,38} = 4\cdot29$, $P = 0\cdot045$, Fig. 5b), but not for other bacterial counts ($F_{1,38} < 1\cdot99$, $P > 0\cdot17$). It is worthy to mention here that the detected association between density of enterococci in the females' secretion and on the eggshells was not due to a possible association between volume of secretion and density of bacteria. Partial correlation coefficients showed no effect of secretion volume of control females, neither at the beginning (partial correlations, density of bacteria in secretion, $R = 0\cdot66$, $n = 21$, $P = 0\cdot001$; volume of secretion, $R = 0\cdot30$, $n = 21$, $P = 0\cdot210$) nor at the end of the incubation period (partial correlations, density of bacteria in secretion, $R = 0\cdot61$, $n = 21$, $P = 0\cdot004$; volume of secretion, $R = -0\cdot23$, $n = 21$, $P = 0\cdot334$). Therefore, all
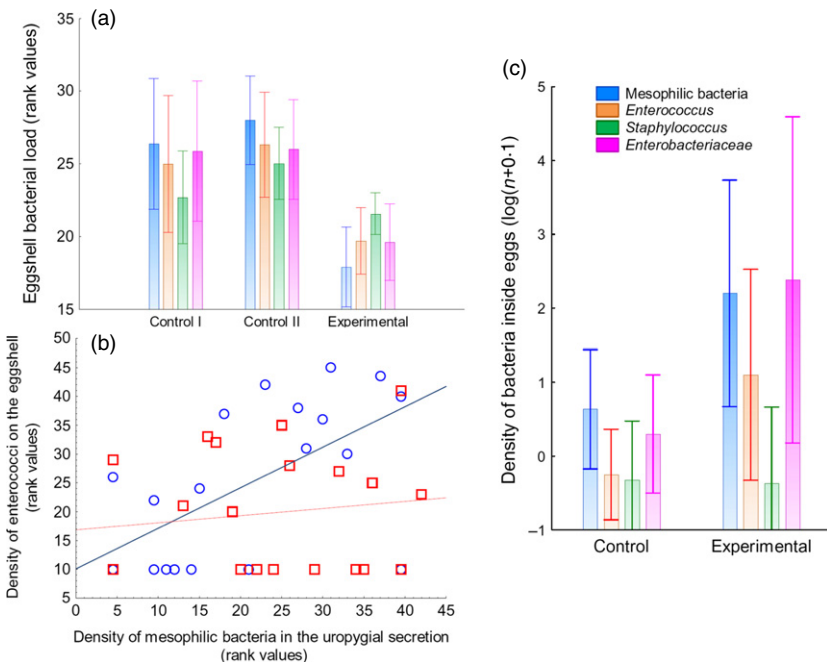

Fig. 5. (a): Bacterial loads (± SE) of hoopoe eggshells with (control I and control II) and without (experimental) access to uropygial gland secretion. (b) Relationship between density of aerobic bacteria in the uropygial gland of hoopoe females at the beginning of laying (i.e. before the manipulation of the experiment to impede access to secretion) and density of *Enterococcus* on eggshells of experimental (red squares and lines) and control (blue circles and lines) females at the end of incubation (i.e. in response to the experiment). (c): Density (i.e. colony-forming units per 100 μL of egg contents) of bacteria inside hoopoe eggs that failed to hatch and that were incubated by females with (control) or without (experimental) access to uropygial gland secretion.

these results suggest a link between symbiotic bacteria found on the eggshells of hoopoes and those in the uropygial gland.

BACTERIA INSIDE UNHATCHED EGGS OF HOOPOES AND ITS RELATIONSHIP WITH UROPYGIAL SECRETION

The experimental prevention of access to uropygial secretion by female hoopoes affected the risk of trans-shell contamination of their eggs by bacteria. Eggs that failed to hatch in nests of females without access to uropygial secretions contained higher bacterial density than did those of control females, for mesophilic bacteria ($F_{1,21} = 4.93$, $P = 0.037$), *Enterococcus* ($F_{1,21} = 6.54$, $P = 0.018$) and *Enterobacteriaceae* ($F_{1,21} = 5.44$, $P = 0.030$), but not for *Staphylococcus* ($F_{1,17} = 0.01$, $P = 0.94$, Fig. 5c).

RELATIONSHIP BETWEEN SYMBIOTIC BACTERIA AND HATCHING SUCCESS

Hatching success of females increased with density of symbiotic bacteria in their uropygial secretion at the beginning of the incubation period for control females ($R = 0.55$, $n = 20$, $P = 0.012$), but not for those with experimentally restricted access to the uropygial gland ($R = -0.24$, $n = 17$, $P = 0.346$) (Fig. 6a). Therefore, the manipulation successfully broke the positive effect of symbionts from the uropygial gland on egg viability (interaction between treatment and density of symbionts in the secretion GLM: $F_{1,33} = 6.35$, $P = 0.017$). Similar effects were detected when considering eggshell bacterial loads of symbiotic bacteria (enterococci) at the end of incubation (controls, $R = 0.43$, $n = 23$, $P = 0.040$; experimental: $R = -0.02$, $n = 17$, $P = 0.949$, Fig. 6b), although in this case, the interaction between treatment and eggshell loads of enterococci did not reach statistical significance (GLM: $F_{1,36} = 1.38$, $P = 0.248$). However, we failed to find an effect of treatment on hatching success for females that during the same season experienced different experimental treatments (GLM, experimental treatment: $F_{1,13.4} = 1.40$, $P = 0.26$; female identity nested within study year: $F_{36,10.9} = 2.06$, $P = 0.10$; interaction between treatment and females identity nested within study year: $F_{13,13} = 1.44$, $P = 0.26$).

The relative abundance of the different groups of bacteria present on eggshells at the end of the incubation period did not explain a significant proportion of variance in hatching success considering all clutches together (the strongest relationship was for enterococci: $R = 0.262$, $n = 40$, $P = 0.10$). Interestingly, when only considering clutches with enterococci on the eggshells, the density of this group of bacteria and hatching success were positively related ($R = 0.53$, $n = 23$, $P = 0.010$). This relationship was not due to a general effect of bacterial density on eggshells because it persisted when using residuals of enterococci corrected for density of total mesophilic bacteria,
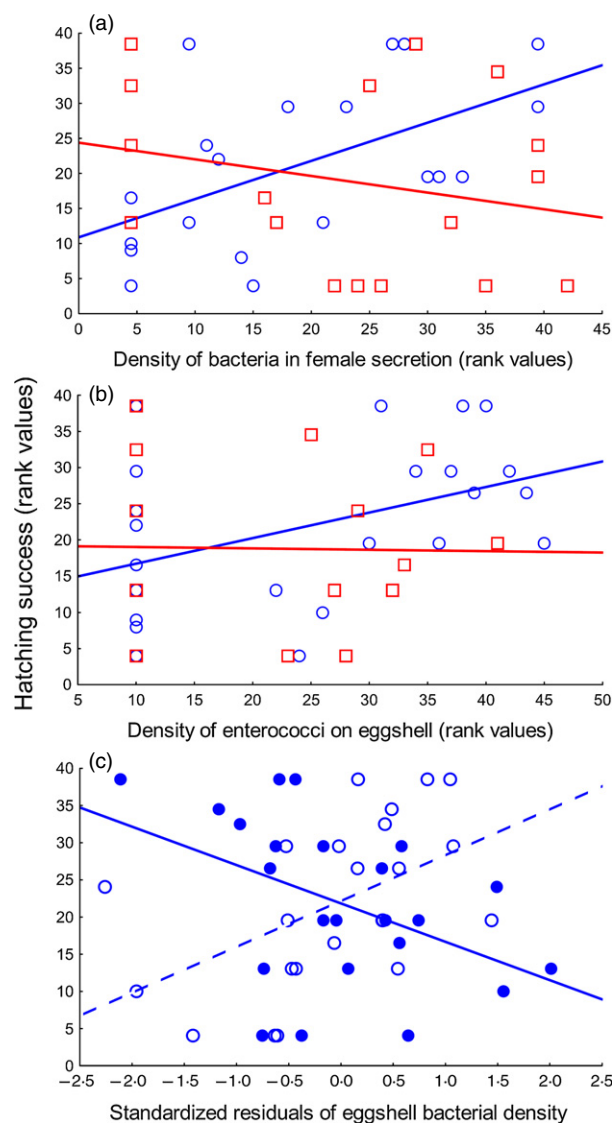

Fig. 6. Relationships between density of symbiotic bacteria in female secretions (a) and on eggshells (b) and hatching success for control (blue circles and regression line) and experimental females (red squares and regression line). Subfigure c shows the relationship between hatching success and eggshell density of *Enterococcus* (blue empty circles and dashed regression line) and *Enterobacteriaceae* (blue-filled circles and continuous regression line) (i.e. standardized residuals of colony-forming units after controlling for estimates of other kinds of bacteria) for nests with detected bacterial growth in the selective medium.

staphylococci and *Enterobacteriaceae* (i.e. semipartial correlation, $R = 0.52$, $n = 23$, $P = 0.012$; Fig. 6c). Furthermore, residuals of eggshell density of *Enterobacteriaceae* (corrected for density of total aerobic bacteria, enterococci and staphylococci), which comprise some of the most severe pathogenic bacteria of avian embryos, were negatively associated with hatching success for nests with detected bacterial growth in the enterococci selective medium ($R = -0.44$, $n = 23$, $P = 0.034$, Fig. 6c). Residuals of aerobic bacteria in general ($R = 0.38$. $n = 23$, $P = 0.075$) or those of *Staphylococcus* ($R = 0.14$, $n = 23$, $P = 0.520$) were not significantly associated with hatching success.

Discussion

Detecting and exploring animal adaptations favouring mutualistic relationships with antibiotic-producing micro-organisms as a strategy to fight against pathogens is of prime importance for evolutionary ecologists. Host characteristics favouring these mutualistic relationships have been mainly studied in invertebrates, and our results here suggest for the first time that it also occurs in birds. Our main findings are (i) that the eggshells of hoopoes are full of crypts, which soon after egg laying (ii) are filled with uropygial secretion. We also detected (iii) an experimental link between uropygial secretion reaching eggshells and level of trans-shell contamination of hoopoe eggs. Finally, we also found positive relationships between hatching success and density of bacteria (iv) in the uropygial secretion and (v) on the eggshell. Below, we discuss the novelty and the importance of such findings in a scenario of long-term evolutionary history between hoopoes and mutualistic enterococci living in their uropygial gland.

SPECIAL HOOPOE EGGSHELL STRUCTURE RETAINS UROPYGIAL SECRETIONS WITH SYMBIONTS

The eggshells of hoopoes are full of crypts that had never been described for birds. Moreover, hoopoe eggshells differ from those of most birds except turtledoves and pigeons (Tullett 1984; Mikhailov 1997) in lacking the typical organic cuticle or external inorganic layers that protect embryos against trans-shell contamination (Sparks 1994; Wellman-Labadie, Picman & Hincke 2008). The crypts, which were empty at laying, became full of a doughy material containing bacteria at hatching. When female access to the uropygial gland was experimentally prevented, the crypts stayed almost empty, demonstrating a link between the uropygial secretion and the material that fills the crypts on eggshells. In addition, the video-recordings showed that incubating females directly inoculated eggshells with uropygial secretion collected from the gland with the bill. While the use of uropygial secretions on eggs had previously been hypothesized (Reneerkens, Piersma & Sinninghe Damsté 2006; Martín-Vivaldi *et al.* 2009; Møller, Erritzøe & Rózsa 2010; Soler *et al.* 2012), to our knowledge, this is the first experimental demonstration of such function.

We have previously shown that hoopoe breeding females host symbiotic bacteria in their uropygial glands (Soler *et al.* 2008; Martín-Vivaldi *et al.* 2009) that produce several antimicrobial chemicals (Martín-Platero *et al.* 2006; Martín-Vivaldi *et al.* 2010), suggesting that the bacteria found in the crypts may be symbionts of the uropygial gland that together with the secretion are transferred onto eggshells to protect embryos. Thus, the presence of abundant shallow eggshell crypts is likely a specialized trait of hoopoes that increases the amount of uropygial secretion, symbiotic bacteria and antimicrobial substances that eggshells can retain to protect eggshells from

colonization by pathogens and therefore from trans-shell embryo infection.

ENTEROCOCCI ON HOOPOE EGGSHELLS COME FROM FEMALE UROPYGIAL SECRETIONS

Enterococci growing in the uropygial gland of hoopoes produce antimicrobial substances of the preen secretion (see Introduction), and two pieces of information are in accordance with the hypothesis that enterococci on the eggshells of hoopoes are derived, at least partly, from bacteria in the uropygial gland. First, eggshells of females experimentally prevented from accessing the gland had lower bacterial densities than those of control females. Secondly, there was a positive association between bacterial density of the uropygial gland secretion of non-manipulated females and eggshell bacterial density when considering either total aerobic bacteria or enterococci. Finally, the positive correlations between densities of bacteria in the uropygial secretion and on eggshells when considering enterococci, but not other groups of bacteria found on the eggshell, were detected in control but not in experimental nests. These results support the hypothesis that the enterococci found on eggshell of hoopoe eggs at least partially derive from those present in the uropygial gland secretion of incubating females, and consequently, eggshell crypts would enhance adhesion of symbiotic bacteria from the females' secretion to the eggshell.

UROPYGIAL SECRETIONS WITH SYMBIONTS ON HOOPOE EGGSHELLS REDUCE TRANS-SHELL CONTAMINATION AND IMPROVE HATCHING SUCCESS

The density of bacteria inside unhatched eggs was higher in nests of experimental females than in those of control females indicating that the absence of uropygial gland secretion on hoopoe eggshells makes eggs more permeable to bacterial infection. The effect of uropygial secretion on trans-shell contamination could be due to physical properties of eggshells if, by filling crypts, the secretion acted as a barrier to water penetration thus reducing the risk that bacteria were transported by water (Cook *et al.* 2003). Although we cannot discard this possibility, several lines of evidence suggest that symbiotic bacteria living in the secretion on the eggshell also play a major role in reducing trans-shell contamination and increasing hatching success. First, density of enterococci on the eggshell was related to density of bacteria in the secretion, and not to the amount of secretion produced by females, which suggest that density of symbionts on the eggshell is not an index of the amount of secretion transferred to eggs, but of the abundance of symbionts in the secretion that are transferred to eggshells. Secondly, the density of bacteria in the secretion and of enterococci on the eggshells was positively correlated with hatching success of the clutch in control but not in experimental females (Fig. 6a,b).

UROPYGIAL SECRETIONS WITH SYMBIONTS ON HOOPOE EGGSHELLS AND HATCHING SUCCESS

The experimental impediment of the use of uropygial secretion by females did break the detected positive relationship between abundance of enterococci symbionts in the gland and on the eggshells and hatching success (Fig. 6a,b), which agrees with a direct fitness benefit of the impregnation of eggs with the UGS of hoopoes. However, hatching success of clutches of experimental females did not differ significantly from those of control females and, even for some clutches of females with non-detected enterococci in the uropygial secretion or on the eggshell, none of the eggs failed to hatch (Fig. 6). This result is not the expected under the hypothesis that the uropygial secretion of hoopoes with their symbionts enhances hatching success. However, because the associated probability of erroneously accepting the null hypothesis of no differences is quite high ($P = 0.74$), we can neither conclude in favour of it. Alternative explanations include insufficient sample size for detecting a possible reduced fitness benefit of UGS, or that the expected fitness benefits accrue later in life. Dozens of factors related to egg characteristics and adult incubation behaviour are known to affect hatching success (Deeming 2004) and, if the effect of UGS on hatching success of hoopoes is not relatively high, a much larger experiment may be required to detect differences among experimental groups. Variation in pathogenic microbial environment of nests of hoopoes could also hinder the predicted experimental effect on hatching success. We would expect high effects of our manipulation in environments with a high risk of infection, while in subpopulations where the probability of hatching failures is low, such effect would be minimized. Moreover, we can also speculate with the possibility that hoopoes enhance growth of beneficial enterococci in risky pathogenic environments differentially. In accordance with this possibility, we found that density of symbiotic *Enterococcus* and that of the potentially pathogenic *Enterobacteriaceae* on the eggshells of control hoopoes were strongly positively related ($R = 0.891$, $n = 23$, $P < 0.001$). This result might suggest that protective enterococci are more abundant in nest environments with higher risk of embryo infection, and therefore, antimicrobial characteristics of uropygial secretion might be adjusted to pathogenic characteristics of nest environment. Experiments (i.e. augmenting density of pathogenic bacteria on the eggs) breaking the relationship between symbiotic and potential pathogenic microorganisms are necessary for further exploring the protective role of enterococci from the uropygial gland of hoopoes impeding trans-shell embryo infection.

EGGSHELL CRYPTS AND UROPYGIAL SECRETION OF HOOPOES

Together with the detection of special structures of the eggshell of hoopoes, all these results suggest that eggshell crypts harbouring secretions function enhancing protection of eggs (i.e. preventing trans-shell contamination) by accumulating antimicrobial secretions and symbionts on eggshells as has been described for bacteria on eggs of squids (Barbieri *et al.* 1997) and shrimp (Gil-Turnes, Hay & Fenical 1989). Aquatic invertebrates are exposed to high infections risks, which would explain the evolution of the symbiotic association with protective bacteria (Gil-Turnes, Hay & Fenical 1989). Hole-nesting birds develop in environments of high risk of infection (Peralta-Sánchez *et al.* 2012), and this may be specially the case for hoopoes. Contrary to most hole-nester, hoopoes use faeces in antipredation defensive behaviour (Krištín 2001), but do not keep their nest clean of faeces or rests of food (Soler *et al.* 2008). Thus, the especially high risk of infection experienced by hoopoes during the nesting stage might be the cause of the evolution of eggshell crypts enhancing the antimicrobial function of uropygial secretion and symbionts on the eggshell.

An additional non-exclusive embryo protecting function of smearing uropygial secretion on eggshells crypts by hoopoe females could be related to the possible deterrent effect that the malodours secretion may have for mammalian and reptilian predators; effects that have been shown for the similar secretion of the close relative green woodhoopoes (*Phoeniculus purpureus*) (Burger *et al.* 2004). Another alternative functional explanation of the exclusive crypts of hoopoe eggshells is that it may allow females to regulate gas exchange and therefore rate of embryo development. This possibility seems, however, improbable since, as shown by eggshell colour change, crypts became filled with secretion soon after laying and remained filled until hatching (Pers. Obs.). Moreover, the crypts, which do not completely pierce the eggshell, get filled with secretion, while the true pores do not retain secretion (Fig. 2f).

GENERAL CONCLUSIONS AND INFERENCES

We here describe morphological traits in eggshells of hoopoes (i.e. crypts) that function to increase the adhesion of secretion of incubating females containing antimicrobials and mutualistic bacteria on eggshells. Moreover, the detected association between hatching success and density of symbiotic bacteria in the uropygial secretion of incubating control females and on their eggshells suggests that crypts enhancing the adhesion of symbiotic-carrying uropygial secretion likely protect embryos against infections. Furthermore, because hoopoes are unique among birds showing crypt in their eggshells, our results suggest a long-term evolutionary history between hoopoes and mutualistic enterococci living in their uropygial gland.

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Authorship statement

MM-V. and JJS. designed the study with considerable assistance from JMP-S, AMM-P, MM-B, MR-R. and EV for the several microbiological aspects of the article. JMP-S and LA performed most of the fieldwork with assistance by MM-V and JJS and greatly contributed to improve the experimental protocol. MM-V and JJS performed all the analyses and wrote the manuscript with substantial contribution from all authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Video S1. Female hoopoe using the dark uropigial secretion to preen feathers during incubation.

Video S2. Female hoopoe impregnating eggs with dark uropygial secretion taken with the beak.