Special structures of hoopoe eggshells enhance the adhesion of symbionts-carrying uropygial secretion to prevent embryo infection

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Summary

1. Detecting and exploring animal adaptations favouring mutualistic relationship with antibiotic producing bacteria as a strategy to fight against pathogens is 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 and here, we investigated the microscopic structure of hoopoe eggshells looking for special features favouring the adhesion of antimicrobial uropygial secretions.
3. By impeding female access to the uropygial gland and comparing microscopic characteristics of eggshells, bacterial loads of eggs and of uropygial secretion, as well as hatching success of experimental and control females, 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) and 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 positively affects permeability of eggshells by several groups of bacteria and successfully broke the positive relationship between hatching success and density of symbiotic bacteria either, in the uropygial secretion of females or on the eggshell.
5. We video recorded females smearing secretion onto the eggshells. Taken together, our results strongly suggest morphological adaptations in hoopoe eggshells that

function to retain uropygial secretions with mutualistic bacteria for the protection of embryos against infections.

Key-words Antimicrobial defences, Birds, Coevolution, Mutualism, Symbiotic bacteria, Uropygial gland

Introduction

Bacteria produce an extraordinary diversity of antimicrobial compounds to inhibit other microorganisms (Ji, Beavis & Novick 1997; Riley & Wertz 2002). Some animals use such chemicals from metabolism of symbiotic bacteria as defences against pathogenic microorganisms and parasites, and in some cases have even evolved specialized crypts (depressions in the tegument) for bacterial growth as those described for fungus-growing ants (Currie *et al.* 2006). Chemicals produced by symbiotic bacteria are known to protect ants' gardens, wood galleries of beetles and embryos of shrimp, lobsters, squid, wasps and some salamanders from pathogenic bacteria and/or competitor fungi (Gil-Turnes, Hay & Fenical 1989; Barbieri *et al.* 1997; Currie *et al.* 1999; Barbieri *et al.* 2001; Kaltenpoth *et al.* 2005; Cardoza, Klepzig & Raffa 2006; Banning *et al.* 2008), and aphid hosts from their parasitoids (Oliver *et al.* 2003). All these cases are good examples of the importance of symbiotic associations between animals and microorganisms for which animal behaviour related to the acquisition and use of antimicrobials play a central role in the establishment and regulation of the microbial assemblage (Ezenwa *et al.* 2012). Animals live in a bacterial world and exploring physiological, morphological and behavioural characteristics of animals facilitating microbial colonization is of prime importance for the life science (McFall-Ngai *et al.* 2013). The hoopoe (*Upupa epops*, Linnaeus) is the only bird for which the use of such substances has been suggested (Soler *et al.* 2008) and we here explored possible characters in hoopoes favouring the use of antimicrobials from symbiotic bacteria for protecting embryos against pathogenic infections.

The hoopoe is an upupiform bird that nests in holes with no nest material. Nesting hoopoe females and nestlings but not males secrete brown and malodorous uropygial

secretion that harbours bacteria with antimicrobial capabilities at a high density (Soler *et al.* 2008; Martín-Vivaldi *et al.* 2010). Preen secretion are deposited onto the plumage and protects feathers from bacterial degradation (Ruiz-Rodriguez *et al.* 2009). This secretion, when assayed *in vitro* in Petri dishes, inhibits growing of the feather-degrading bacteria *Bacillus licheniformis* (Soler *et al.* 2008), and the symbiotic bacteria produce several antimicrobial chemicals (Martín-Platero *et al.* 2006; Martín-Vivaldi *et al.* 2010; Ruiz-Rodríguez *et al.* 2012) against potential pathogens. Most of the cultivable bacteria growing in the uropygial gland secretion (UGS) of hoopoes are *Enterococcus*, mainly *E. faecalis* and *E. faecium*; bacteria that produce several sorts of bacteriocines (Ruiz-Rodríguez *et al.* 2012). Interestingly, the strains with higher antimicrobial activity were the most frequent and abundant in hoopoe's UGS (Ruiz-Rodriguez *et al.* 2013). These strains, or their antimicrobial substances, are active against keratinolytic bacteria (Ruiz-Rodriguez *et al.* 2009) and some potential pathogens of embryo infection such *Staphylococcus* sp., *Listeria sp*. and some other bacteria (Martín-Platero *et al.* 2006) (Ruiz-Rodríguez *et al.* 2012).

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, hoopoe eggs change from bluish to a brown colour within a few days, which may be caused by the deliberated impregnation of 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 added to the shell, which would also serve to enhance protection of embryos against pathogenic infection mediated by uropygial secretion (Soler *et al.* 2012).

Thus, because of the antimicrobial properties of UGS and/or of the symbiotic enterococci bacteria living therein, UGS on the eggshell of hoopoes may confer protection against trans-shell embryonic infection as suggested by the experimental deactivation of proteins in nests of hoopoes (Soler *et al.* 2008). If that was the case, a detailed analysis of the eggshell of hoopoes is worth because, similarly to other animals, may reveal special traits that enhance the adhesion of uropygial secretion to the egg.

The aims of the study were therefore (1) to explore the hypothesis that hoopoes use their uropygial gland secretion onto the eggshells likely to protect embryo from pathogenic infection, and (2) 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 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 (a) a different microscopic structure due to the absence of UGS, (b) less abundant symbiotic bacteria (enterococci) on eggshells, (c) increased trans-shell bacterial contamination, and (d) 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 *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 (e) abundance of mutualistic enterococci on the eggshell should be positively related to hatching success, while (f) 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 garrulous*, 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 3m x 2m x 2m 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 to 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, de 10 de Octubre) and under the permission of Junta de Andalucía, Dirección General de Gestión del Medio Natural which authorized the establishment and maintenance of the captive breeding population (Resolución de 14 de Abril de 2008) and field protocols (Resoluciones de 14 de Abril de 2008 and 23 de Marzo de 2010).

FIG. 1 ABOUT HERE

Microscopic study of eggshells

During the breeding seasons 2009 - 2010 we collected recently laid (less than 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 12h 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 per clutch. In addition, eggshells from experimental hoopoe nests

198 at the end of incubation (i.e., eggshells from hatchlings) were also collected for
199 microscopic study of the effect of the experiment (N = 80 eggshells from 43 clutches).
200 A small fragment (about 3 mm x 3 mm) of the equator of each egg was fixed in 2.5%
201 glutaraldehyde in PBS for 24 h, rinsed three times in PBS and stored at 4ºC in
202 distilled water to avoid crystalline mineral precipitation until the microscopic
203 analyses. Afterwards, samples were air dried overnight and coated with approximately
204 10 nm of gold/palladium using a Nanotech Semprep2 sputter coater. Observations
205 were made with a Hitachi S-510 scanning electron microscope (SEM) at the Scientific
206 Instrumental Services of the University of Granada and images digitalized with Scan
207 Vision. Three randomly selected fields of each piece of eggshell were photographed at
208 900x magnification. In the case of hoopoe eggshells the photographs were used to
209 evaluate where there exists any kind of crypts, their abundance and the percentage of
210 them filled with secretion.
211 The SEM images of recently laid eggs were examined looking for shell textures
212 identifiable as cuticle (a fissured external layer with the appearance of dried mud
213 (Becking 1975) or other shell accessory material covering pores (Tullett 1984). The
214 first two images of eggshells of ten randomly selected hoopoe females (five wild and
215 five captive) were processed with Adobe Photoshop 7.0 to estimate the percentage of
216 eggshell surface occupied by crypts (i.e. in units of pixels). These estimates were
217 repeatable within eggs (R = 0.78, $F_{9,10}$ = 7.93, P = 0.002) and, therefore, we used the
218 mean values for eggs in subsequent analyses. Furthermore, eggshells of wild and
219 captive females did not differ in the percentage of their surface occupied by crypts at
220 laying (wild females: mean(SE) = 31.9(3.22); captive females: mean(SE) =
221 33.9(1.68); $F_{1,8}$ = 0.30, P = 0.60) and, consequently, we pooled data of these nests to
222 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 4ºC until processed in the laboratory within the following 24 hours. In the lab we added 45 µl of sodium phosphate buffer (0.2 M, pH 7.2) and vigorously mixed it by repeated pipetting before

247 inoculating growth media. The total volume of secretion available in the gland was
248 also estimated (in µl) by using a 1-10 µl micropipette (Finnpipette).
249
250 Eggshells
251 We sampled a single egg per clutch at the end of incubation (day 15 after the first egg
252 was laid) during the 2010 breeding season by cleaning the entire eggshell with a
253 sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M, pH 7.2). The
254 swab was preserved in an eppendorf tube at 4ºC containing the sterile buffer until lab
255 analyses during the following 24 hours. Estimates of bacterial load were standardized
256 to eggshell surface sampled, which were estimated according to the formula
257 $S=3L^{0.771}B^{1.229}$ following Narushin (2005), where S is the surface in cm^2, L the egg
258 length and B the egg breadth.
259
260 Unhatched egg contents
261 To estimate the effects of experimental manipulation on trans-shell bacterial
262 contamination, during the 2009 - 2010 breeding seasons we collected all eggs (N = 60
263 from 23 different clutches) that failed to hatch in nests involved in the experiment and
264 that did not show any sign of breakage. These eggs were collected two or three days
265 after the expected hatching date for the last egg in the clutch. After disinfection of the
266 eggshell surface with 70% ethanol, unhatched eggs were broken in a laminar flow
267 cabinet and after carefully cutting egg membranes, whenever possible, 300 µl of yolk
268 and 300 µl of egg white were collected and homogeneously mixed using a sterile
269 single-use inoculation loop. Afterwards, 300 µl of this mix were used for the final
270 dilution. When egg membranes were deteriorated and it was impossible to extract
271 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 (see below).

Culturing bacteria

In the lab, samples were collected from eppendorf tubes after vigorously shaking them in vortex for at least three periods of 5 seconds, 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 Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnson Agar (VJ) added of potassium tellurite for growing bacteria of the genus *Staphylococcus*; and Hecktoen 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; Soler *et al.* 2010).

297 Egg samples were inoculated both in the general (TSA) and the three selective media
298 (KF, VJ and HK), while the uropygial secretions were inoculated only in TSA (we
299 have previously shown that most aerobic cultivable bacteria in hoopoe UGS are
300 enterococci (Soler *et al.* 2008) so we can assume growth in TSA from these samples
301 to correspond mostly to enterococci). Cultures were incubated aerobically, in the case
302 of uropygial secretions at 28ºC for 12 h, and egg samples at 37º C for 72 h. When the
303 number of bacterial colonies was too dense to count, we performed serial dilutions to
304 obtain isolated colonies allowing us to estimate the bacterial density of the sample.
305 Bacterial density was expressed as CFU (Colony Forming Units) per cm^2 (for
306 repeatability estimates of intraspecific variation of eggshell bacterial loads see
307 Peralta-Sánchez *et al.* 2012), or as CFU per μl of sample (secretions and unhatched
308 egg contents).
309
310 **Hatching success**
311 We estimated hatching success of nests as the percentage of eggs that successfully
312 hatched among those that remained in the nests at hatching. Some nests were deserted
313 after sampling the eggs at the end of incubation and therefore before hatching. Thus,
314 sample sizes in tests analysing hatching success differ from those involving only
315 bacterial loads (see degrees of freedom associated with different statistical models in
316 the text).
317
318 **Statistical analyses**
319 Statistical analyses were performed using the software Statistica 10 (Statsoft Inc.
320 2011). We did not collect all information for all reproductive attempts of experimental
321 females and, thus, sample sizes used in different analyses differ.

We estimated the effects of early versus 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

347 factors and sampling date as covariate; effect of treatment: *Wilks* $\lambda = 0.84$, $F = 0.20$, P
348 $= 0.93$)). Thus, we combined data from control I and Control II treatments to improve
349 statistical power of the analyses.
350 The effects of experimental manipulation of the uropygial gland on eggshell bacterial
351 load were analysed using two different approaches. First we used GLMMs with the
352 four different bacterial counts as dependent variables, experimental treatment as the
353 fixed factor and female identity as a random factor to account for the repeated
354 measures nature of our data set. The interaction between treatment and female identity
355 was also included in the model for testing whether the experiment resulted in a similar
356 effect on different females. Secondly, by mean of MANCOVAs we analysed whether
357 the relationship between eggshell bacterial loads and bacterial density of the uropygial
358 gland secretion differed for experimental and control females. Therefore, the models
359 included eggshell bacterial densities as dependent variables, treatment as the fixed
360 factor, bacterial density of uropygial gland secretion of females at the beginning of the
361 incubation as covariate, and the interaction between treatment and the covariate.
362 Next, we analysed the bacterial loads of contents of unhatched eggs. Log-transformed
363 bacterial loads of contents of unhatched eggs did not differ from normal distributions
364 (Kolmogorov-Smirnov test, $P > 0.05$), and we therefore used parametric statistics.
365 Moreover, among nests variation in bacterial loads of unhatched eggs was
366 significantly larger than the within nest variations ($F_{25,32} > 2.14$, $P < 0.033$), and thus
367 we used mean values of experimental nests in subsequent analyses. In addition, we
368 did not find evidence of between-years variation ($F_{1,10} > 0.45$, $P > 0.51$), and thus we
369 did not include year as an independent factor in subsequent analyses. Finally, bacterial
370 loads of unhatched eggs in nests of control I and control II females harboured similar
371 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 semi-partial 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 1 in Supporting Information), or to smear the eggshells by opening the bill tip holding a drop of secretion when it contacts to the egg (Video 2 in 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).

FIG.2 AND FIG. 3 ABOUT HERE

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, Figs 2C and 2E). Close examination of crypts in eggs of un-manipulated females at the end of the incubation period (i.e. SEM images at greater magnification) let to recognize bacteria within the matrix of the filling substance (Figs 2G and 2H).

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 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.

FIG. 4 ABOUT HERE

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 those of control females. Interestingly, these differences were especially pronounced for total mesophilic bacteria (GLM, treatment effect: $F_{1,11.4}$ = 12.31, P = 0.005) and *Enterococcus* (GLM, treatment effect: $F_{1,\ 11.5}$ = 7.64, P = 0.018), but not for *Staphylococcus* (GLM, treatment effect: $F_{1,11.4}$ = 0.03, P = 0.86) and did not reach statistical significance for *Enterobacteriaceae* (GLM, treatment effect: $F_{1,11.2}$ = 4.16, P = 0.072) (Fig. 5A). These results suggest that the experiment limited the access of symbiotic enterococococci from the uropygial secretion to the eggshells.

Several pieces of evidence are in accordance with that interpretation. First, the variation in abundance of total mesophilic bacteria on the eggshells of control females (control I and II together) was closely associated to abundance of enterococci (Beta (SE) = 0.74 (0.14), t_{20} = 5.41, P < 0.0001), and abundance of staphylococci and *Enterobacteriaceae* did not explain significant additional variance (P > 0.2) suggesting that most bacteria detected in eggshells of control females are enterococci. Second, the group of bacteria more closely associated to mesophilic bacteria abundance in eggshells of experimental females was *Enterobacteriaceae* (Beta (SE) =

0.82 (0.15), t_{15} = 5.65, P < 0.0001) with no other group of bacteria explaining additional significant variance suggesting that the experiment prevented the access of symbiotic enterococci to the eggshell. Finally, and more importantly, a positive association between bacterial density of the uropygial gland secretion at the beginning of the incubation period (estimated with TSA culture media) and eggshell bacterial density of total aerobic bacteria ($F_{1,38}$ = 7.01, P = 0.012) and *Enterococcus* ($F_{1,38}$ = 8.74, P = 0.005) were detected for control but not for experimental females, while this was not the case for *Staphylococcus* ($F_{1,38}$ = 1.42, P = 0.24) and *Enterobacteriaceae* ($F_{1,38}$ = 3.81, P = 0.058). Slopes of such relationships for control and experimental females differed significantly for *Enterococcus* (interaction between experimental treatment and bacterial density of secretion, $F_{1,38}$ = 4.29, P = 0.045, Fig. 5B), but not for other bacterial counts ($F_{1,38}$ < 1.99, P > 0.17). It is worth mentioning 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= 0.66, N = 21, P = 0.001; volume of secretion, R= 0.30, N = 21, P = 0.210) or at the end of the incubation period (partial correlations, density of bacteria in secretion, R= 0.61, N = 21, P = 0.004; volume of secretion, R= -0.23, N = 21, P = 0.334).

FIG. 5 ABOUT HERE

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, staphylococci and *Enterobacteriaceae* (i.e. semi-partial 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, was 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 staphylococci (R = 0.14, N = 23, P = 0.520) were not significantly associated with hatching success.

FIG.6 ABOUT HERE

Discussion

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 hypothesised (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

546 in accordance with the hypothesis that enterococci on the eggshells of hoopoes are
547 derived, at least partly, from bacteria in the uropygial gland. First, eggshells of
548 females experimentally prevented from accessing the gland had lower bacterial
549 densities than those of control females. Second, there was a positive association
550 between bacterial density of the uropygial gland secretion of non-manipulated females
551 and eggshell bacterial density when considering either total aerobic bacteria or
552 enterococci. Finally, our experiment successfully broke the positive correlation
553 between densities of bacteria in the uropygial secretion and on eggshells when
554 considering enteroccoci, but not other groups of bacteria found on the eggshell. These
555 results support the hypothesis that the enterococci found on eggshell of hoopoe eggs
556 at least partially derive from those present in the uropygial gland secretion of
557 incubating females and, consequently, eggshell crypts would enhance adhesion of
558 symbiotic bacteria from the females' secretion to the eggshell.
559
560 **Uropygial secretions with symbionts on hoopoe eggshells reduce trans-shell**
561 **contamination and improve hatching success**
562 The density of bacteria inside unhatched eggs was higher in nests of experimental
563 females than in those of control females indicating that the absence of uropygial gland
564 secretion on hoopoe eggshells makes eggs more permeable to bacterial infection. The
565 effect of uropygial secretion on trans-shell contamination could be due to physical
566 properties of eggshells if, by filling crypts, the secretion acted as a barrier to water
567 penetration thus reducing the risk that bacteria were transported by water (Cook *et al.*
568 2003). Although we cannot discard this possibility, several lines of evidence suggest
569 that symbiotic bacteria living in the secretion on the eggshell also play a role in
570 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. Second, 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 and 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 enteroccoci symbionts in the gland and on the eggshells and hatching success (Fig. 6a and 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 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

596 required to detect differences among experimental groups. Variation in pathogenic
597 microbial environment of nests of hoopoes could also hinder the predicted
598 experimental effect on hatching success. We would expect high effects of our
599 manipulation in environments with a high risk of infection, while in subpopulations
600 where the probability of hatching failures is low such effect would be minimized.
601 Moreover, we can also speculate with the possibility that hoopoes enhance growth of
602 beneficial enterococci in risky pathogenic environments differentially.. In accordance
603 with this possibility we found that density of symbiotic *Enterococcus* and that of the
604 potentially pathogenic *Enterobacteriaceae* on the eggshells of control hoopoes were
605 strongly positively related (R = 0.891, N = 23, P < 0.001). This result might suggest
606 that protective enterococci are more abundant in nest environments with higher risk of
607 embryo infection. This can happen, for example, if symbionts and pathogens are more
608 abundant in nest holes used by hoopoes the previous breeding season. This possibility
609 opens a hypothetical adjustment of antimicrobial characteristics of uropygial secretion
610 to pathogenic nest environment, which we know increase for delayed reproductive
611 attempts (Martin-Vivaldi et al unpublished results). Experiments (i.e., augmenting
612 density of pathogenic bacteria on the eggs) breaking the relationship between
613 symbiotic and potential pathogenic microorganisms are necessary for further
614 exploring the protective role of enterococci from the uropygial gland of hoopoes
615 impeding trans-shell embryo infection.
616
617 **Eggshell crypts and uropygial secretion of hoopoes**
618 Together with the detection of special structures of the eggshell of hoopoes, all these
619 results support the hypothesis that eggshell crypts harbouring secretions function
620 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 *et al.* 1989). 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 wood-hoopoes (*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. However, this possibility seems improbable, given that eggs change in colour occurs soon after laying as a consequence of smearing with the secretion eggshells without accessory material (i.e. cuticle cover) and crypts (Martín-Vivaldi et al. unpublished data), and there is not reversion in its appearance along incubation (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 adaptive 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, thereby, reducing the probability of trans-shell microbial contamination of eggs and, therefore, of embryo infection as shown by the detected positive association between abundance of enterococci and hatching success that disappeared for females with experimental restricted access to the secretion. Our results therefore further support the mutualistic relationship between hoopoes and these symbionts (Soler *et al*. 2008; Ruiz-Rodriguez *et al.* 2009;

2012) and suggest a long-term evolutionary history between hoopoes and mutualistic enterococci living in their uropygial gland.

Acknowledgements

We thank Estefanía López Hernández for help in laboratory work; Ana Belén García, Jonathan Romero, Manuel Soto and Jorge Doña for help in caring for captive hoopoes; Juan de Dios Bueno for advice in preparation of eggshell samples; and Alicia González, Isabel Guerra and Rocío Márquez for help with SEM observations. Anders Pape Møller, Juan Moreno, Manuel Soler and Carmen Zamora gave helpful suggestions. Support by funding was provided by Spanish Ministerio de Ciencia e Innovación, European funds (FEDER) (CGL2009-14006, CGL2010-19233-C03-01 and CGL2010-19233-C03-03) and Junta de Andalucía (P09-RNM-4557).

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 field work 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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809 Figure legends

810

811 Figure 1. The experiment consisted of impeding (Experimental, A) the access by
812 some females to uropygial secretion with flexible silicone tubes (D). Other females
813 wore silicone tubes that did not covered gland entrance thus they had access to the
814 secretions (Control I, B), while a third group of females did not wear silicone tubes
815 (control II, C).

816

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

834

835 Figure 3. SEM images of the eggshell surface of (**a**) scops owl, (**b**) roller, (**c**) rock
836 dove, (**d**) spotless starling, and (**e**) house sparrow, at laying (left, numbered 1) and at
837 the end of incubation (right, numbered 2). Eggshells of these species do not have
838 crypts, and in some (a-1, d-1, d-2, e-1, e-2), but not in other pictures (b and c) the
839 cuticle or an organic cover can be detected as a thin layer usually fractured with the
840 appearance of dried mud.

841

842 Figure 4. Percentage (± 95% confidence intervals) of crypts of eggshells of
843 experimental hoopoes that were filled with material at hatching. SEM pictures show
844 empty (0%) and filled (100%) eggshell crypts.

845

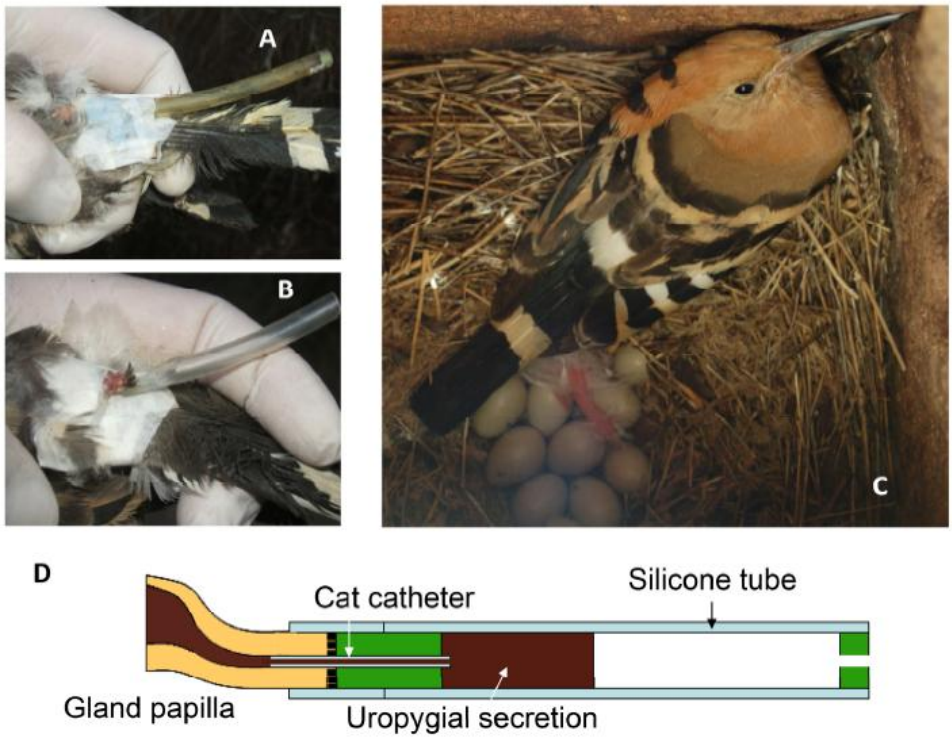
846 Figure 5. **a**: Bacterial loads (± SE) of hoopoe eggshells with (control I and control II)
847 and without (experimental) access to uropygial gland secretion. **b:** Shows the
848 relationship between density of aerobic bacteria in the uropygial gland of hoopoe
849 females at the beginning of laying (i.e. before the manipulation of the experiment to
850 impede access to secretion) and density of *Enterococcus* on eggshells of experimental
851 (red squares and lines) and control (blue circles and lines) females at the end of
852 incubation (i.e. in response to the experiment). **c**: Density (i.e. CFU per 100 µl of egg
853 contents) of bacteria inside hoopoe eggs that failed to hatch and that were incubated
854 by females with (control) or without (experimental) access to uropygial gland
855 secretion.

856

857 Figure 6. Relationships between density of symbiotic bacteria in female secretions (a)
858 and on eggshells (b) and hatching success for control (blue open circles and dashed

regression line) and experimental females (blue filled circles and continuous regression line). Subfigure c shows the relationship between hatching success and eggshell density of *Enterococcus* (blue circles and regression line) and *Enterobacteriaceae* (red circles and regression line) (i.e. standardized residuals of CFU after controlling for estimates of other kinds of bacteria) for nests with detected bacterial growth in the selective medium.

Fig 1


A
B
C
D
Cat catheter
Silicone tube
Gland papilla
Uropygial secretion

866

867

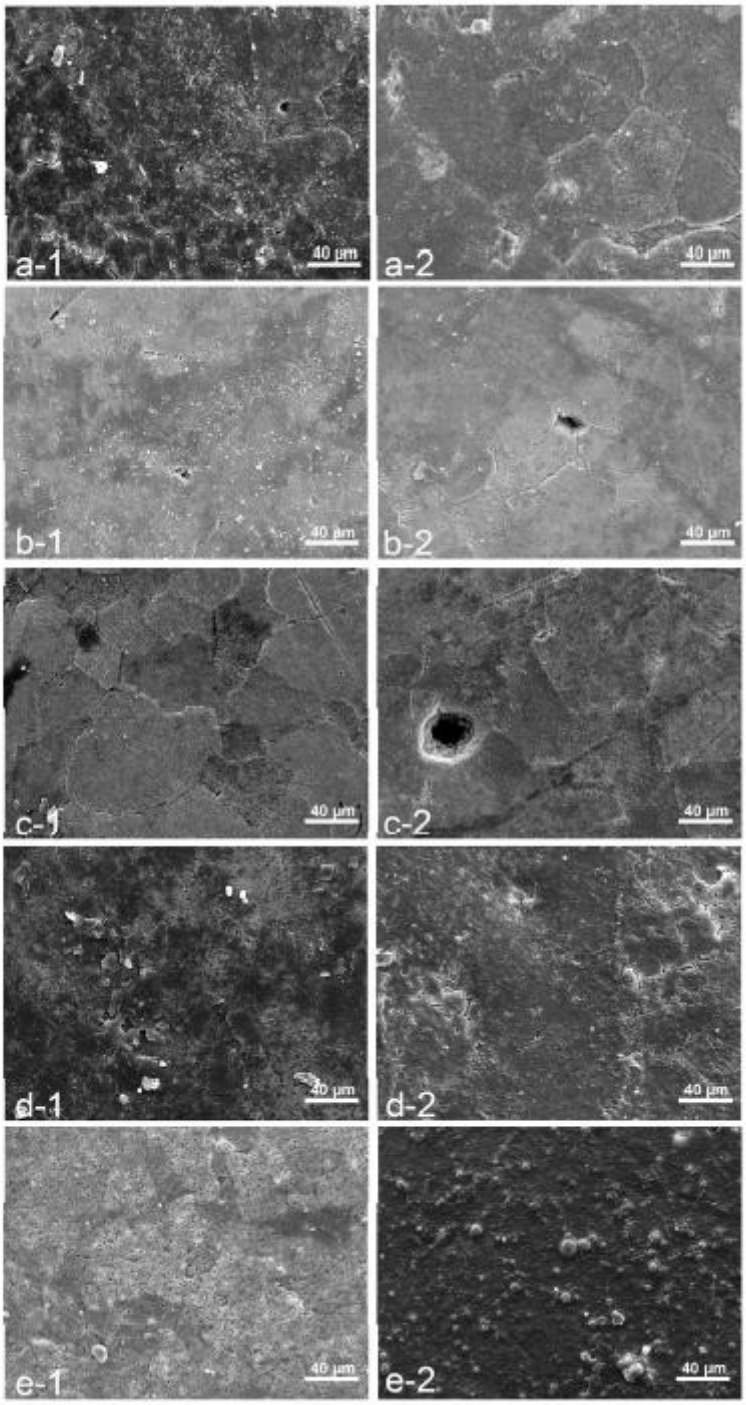
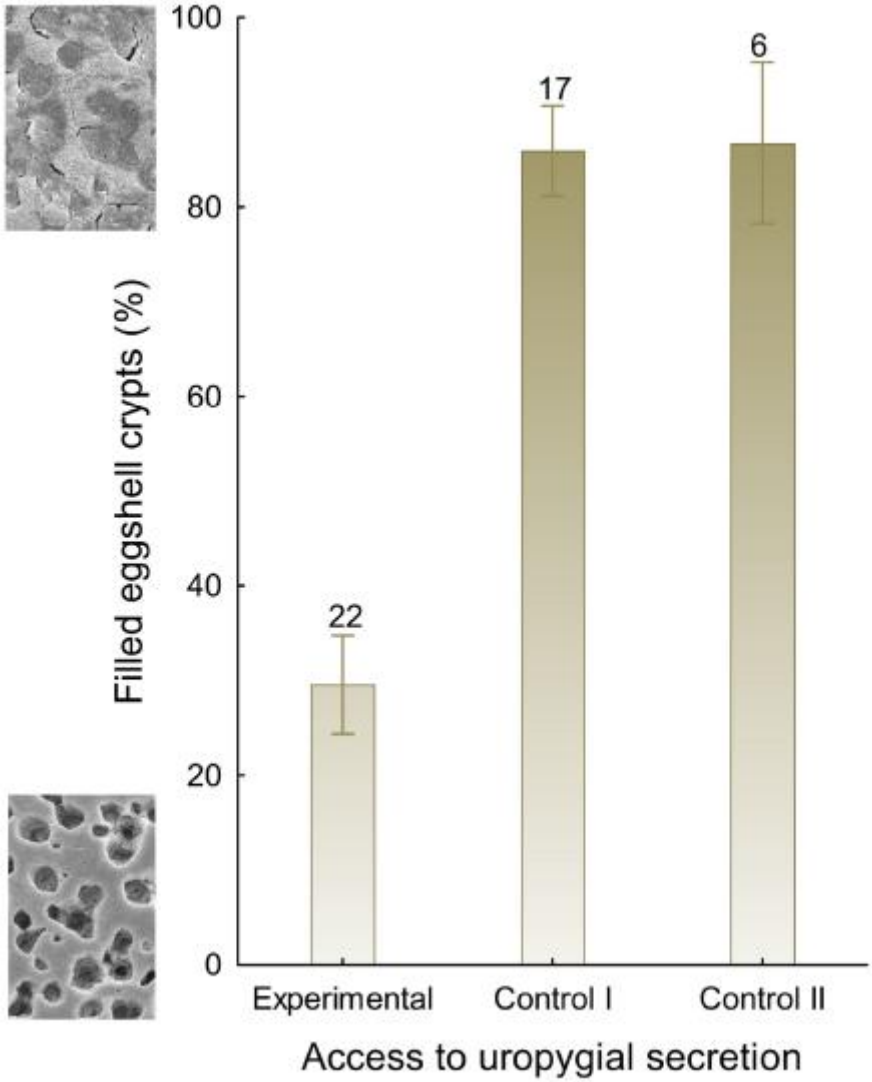

a-1
40 μm
a-2
40 μm
b-1
40 μm
b-2
40 μm
c-1
40 μm
c-2
40 μm
d-1
40 μm
d-2
40 μm
e-1
40 μm
e-2
40 μm

Fig 3

868

869

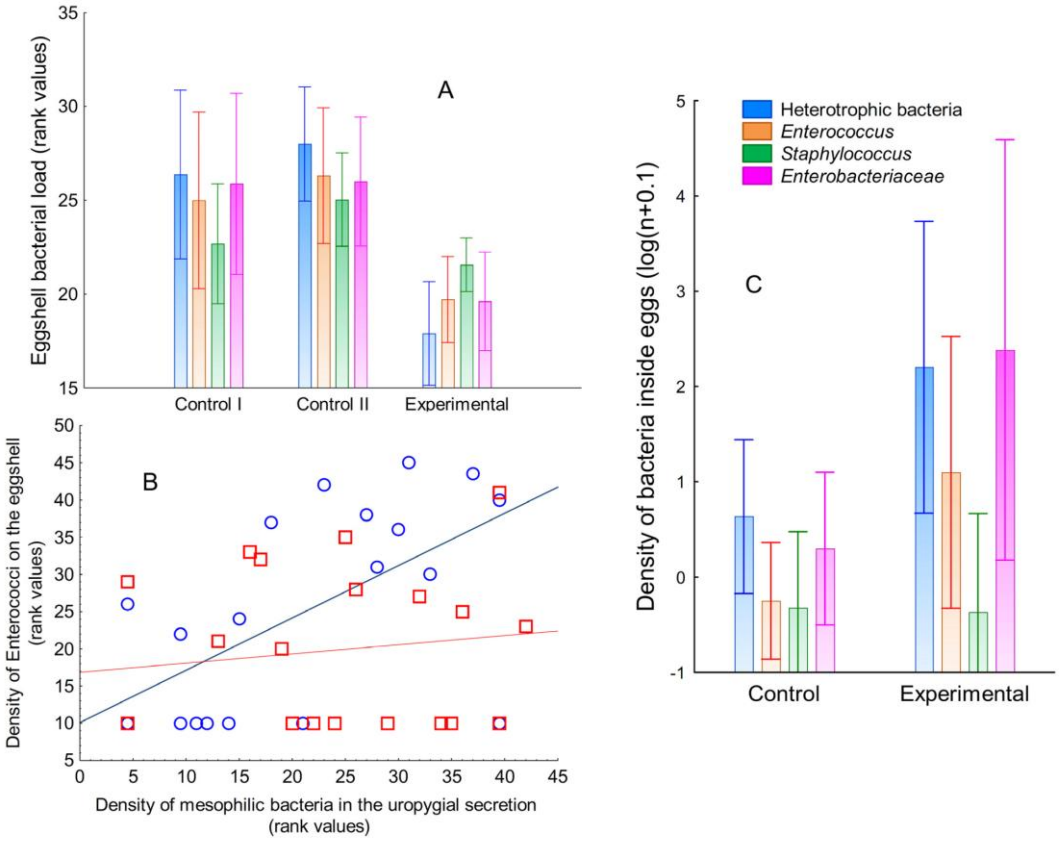
Fig 4


100
80
60
40
20
0
Filled eggshell crypts (%)
22
17
6
Experimental
Control I
Control II
Access to uropygial secretion

870

871

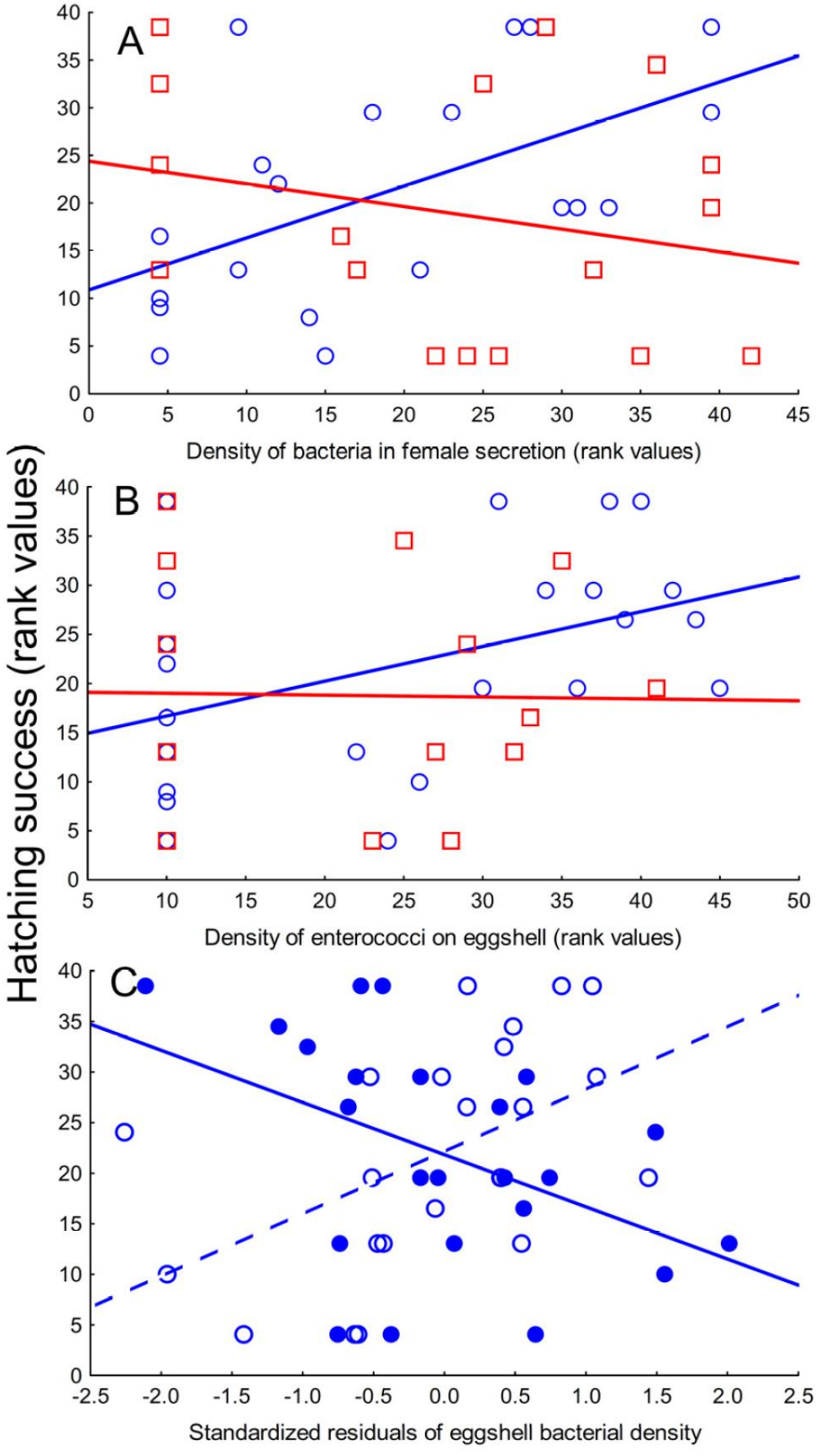
Fig 5


A
Eggshell bacterial load (rank values)
Control I
Control II
Experimental
B
Density of Enterococci on the eggshell (rank values)
Density of mesophilic bacteria in the uropygial secretion (rank values)
C
Heterotrophic bacteria
Enterococcus
Staphylococcus
Enterobacteriaceae
Density of bacteria inside eggs (log(n+0.1)
Control
Experimental

872

873

Fig 6:


A
B
C
Hatching success (rank values)
Density of bacteria in female secretion (rank values)
Density of enterococci on eggshell (rank values)
Standardized residuals of eggshell bacterial density

874