

Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland

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Summary

1. It has been recently showed that one bacterial strain isolated from the uropygial gland of a nestling hoopoe *Upupa epops* produced antimicrobial peptides active against a broad spectrum of pathogenic bacteria. These bacteria might thus mediate antimicrobial properties of the uropygial secretions as a consequence of the symbiotic association with hoopoes.

2. We study antimicrobial properties of white (from males and non-breeding females) and brown (from nestlings and breeding females) uropygial gland secretions of hoopoes *Upupa epops*, as well as the association with the presence of bacteria living inside their uropygial gland.

3. We found that brown, but not white secretions contained bacteria and showed antimicrobial activity against the feather degrading bacterium *Bacillus licheniformis*. The antagonistic activity of bacterial colonies was mediated by antimicrobial peptides because protease inhibited antimicrobial properties.

4. All except one identified bacterium in aerobic cultures were of the genus *Enterococcus*, and the microscopic study of uropygial secretions and glands confirmed a high density of bacteria within the gland.

5. Furthermore, we studied potential benefits of antimicrobial peptides produced by symbiotic bacteria of hoopoes by adding protease to incubating nests.

6. The experiment increased bacterial growth and hatching failures in hoopoes but not in spotless starlings *Sturnus unicolor*, a species that does not harbour bacteria in its uropygial gland.

7. Thus, microbiological, anatomical and ecological results suggest a tight symbiotic interaction between bacteria that produce antibiotic substances and the hoopoes.

Key-words: antibiotic producing bacteria, mutualism, preening behaviour, symbiotic interactions, uropygial glands

Introduction

Bacteria associated with the uropygial gland secretions have recently been detected in two different species of Coraciiformes, the hoopoe *Upupa epops* (Martín-Platero *et al.* 2006) and in the closely related red-billed woodhoopoe *Phoeniculus purpureus* (Law-Brown & Meyers 2003). For the former, a bacterial strain of the species *Enterococcus faecalis* that produced at least two different kinds of peptides with strong antibiotic properties (bacteriocins) has been isolated (Martín-Platero *et al.* 2006). For the latter, a new bacterial species (*E. phoeniculicola*) was isolated in their uropygial

gland secretions (Law-Brown & Meyers 2003), where some chemical components with known antimicrobial activity also exist (Burger *et al.* 2004). The secretions of the woodhoopoes, as well as those of nestling and female hoopoes (but not those of males), during the breeding season are very similar and peculiar. Both species secrete brown and malodorous oils that are believed to function as predator repellents (Cramp 1985; Ligon 2001). The secretions may also prevent infections given the presence of antimicrobial chemical components and/or bacteriocin-like inhibitory substances-producing bacteria.

Bacteria produce compounds that inhibit antagonistic-competing micro-organisms (Riley & Wertz 2002) and, therefore, they may provide hosts with protection against pathogens and parasites due to bacterial interference (Ji,

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Beavis & Novick 1997). Accordingly, there are reports of highly integrated symbiotic associations (*sensu*, Herre *et al.* 1999; Thompson 2005) between animals and antibiotic-producing bacteria (Gil-Turnes, Hay & Fenical 1989; Currie *et al.* 1999; Oliver *et al.* 2003) that have been reviewed recently elsewhere (Moran 2006; Lombardo 2008). Thus, bacteria associated with the uropygial gland of hoopoes and/or the antimicrobial peptides they produce might impede pathogenic infections at the nests.

Here, we study the antimicrobial properties of the brown, malodorous uropygial gland secretion of nestling and breeding female European hoopoes, but also of the white and odourless secretion of males and non-breeding females. Soon after laying hoopoe eggs become completely smeared with the secretion of females, changing the colour of eggshells from pale blue to completely brown (Cramp 1998), suggesting a potential antimicrobial function. We experimentally deactivate antimicrobial peptides from the uropygial gland secretion by adding protease to the eggshells and study the effect on bacteria growth on eggshell, and on hatching success. We also identify bacteria isolated from secretions and explore the presence of bacteria within both the organ and the secretion.

Material and methods

STUDY SPECIES

The hoopoe is a coraciiform bird that nests in holes without adding nest material. As other species (Reneerkens, Piersma & Damste 2002; Reneerkens *et al.* 2008), hoopoes experience seasonal changes in the properties of the uropygial gland secretion of females and nestlings, but not in that of males (Cramp 1985). While male secretion is invariably white and oily, that of females and nestlings is, at least partially, water soluble, brown, and malodorous during their stay in the nest, but not at other times of the year when the secretions of females and males appears not to differ (Cramp 1985).

STUDY AREA

The fieldwork was performed during the breeding seasons 2003–2007 in the Hoya de Guadix (37°18'N, 38°11'W), southern Spain, where hoopoes breed in crops, forests and gullies within nest-boxes placed in trees or buildings (for a more detailed description of the study area see Martín-Vivaldi *et al.* (1999)). Uropygial gland secretions from adults and nestlings were collected in all years, while the protease experiment and quantification of bacterial colonies on the eggshell were performed during 2005 in a population of hoopoes and starlings breeding in nest boxes (see below).

Sampling of uropygial gland secretion and bacterial determination

We sampled uropygial secretions of both adults and nestling hoopoes. Adults were caught in mist-nets or within nest boxes while incubating or brooding hatchlings and nestlings were sampled at the age of ringing (19–23 days). Samples were collected with a micropipette directly from the inside of the uropygial gland after feathers around the gland were separated and washed with ethanol to avoid contamination. We obtained up to 100 µL of secretion for brown samples, but

only about 10–20 µL for white samples because of the smaller available volume. 5 µL of both brown and white secretions were used for detection of antimicrobial activity (see below) and the remainder sample were introduced in a sterile Eppendorf tube (1.5 mL) and stored at 4 °C until processed in the laboratory shortly after collection.

We added 20 µL of sterile distilled water to each sample and vigorously mixed by repeated pipetting, and then smeared 5 µL of this solution onto two different cultures. As a general bacterial growth medium, we used tripticase soy agar. All cultures were incubated aerobically at 32 °C for 24–48 h. When the number of bacterial colonies was too dense to count, we performed serial dilutions of the uropygial secretion until the cultured sample had isolated colonies allowing us to estimate the bacterial density of the sample.

For bacterial determination we analysed a first set of 23 randomly selected samples including seven nestlings from six different nests, eight females, and eight males. In females, three of the samples were brown, all from incubation and the first 10 days of nestling period, and five were white, from before incubation, or after the nesting phase. All samples from nestlings were brown, and all from males were white. Bacteria only grew in the ten brown samples from which five different colonies were used for bacterial identification. In nine of them, all five bacterial isolates from each sample were initially assigned to the genus *Enterococcus* according to the classification by Schleifer & Kilpper-Bälz (1984) and the criteria of Orvin (1986). To type the isolates from each sample, we used RAPD methodology (Williams *et al.* 1990), which allows us to detect genomic DNA changes between samples, even of closely related strains of the same species (Welsh & McClelland 1990). Bacterial genomic DNA was extracted from overnight cultures of isolated colonies at 37 °C in 1 mL of Brain Heart Infusion broth using the AquaPure Genomic DNA isolation kit (Bio-Rad). DNA amplification was made using the following primer of arbitrary nucleotide sequence M13: 5'-GAG-GGTGGCGGTTCT-3' at a concentration of 1 µM in the reaction mixture. Amplified fragments were visualized on 1.5% agarose gels containing 1 µg mL⁻¹ ethidium bromide, using the 1-kb pair ladder (Biotools, Madrid, Spain) as the molecular weight standard. Isolates of each RAPD pattern were identified firstly to the genus and species level by API 20 Strep strip (Biomérieux, Lyon, France). Genus and species were designated according to phenotypic characteristics and confirmed by PCR amplification using specific primers. For confirmation of the genus *Enterococcus* we used the oligonucleotides 'entero 1' (5'-CCCGGCTCAACCGG-3') and 'entero 2' (5'-CTCTA-GAGTGGTCAA-3'), at concentrations of 2.5 µL each in the reaction mixture (50 µL), to amplify a 500 bp fragment for the identification of enterococci. PCR was carried out as previously described by Deasy *et al.* (2000). We used a multiplex PCR assay based on the specific detection of genes encoding d-alanine : d-alanine ligase (*ddl*) to confirm the identification of *E. faecalis* and *E. faecium* (Dutka-Malen, Evers & Courvalin 1995; Cheng *et al.* 1997). The DNA sequences (5'–3') for the primers used in this study and their corresponding specificities were as follows: the pair of 21-mer primers EM1A (5'-TTGAGGCAGACCAGATTGACG-3') and EM1B (5'-TATGA-CAGCGACTCCGATTCC-3') were used to confirm the identification of *E. faecium* (Cheng *et al.* 1997); *ddl*-E1 (5'-ATCAAGTACAGT-TAGTCTT-3') and *ddl*-E2 (5'-ACGATTCAAAGCTAACTG-3') for *E. faecalis* (Dutka-Malen *et al.* 1995). The specific amplicons were 658 and 940 bp, respectively.

In a second set of samples we used brown uropygial secretions from 7 females and 19 nestlings and RAPD patterns were obtained for all samples, as described above. Resulting fingerprints were analysed with the Fingerprints II Informatix Software 2000 (Bio-Rad, Hercules, CA). Similarity between bacterial samples was calculated

by using the Pearson product moment correlation coefficient, with clustering by the UPGMA algorithm. The identity level for strain discrimination was set at 80% similarity between different genotypes. For 30 of the 41 established genotypes, at least a representative strain was identified by partial 16S gene sequencing. A 700 bp fragment of the 16S ribosomal RNA gene, which includes V1 to V4 variable regions, was amplified in representative strains of each RAPD genotype for subsequent sequencing. PCR was carried out in a total volume of 50 μ L containing 5 μ L of 10 \times Taq reaction buffer, 10 μ L of 5 \times Taq Enhancer, 1.5 mmol of (OAc)₂Mg, 400 μ M of dNTPs, 0.4 μ M of the primers WO1 (5'-AGA GTT TGA TC(AC) TGG CTC-3') and WO12 (5'-TAC GCA TTT CAC C(GT) C TAC A-3'), 1 U of Eppendorf Master Taq polymerase and 1 μ L of template DNA (30–70 ng). The amplification programme consisted of an initial denaturing step at 94 °C for 4 min followed by an amplification step of 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C, and a final extension of 2 min at 72 °C. PCR products were purified with a Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany) and sequenced using an ABI PRISM dye terminator cycle-sequencing ready-reaction automated sequencer (ABI 3100, Applied Biosystems). Homologies were searched in the BLASTN database (National Center for Biotechnology Information) using BLAST (Altschul *et al.* 1997).

Detection of antimicrobial activity

During the breeding seasons of 2004, 2005, 2006 and 2007 we performed tests of uropygial secretion activity against *Bacillus licheniformis* directly in the field. For these tests, we used *B. licheniformis* D13 (Galvez *et al.* 1994). We cultured this strain in 5 mL BHI broth and after overnight growth it was mixed with 15 mL BHA and poured the blend into an empty plate. In the field, the activity test was performed by putting on 5 μ L of the secretion from males, females and nestlings, on the surface of the pre-inoculated BHA plates, directly after extraction from the bird. Plates were incubated at 32 °C for 12 h. Then, we checked for inhibition of growing *B. licheniformis* by detections of clearing zone around the drop of uropygial secretion.

For detection of antimicrobial activity by bacteria isolated from UGS we used BHA, prepared in sodium phosphate buffer 0.1 M, pH 7.2 (BHA-B) as culture medium. All five isolates from each analysed sample were replicated by spotting on BHA-B plates. They were incubated at 37 °C for 24 h and then overlaid with 5 mL of BHI plus 0.8% agar inoculated with 0.1 mL of an overnight culture of *E. faecalis* S-47 (from our collection), *Listeria innocua* CECT 4030 or *B. licheniformis* D-13.

Microscopic study

Uropygial secretions were obtained from live individuals with a micropipette as explained above. Glands were from fresh individuals killed by predators. After extraction, samples used for electronic microscope were immediately fixed in 2.5% glutaraldehyde, washed and post-fixed in osmium tetroxide, dehydrated and embedded in Embed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a C. ZEISS EM 902 transmission electron microscope. Semi-thin sections of the same samples were stained with toluidine blue and observed in an OLYMPUS BX51 microscope. Samples used for fluorescence microscopy consisted of 2 μ L of the secretion that were transferred directly from the gland of live birds to a gelatinized excavated-slide, air-dried, fixed in 4% paraformaldehyde, dehydrated, stained with hoescht and examined with a blue filter.

Protease-field experiments

During the breeding season of 2005, before laying, we covered the bottom of nest boxes with a permeable carpet that was permanently punctured with a butterfly needle and connected to the exterior by a plastic tube of 3 mm diameter. Carpets were partially covered by nest material (small pieces of bark that are commercialized for gardens and that were added to the nest boxes when installed), but assuring that eggs were in contact with the carpet. Each occupied nest box was randomly assigned to one of three treatments, namely, (i) protease; (ii) water and (iii) untreated control. After clutch completion, all nests were visited every second day to perfuse the carpet with 50 mL of protease (Proteinase-K) dissolved in distilled water (dilution 5 g/200 mL) or distilled water respectively, at experimental (protease) and control 1 (water) nests. Previous experience in unoccupied nests allowed us to estimate the appropriate amount of water to assure proper nest moistness (i.e. avoiding carpet inundation or total desiccation) during 48 h. The untreated control nests (natural) were also visited every two days. Inhibitors of proteases are common components of bird eggs (Board *et al.* 1994) and, thus, it is unlikely that the protease directly affected the embryo or hatchability. In any case, to rule out this possibility, we also performed the experiment on spotless starling as a control species, as there is no evidence of bacteriocin-like inhibitory substances-producing bacteria living in the uropygial gland in this species. We directly applied protease, water, or nothing to the eggs on every second day and explored the effects on bacteria living on the eggshell and on hatching success.

Bacteria sampling on eggs of hoopoes and starlings

After clutch completion, all eggs within a nest were sampled for bacterial contamination. Briefly, we cleaned each eggshell with a swab that was subsequently stored in an eppendorf tube with sterilized water (0.6 mL). Swabs with the bacterial sample were transported to the lab in the Eppendorf tube with the rest of the sterilized water at 4 °C. In the afternoon, 50 μ L of bacterial suspensions (water in the eppendorf tubes where the cottons with bacteria were transported to the lab) were spread on Hektoen plates, a selective medium for Enterobacteria, and incubated during 72 h at 37 °C. After incubation, the number of colonies in the Petri dishes were counted. When bacterial density in the plate did not allow counting the number of colonies properly, we performed serial dilutions up to having isolated colonies in the plates. The absolute number of colonies was used when < 300 colonies were detected. Otherwise, we used an estimate (i.e. 500, 1000, 2000).

The collection and laboratory protocol were repeated after 9 days, therefore, allowing estimation of the change in number of bacterial colonies in relation to experimental treatment. Before the analyses, bacterial counting was log-transformed, and nest identity was introduced as a random factor nested within experimental treatment to avoid pseudo-replication. All statistical test were two tailed and performed with the Statistica 7.1 software (Statsoft 2006).

Results

ANTIMICROBIAL ACTIVITY AND BACTERIAL DETECTION

While white secretions from adults ($N = 26$) did not inhibit growth of *B. licheniformis* (percentage of samples with activity: 0%), brown secretions from females ($N = 35$; percentage

of samples with activity: 74.3%, comparison with odourless secretions Fisher exact test, $P < 0.0001$) and nestlings did ($N = 114$; percentage of samples with activity: 92.5%, comparison with odourless secretions Fisher exact test, $P < 0.0001$). When considering one randomly selected nestling per brood, results did not change ($N = 16$, percentage of samples with activity 94.0%). Moreover, bacteria grew more often and produced more colonies per standardized volume of secretion when using malodorous brown secretions from females (prevalence of bacteria: 91.9%, $N = 37$; average number of colonies per 1 μL of secretion = 1.3×10^3) or nestlings (prevalence of bacteria: 95.9%, $N = 74$; average number of colonies per 1 μL of secretion = 7.5×10^5) than when using white odourless secretions from males (prevalence of bacteria: 30.8%; $N = 13$; Number of colonies < 5) (Fisher exact test, $P < 0.0001$). Therefore, we detected antimicrobial activity for brown but not for white secretions and a significant association between secretion properties and the presence and abundance of bacteria in the secretion.

Spots of *E. faecalis* isolated from uropygial gland of three different females and seven nestlings from six different nests demonstrated clear growth-inhibition activity against *E. faecalis* S-47, *L. innocua* CECT 4030 and *B. licheniformis* D13. The detected antimicrobial activity was due to bacteriocin-like inhibitor substances produced *E. faecalis* because the antimicrobial effects of such colonies were suppressed by the addition of proteases to the culture media in all cases ($N = 20$; two samples per individual). This last result confirms the use of protease as a valid experimental approach for inhibition of activity of bacteriocin-like substances produced by bacteria living in the uropygial gland of hoopoes.

In accordance with the association between characteristics of the uropygial gland secretion and the presence of bacteria, the microscopic study of the uropygial gland of hoopoes that produced brown secretions, as well as the magnification of the uropygial secretion, clearly showed an exaggerated bacterial density, both in the secretion (Fig. 1) and inside the papilla of the uropygial gland (Fig. 2). Moreover, dissection of the papilla wall allowed the observation of aggregations of bacteria among epithelial layers that are also detected in the secretions under the electron microscope (Fig. 2). This visual detection of bacteria at high density within the uropygial gland confirms that the association between the bacteria and the secretion occurs within the gland.

BACTERIAL IDENTIFICATION

Interestingly, the identification of colonies isolated from ten randomly selected brown uropygial gland secretions in 2003 (i.e. females or nestlings) analysed for this purpose, invariably resulted in a single specific bacterium, *E. faecalis*, (see Material and Methods). Further, all five isolates from each birds usually belong to the same bacterial strains as it is demonstrated by the identical RAPD profile (see Fig. 3). On the other hand, the few bacterial colonies isolated from the few white secretions harbouring them in the sample of 2003 (4 out of 13, in all cases < 5 colonies per sample), were never enterococci. Further, BLAST analyses (Altschul *et al.* 1997) of the 700-nt 16S rRNA gene sequences of 107 strains from seven females and 19 nestlings revealed that the top hits were *E. faecalis* (79.4% of the strains), *E. faecium* (6.5%), *E. mundtii* (5.6%), *E. avium* (3.7%) and *E. gallinarum* (2.3%). Only one strain was

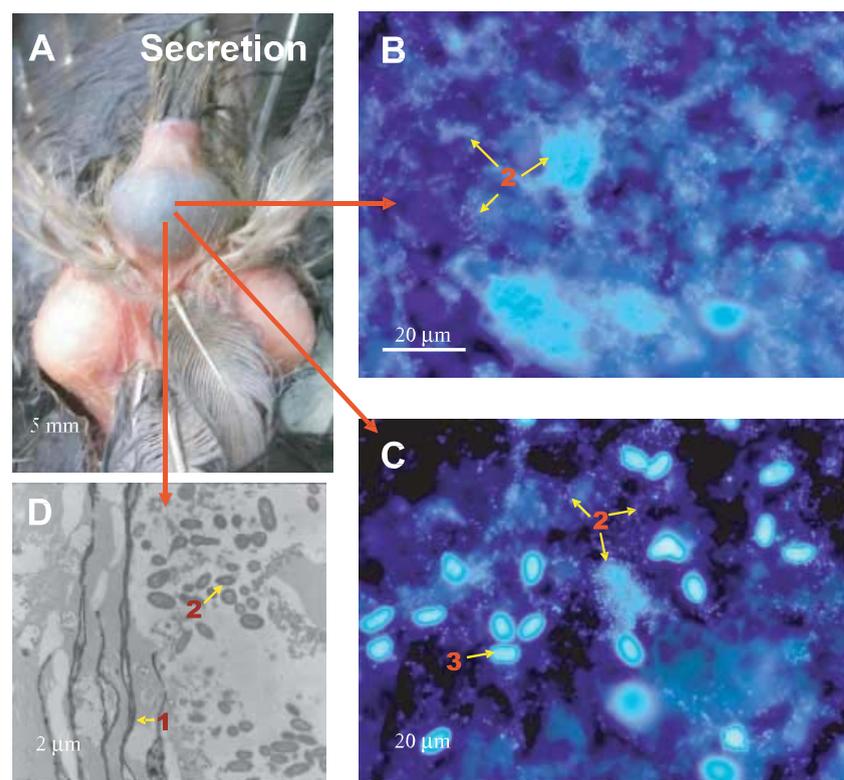


Fig. 1. Location of bacteria living within hoopoe uropygial glands: observation of secretion. (A) Uropygial gland of a female hoopoe with the papilla filled with brown secretion. (B and C) Brown UGS from two different breeding females visualized under a fluorescence microscope with a blue filter. Bacteria stained with Hoescht (2) are abundant throughout the whole secretions, and in some places form dense aggregates. In image C there are also stained nuclei from the bird cells (3). (D) Electron microscope image of the secretion of another breeding female where bacteria (2), but also remains of epithelial layers (corneum) (1) and secretory cells are visualized.

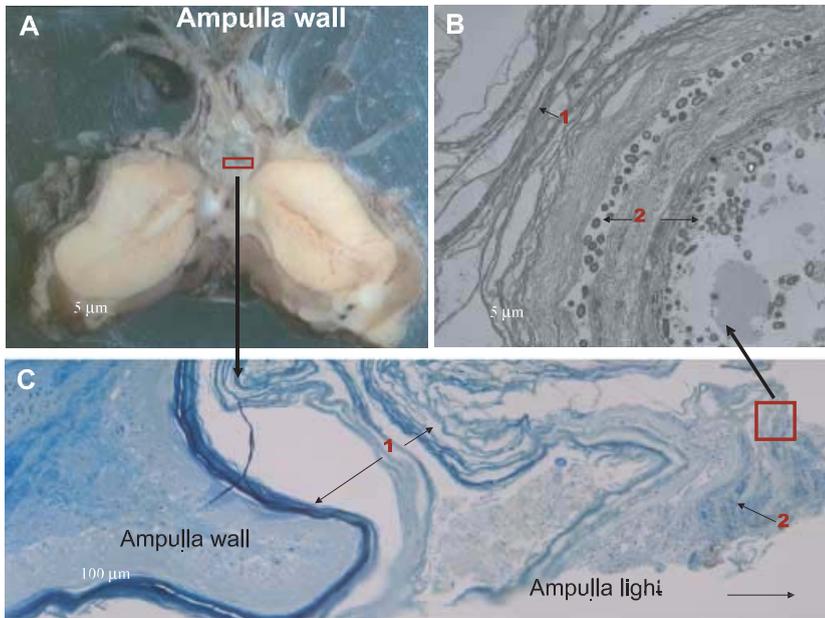


Fig. 2. Location of bacteria living within hoopoe uropygial glands: observation of gland sections. (A) Transversal dissection of the uropygial gland of a nestling hoopoe. The square represents the area of the papilla wall that was serially slashed and prepared for observation under both optic (C) and electron (B) microscope. In both pictures the aggregation of bacteria (2) among corneum layers at the limit between the papilla wall and the cavity are patent.

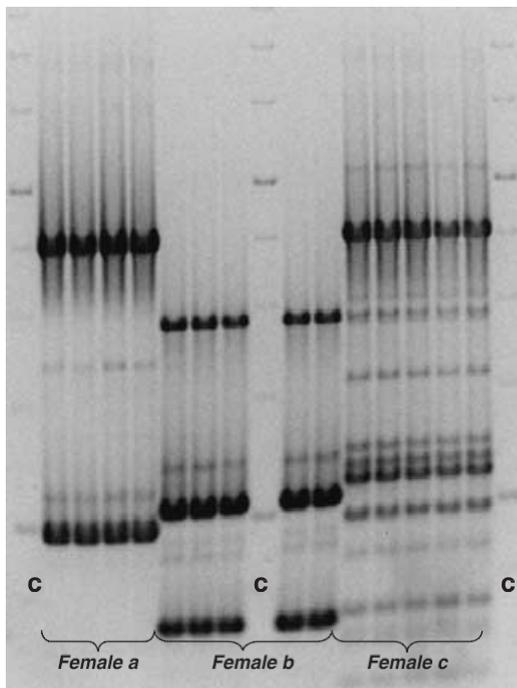


Fig. 3. RAPD patterns of the 14 strains isolated from three different hoopoe females. Lanes C show lambda ladder used as DNA marker and consists of seven fragments ranging in size from 564 to 23 130 bp (564, 2027, 2322, 4361, 6557, 9416, 23 130).

not enterococci and that was identified as *Micrococcus luteus* (0.9%).

PROTEASE EXPERIMENT

The experimental addition of protease to nests of hoopoes resulted in a higher eggshell bacterial colonization in comparison with that of eggshells from natural nests or from

those with experimental addition of water (Fig. 4a) (GLM, log-transformed differences in number of colonies between the two sample dates as dependent variable; experimental treatment as the fixed factor, and nest identity as random factor; 22 nests, 155 eggs; post-hoc comparisons (LSD): protease vs. water, $P < 0.0001$; protease vs. non-manipulated, $P < 0.0001$; water vs. non-manipulated, $P > 0.95$). Although with lower sample size, we did not detect the same experimental effect in nests of spotless starling (Fig. 4b) (GLM, log-transformed differences in number of colonies between the two sample dates as dependent variable; experimental treatment as the fixed factor, and nest identity as random factor; 8 nests, 32 eggs; post-hoc comparisons (LSD): protease vs. water, $P = 0.34$). In addition, the effect of the experiment was not due to the water used to perform protease dilution (i.e. increase in nest humidity) because bacteria increased at a similar rate in natural and water treated nests (Fig. 4).

Further, we detected a marginally non-significant effect of our protease experiment on hatching success of hoopoes (Kruskal-Wallis test: $H_2 = 1.91$; $P = 0.059$) but not on that of starlings (Kruskal-Wallis test: $H_2 = 1.19$; $P = 0.55$) (Fig. 4c). In hoopoes, hatching success of control-treatment nests did not differ ($N_{(\text{non-manipulated})} = 6$, $N_{(\text{control-water})} = 4$; Mann-Whitney U -test, $Z_{\text{adj}} = 1.38$; Exact P for small samples = 0.26). Further, the addition of protease to experimental nests significantly increased hatching failure when comparing with that of control-water group ($N_{(\text{Protease})} = 5$, $N_{(\text{control-water})} = 4$; Mann-Whitney U test, $Z_{\text{adj}} = 2.29$; Exact P for small samples = 0.032), but comparison with natural nest did not reach statistical significance ($N_{(\text{Protease})} = 5$, $N_{(\text{non-manipulated})} = 6$; Mann-Whitney U test, $Z_{\text{adj}} = 1.23$; Exact P for small samples = 0.25) (Fig. 4c). Again, the experiment had no statistically significant effect on hatching failure of starlings (protease treatment, $N = 8$; hatching failure in only one nest), vs. water treatment ($N = 7$; hatching failure in only one nest)

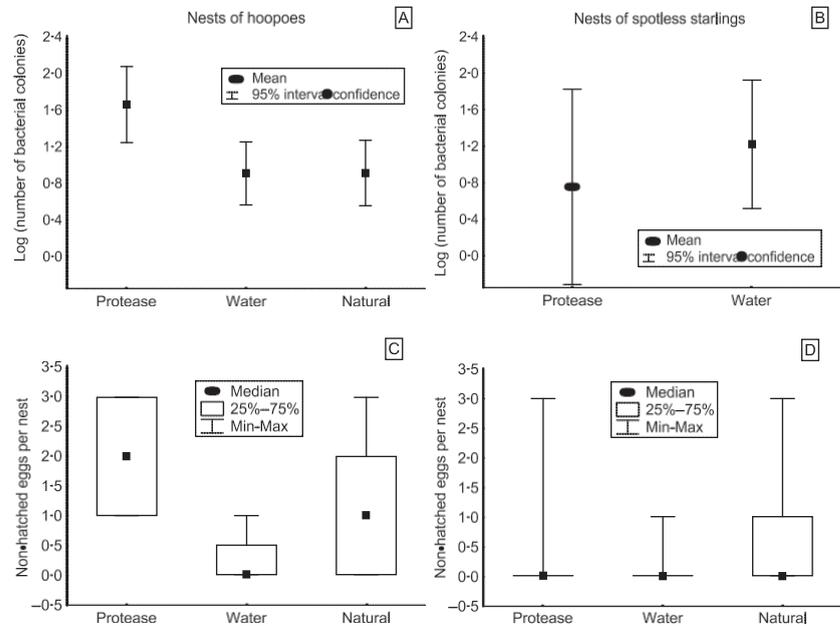


Fig. 4. Differences in number of bacterial colonies grown from egg samples before and 9 days after treatment with protease or water, and in untreated-control nests of hoopoes (A) and spotless starlings (B). Median, 25–75% quartile, and minimum-maximum values of number of eggs that failed to hatch in nests of hoopoes (C) and starlings (D) under different experimental treatments are also shown.

and vs. natural-control treatment ($N = 38$; some egg(s) from 11 nests failed to hatch); Kruskal-Wallis test: $H_2 = 1.19$; $P = 0.55$ (Fig. 4d). These results are unlikely the consequence of the protease diluent because nests that were treated with water experienced the lowest rate of hatching failure in both hoopoe and starling nests (Fig. 4).

Discussion

The main findings of this study were that (i) the brown, but not the white uropygial secretions of hoopoes demonstrated antibiotic properties against pathogenic bacteria; (ii) there was an association between antibiotic properties of the secretion and the presence of bacteria at high density; (iii) colonies isolated from brown secretions were in most cases identified as *E. faecalis*, and such isolates demonstrated antibiotic properties, likely mediated by bacteriocin production; and (iv) microscopic observation of brown secretions and uropygial glands revealed high-density aggregations of bacteria within the papilla of the gland. All these results taken together strongly suggest a symbiotic association between hoopoes and bacteria that live in their uropygial gland during the nesting phase. Furthermore, (v) the inactivation of antimicrobial peptides in nests of hoopoes resulted in a relatively higher egg bacterial infection and hatching failures than in control nests where only water was added. We will briefly discuss these findings and their possible implications in the symbiotic association between bacteria and hoopoes. It should be noted here that, although the term ‘symbiont’ is typically applied to mutualistic microorganisms, we use this term in its broad sense, which includes associates for which the full spectrum of effects on hosts is not known (see, Moran 2006).

It is well known that the uropygial secretions of birds may contain antibiotic substances (Jacob & Ziswiler 1982; Shawkey, Pillai & Hill 2003) and, in accordance, we here found that brown but not white secretion of hoopoes had

antimicrobial properties. In addition, in contraposition to the scarce white secretions, brown secretions are abundant, malodorous, and contain bacteria. The differences in antimicrobial properties of brown and white secretions of hoopoes are not due to differences in volume of secretion since tests of antagonism were performed with similar volumes of white and brown secretions. The presence of bacteria is likely an important factor explaining properties of brown secretions since the experimental injection of antibiotics affected properties of the uropygial gland (i.e. size) of hoopoes as well as the colour, stored volume, and odour of the secretion (Martin-Vivaldi *et al.* submitted). The injection of antibiotic did not clarify whether bacteria are responsible of the antimicrobial property of the secretion because obviously the exogenous commercial antibiotic used in the experiment conferred antimicrobial properties to the uropygial secretion of experimental individuals.

Most colonies isolated from brown secretions in this study were identified as *E. faecalis*, and only one analysed strain was not within the Genus *Enterococcus*. We have however analysed only bacteria that grew aerobically in trypticase soy agar, the most usual general medium for growing bacteria. However, it is known from molecular methodologies that some others bacteria cannot be detected in general media (Pace 1997; Bisson *et al.* 2007) and, thus, we cannot discount the possibility that others anaerobic and/or unculturable bacteria were also present within the uropygial gland of hoopoes. In any case, the very high prevalence of *E. faecalis* in particular and of the genus *Enterococcus* in general suggests a symbiotic relationship between hoopoes and enterococci. Enterococci are facultatively aerobic and, thus, can and do grow under anaerobic conditions (e.g. the uropygial gland of hoopoes).

Could the apparent association between enterococci and the uropygial gland of hoopoes be the result of contamination of the secretion after extraction from the uropygial gland of hoopoes? Several results contradict this interpretation. First, we only detected bacteria in the brown secretion samples and

the risk of contamination should have been equal for both brown and white. Second, we observed at the microscope bacterial aggregations at a high density in secretions fixed immediately after extraction (Fig. 1) as well as inside the uropygial gland of a female hoopoe (Fig. 2). Finally, accidental contamination of the uropygial gland or its secretions would predict for a low prevalence, but we detected this association in most brown samples collected during the breeding season.

Mechanisms explaining colonization of the gland by the bacteria may include immune depression of hoopoes during reproduction. The elevated energetic costs of reproduction and the production of sexual hormones at this stage implies a physiological stress that may have immune suppressive effects (Alonso-Alvarez *et al.* 2004). *Enterococcus* sp. are abundant in the digestive tract and faeces of birds and, contrary to most hole-nesters, hoopoes do not keep their nest clean of faeces. Thus, the faecal contamination of nests of hoopoes, together with a possible worsened immune system during the nesting phase, would facilitate the colonization of the gland of hoopoes by *Enterococcus* sp. However, immune responses of females toward injections of the mitogenic phytohaemagglutinin (PHA) were significantly larger during the nesting phase (incubation ($N = 3$): mean PHA response (SE) = 1.18 (0.16); brooding ($N = 6$): mean PHA response (SE) = 1.34 (0.11)) than when UGS of females were of white colour (pairing ($N = 12$): mean PHA response (SE) = 0.43 (0.08); after abandoning the nest for feeding nestlings ($N = 19$): mean PHA response (SE) = 0.68 (0.06)) (Martin-Vivaldi, Soler & Ruiz-Rodriguez unpublished data), which suggests that females have an enhanced immune system during the nesting phase of reproduction instead of suffering immunosuppression.

Colonies of *Enterococcus* isolated from hoopoe glands, as well as the brown secretions, demonstrated clear growth-inhibition activity against different bacteria including *B. licheniformis*, a common feather-degrading bacterium (Burt & Ichida 1999). Moreover, the addition of protease inhibited the antimicrobial activity of colonies, which suggests that the antimicrobial properties of *Enterococcus* isolated from hoopoes are mediated by production of peptide substances. Therefore, although the brown secretion of hoopoes may contain some other antibiotic substances not directly related to the associated enterococci, due to the antimicrobial properties of the bacteriocin-like inhibitor substances produced (Martín-Platero *et al.* 2006), it is likely that the antimicrobial properties of the uropygial secretion of hoopoes were, at least partially, the product of symbiotic bacteria.

The beneficial antimicrobial-effect of enterococci, due to the production of bacteriocin-like inhibitor substances, is known from research on poultry and food conservation (Foulquié Moreno *et al.* 2006). Among the two species of enterococci that have been intensively studied, *E. faecium* and *E. faecalis*, the beneficial effects are clearer for the former than for the latter. For instance, although strains of both species have been applied in human and veterinary probiotic supplements, nine different strains of *E. faecium*, but none of *E. faecalis*, are authorized by the European Union as additive to feeds (see, Foulquié Moreno *et al.* 2006). For *E. faecalis*, although it is considered an opportunistic pathogen responsible

of nosocomial infections (Franz, Holzapfel & Stiles 1999), some clear benefits due to competitive exclusion of more pathogenic bacteria have been detected (Wagner, Holland & Cerniglia 2002). Moreover, in a wild population of flycatchers, prevalence of both enterococci species in the cloaca of nestlings were negatively associated and that of *E. faecium* was strongly and positively associated with fledging body size and mass (Moreno *et al.* 2003).

To test the hypothetical role of bacteriocin-like inhibitor substances produced by symbiotic bacteria, by means of protease experiments, we deactivated possible antimicrobial peptides on the eggshells of hoopoes and explored the effect on bacterial growth and hatching success. In accordance with the hypothesis we found that, in comparisons with control nests, eggs in experimental nests experienced higher bacterial infection and hatching failures. These effects were not due to protease directly affecting both bacterial infection and hatching failure because avian eggs contain natural inhibitors of proteases (Board *et al.* 1994; Mine 2007), and the experiment had no effect in nests of spotless starling where bacteriocin-like inhibitor substances have not been detected. These results suggest a mutualistic association between hoopoes and *E. faecalis* living in the uropygial gland that provide hosts with substances that inhibit growth of pathogenic bacteria. Alternatively, our experiments might have also deactivated antimicrobial peptides that are directly produced by hoopoes. However, chemical composition of the uropygial secretions of birds do not usually include peptides (Burger *et al.* 2004; Gebauer *et al.* 2004; Montalti *et al.* 2005) and, thus, this explanation is unlikely. In any case, and whatever the origin of deactivated peptides, the association between the experimental effects on bacterial growth and hatching failures detected in hoopoes, at least indicated that protease on the eggshell provoked an increased probability of bacterial infection that might be the cause of the increase in hatching failures.

The use of the spotless starling as a control species for the effect of the protease experiment has also some caveat. Different effects for different species could be explained by interspecific differences not only in the production of bacteriocin-like inhibitor substances by associated bacteria, but also in nest's environmental conditions and eggshell characteristics. However, that problem would occur independently of the species used. Moreover, sample size of starlings is quite low and, thus, the absence of significant effects of the experiment in this species should be cautiously interpreted. Thus, the use of starlings as a control species should be interpreted as a first approach trying to evaluating the effect of protease in eggs or embryos of species other than hoopoes.

Summarizing, we present strong evidence of an association between the presence of enterococci and characteristics of the uropygial gland and its secretion, including the antimicrobial properties, which suggests a symbiotic relationship between hoopoes and these bacteria. Furthermore, the experimental deactivation of peptides on the eggshells of hoopoes resulted in increased hatching failure. Although those results suggest a mutualistic relationship, more studies of potential benefits and costs associated with this symbiotic association are

needed. Furthermore, questions related to the acquisition of the bacteria and its functioning within the uropygial gland of hoopoes are necessary for a better understanding of the symbiotic association and, therefore, for concluding that bacteria are beneficial for hoopoes.

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