Preening as a vehicle for key bacteria in hoopoes

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ABSTRACT

Oily secretions produced in the uropygial gland of incubating female Hoopoes contain antimicrobial-producing bacteria that prevent feathers from degradation and eggs from pathogenic infection. Using the beak, females collect the uropygial gland secretion and smear it directly on the eggshells and brood patch. Thus, some bacterial strains detected in the secretion should also be present on the eggshell, beak, and brood patch. To characterize these bacterial communities, we used Automatic Ribosomal Intergenic Spacer Analysis (ARISA), which distinguishes between taxonomically different bacterial strains (i.e. different Operational Taxonomic Units [OTUs]) by the size of the sequence amplified. We identified a total of 146 different OTUs with sizes between 139 bp and 999 bp. Of these OTUs, 124 were detected in the uropygial oil, 106 on the beak surface, 97 on the brood patch, and 98 on the eggshell. The highest richness of OTUs appeared in the uropygial oil samples. Moreover, the detection of some OTUs on the beak, brood patch, and eggshells of particular nests depended on these OTUs being present in the uropygial oil of the female. These results agree with the hypothesis that symbiotic bacteria are transmitted from the uropygial gland to beak, brood patch, and eggshell surfaces, opening the possibility that the bacterial community of the secretion plays a central role in determining the communities of special hoopoe eggshell structures (i.e. crypts) that, soon after hatching, are filled with uropygial oil, thereby protecting embryos from pathogens.

INTRODUCTION

Symbiotic bacteria are fundamental for animal life. For instance, they are essential to the digestive system of animals (Nalepa 1994, Hill 1997, Ley et al. 2008), play an important role in training the immune system (Umesaki et al. 1999, Macpherson and Harris 2004), and protect the respiratory and gastroinstestinal tracks of animals from pathogenic infections (Fons et al. 2000, Dillon et al. 2005). Some bacteria establish more intimate mutualistic associations with animals harboring them in specialized glands or compartments (Barbieri et al. 2001, Currie et al. 2006), and may protect hosts or their offspring from particular parasites (Moran 2006). For example, such mutualistic associations have been described in in marine isopods (Lindquist et al. 2005), shrimps and lobsters (Gil-Turnes et al. 1989, Gil-Turnes and Fenical 1992), ants (Currie et al. 1999), aphids (Oliver et al. 2003), salamanders (Banning et al. 2008), and birds (Soler et al. 2008, Martín-Vivaldi et al. 2014).

The only cases of mutualism between bacteria known to produce antimicrobials and birds have been described from the uropygial gland of the European hoopoe *(Upupa epops)* (Martín-Platero et al. 2006, Soler et al. 2008) and red-billed woodhoopoe (*Phoeniculus purpureus*) (Law-Brown and Meyers 2003), two closely related species (Mayr 2008). Unlike the red-billed woodhoopoes, symbiotic bacteria of European hoopoes (hereafter hoopoes) appear only in nesting females and chicks, but apparently never in males (Soler et al. 2008). Moreover, the uropygial oil of nesting female hoopoes, which is malodorous and brown in color, is used to coat their eggs (Martín-Vivaldi et al. 2009, Soler et al. 2014). Consequently, it is quite likely that bacteria from the uropygial oil reach eggshells and help protect embryos against trans-shell bacterial contamination (Martín-Vivaldi et al. 2014). In this case, bacterial communities of the secretion and eggshells should have some bacterial strains in common.

The uropygial gland is the only exocrine gland of birds. Located dorsally at the base of the tail, it produces oily secretions that birds use for preening (i.e. to clean their feathers and make them more waterproof and flexible (Jacob and Ziswiler 1982)). Using the beak, birds collect the uropygial oil and spread it over the plumage to prevent physical abrasion and bacterial contamination of feathers (Reneerkens et al. 2002, Delhey et al. 2007, 2008, Ruiz-Rodríguez et al. 2009, Lopez-Rull et al. 2010). Incubating hoopoes smear uropygial oil on the eggshells and the brood patch (Martín-Vivaldi et al. 2014, Soler et al. 2014), and the eggshells of this species are full of crypts of different sizes and depths that end at the spongy palisade layer (i.e. they do not pierce the eggshell) and that become filled with uropygial oil and symbiotic bacteria throughout the incubation period (Martín-Vivaldi et al. 2014, Soler et al. 2014). Since hoopoes handle the uropygial oil with the beak and spread it on their body and

eggs, some bacterial strains in the uropygial oil should appear in bacterial communities of the beak, brood patch, and eggshells (the two latter are in contact during incubation). Some of the symbiotic bacteria from uropygial oil of hoopoes and their antimicrobial products are known to protect feathers (Ruiz-Rodríguez et al. 2009) and embryos (Soler et al. 2008, Martín-Vivaldi et al. 2014) from pathogenic infection. In addition to the uropygial oil, there are many more possible sources of microbes for the eggshells, brood patch, and beak, but the antimicrobial properties and the bacterial symbionts of the uropygial oil should affect microbial communities of beak, brood patch, and eggshells. Characterization of bacterial communities of uropygial oil, beak, brood patch, and eggshells and determination of the relationships among them will help us understand the effect of the symbiotic bacteria of hoopoes. In particular, the determination of the frequency at which uropygial oil bacterial strains are present on the eggshells, beak, and brood patch of female hoopoes would help to identify strains that may act outside the uropygial gland. Current knowledge of the bacterial community from hoopoe uropygial oil comes from studies with traditional culture methods for bacterial isolation, and only a few species, most belonging to the genus *Enterococcus*, have been detected (Soler et al. 2008, Ruiz-Rodríguez et al. 2014). In the present study, using ARISA (Automatic Ribosomal Intergenic Spacer Analysis), we characterize the microbial biodiversity of bacterial communities in hoopoes and the places where the samples were taken were the uropygial gland, beak, brood patch, and egg (hereafter, sampled sites). ARISA, which has been broadly used to investigate complex symbiotic relationships among microorganisms and their hosts (Sepehri et al. 2007, Schöttner et al. 2009, Welkie et al. 2010, Porporato et al. 2013), identifies different bacterial strains as Taxonomic Operational Units (OTUs).

MATERIALS AND METHODS

Study species, study area, and general methods

The hoopoe is distributed throughout Europe, Asia, and Africa, inhabiting open woods or open areas as steppes, grasslands, pastures, semi-deserts, or field crops with scattered trees, walls or buildings providing holes for nesting and soil without tall vegetation for feeding (Rehsteiner 1996, Barbaro et al. 2008, Schaub et al. 2010). Females lay one or two clutches of 6-8 eggs over the breeding season, between February and July (Martín-Vivaldi et al. 1999). Incubation lasts 17 days and starts with the first or second egg, which results in eggs hatching asynchronously at 24 h or even greater intervals (Bussman 1950, Gupta and Ahmad 1993, Cramp 1998).

The fieldwork was performed during the breeding seasons of 2010-2011 in a wild population located 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. In 2011, hoopoes were also sampled in a captive population that descended from our wild population and that have been breeding in captivity since 2008. The captive pairs were distributed in two different subpopulations, one at facilities of the University of Granada in Hoya of Guadix (Granada) and the other at the facilities of Estación Experimental de Zonas Áridas (CSIC) in Finca Experimental Lla Hoya in Almería (36º50´N, 2º28´W), both in southeastern Spain. All females were ringed with both numbered and color rings for individual recognition.

A total of 117 nests were sampled (wild population in 2010, N=31; wild population in 2011, N=33; captivity population in 2011, N=53). For 97 nests, we recorded information from the four sampled sites (uropygial oil, beak, brood patch, and eggshells). For the remaining 20 nests, one or more of the samples was missing. We successfully collected information on 87 females; 25 of which were sampled twice; in three cases the samples were from the first brood of two different years, and in the remaining 22 cases they were from two clutches of the same season (on five of these 22 occasions, females laid in two different nest boxes). Five additional females were sampled three times in the same nest box and year. The 52 remaining females were sampled only once during their first breeding attempt.

Nest-boxes in the wild were visited twice per week from mid-February to the end of July to record laying date, clutch size and hatching date. Pairs of hoopoes breeding in captivity were housed in independent cages at least 3m x 2m x 2m installed in the open, scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages were visited daily, and the hoopoes had access to soil and were provided *ad libitum* access to live food (crickets, vitamin-enriched fly larvae) and meat (beef heart).

Incubating females were caught 14 days after laying the first egg within the nest-box by hand, briefly sampled and released again within the nest to reduce disturbance. For each capture, we wore new latex gloves cleaned with 96% ethanol for the whole process in order to avoid external bacterial contamination and ensure correct sampling. Before collecting samples from uropygial oil, we gently washed the circlet of feathers and skin surrounding the uropygial gland with a cotton swab dipped in ethanol to reduce the risk of contamination with external bacteria. After evaporation of the alcohol, a sterile micropipette tip (1-10 µl micropipette [Finpipette]) was inserted into the gland papilla after opening the circlet of feathers that covered the gland entrance. The papilla was pressed softly with a finger and the uropygial oil collected

was transferred to a sterile microfuge tube. Afterwards, 5µl were separated and placed in a different sterile microfuge tube for the analyses.

Bacterial samples from beak, eggshells, and brood patch were collected by rubbing the complete surface with a sterile swab slightly wet with sterile phosphate buffer (Na_2HPO_4, 0.1 M and NaH_2PO_4 0.1 M, pH 7.2). These samples were individually stored in sterile microfuge tube with 1.2 ml of buffer solution (see Peralta-Sánchez et al. 2012) All samples were kept cool (i.e. 1-3º C) until being stored in the lab at -20º C the same day of sampling for further molecular analyses.

Laboratory work

Bacterial genomic DNA was extracted in two different ways depending on the sampled sites: those from the beak, brood patch, and eggshells were extracted with a specific procedure to obtain genetic material from swabs, called Chelex-based DNA isolation (Martín-Platero et al. 2010). On the other hand, the viscous uropygial oil samples were extracted with a commercial KIT (The FavorPrep™ Blood Genomic DNA Extraction Kit, Favorgen).

Automated rRNA Intergenic Spacer Analysis (ARISA) (Fisher and Triplett 1999) was used to characterize the composition of bacterial communities inhabiting the different samples. ARISA amplifies an intergenic transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. This region is highly variable both in size and sequence between species, offering higher taxonomic resolution than do other techniques (Danovaro et al. 2006). The ITS was amplified using the primer pair ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITSReub (5´-GCCAAGGCATCCACC-3´) (Cardinale et al. 2004). The primer ITSReub was labeled fluorescently with 6-FAM. The primer ITSReub was labeled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 20 µl of 5 PRIME MasterMix (2.5x) including 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase 0.2 µM of primers and 5µl of diluted DNA 1:10. PCRs were conducted in the Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94°C 2 min, followed by 30 cycles with denaturation at 94°C 45 s, annealing at 52°C 45 s, and extension at 72°C 1 min, with a final extension at 72°C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by automated fluorescent capillary electrophoresis in a 3130 Genetic Analyzer. Electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard

(both Applied Biosystems). These analyses were performed in the Scientific Information Center of Granada University.

Statistical analysis

Peak Scanner 1.0 (Applied Biosystems) was used to determine fragment length in terms of base pairs of each peak that enables the identification of different bacterial strains (i.e. OTUs) within each site. For methodological reasons, the estimated length of the same bacterial strain from different samples may differ slightly. Thus, binning DNA fragment lengths from different samples is necessary before comparing bacterial communities. We did so by using available scripts in R-environment [http://cran.r-project.org/] at http://www.ecology-research.com (Ramette 2009) with a window size of 4 base pairs (bp) and a distance of two consecutive binning frames (i.e. shift) of 0.1. The algorithm rearranges the data and calculates the relative fluorescence intensity (RFI) of each peak by dividing individual peak areas by the total peak area for the respective sample. All peaks with RFI values of < 0.09% were not included in further analyses since they consisted of background peaks. Only fragments above a threshold of 50 fluorescence units and ranging between 100 and 1.000 bp were taken into consideration so as to include the maximum number of peaks while excluding background fluorescence (Ramette 2009). We used the presence-absence matrix generated after the binning process for all analyses. Molecular fingerprinting techniques are highly reproducible, robust, and have proven useful for comparative analysis of microbial community structure (Loisel et al. 2006, Bent and Forney 2008).

The number of OTUs detected per sample did not differ from a normal distribution after log-transformation (Kolmogorov-Smirnov test for continuous variables, $p > 0.15$). The random effect of individual females did not explain additional significant variance of species richness of uropygial oil ($F = 1.46$, $df = 76.32$, $p = 0.12$), beak ($F = 0.83$, $df = 79,35$, $p = 0.75$), brood patch ($F = 1.34$, $df = 80,32$, $p = 0.18$), or eggshells ($F = 1.49$, $df = 77,32$, $p = 0.051$). Thus, this random factor was not included in subsequent models. Rather, because some females were sampled during different breeding attempts, we included information on breeding attempt in the models as a fixed factor.

We used general lineal models (GLMs) to explore the effects of population (captive or wild) and study year on species richness (i.e., number of OTUs per sample) at different sampled sites (uropygial oil, beak, brood patch, and eggshells). The captive population was sampled only in 2011 and, thus, the effects of year were explored with samples from the wild population, while the effects of captivity were explored with samples from 2011. Models explaining species

richness therefore included sample site, breeding attempt, and either population or year, as well as the interaction between these two factors as fixed effects. Estimating main effects in models without the interaction did not affect the results and, consequently, we report results from models that included the interaction as a fixed factor. Breeding attempt did not explain a significant proportion of variation of species richness (all models explained below, $p > 0.55$) and, thus, we removed this factor from all subsequent models. *Post hoc* comparisons (i.e. LSD Test) were used to explore differences between pairs of sampled sites depending on years and populations (captivity *vs*. wild) differences.

Information from different study years and populations were pooled to explore possible differences in bacterial prevalence in samples of the uropygial oil, beak, brood patch, and eggshells. Moreover, trying to reduce the probability of detecting significant differences among sampled sites due to rare OTUs, we considered only the most abundant, i.e. those that appeared in more than 30% of the samples in at least one site (uropygial oil, beak, brood patch or eggshells). Comparisons were performed by means of Log-linear analyses, and FDR (False Discovery Rate) method was used to adjust p-values for multiple comparisons. To explore the within-individual association in OTU prevalence at different sampled sites, we built 2x2 contingence frequency tables with a target OTU absent or present at two different sites. Again, we considered only the most frequent OTUs (i.e. those that appeared at least in 20 different females). All the analyses were performed with STATISTICA 8 software (StatSoft 2006) except FDR adjustment, which was conducted by p-adjust function of stats package in R 3.1.2 [54] (http://www.r-project.org/).

We analyzed differences in OTU composition among sampled sites taking into consideration the most abundant OTUs by one-way NPMANOVA based on the Jaccard distance with 9999 permutations using PAST Paleontological Statistics Software (Hammer et al. 2001). We used classical multidimensional scaling analysis (Multidimensional Scaling (MDS), Principal Coordinates Analysis (PCoA)) to represent graphically the relationships between bacterial communities of the uropygial oil, beak, brood patch, and eggshells. This technique represents the communities on a plot with canonical axes, where the relationship between communities shows their underlying similarity (Legendre and Legendre 1998). We used Jaccard's coefficient to estimate the similarity between bacterial communities of different sampled sites. Statistical analyses were conducted by "vegdist" function of "vegan" package, "cmdscale" function of "stats" package and function "ordiplot3d" of "vegan3d" package in R 3.1.2 (R Core Team 2014) (http://www.r-project.org/).

RESULTS

Richness of bacterial communities

We identified a total of 146 different OTUs (sizes between 139 bp and 999 bp) in the bacterial communities of hoopoe sampled sites. Of these, 124 OTUs were detected in the uropygial oil, 106 on the beak surface, 97 on the brood patch, and 98 on the eggshell. We recorded complete information (uropygial oil, beak, brood patch, eggshells) from 97 nests with the richness of OTUs per nest (i.e. considering all sites together) ranging from 11 to 60 (Mean (SE) = 33 (1.1), Mode = 40). Within individuals, the highest richness in terms of number of detected OTUs appeared in the uropygial oil samples independently of the study year and whether samples were from wild or captive populations (Fig. 1, Table 1). *Post hoc* comparisons revealed that species richness of the beak differed significantly between captive and wild populations and that values for eggshells varied between years in wild populations (Table 1). Thus, study year and population (captivity or wild) had a relatively weak effect on estimated species richness and, consequently, the general effect of site in Table 1 was due to characteristics of the uropygial oil bacterial community.

Samples of uropygial oil, brood patch, and eggshells from the wild population were more diverse than those from captivity, but *post hoc* analyses revealed statistical significant differences only when comparing samples from the brood patch (Table1, Fig. 1a). Similarly, study year significantly affected species richness (Table 1), samples from 2011 being more diverse than those from 2010 (Fig.1b), for the uropygial oil and the eggshells (Table 1). Finally, the variation in OTUs' richness among sampled sites did not depend on population (wild vs. captivity), but on the study year. Community of the uropygial oil was more diverse than those of beak, brood patch and eggshells, especially in 2011(see *post hoc* analyses associated to the interaction terms in Table 1, Fig. 1).

Prevalence of bacterial strains in different bacterial communities

When the four sampled bacterial communities (146 OTUs) were considered, the estimated prevalence of most OTUs proved very low (mode = 0) ranging from 0.87% (OTU with 999 bp) to 85% (OTU with 183 bp). However, trying to reduce the effect of rare bacterial strains when exploring similarities between different bacterial communities, we considered 27 OTUs that appeared on at least one site in more than 30% of individuals. Length of the ITS fragment of these OTUs ranged between 139 bp and 567 bp (Fig. 2a). All the 27 OTUs selected were present in the uropygial oil samples, and three of them were exclusive to this site (sizes 139 bp, 171 bp, and 219 bp, Fig. 2). Moreover, two OTUs (sizes 307 bp and 367 bp) showed high

prevalence (> 50%) in beak, brood patch, and eggshell, while being rarer (< 30%) in uropygial oil samples (Fig. 2a), suggesting that a few strains could be typical of each site.

For the OTUs considered, the prevalence in samples from the uropygial oil, beak, brood patch and eggshells significantly differed (Log-linear analysis, $\chi^2 = 894.5$, df = 78, $p < 0.001$). These differences were due mainly to higher species richness in the uropygial oil (Fig. 2a), although differences were also detected when considering the other three sampled sites (beak, brood patch, and eggs) (Log-linear analysis, $\chi^2 = 96.31$, df = 52, $p < 0.001$).

Differences in prevalence of each of the 27 most frequent OTUs revealed that only two of them (535 bp and 567 bp) did not differ significantly among sampled sites (Log-linear analysis, $\chi^2 > 3.04$, df = 3, $p > 0.34$), while the remaining 25 did (Log-linear analysis, $\chi^2 > 14.3$, df = 3, $p < 0.01$). Prevalence of two additional OTUs (311 bp and 407 bp) did not differ among samples from beak, brood patch, and eggshells ($\chi^2 > 2.68$, df = 2, $p > 0.3$, comparison for the remaining 23 OTUs, $\chi^2 > 8.2$, df = 2, $p < 0.05$) (Fig. 2a).

When exploring the association between pairs of bacterial communities connected by the preening behavior of hoopoes (i.e. uropygial oil *vs*. beak, beak *vs*. brood patch, beak *vs.* eggshells and brood patch *vs*. eggshells), we found that, in the prevalence of different OTUs, two of them appeared to be significantly related for all pairs of sampled sites. The detection of 535 bp and 567 bp in the eggshells was more likely when detected in the brood patch; detection in the latter was predicted by the detection in samples from the beak, while detecting these OTUs in beak samples were more likely when detected in samples from the uropygial oil (Fig. 3; Appendix 1). In addition, the prevalence of three more OTUs (307 bp, 367 bp, 407 bp) in samples from the beak and brood patch, brood patch, and eggshell, and from the beak and eggshells were significantly associated (Fig. 3; Appendix 1).

Composition of bacterial communities

The ordination of sampled sites by PCoA was represented in three dimensions (Fig. 2b). The three axes explained 15.8%, 11.1%, and 9.2% of variance, respectively. These axes clearly separated the uropygial oil community from those of all the other sampled sites (NPMANOVA, $F > 23.39$, $p = 0.0001$; Fig. 2b). In addition, the bacterial community of the eggshell also differed from those of the beak and brood patch (NPMANOVA, $F > 23.39$, $p < 0.001$), but those of the beak and brood patch did not significantly differ (NPMANOVA, $F = 23.39$, $p = 0.266$; Fig. 2b).

DISCUSSION

In the present work, for the first time, the entire bacterial community (including non-culturable species) of hoopoe uropygial oil has been characterized by means of molecular techniques. It has previously been suggested that, because of preening, the uropygial oil including antimicrobial components (or antibiotic producing symbionts) may reach the eggshells of birds and protect the embryo from trans-shell infection (Cook et al. 2005, Soler et al. 2010, 2012, Møller et al. 2010, Martín-Vivaldi et al. 2014), but see (Giraudeau et al. 2014). Thus, since incubating hoopoes harbor symbiotic bacteria in their uropygial oil inside the uropygial gland, the bacterial communities of the beak, brood patch, and eggshells may share some of their bacterial strains with the uropygial oil. In accordance with this possibility, we found that a majority of the bacteria detected in the uropygial oil were also present in the other sampled sites, and that for some bacterial strains, their detection on the beak, brood patch, and eggshells depended on their presence in the uropygial oil. There are several sources of bacteria that colonize the beak, brood patch, and eggshells of hoopoes and, thus, our results strengthen the idea that symbiotic bacteria of the uropygial gland help determine bacterial communities of hoopoes. Below, we discuss alternative hypotheses that seek to explain such relationships between bacterial communities of hoopoes, and we speculate on possible implications on mutualistic bacteria found on the eggshells.

The community of aerobic-cultivable bacteria in hoopoe uropygial oil includes mainly few species of *Enterococcus* (Soler et al. 2008, Ruiz-Rodríguez et al. 2014). Our results suggest a more complex community of bacteria that is even more diverse than those of the beak, brood patch, and eggshells. These differences may be due to the presence of strict anaerobic bacteria that do not survive outside the uropygial gland, but also to environmental conditions such as temperature and humidity that would differentially affect bacteria on the body surfaces of animals (Ley et al. 2008, Ding and Schloss 2014). Notably, we detected a significant effect of study year on species richness but only for that of the uropygial oil, which is consistent with previous results of environment influencing the enterococci strains present in the hoopoe uropygial gland (Ruiz-Rodríguez et al. 2014) and the symbiotic bacteria found inside squid light organs (Guerrero-Ferreira et al. 2013).

Most of the 146 OTUs found were only sporadically detected, but 27 of them were present in more than 30% of the females. This pattern with a mixture of many rare species but a few highly prevalent ones is common in bacterial communities (Hulcr et al. 2012, Roggenbuck et al. 2014). Most OTUs with high prevalence (24 of 27 OTUs) were detected both inside the uropygial gland and on external sampled sites. This group includes antibiotic-producing

386 enterococci strains (OTU307 and OTU407 for *Enterococcus faecalis*) (Martín-Platero et al.
387 Unpublished data) that help hoopoes in their antimicrobial defense (Soler et al. 2014, Martín-
388 Vivaldi et al. 2014, Ruiz-Rodríguez et al. 2014). These may also include other mutualistic
389 bacteria responsible for antibiotic production within the uropygial gland (Martín-Vivaldi et al.
390 2010) that would reach and be hosted in the special structures of hoopoe eggshells adapted to
391 accumulate uropygial oil (Martín-Vivaldi et al. 2014). The eggshells of hoopoes are full of
392 crypts (Martín-Vivaldi et al. 2014) and lack the organic cuticle that in some other species
393 protects embryos from trans-shell infection (Sparks 1994, Wellman-Labadie et al. 2008). Crypts
394 of eggshells became filled with uropygial oil during early incubation, and the secretion and/or
395 symbionts that accumulate there protect embryos from pathogenic infection (Martín-Vivaldi et
396 al. 2014). Therefore, we expected the mutualistic bacterial strains to be transmitted from the
397 uropygial gland to the eggshells when females take uropygial oil with the beak to smear eggs
398 directly (Martín-Vivaldi et al. 2014) or to impregnate skin and body feathers that may make
399 contact with eggs during incubation (brood patch); i.e. an association among the microbial
400 communities of those sites. Actually, we found that some OTUs which were more frequently
401 detected on the beak of females were also detected in their uropygial oil as well as on the eggs
402 when the OTUs were also detected in the brood patch or beak of females (Fig.3). These strains
403 will be crucial in further studies such as the direction of transmission and as key mutualistic
404 species involved in protecting hoopoes from infections outside the uropygial gland (i.e.
405 eggshells or feathers).
406
407 Contrary to what should be expected if the uropygial secretion was the main source of
408 bacteria for the other sampled sites, the detected associations were stronger among bill, brood
409 patch and eggshells than those between uropygial secretion and all other sampled sites (Fig. 3).
410 This apparently unexpected result may be explained if some strains commonly detected in the
411 uropygial secretion were also present in nest remains and cloacal samples of hoopoes as it look
412 to be case (Martínez-García et al. Unpublished). Thus, we can speculate with the possibility that
413 some of the strains in Fig. 3 could have reached bill, brood patch or eggshell of hoopoes directly
414 from nest materials or cloacal environment but did not successfully colonized (or were not
415 detected in) the uropygial gland of some birds. In addition, brood patch, bill and eggshell are in
416 close contact to each other and, consequently, the explored relationships would more easily be
417 detected among these sites. In any case, since the bacterial community of the uropygial oil was
418 not experimentally manipulated in this study, we cannot infer causation for the relationships
419 detected nor can we establish the direction of the colonization. Different scenarios include the
420 possibility of non-directional transmission among the different body parts, and differential
421 effects of incubation on bacterial strains. Brood patch and eggshells are in contact, and brooding
422 birds move and turn the eggs with their beak during incubation. Moreover, eggs, as well as the

female's body, are in contact with the nest and, thus, bacterial communities may share some strains with nest material (Brandl et al. 2014). Moreover, it is known that incubation activity affects bacterial assemblage on the eggshells of several bird species (Shawkey et al. 2009, Brandl et al. 2014, Lee et al. 2014), for which the associations detected in only few strains could partially result from the differential effect of incubation on the communities at different sampled sites. Experimental studies manipulating bacterial presence are needed to firmly establish the causes of the composition of these communities. We hypothesize that transmission from the most diverse community of the uropygial oil of uropygial gland to beak, brood patch, and eggshells is the most likely explanation because of the antimicrobial potential of hoopoe uropygial oil (Martín-Platero et al. 2006, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013) and also because bacteria living in the uropygial oil have to be resistant to the majority of uropygial oil antimicrobials. Therefore, a likely scenario is that the uropygial oil kills many bacteria on the beak, brood patch, and eggshell, and will therefore facilitate the colonization and growth of some of the symbiotic bacteria from the uropygial gland on hoopoe body surfaces and eggshells.

Our findings that bacterial communities living in eggshell crypts are associated with those found within the uropygial oil open the possibility that each strain has a different role, combining the antimicrobial action within glands and eggshell crypts. Further studies are necessary to fully understand the evolution of the mutualism between hoopoes and its symbionts.

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Table legends

Table 1. Results from General Linear Models explaining variation in species richness (i.e. number of OTUs) in relation to sampled sites [uropygial oil (UO), beak (B), brood patch (BP) and eggshells (E)], year or population [wild *vs*. captive populations (W/C)], and the interaction between site and year/population as fixed effects. *Post hoc* comparison for the effect of year or population on richness of bacterial communities of each site are also shown (normal and italic fonts show results for the wild hoopoe population sampled in 2010 and 2011 (upper sub-table), or wild and captive populations of sampled during 2011 (lower sub-table), respectively.

Figure legends

Figure. 1. Average number of OTUs (species richness) (± 95% CI) found at sampled sites from the uropygial oil (UO), beak (B), brood patch (BP), and eggshells (E) collected from wild and captive hoopoe populations during 2011 (a), and from wild populations during the 2010 and 2011 (b).

Figure. 2. Prevalence (%) of different bacterial OTUs (named by their length in base pairs (bp)) found in samples from the uropygial oil (N=109), beak (N=115), brood patch (N=113), and eggshells (N=110) of female hoopoes (a). Multidimensional space representation (PCoA) based on similarities of the most frequent bacteria communities harbored in uropygial oil, on beak, brood patch, and eggshells is also shown (total variance captured by the three axes = 36.1%) (b).

Figure. 3. Relationships between pairs of sampled sites (uropygial oil, beak, brood patch, and eggshells) within hoopoe females by the co-occurrence of particular OTUs. Broadest arrows indicate high number of OTUs with significance relation between pairs of sites. Bold fonts show OTUs with significant relations to all sampled sites.

Supplementary material legend

Appendix 1. Relationships of OTU co-occurrence between pairs of sampled sites (UO *vs.* B, B *vs.* BP, BP *vs.* E, B *vs.* E, UO *vs.* E, UO *vs.* BP) within females, being UO (uropygial oil), B (beak), BP (brood patch) and E (eggshells). The p-values obtained by means of Log-linear analyses were corrected for multiple tests by using FDR methodology. Three of 27 frequent OTUs (139 bp, 171 bp, 219 bp) were specific of uropygial oil (UO) and were not used for this analysis. N represents the number of females in which each OTU was detected in the two sampled sites compared.