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Article

Experimental old nest material predicts hoopoe *Upupa epops* eggshell and uropygial gland microbiota

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Nest re-use in birds has the potential cost of infection by parasites and pathogens but may also be a source of beneficial symbiotic bacteria transmitted horizontally. Eurasian hoopoes *Upupa epops* host antibiotic-producing bacteria in their uropygial gland but only while breeding, which suggests that the nest-hole may be a source of those symbionts. Interestingly, hoopoes do not build nests, thus might prefer for reproduction nest holes with soft materials from previous reproductions. Here, we tested experimentally this preference by installing in the field new nest boxes that were left empty or filled with either sawdust or a mixture of sawdust and hoopoe's nest material from the previous year. We explored the experimental effect on the composition of the uropygial secretion bacterial community, on eggshell bacterial loads, and on several proxies of reproductive success. Hoopoes bred significantly more often in nest boxes with nest material than in empty ones, but the type of nest material did not affect nest box occupancy. Eggs in nest boxes with old-soft material harbored higher bacterial density on their shells, and the microbiota of the uropygial secretion of nestlings and females in these nest boxes differed from those in nest boxes without old-soft material. Moreover, although the experiment did not affect breeding success or related proxies, several operational taxonomic units from female uropygial secretions were positively associated with hatching success. This is the first experimental evidence showing that re-used nest material affects the bacterial community of the uropygial secretions of hoopoe females. This suggests that the nest material can be a source of strains for their incorporation to both the uropygial gland and eggshell communities, highlighting a possible advantage of nest re-use previously unconsidered.

Keywords: eggshell, horizontal transmission, nest material re-use, symbiotic bacteria, *Upupa epops*, uropygial gland secretion

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Introduction

Territory choice affects individual fitness, so individuals should be very selective when choosing the place to breed to maximize reproductive success (Refsnider and Janzen 2010). This choice might for instance affect probability of parasitism or availability of resources (Sergio and Newton 2003). In addition to territory choice, the choice of the nesting site and, specifically, the choice of nest material is also very important and might even affect acquisition of symbiotic bacteria when they are transmitted horizontally (Peralta-Sanchez et al. 2010, Martínez-García et al. 2016a, van Veelen et al. 2017). In hole-nesting species that do not add any material to the nest, the presence and quality of soft material inside the cavity could be an important nest-site choice criterion, because it could for instance improve the incubation efficiency by increasing thermal insulation and reducing egg heat loss (Mazgajski 2007, Mainwaring et al. 2014, Podofillini et al. 2018). Moreover, nest material could act as a physical barrier against bacterial contamination from the bottom of the nest or, due to their antimicrobial properties, prevent offspring infection (Gwinner and Berger 2005, D'Alba and Shawkey 2015, Ruiz-Castellano et al. 2016, Soler et al. 2017). Secondary hole nesters usually re-use nests from previous breeding seasons (Mazgajski 2007). These nests can contain nest dwelling ectoparasites and pathogens that remain quiescent within old nest material residues (Maier et al. 2000), and that will affect reproductive success of future users (Mazgajski 2007, Møller et al. 2009). Moreover, it can contain remains of previous reproduction such as faeces, discarded food and even dead nestlings that enhance bacterial growth but that might also inform visitors of whether nestlings survived during their nest stage in previous reproductive events (Erckmann et al. 1990, Olsson and Allander 1995, Sumasgutner et al. 2014). This information is especially important in migratory birds, because they have less time to evaluate the quality of the territory before the start of the breeding season (Mazgajski 2007). Many studies have investigated the preference for nest boxes with old nest material in birds (reviewed in Mazgajski 2007). However, they have only focused on the presence or absence of nest material per se, independently of whether it comes from previous reproductions or from artificial sources.

Parental and breeding activity, as well as nest material and ectoparasites, determine bacterial environment of nesting cavities and of eggshells (González-Braojos et al. 2012, Peralta-Sánchez et al. 2012, 2014, Grizard et al. 2015, Tomás et al. 2018). These bacteria can have positive, negative or no effects on the offspring (Singleton and Harper 1996, Moreno et al. 2003, González-Braojos et al. 2012, Soler et al. 2017, Devaynes et al. 2018). For example, some symbiotic bacteria can produce chemicals, such as antimicrobial compounds, that might outcompete or inhibit the colonization by pathogenic microorganisms or parasites, defending and protecting the host against them (Soler et al. 2010, Martín-Vivaldi et al. 2014a). Bacteria on the eggshells and offspring are partly from the nest materials (Brandl et al. 2014, Martínez-García et al. 2016a, van Veelen et al. 2018) and, thus, old nest materials might be the source of potentially beneficial bacteria for breeding birds. Exploring the bacterial load of eggshells in nests with old or artificial new material would therefore help to clarify the effect of nest material on nest bacterial environments.

Here we explore the importance of old nest materials in nest box choice for breeding in a population of Eurasian hoopoes *Upupa epops*. This species is a secondary hole-nester where a mutualistic relationship with bacteria growing in their uropygial gland has been described particularly in breeding females and nestlings (Martín-Platero et al. 2006, Soler et al. 2008, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013). These mutualistic bacteria reach the egg surface when females smear their uropygial secretion on them soon after laying, increasing hatching success (Martín-Vivaldi et al. 2014a, Soler et al. 2014). Even though uropygial gland bacteria have been also described in dark-eyed juncos *Junco hyemalis* and red-billed woodhoopoe *Phoeniculus purpureus* (Law-brown and Meyers 2003, Whittaker and Theis 2016), the behaviour of smearing the eggs has only been demonstrated in hoopoes (Soler et al. 2014). Several sources and mechanisms have been proposed to explain the complex bacterial community of hoopoe uropygial secretion (Rodríguez-Ruano et al. 2018). These bacteria can be transmitted vertically from mother to offspring but others are obtained possibly, from their own gut microbiota (cloaca) and from the nest environment (e.g. nest materials) (Ruiz-Rodríguez et al. 2014, Rodríguez-Ruano et al. 2015a, Martínez-García et al. 2016a, Martín-Vivaldi et al. 2018). Hoopoes do not carry nest materials to the nest (Martín-Vivaldi et al. 2014b). Therefore, by re-using nests, the new female could increase the diversity of strains of her uropygial gland symbiotic bacterial community. In addition to other advantages, this could be one of the explanations of why hoopoes frequently re-use the same holes for many years (Cramp 1998), and why the first individuals arriving to the breeding grounds preferentially occupy previously used nest boxes (van Wijk et al. 2017). A previous study demonstrated an effect of nest bacterial environment on hoopoe eggshells bacterial communities, but no effect on the bacterial community of the uropygial secretion was found (Martínez-García et al. 2016a). However, that experiment was carried out with captive hoopoes, whose microbiome is slightly poorer than that of wild ones (Martínez-García et al. 2015, Rodríguez-Ruano et al. 2015b, 2018) and bacterial community was characterized by means of ARISA fingerprinting technique, that is not able to detect the whole diversity of bacteria (Bentley et al. 2008). Moreover, the experimental nest material used (commercial crushed and mashed olive *Olea europaea* stones) had antimicrobial properties, which could have affected the composition of microbiomes (Martínez-García et al. 2016a).

Within the theoretical background exposed above, and considering previous knowledge on the symbiotic association between hoopoes and bacteria of their uropygial secretion, we experimentally explored nest box choice by wild hoopoes and associated effects on the bacterial load of eggshell and the

bacterial community of the secretion using high throughput sequencing. We tested whether hoopoes prefer to breed in 1) nest boxes with available soft material instead of in empty ones, 2) mainly those used in previous reproduction (i.e. with old hoopoes' nest material inside). We also 3) explored the effect of experimental old material on the bacterial community of the uropygial gland secretion and on the bacterial load of the eggshells of hoopoes. Finally, 4) we explored possible fitness effects of the experimental nest materials in terms of breeding success. Our prediction is that hoopoes should prefer nest boxes with material instead of empty ones and specifically those with old hoopoe nest material. In this way, we expect that the bacterial composition of uropygial secretion and of eggshell will differ depending on the experimental treatment because of the incorporation of the symbionts from old nest material to the communities obtained from other sources (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016a, Martín-Vivaldi et al. 2018, Rodríguez-Ruano et al. 2018). Finally, because of a greater diversity of the composition of the bacterial community in the gland, we expect that females that breed in nests with old material have higher breeding success than those breeding in nests without it.

Material and methods

Study species

The hoopoe is a cavity nester that readily breeds in nest boxes. This species is a migratory bird distributed throughout Europe, Africa and Asia. There can be partial migrant populations in areas with low seasonal differences, like in our study population, where sedentary and migratory specimens reproduce (Reichlin et al. 2013, van Wijk et al. 2018). Hoopoes do not carry nest materials, but if soft materials are present in the cavity selected, females excavate a slight depression where they lay the eggs (Martín-Vivaldi et al. 2014b). These soft materials are usually remains of previous reproduction, soil and/or decomposed wood. Females lay one or two clutches of six to eight eggs between February and July (Martín-Vivaldi et al. 1999, Plard et al. 2018). Incubation starts with the first eggs, resulting in asynchronous hatching, generating a marked size hierarchy within the brood (Cramp 1998). Females stay inside the nest from the start of incubation (which lasts 17 days from the laying of the first or second egg), until nestlings are about eight days old. Nestlings leave the nest after 24–30 d (Martín-Vivaldi et al. 2014a).

Study area and general procedures

The fieldwork was carried out during the 2015 breeding season in the Hoya de Guadix (37 C 18′N, 11′W), Granada (southern Spain). In this area, hoopoes breed in nest boxes situated in trees and in natural cavities. Nest boxes were made in cork with the following dimensions: 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter).

Nest boxes were visited every five days from early March to the end of July. Hoopoes lay one egg per day (Cramp 1998) and, thus, this frequency of nest visiting allowed us to estimate laying and hatching dates, clutch size and number of fledglings that left the nest. Laying date was defined as the day when female laid the first egg. Females were captured by hand inside the nest boxes twice, 15 days after laying the first egg and again when the first nestling was five days old. Nestlings were sampled 19–20 days after hatching of the first egg. We measured the bill, the tarsus and the uropygial gland size using a calliper (accuracy 1 mm), the wing using a metallic ruler, and the body mass with a hanging scale (Pesola 0–100 g, accuracy 1 g). We also extracted uropygial gland secretion by automatic 1–10 µl micropipettes (see below for more details) and collected blood samples in heparinized capillary tubes by puncturing the brachial vein. Individuals were marked with numbered aluminum rings (Spanish Institute for Nature Conservation, ICONA). Afterwards, they were released into their nest box. All the manipulation was made wearing disposable latex gloves previously cleaned with 96% ethanol to prevent contamination among nests.

As proxies of breeding success, we used the following variables: clutch size, hatching success (proportion of eggs hatched), number of fledglings (number of nestlings that survived 20 days in the nest), fledging success (proportion of hatchlings that survived until fledging in successful nests), and clutch productivity (proportion of eggs that produced a fledgling in successful nests). Some nests were predated or deserted before hatching or during the nestling stage and, thus, sample sizes differ depending on the considered variable (see degrees of freedom in Table 1).

Body condition was estimated as the residuals of body mass on tarsus length^3 (Senar and Pascual 1997).

Experimental design

The experiment was performed by installing new nest boxes in the study area at the beginning of spring of 2015. This hoopoe population breeding in nest boxes has been studied for many years and we knew the fate of the nesting attempts of the previous breeding seasons. Old nest boxes in the study area were cleaned of nest material at the beginning of March of 2015 and, in the case of those where hoopoes successfully bred in 2014 (14 nest boxes), materials were kept in plastic bags shortly before added to the experimental nest boxes (one or two weeks later). During this short period of time, they were kept at room temperature and with a small opening in the bag to allow air exchange. Experimental new nest boxes were geographically placed randomly in pairs with a distance from each other of about 25 m, more or less spatially mixed with old nest boxes installed in previous years (Supplementary material Appendix 1 Fig. A1, A2). To investigate the possible effects of old material, one of the nest box in the pair was filled with a 3 cm layer of only commercial sawdust (Allspan® Animal bedding, wood shavings; control nests), and the other with the same volume of 50% mixture of sawdust and hoopoe's nest material from the previous year

Table 1. Results from ANOVA (F-values) or Mann Whitney tests (Z-values in parentheses), means and standard error (SE) explaining the influence of experimental nest material (sawdust or a mixture of sawdust and old hoopoe's nest material) on several dependent variables. We include only the co-variables that explained additional significance variance to those explained by the experimental treatments. Significant p-values are in bold.

	Type of nest material				Comparisons		
	Sawdust		Old hoopoe's nest				
	Mean (SE)	n	Mean (SE)	n	df	F or (Z)	p
Microbial load and antagonism							
Log CFU ml^{-1} eggshell TSA							
Nest material	**2.438 (0.141)**	**11**	**3.018 (0.156)**	**9**	**1,18**	**7.58**	**0.013**
CFU ml^{-1} eggshell KF							
Nest material	113.64 (51.013)	11	93.33 (34.521)	9		(−0.43)	0.669
Log antagonistic activity secretion of females							
Nest material	−0.690 (0.046)	10	−0.636 (0.048)	9	1,16	0.66	0.426
Laying date					**1,16**	**5.55**	**0.032**
Antagonistic activity secretion of nestlings							
Nest material	0.216 (0.032)	32	0.197 (0.026)	41	1,57	0.09	0.773
Nest (random)					**14,57**	**2.48**	**0.008**
Log CFU ml^{-1} secretion TSA of females							
Nest material	3.443 (1.096)	8	1.810 (1.386)	5	1,11	0.85	0.375
Log CFU ml^{-1} secretion TSA of nestlings							
Nest material	4.753 (0.629)	20	4.516 (0.586)	23	1,11	0.08	0.787
CFU ml^{-1} secretion KF of females							
Nest material	84×10^5 (19636700)	10	0.000 (0.000)	5		(−1.03)	0.301
Breeding success							
Clutch size							
Nest material	7.000 (0.385)	11	7.900 (0.403)	10	1,19	2.61	0.123
Hatching success							
Nest material	0.901 (0.175)	8	0.901 (0.151)	8		(0.00)	1.000
Number of fledglings							
Nest material	4.787 (0.305)	7	5.312 (0.285)	8	1,12	1.51	0.242
Laying date					**1,12**	**10.12**	**0.008**
Fledglings success							
Nest material	0.740 (0.058)	7	0.818 (0.054)	8	1,13	0.97	0.342
Clutch productivity							
Nest material	0.639 (0.061)	7	0.735 (0.057)	8	1,13	1.32	0.272
Body condition of nestlings							
Nest material	−1.040 (1.367)	34	0.804 (1.190)	44	1,12	0.96	0.346
Laying date					**1,12**	**7.83**	**0.015**
Number of nestlings					**1,12**	**5.10**	**0.043**

(old nest material). This procedure was performed wearing different new latex gloves for each nest box to avoid contamination of the clean sawdust of controls. A total of 49 pairs of new experimental nest boxes were scattered within the area with old nest boxes, or installed in new surfaces of pine plantations at the edge of this area. To test if there is a preference between new nest boxes with material versus new empty nest boxes, we also installed new empty nest boxes. The new empty nest boxes were placed on the same tree, paired with an old empty (cleaned) nest box (a total of 88 new empty nest boxes) (Supplementary material Appendix 1 Fig. A1).

Microbiological study

Eggshell bacterial loads

To estimate the abundance of cultivable bacteria on the eggshells, the 15th day after incubation started, we cleaned the complete surface of one randomly chosen egg per clutch with a sterile swab (EUROTUBO® DeltaLab) previously moistened with sterile sodium phosphate buffer (PBS, 0.2 M; pH = 7.2). Afterwards, the swab was introduced into a sterile microcentrifuge tube with phosphate buffer and transported in a portable refrigerator at 4–6°C to the laboratory. Samples were stored at 4°C until being processed within the next three days. The microcentrifuge tubes were gently vortexed to facilitate the transmission of bacteria from swabs to the phosphate buffer, as well as its homogenization. Serial tenfold dilutions to 10^{-6} were cultured. Briefly, 100 µl of sample of each serial dilution was spread onto four different solid culture media (Scharlau Chemie S.A., Barcelona): Tryptone Soya Agar (TSA), a broadly used general medium to grow heterotrophic bacteria; Kenner Fecal Agar (KF), a selective medium for *Enterococcus*, and two specific media for potentially pathogenic bacteria: *Enterobacteriaceae* (Hektoen Enteric Agar, HK) and *Staphylococcus* (Vogel Johnson Agar, VJ). In these two last specific media we hardly found growth

(only in 1 of 19 nests in both cases) so we will show the results of the first two media (TSA and KF). The plates were incubated aerobically at 37°C for 24–72 h before colony counting. Estimates of bacterial loads were standardized to number of colony forming units (CFU) per milliliter (no. colonies × $10^{\text{dilution factor}}$ ml^{-1} spread).

Uropygial secretion bacterial loads

The available uropygial gland secretion was collected using automatic 1–10 µl micropipettes. First, with gloves previously disinfected with ethanol, we cleaned the surroundings of the gland with a cotton swab soaked in 96% ethanol. Second, we gently introduced a previously autoclaved tip into the opening of the papilla of the uropygial gland and directly pipetted the secretion. Finally, we introduced the secretion into a sterile microcentrifuge tube. This procedure was repeated until the papilla got empty. The samples were kept cold in a portable fridge after collection until storage in a fridge in the lab. The samples were processed within the following 24 h to estimate bacterial loads and afterwards stored in a freezer until DNA extraction. Briefly, 5 µl of the secretion were homogenized with 45 µl of PBS in a sterile microcentrifuge tube and 5 µl of the mixture of each serial dilution (tenfold dilutions to 10^{-4}) was spread onto TSA and KF media. The plates were incubated aerobically at 37°C and colonies were counted after 24 h (TSA plates) and 72 h (KF plates). Estimates of bacterial loads were standardized to CFU ml^{-1} as explained before.

Nest material effect on hoopoe bacterial community

For the study of the composition of the bacterial community of uropygial secretions, we used Illumina high-throughput sequencing (HTS). The total DNA was extracted from 80 samples (20 µL) using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech, Ping-Tung, Taiwan) according to manufacturer's instructions, adding a lysozyme treatment (10 mg ml^{-1}, at 37°C for 30 min).

The libraries for sequencing were obtained amplifying a fragment of approximately 400 bp of the 16S ribosomal DNA (rDNA) V6–V8 hypervariable regions. The universal primers for that region, B969F and BA1406R, were modified to include the standard Illumina Nextera adapters and 8 bp barcodes (S500 + N700 series) in the forward and reverse primers to allow for dual-indexing, as follows: B969F (5′ AATGATACGGCGACCACCGAGATCTACAC-NNNNNNNN-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACGCGHNRAACCTTACC**-3′) and BA1406R (5′-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNN-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACGGGCRGTGWGTRCAA**-3′), where the Nextera adaptors (L and R arm) are in normal font (to either side of the barcodes), the barcodes are represented by NNNNNNNN, and the specific primer regions are in bold and underlined. Then the libraries were sequenced in a single run of Illumina MiSeq (2 × 300 bp output mode) sequencer. All this processing was carried out at the Integrated Microbiome Resource, Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB), University of Dalhousie (Canada).

Original sequences are available in the NCBI repository (BioProject PRJNA559797, accession: <http://www.ncbi.nlm.nih.gov/bioproject/559797>). Sequence processing to get an operational taxonomic units (OTU) table was performed using QIIME software ver. 1.9.1 (Caporaso et al. 2010) and following recommendations on genomic data processing (Navas-Molina et al. 2013, Dumbrell et al. 2017, Knight et al. 2018). Briefly, sequences of paired-read amplicon libraries were paired-end aligned using fastq-join method (Aronesty 2011), with a minimum overlap of 100 base-pairs and maximum 10% difference in the overlapping region. Then, demultiplexing and quality filtering (at Phred ≥ Q20) was performed, and sequences trimmed to 400 base-pairs with Usearch. The subsampled open-reference OTU picking procedure (Rideout et al. 2014) was applied to generate an OTU table, clustering sequences against Greengenes database ver. 13_8 at 97% similarity (DeSantis et al. 2006, McDonald et al. 2012), with a minimum OTU size of 10 sequences, the reverse-strand-match option enabled, and suppressing step 4 (a second round of de-novo picking after the first one). Subsequently, the OTU table was filtered to remove Archaea, chloroplast, mitochondria, non-phylum assigned OTUs, singletons and OTUs with frequency lower than 0.005% of the total sequence count (Bokulich et al. 2013). After filtering we obtained a total of 1 696 144 valid sequences (number of sequences per sample, n samples = 80, mean(min, max) = 21201.8(1116, 38 458)). In order to control for the sequencing effort, we performed a multiple rarefaction (10 random repetitions) at 1500 sequences (this caused discarding one sample from a non-experimental female), and performed analyses with each of the rarefied files to obtain mean (SE) estimates of the parameters of the statistical models applied. The normalized number of sequences per OTU after rarefaction was used as an estimate of their rarefied abundance (out of 1500 sequences) for the analyses and graphs.

One of the most discussed issues on the processing of HTS data, is the method for clustering and assigning sequences to OTUs, so that any approach may be questioned and therefore, should be justified. The open-reference picking method has been shown to produce more stable OTU assignation that the alternatives de-novo or closed reference (He et al. 2015) and the labels of reference-based methods are comparable among studies, while this is not possible with the de-novo approach (Callahan et al. 2017). Moreover, although reference-based methods are subject to more errors when assigning taxonomies to OTUs than de-novo approaches (Westcott and Schloss 2015), recent clustering methods improve the accuracy of OTU assignations and preserve estimated diversity also for reference based approaches, and several of them are implemented in QIIME (Kopylova et al. 2016). Any bioinformatic tool used in microbiome analyses

has its own advantages and disadvantages and, surprisingly, all have low accuracy in the assignation of taxonomies, with small changes in the parameters used profoundly affecting results (Golob et al. 2017). Such tools are being improved continuously and the best available approaches change from year to year (Callahan et al. 2017, Knight et al. 2018), being difficult to work updated. In this situation, researchers should evaluate their chosen pipeline and settings to confirm if it can adequately answer the research question, as well as provide the details of their methods (Golob et al. 2017). In this sense we believe that the open-reference OTU based approach used here with the pertinent quality and abundance filtering applied, that reduces the possibility of false positives (Bokulich et al. 2013, Golob et al. 2017), is adequate for the comparison of the microbiomes of individuals of the same bird species at the OTU level for bacteria.

Antagonistic activity of uropygial secretion

The antagonistic activity of uropygial secretion was estimated against *Bacillus licheniformis* D13, a very common feather degrading bacteria. To test the antimicrobial activity, 5 µl of secretion was deposited on the surface of pre-inoculated BHA plates (prepared the same morning), directly after extraction from the bird, in the field. We deposited also 2 µl of amoxyciline (714 µg ml^{-1}) diluted in sterile sodium phosphate buffer as positive control. Plates were incubated at 28°C for 12 hours. The antimicrobial activity was detected by the appearance of clear growth-inhibition halos around the drop of uropygial secretion. Then, the external diameter of the halo and that of the drop of secretion were measured. The diameter of the secretion was subtracted from the diameter of the halo to obtain the width of the inhibition zone.

Statistical procedures

Nest box preference

For these analyses, we used only the first time that a nest box of each experimental pair was occupied (i.e. first female choice).

Hoopoe preference for breeding in nest boxes under different experimental treatments (with versus without soft nest material, or with versus without old nest material) were analysed by means of contingency tables and two-tailed Chi-square tests.

Effect on breeding parameters

For these analyses we used only the first clutch of each female.

The variables that approximately followed a normal distribution were: antagonistic activity (nestlings) and all the variables of breeding success except hatching success. The variables that followed a normal distribution after $\log_{10}$ transformation + 0.1 were: CFU ml^{-1} of eggshells in TSA medium, antagonistic activity (in females) and CFU ml^{-1} in the uropygial secretion in TSA. The variables that were not normally distributed, even after logarithmic transformation, were: CFU ml^{-1} of eggshells and of secretion in KF medium and hatching success (Table 1).

To explore the effect of hoopoe old nests material on bacterial load and breeding success, we used ANOVA with experimental treatment (type of nest material) as fixed factor and laying date as covariable (when it was not statistically significant, it was eliminated from the model). When considering information from individual nestlings, we considered nest identity as a random factor. Whenever laying date explained a significant proportion of variance, the statistical model does not allow to include nest identity as random factor (i.e. laying date does not vary within nest identity). In these cases we calculated F-values from models, but df were adjusted to the number of nests considered in the analyses to estimate p-values. When variables were not normally distributed, even after transforming them, we used non-parametric analysis (Mann Whitney tests).

All these statistical tests were performed with STATISTICA ver. 7 (StatSoft).

Bacterial community composition

QIIME was used to generate alpha-diversity estimates for samples (Shannon index), and beta-diversity matrices of distances among samples (weighted and unweighted UniFrac distances (Lozupone and Knight 2005)). For this approach, the phylogenetic tree built in the subsampled open-reference OTU picking procedure (Rideout et al. 2014) by means of FastTree (Price et al. 2009) was used to estimate branch lengths (see the phylogeny obtained in Fig. 1). Comparisons among types of samples were performed with Primer 7.0.13 (PRIMER-e) and Statistica ver. 7.1. Comparison of Shannon index was performed by means of ANOVA, since the distribution of values did not differ from Gaussian distribution (Kosmogorov Smirnov test $p > 0.2$) and there were homogeneity of variances among groups (Levene test, Shannon index $F(1,34) = 1.47$, $p = 0.236$). The composition of bacterial community were compared among types of samples by means of PERMANOVA in Primer ver. 7.0.13 using weighed UniFrac distance matrices generated with QIIME for the OTU level. Principal coordinates analyses (PCoA) were used to visualize the relative position of the two types of samples in the multidimensional space of bacterial community composition.

In order to determine which particular OTUs or higher taxonomic level were affected by the experiment, we used two different approaches: 1) A linear discriminant effect size analysis (LEfSe, Segata et al. 2011) which determines the taxa most likely to explain differences between groups of samples (possible 'biomarkers'). This method relies on three steps testing statistical significance (Kruskal Wallis sum-rank test among classes), consistence (unpaired Wilcoxon rank-sum test among subclasses) and effect relevance (Linear Discriminant effect size). The LEfSe analyses was performed in the Galaxy platform (<http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=testtoolshed.g2.bx.psu.edu/repos/george-weingart/lefse/LEfSe_run/1.0>) with the default parameters (per sample normalization of raw counts to 1M, threshold of LDA score = 2.0 and significant level 0.05). We used treatment as 'class', type of sample (female or nestling) as 'subclass', and sample as 'subject'. The analysis was performed

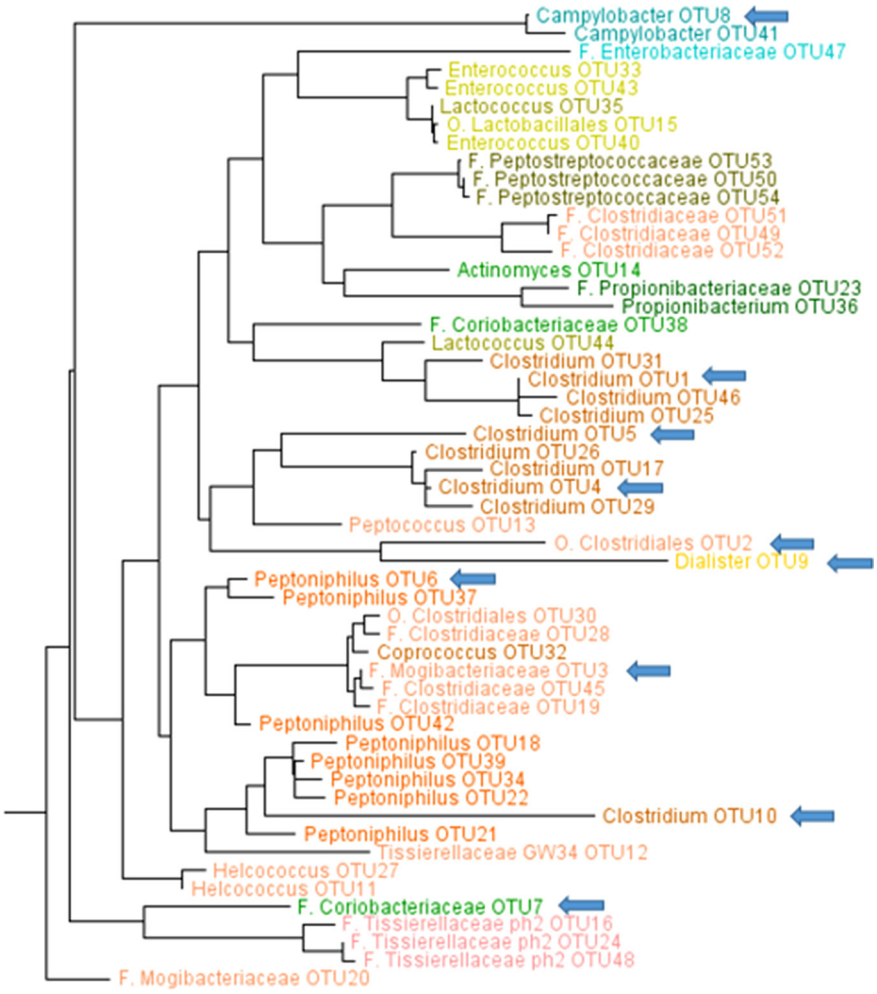

Figure 1. Phylogenetic tree reflecting the similarities of the 16S sequences for the 54 OTUs identified by Illumina HTS in the uropygial secretions of female and nestling hoopoes. Names in tips reflect the taxonomic level of identification reached by comparison with Greengenes database, and OTUs are numbered in decreasing abundance as for Fig. 4. Blue arrows mark the ten OTUs with the highest abundances. Taxa in blue characters are Proteobacteria, those in dark green are Actinobacteria, and the remaining are Firmicutes.

both a) restraining Wilcoxon comparisons to the same subclass and b) letting all-two-all comparisons. The two ways of comparing subclasses only differed in one taxon (lost in the second approach), so we have included results only of the first one to show the whole set of possible biomarkers of groups detected by LEfSe. 2) Non-parametric t-tests comparing between treatments the rarefied abundance (counts rarified to 1500 sequences per sample, see above), of all OTUs or higher order levels present. In order to control for the consistence of the results, the tests were repeated with ten rarefactions and t and p values were averaged. Additionally, we included a false discovery rate correction for multiple tests.

Analyses of the co-occurrence of OTUs in the same sample were performed by means of SparCC correlations (sparse correlations for compositional data) to avoid compositional bias (Friedman and Alm 2012). SparCC estimates the linear Pearson correlations between the log-transformed components (OTUs abundances), based on the log-ratio transformation of the fractions of OTUs estimated from observed counts of sequences by a bayesian approach (Friedman and Alm 2012). Therefore, for the analysis we preprocessed OTU counts by summing 0.1 to all counts to avoid zero values (Friedman and Alm 2012). SparCC generates a matrix of inferred correlations and p-values estimated by a bootstrap procedure for all pairs of OTUs.

Finally, a stepwise BEST analysis on bacterial community of female uropygial secretions (100 restarts) was used to select the best combination of OTUs explaining hatching success of clutches. The BEST analysis selects environmental variables, or species 'best explaining' community pattern, by maximizing a rank correlation between their respective resemblance matrices. For this, a matrix of Euclidean distances in hatching success among females (both including and not-including un-hatched non-embryonated eggs) was used as the resemblance matrix for the analysis. The OTUs included in the best model were tested for bivariate Spearman correlations with hatching success. In addition to the experimental nest boxes (with and without old hoopoe nest material), we analyzed all

the nest boxes that were used during that breeding season (all nests). For these analyses we excluded three clutches without information on the presence of embryos for un-hatched eggs.

The main analyses were conducted independently with the 10 rarefied OTU tables, and mean (SE) values of parameters are presented. However, the post-hoc analyses were performed only with the first rarefaction, and for the analysis of best subset of OTUs explaining hatching success, the mean abundance of OTUs across the ten rarefactions was used.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8574ft0> (Díaz Lora et al. 2019).

Results

Nest box preference

Hoopoes significantly preferred to breed in nest boxes with material over empty nest boxes (Chi-square test: $\chi^2 = 16.49$, df = 1, $p < 0.001$, Fig. 2). However, there was no preference for the nest boxes with hoopoe old nest material experimentally added over those only with sawdust (Goodness of fit Chi-square: $\chi^2 = 0.182$; df = 1; $p = 0.670$; occupied nests with sawdust = 12; occupied nests with sawdust and hoopoe old nest material = 10; total nest boxes = 98 (49 pairs of nest boxes)).

Microbial load and uropygial secretion's properties

Eggshell

Eggshells in nest boxes with hoopoe old nest material harboured higher mesophilic bacterial density than those in nests with only sawdust (Fig. 3, Table 1). No differences were detected for enterococci (Table 1).

Uropygial gland secretion

Neither in females nor in nestlings did the antagonistic activity of secretions or their bacterial loads (mesophilic bacteria and enterococci) depend on experimental treatment. The antagonistic activity of secretions was negatively related to laying date in females (Table 1).

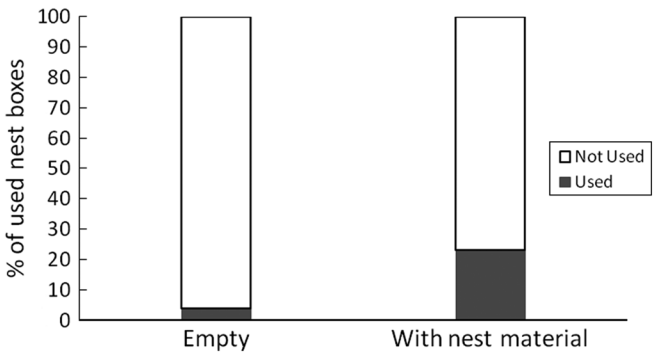

Figure 2. Differences in the frequency of use by hoopoes of new nest boxes depending on the presence (n = 100, 23% occupation) or absence (n = 105, 3.81% occupation) of experimental nest material.

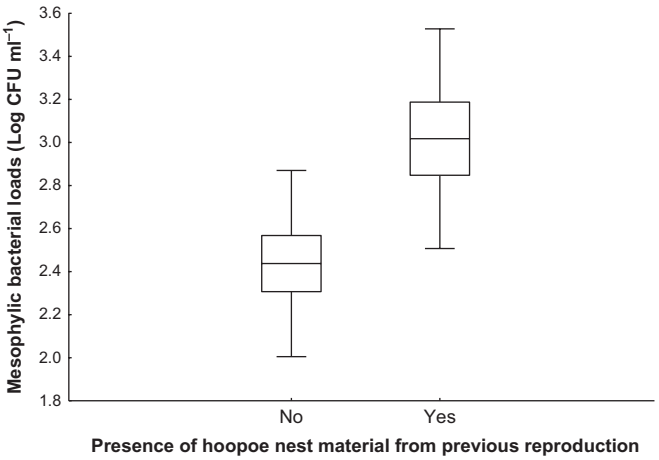

Figure 3. Mean ± 95% CI of bacterial growth (log(CFU ml^{-1})) in TSA medium of samples from hoopoe eggshells, depending on type of experimental nest material added to nest boxes.

Breeding success

Experimental treatment did not affect any of the variables used as proxy of breeding success of hoopoes. Laying date explained a significant proportion of variance only in the case of number of fledglings and body condition of nestlings, that decreased significantly with it (Table 1).

Uropygial gland bacterial community

When considering the two types of nest material together (with and without old hoopoe nest material), the uropygial secretion bacterial community consisted of 54 different OTUs. The bacterial community of uropygial secretion of incubating females and nestlings were very similar except for the presence in nestlings of six OTUs not present in females (Fig. 4a–b respectively). In both kinds of secretions, the same three OTUs clearly dominated the community, comprising together 70% of the analysed sequences per sample (Fig. 4).

When we distinguish between the two types of nest material, the experimental addition of hoopoe old nest material did not affect the alfa-diversity of the bacterial community inside hoopoe glands (Shannon index, ANOVAs mean (SE) across 10 rarefactions, $F(1,34) = 1.45$ (0.10), $p = 0.243$ (0.017), sawdust: mean (SE) = 2.89 (0.08), n = 16, sawdust + old nest material: mean (SE) = 2.77 (0.06), n = 20). However, the composition of the bacterial community in these two kinds of samples was different. The experiment manipulating the presence of hoopoe old nest material in nest boxes affected the bacterial community established in female glands, both considering weighted and unweighted Unifrac distances (Permanovas averaged across 10 rarefactions, weighted Unifrac: mean (SE), pseudoF(1,15) = 4.51 (0.10), $p = 0.0036$ (0.0007), Fig. 5a; unweighted Unifrac: mean

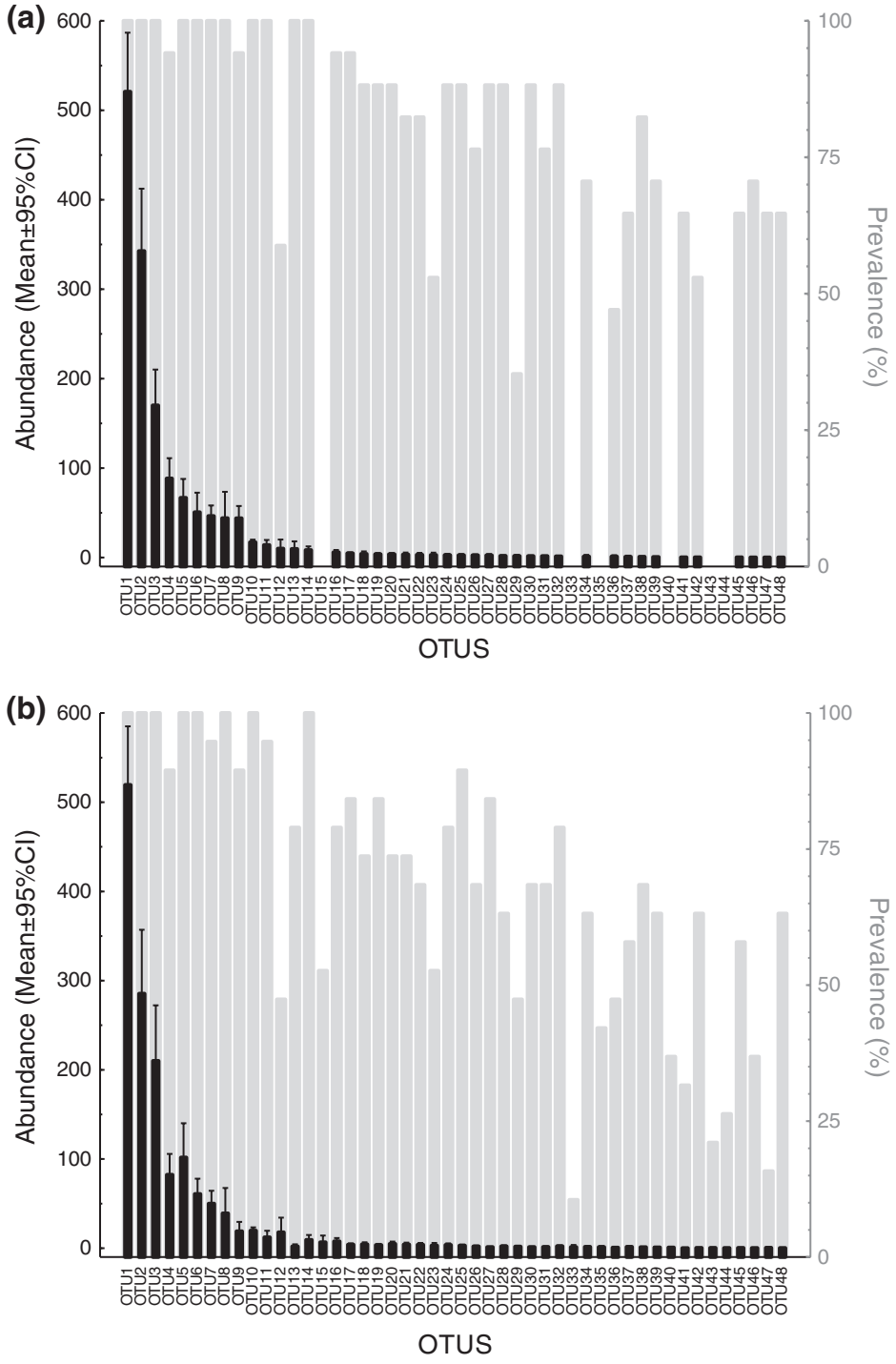

Figure 4. Rarefied abundance (number of sequences out of 1500 per sample after rarefaction, mean ± 95% CI; black bars for the left Y axis) and prevalence (percentage of samples where a target OTU was detected; grey bars for the right Y axis) of the OTUs detected by Illumina HTS in the uropygial gland secretions of hoopoes. Both types of nest material (with and without old hoopoe nest material) are considered together. Only the 48 (out of 54) most prevalent OTUs (those present in at least three individuals) are included in the graphs. (a) Data for females. (b) Data for nestlings.

(SE), pseudoF(1,15) = 2.78 (0.14), p = 0.023 (0.007). On the other hand, the communities present in nestling glands did not differ between treatments (Permanovas averaged across 10 rarefactions, after controlling for nest identity as a random factor, weighted Unifrac: mean (SE), pseudoF(1,17) = 1.07 (0.21), p = 0.3791 (0.0097), Fig. 5b; unweighted Unifrac: mean (SE), pseudoF(1,17) = 0.89 (0.11), p = 0.502 (0.066)).

The LefSe analysis identified several markers for the two experimental groups of samples (Fig. 6). Two OTUs (OTU29 and OTU37) were markers for the group of

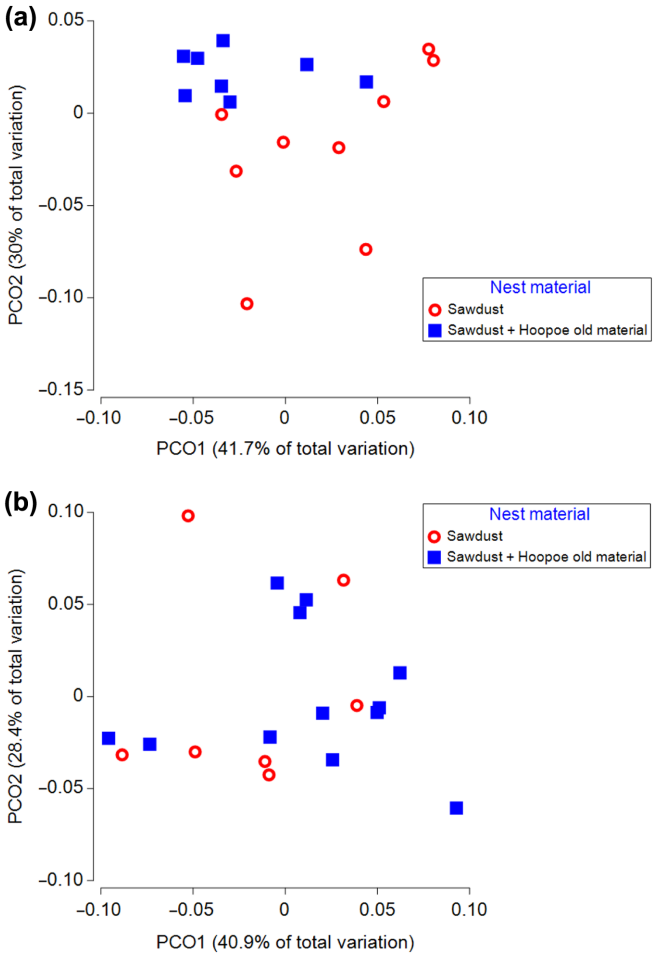

Figure 5. Comparison, based on weighted unifrac distances, between the microbial communities found in the uropygial glands of hoopoes breeding in nest-boxes with sawdust or a mix of sawdust and old hoopoe nest material. (a) Females and (b) nestlings.

samples from nests with old hoopoe nest material, while other three were markers of the control group only with sawdust in the nest (OTU12, OTU13 and OTU23). At higher taxonomic levels, the Fam. Propionibacteriaceae including OTU23 + OTU38, and the whole Class Actinobacteria including additionally OTU14, resulted more typical of the control group. On the other hand, the use of non-parametric t-tests on samples rarified to 1500 reads, resulted in non-significant differences between experimental groups for the Fam. Propionibacteriaceae (non-parametric t (mean (SE)), $t = -1.31$ (0.10), $p = 0.234$ (0.041), FDR-p across all classes = 0.778 (0.043)) and for Actinobacteria (non-parametric t (mean (SE)), $t = -0.96$(0.07), $p = 0.409$ (0.270), FDR-p across all classes = 0.758 (0.596)). With this approach, differences in rarefied abundance between groups only appeared for two OTUs: OTU29 and OTU12 (Fig. 7a, both females and nestlings together to increase the power of the analyses), with mean p-values across 10 rarefactions >0.1 for the other three OTUs identified as biomarkers by the LeFse analysis (data not presented). OTU29 (unidentified *Clostridium*) was completely absent from samples from nest boxes without old nest material, while it appeared at high prevalence in the experimental group with it (prevalence: experimental nests = 75%, n = 16; control nests = 0%, n = 20, comparison between control and experimental nests, two-tailed Fisher exact test, $p < 0.001$; rarefied abundance: non-parametric t(mean (SE)), $t = 4.27$ (0.09), $p = 0.0010$ (0.0000) FDR-p across all OTUs $p = 0.051$ (0.007)). Moreover, OTU12 (identified as *Tissierellaceae* GW-34) was more prevalent and abundant in samples from nests without experimental old nest material (prevalence: experimental nests = 35%, control nests = 75%, comparison between control and experimental nests, two-tailed Fisher exact test, $p = 0.023$; rarefied abundance: non-parametric t (mean (SE)), $t = -2.65$ (0.04), $p = 0.0020$ (0.0003), FDR-p across all OTUs = 0.054 (0.008)). Interestingly, these two OTUs were never simultaneously present in the same sample (Fig. 7b). When we analyzed the relationships among abundances of OTUs in samples with SparCC, these two OTUs resulted significantly correlated with several other components of the community, including some of those retained in the best subset of the components of female secretion bacterial community that explained hatching success with the BEST method (Table 2). Moreover, the abundance of OTU29 was positively related to that of three of the ten most abundant OTUs of the community, and OTU12 to one of them.

The BEST analysis also identified several female secretion OTUs positively correlated with the hatching success of embryonated eggs in their clutches. These included an unidentified Coriobacteriaceae (Ph. Actinobacteria), five closely related Firmicutes in a clade including one *Coprococcus* and one *Peptoniphilus*, another Mogibacteriaceae (probably also *Peptoniphilus*) and Enterococcus strains (Table 2, Fig. 1).

Discussion

Our results indicate that hoopoes preferred for reproduction nest boxes with experimental nest material regardless of whether the material added came from previously used hoopoe nests or not. Characteristics of nest material did not affect reproductive success of hoopoes but affected the bacterial loads of the eggshell and the uropygial gland bacterial community composition. Several OTUs from female uropygial secretions were positively associated with hatching success. These results suggest that a main function of the uropygial secretion of females is to protect the eggs from infection as previously mentioned in Martín-Vivaldi et al. (2014a) and Soler et al. (2014).

As predicted, hoopoes prefer nest boxes with material instead of empty ones. Associated advantages of reusing nest boxes should be even more important for this species that do not built nests because old nest material may help to improve water absorption, thermo-insulation and, thus, incubation efficiency (Mazgajski 2007, Mainwaring et al. 2014, Podofillini et al. 2018). In addition, it could minimize the risk of egg breakage (Podofillini et al. 2018) and the

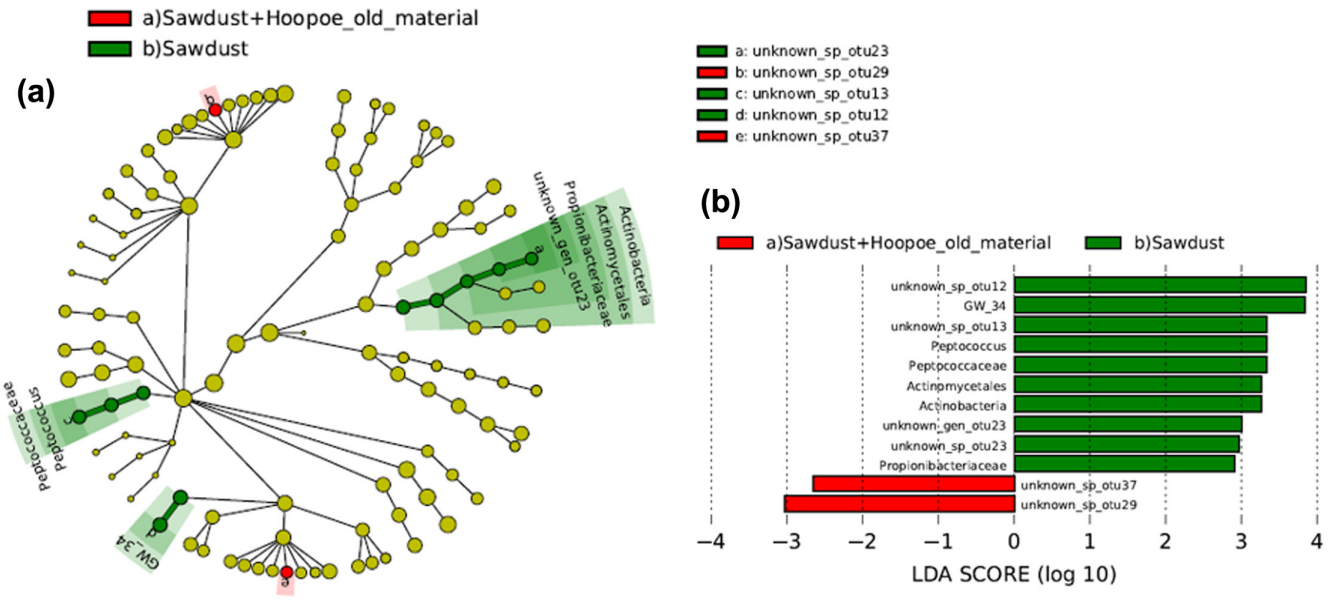

Figure 6. Results of the LefSe analysis comparing the bacterial microbiome of the hoopoe uropygial secretion samples of individuals from nests with sawdust versus sawdust + hoopoe nest material added. (a) Cladogram of the 54 OTUs found in hoopoe uropygial secretions highlighting in colors the bacteria identified by the LefSe method as biomarkers of the two experimental groups (those with a LDA (linear discriminant analysis effect size) > 2). (b) LDA scores for the different biomarkers.

rest of previous breeding activities within the nest material could act as an indicator that it is a good nesting place (Orell et al. 1993).

We hypothesised that hoopoes should prefer for reproduction nest boxes previously used by conspecifics and thus predicted a higher usage of experimental nest boxes with old hoopoe material added. However, our experimental results do not agree with such prediction and hoopoes used at similar rates nest boxes with or without material taken from nests where hoopoes reproduced the previous season. This prediction was based on the possibility that old nest material was a reservoir of mutualistic bacteria for the uropygial gland of nesting hoopoes. Previous studies have pointed out that hoopoes commonly re-use cavities for reproduction (Cramp 1998, Martín-Vivaldi et al. 1999, van Wijk et al. 2017). Our results suggest that this preference for particular nest sites is not mediated by particularities of materials from previous hoopoe reproduction, i.e. by the presence of symbiotic bacteria, because hoopoes reproduced at similar rates in nest-boxes of different experimental treatment. Nest box choice could also depend on the availability of appropriate nest sites (Stanback and Rockwell 2003) and the characteristics of the territory (Tschumi et al. 2014). This possibility would unlikely explain our results since experimental nest boxes were new (never used before) and installed in locations in a pairwise experimental design. Age and previous breeding experience could affect nest-box choice by hoopoes. We do not have a reliable criterion for estimating age in the females of this breeding season so we cannot control for it in the analyses. However, we have very few recruits (11.36% in 2015; 4 of 44 females) due to high mortality and high dispersion of the species (van Wijk et al. 2018). Thus, most breeders were new individuals arrived from other places to our study area. Moreover, the new experimental nest boxes were new for all hoopoes that arrived to the study area regardless of their age (recruits or not recruits) and both, old and new individuals, used new (experimental) and old nest boxes. Another possibility could be that nest box choice was affected by the bacterial community of the individual hoopoes. Thus, although we think this alternative explanation is improbable, we cannot rule it out.

Despite not showing preference for nests with old hoopoe nest material, we found support to the expected effect of the experimental nest material on the composition of females' uropygial secretion, and on the bacterial load of the eggshells. Whittaker et al. (2016) found in dark-eyed junco that shared environments had an influence in shaping bacterial and volatile profiles of the uropygial gland skin and secretion. In our case, the bacteria were only stemmed from the uropygial secretion. This result shows, for the first time in hoopoes, that the nest material could be a source of strains for their incorporation to the uropygial gland that can interact with other components of the community.

Results from the two statistical approaches used for the comparison of bacterial communities of experimental groups agreed in the identity of the two OTUs whose rarefied abundances were more strongly affected by the experiment. LEfSe analysis is more sensitive and detected additional biased taxa that deserve to be investigated as possible biomarkers of these environmental conditions. Our relative low sample size and the relatively high inter-individual variation detected, impede

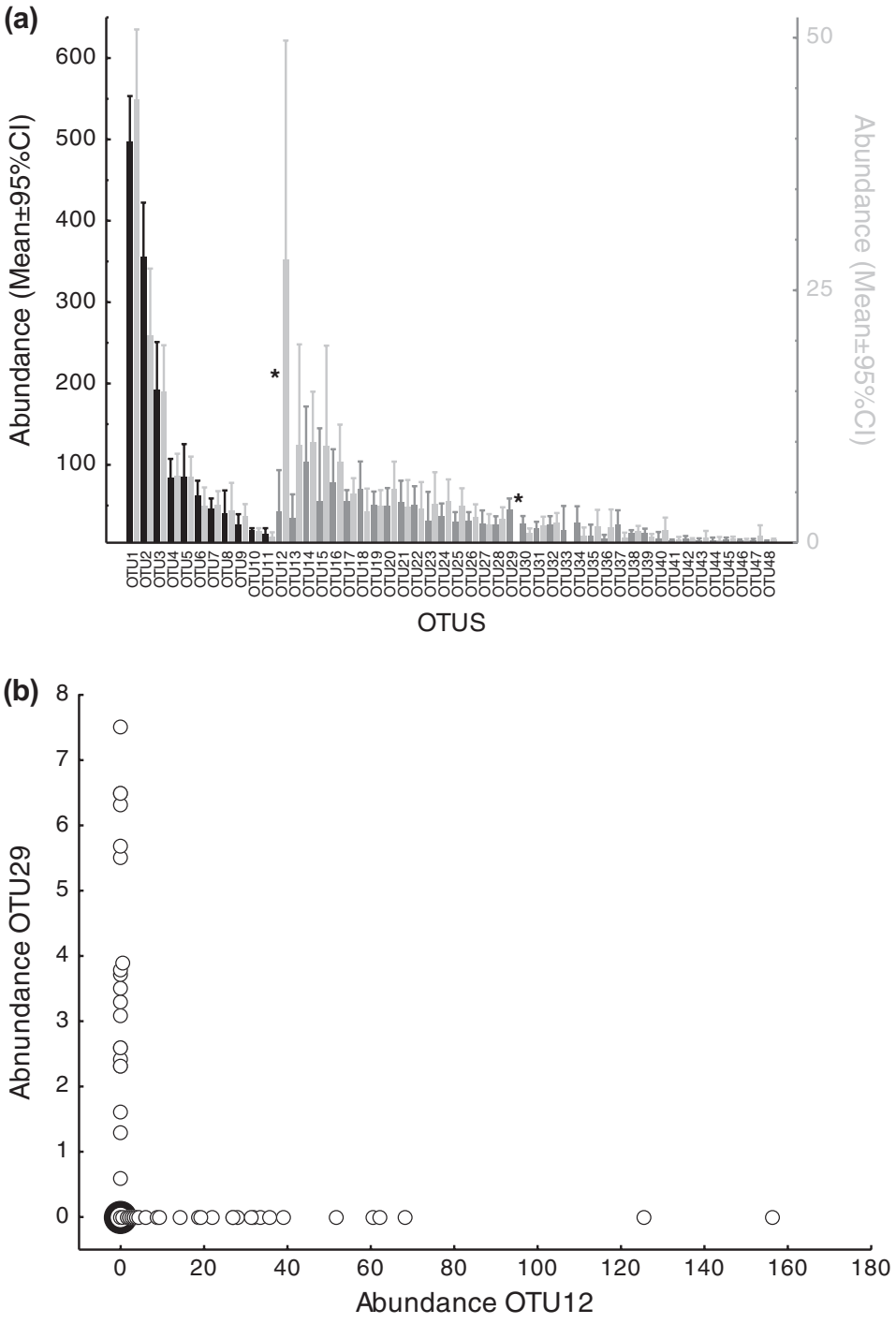

Figure 7. Influence of presence of hoopoe old nest material in nest boxes on the rarefied abundance (number of sequences out of a total 1500 counts per sample) of bacterial OTUs in the uropygial secretion of hoopoes (female and nestling samples together). (a) Comparison of the abundance of OTUs between nests with sawdust + hoopoe nest material (black and dark gray bars for the left and right y-axes, respectively) and nests only with sawdust (light grey bars). Significant comparisons for a particular OTU (non-parametric t-tests after FDR correction) are marked with asterisks. The bars from OTU12 to OTU48 are scaled for the right y-axis. Only the 48 (out of 54) most prevalent OTUs (those present in at least three individuals) are presented in the graph. (b) Relationship between the rarefied abundance of OTU29 and OTU12 in the same hoopoe uropygial secretion samples, including all types of nest boxes. Point sizes are in log-scale.

to point out statistical support when considering averaged repeated t-tests on random repetitions of rarefactions.

There were two particular OTUs of glands that were greatly affected by the presence of hoopoe old nest material, but in opposed directions. OTU29 was only present in nests with hoopoe old nest material, while OTU12 was much more prevalent in nests without such old material. This suggests that OTU29 will only be incorporated to the

Table 2. List of the significant relationships between the two OTUs affected by the nest material experiment (OTU12 and OTU29) and other OTUs of the hoopoe secretion microbiota (SparCC sig. correlations, only for those with $r \geq 0.20$ and $p < 0.05$, the sign of the relationship is indicated). The groups of OTUs from female secretions best explaining the hatching success of their clutches are shown (BEST stepwise search with Primer7, R value of the final model indicated in the columns), with the corresponding bivariate Spearman correlations (rs(p)) with hatching success for each OTU. Significant p-values are in bold.

	(SparCC sig. correlations)		Best subsets explaining hatching success				
			All nests (R = 0.220, n = 29)	All nests, embrionated eggs (R = 0.486, n = 29)	Exper. nests (R = 0.516, n = 13)	Exper. nests embrionated eggs (R = 0.546, n = 13)	
	OTU12	**OTU29**	r_s(p)	r_s(p)	r_s(p)	r_s(p)	Identification
OTU30	–						(Clostridiales)
OTU28				**0.42** (0.024)			(Clostridiaceae)
OTU32	–			**0.42** (0.025)		0.52 (0.066)	*Coprococcus*
OTU19		–	0.14 (0.469)	**0.40** (0.025)		**0.59** (0.035)	(*Clostridiaceae*)
OTU45				−0.10 (0.619)		0.20 (0.512)	(*Clostridiaceae*)
OTU42				0.11 (0.569)	0.54 (0.055)	0.47 (0.102)	*Peptoniphilus*
OTU20			0.05 (0.795)	**0.46** (0.012)		0.51 (0.078)	(*Mogibacteriaceae*)
OTU38			0.30 (0.118)	**0.47** (0.011)		**0.57** (0.041)	*(Coriobacteriaceae)*
OTU40					0.37 (0.211)	0.23 (0.449)	*Enterococcus*
OTU43					0.24 (0.435)	0.29 (0.330)	*Enterococcus*
OTU27			−0.26 (0.181)	−0.11 (0.569)		−0.47 (0.106)	*Helcococcus*
OTU46			−0.23 (0.235)				*Clostridium*
OTU39						−0.02 (0.956)	*Peptoniphilus*
OTU12		–					*Tissierellaceae*
OTU24		–					*Tissierellaceae*
OTU41		–					*Campylobacter*
OTU6		+					*Peptoniphilus*
OTU2		+					(Clostridiales)
OTU31		+					*Clostridium*
OTU26		+					*Clostridium*
OTU17		+					*Clostridium*
OTU4		+					*Clostridium*
OTU29	–			−0.32 (0.094)			*Clostridium*
OTU37	–						*Peptoniphilus*
OTU8	+						*Campylobacter*
OTU47	+						(Enterobacteriales)
OTU36	+						*Propionibacterium*

secretion bacterial community when hoopoes use cavities where conspecifics previously reproduced. We do not know to what extent the incorporation of these bacteria in the secretion is beneficial or not to the host, or even if any of them is potentially pathogenic, since we have not found effects of the experiment on breeding success or antimicrobial activity of secretions. Nevertheless, since both are correlated with the abundance of other components of the community, they may have a role in the uropygial gland microbiota dynamics.

Moreover, our findings show that old nest material could affect the microbial community established in female glands. In this way, females could be able to acquire bacteria from the old nest material. This could also explain the differences found in the bacterial community of uropygial secretion of females and nestlings since the latter had six OTUs not present in females. Another non-exclusive possibility is that nestlings could acquire it from the nest after female leaves it, but not from sources present in the old material. Females stay inside the nest until the first nestlings are eight days old and nestlings leave the nest after 24–30 d (Martín-Vivaldi et al. 2014b). In this way, these OTUs present only in nestlings glands could be obtained from the diverse kind of food remains, faeces, dead nestlings, etc. accumulated in the nest along the nestling period. Alternatively, nestlings' glands may be less selective in the acquisition of strains from the environment than females. The association of age with the maturity of the immune system might determine or control the microbiota of the uropygial gland. This possibility is also consistent with the idea that young animals host more rare and transient symbionts than adults (Palmer et al. 2007). Whatever the reason, some other studies also found a greater number of total bacterial OTUs in nestlings than in adults (van Dongen et al. 2013, Whittaker et al. 2016) and, thus, our result might adjust to a general trend in birds. However, the microbiota of the nest materials has not been analyzed. Therefore, it is possible that the results found are due to indirect effects of the nest material or environmental conditions. Many studies have found that there is an important role of environmental conditions for bacterial load that could influence the final microbiome (Palmer et al. 2007, Peralta-Sánchez et al. 2012, Brandl et al. 2014, D'Alba and Shawkey

2015, Ruiz-Castellano et al. 2016, Whittaker et al. 2016, Martín-Vivaldi et al. 2018).

Regarding bacterial load of the eggshell, previous experimental studies detected the influence of nest materials on eggshell microbiota in several bird species (Brandl et al. 2014, Grizard et al. 2014, Martínez-García et al. 2016a, Ruiz-Castellano et al. 2016, van Veelen et al. 2018). These bacteria are mostly derived from faeces, digestive tract and bare skin of the female, feathers and nest material itself (Peralta-Sanchez et al. 2010, van Veelen et al. 2017, 2018). Moreover, uropygial secretion might reach eggshells and, thus, because of its antimicrobial properties, determine at least partially their microbiota (Peralta-Sánchez et al. 2012). In the case of hoopoes, uropygial secretion also contains bacteriocin-producing bacteria, and females actively smear eggshells with their uropygial gland secretion (Soler et al. 2014) and fill up special structures (i.e. crypts) that enhance the adhesion of the secretion full of symbiotic bacteria (Martín-Vivaldi et al. 2014b). Therefore, since we have found an effect of experimental nest material on the bacterial community of uropygial secretion of female hoopoes, the detected effects on the bacterial load of the eggshell might not only be directly determined by the microbiota of nest material, but indirectly by its effect on the bacterial community of female secretion.

The absence of effects of the type of nest material on breeding success suggests that the bacterial strains acquired from the nest by hoopoes are not especially important for them or that these bacteria are functionally redundant. The core components of the usual microbiome might be acquired vertically, probably maintained from one to another breeding season on reservoirs on the body, or can be obtained horizontally from environmental sources others than nest materials (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016b, Martín-Vivaldi et al. 2018, Rodríguez-Ruano et al. 2018). This result may further explain why hoopoes have not evolved preference for nests with old hoopoe nest material. The effects of nest re-use on nestling fitness and reproductive success are still unclear. Some studies found negative effects (Tomás et al. 2007, González-Braojos et al. 2012). However, most studies agree with our results, and did not find that nest re-use affects hatching or breeding success (review on Mazgajski 2007, Martínez-García et al. 2016a, Podofillini et al. 2018). Even though the experiment did not affect breeding success of pairs, we have found that several OTUs from female uropygial secretions were positively associated with hatching success. These OTUs belong to the phylum Firmicutes, as enterococci, that produce bacteriocins defending from feather degrading bacteria and trans-shell infection of embryos (Martín-Platero et al. 2006, Soler et al. 2008, Martín-Vivaldi et al. 2014b). The experiment did not affect these bacteria and, thus, it is unlikely that they came from the nest material, so it is worthwhile to continue exploring their origin. Another explanation for the absence of a significant effect on breeding success could be the small sample size obtained, being insufficient for detecting statistically significant effects.

In summary, the existence of nest material seems more important for hoopoes' nest-site selection than the possibility of obtaining a reservoir of beneficial bacteria from it. Nevertheless, the experiment confirms an important effect of such re-used nest material on the bacterial loads of the eggshells, and this is the first time that it is shown that it affects also the composition of the uropygial gland secretion bacterial community. More importantly, several particular OTUs resulted related to hatching success. This result highlights the possibility that a main function of the uropygial secretion of female hoopoes is to protect the eggs from infection, using a variety of cultivated bacterial strains, as suggested by previous results mainly for enterococci (Ruiz-Rodríguez et al. 2013, Peralta-Sánchez et al. 2014, Martín-Vivaldi et al. 2014a). The information provided by the description of the whole microbial community inhabiting these glands (Rodríguez-Ruano et al. 2018 and the present study), will lead to the next interesting step of studying the complete set of interactions among them and their effects on bird health and breeding success using network analyses. In addition, it would be worth to analyze the microbiota of the nest material itself, to understand how it interacts with the bacterial composition of the uropygial secretion and to reinforce the results and conclusions derived from this experiment.

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Permits – The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. The protocol was approved by the ethics committee of the Univ. of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.: 785), and all necessary permits for hoopoe's manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for the experiment.

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Supplementary material (available online as Appendix jav-02083 at <www.avianbiology.org/appendix/jav-02083>). Appendix 1.