Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell

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Abstract

How microbial symbionts are established and maintain on their hosts is a leading question with important consequences for the understanding of the evolution and functioning of mutualistic relationships. The acquisition by hosts of mutualistic microbial symbionts can be considered as colonization processes of environments (i.e., host) by symbionts. Colonization processes can be explored by characterizing nestedness of communities, but this approach has rarely been applied to communities of microbial symbionts. We here used this approach, and estimated nestedness of bacterial communities of hoopoes (*Upupa epops*), a species with symbiotic bacteria in their uropygial gland that are expected to colonize eggshells where they protect embryos from pathogens. Bacterial communities were characterized by ARISA and studied nestedness characteristics of bacterial communities living in the uropygial secretion, bill, belly and eggshells of each sampled female hoopoes. We detected a consistent nested pattern of bacterial communities of hoopoes; from the uropygial gland to the eggshell. We also found evidence of study year and reproductive event influencing level of nestedness of bacterial communities of hoopoes. These results indicate that bacterial communities of eggshells and body parts of female hoopoes are at least partially conditioned by the symbiotic community in the uropygial gland. We discuss the importance of these results for understanding this host-microbial mutualism functioning and evolution.

Keywords: bacteria meta-community, birds, ecological network, eggshells, mutualistic bacteria, symbiosis, preening, uropygial secretion

Introduction

Host species may receive from their microbial symbionts a multitude of benefits (mutualistic) and costs (parasitic), and understanding how microorganisms are established and maintained within their hosts is a leading question in evolutionary biology that is being explored from different perspectives such as molecular biology, behavioral ecology, community ecology and evolutionary game theory (Bright & Bulgheresi, 2010; Archie & Theis, 2011; Ezenwa et al., 2012; Scheuring & Yu, 2012; McFall-Ngai et al., 2013). Mainly for horizontally acquired mutualistic symbionts, authors have traditionally dealt with this question by considering antagonistic characteristics of bacterial strains driving competitive exclusion within bacterial communities; and hosts may even be able to drive this interference competition and favor the recruit of appropriated microbial symbionts (Scheuring & Yu, 2012).

Bacterial communities are not isolated from each other and sometimes come in direct contact due to their expansion or because of migration of some species or strains with particular antagonistic characteristics (Long & Azam, 2001; Prasad et al., 2011; Long et al., 2013). Such interactions would influence functionality (i.e. antibiotic production and resistance) of bacterial communities as a whole (Cordero et al., 2012) and, in the case of including mutualistic bacteria, the adaptive value for their hosts. In this scenario, hosts can acquire beneficial bacteria from the surrounding communities and recruit them into the mutualistic community, which produces antimicrobials that impede or limit proliferation of pathogenic strains at particular body locations. Thus, identifying the degree of connection among different bacterial communities of animal or plant hosts in a meta-community framework would help to understand the mechanisms by which particular symbionts help their hosts and therefore the evolution of mutualistic relationships (Chagnon et al., 2012). This exercise, which is lately approached within

frameworks derived from network theory, has recently been applied to ecological studies of several mutualistic systems including those of plants and mycorrhizals (Chagnon et al., 2012; Montesinos-Navarro et al., 2012; Jacquemyn et al., 2015). However, it has largely been ignored in studies exploring mutualistic associations between bacteria and animal hosts.

Some mutualistic symbionts or their produced antimicrobial chemicals protect ant gardens, wood galleries of beetles and embryos of shrimp, lobsters, squid, wasps, salamanders and birds from pathogenic bacteria and/or competitor fungi (Gil-Turnes et al., 1989; Barbieri et al., 1997; Currie et al., 1999; Barbieri et al., 2001; Kaltenpoth et al., 2005; Cardoza et al., 2006; Banning et al., 2008; Scott et al., 2008; Martín-Vivaldi et al., 2014b). Microbial communities growing in ant gardens or on embryos coverings, should be interconnected with, and at least partially determined by, the ones inhabiting the body of host individuals. This is for instance the case of hoopoes (*Upupa epops*) harboring beneficial bacteria with high antimicrobial potential in their uropygial gland (Martín-Platero et al., 2006; Soler et al., 2008; Martín-Vivaldi et al., 2010; Ruiz-Rodríguez et al., 2012; 2013; 2014). In this species, incubating females collect the uropygial secretion with the bill and, then, use it to either preen feathers (Ruiz-Rodríguez et al., 2009) including those of the belly, or to directly smear the eggshells (Martín-Vivaldi et al., 2009; Soler et al., 2014; Martín-Vivaldi et al., 2014b). In this way, the bacteria hosted in the female uropygial gland can reach the eggshell indirectly by mean of the secretion on the bill surface, or during incubation by mean of secretion impregnated on belly skin and feathers. Thus, the bacterial community on the eggshells should be conditioned by that on the bill and/or belly; which in turn should depend on the bacterial community in the uropygial gland of females. We previously have shown that some of the bacterial strains detected in the uropygial gland are also detected on the

bill, brood patch and eggshell of hoopoes, and that finding some of them in one of these location (i.e. uropygial oil) increase the probability of detecting the same bacteria in some other location (i.e. eggshells) of the same female (Martínez-García et al., 2015). Finding evidence of such hypothetical hierarchized relationship among bacterial communities from the gland to the eggshells would suggest a causal explanation (i.e. direction of colonization) for the relationship between bacterial community of the uropygial secretion and that living on the eggshell of hoopoes. Furthermore, because the bacterial symbionts are of adaptive value for hosts, it would contribute to understand functionality and evolution of the mutualistic relationship.

One useful approach to detect interactions affecting the distribution pattern of multiple species across multiples localities is nestedness analysis (Ulrich et al., 2009; Ulrich & Almeida-Neto, 2012; Traveset et al., 2014). The nestedness concept originated in the context of explaining insular biotas as result of colonization by a source pool of species from mainland and has two different components. The first one estimates nestedness among species; i.e., better dispersers are expected to colonize the majority of islands, including the most distant ones, whereas poor dispersers would be restricted to the less isolated island, which results in a nested pattern of species occurrence on islands (Ulrich & Almeida-Neto, 2012). The second component of nestedness detects non-random patterns of variability of species composition along environmental gradients (Ulrich & Almeida-Neto, 2012). Thus, in meta-communities, the presence of strong components of nestedness is a clear indication of coupled gradients of site environmental characteristics and species traits (Ulrich et al., 2009). Nested patterns are also common in ecological networks of interacting species (Bascompte et al., 2003; Fortuna et al., 2010) and have rarely been explored in bacterial communities (Poisot et al., 2011; Aguirre-von-Wobeser et al., 2014). Knowledge of the nestedness of symbiotic

meta-communities will consequently help the comprehension of the dynamic and stability of microbial communities of animals including those of adaptive value.

Here, we study nestedness characteristics of bacterial communities living in the uropygial secretion, bill, belly and eggshells of hoopoes, which correspond to the second nestedness component exposed above. Before establishing on the eggshells, bacteria from the uropygial gland should be detected in the bill and/or the belly of females. Thus, finding statistical support of bacterial communities of hoopoes being nested in that direction would suggest that some of the bacteria in the bill, belly and eggshell of hoopoes came from those in the uropygial gland. There is strong experimental evidence suggesting that environmental conditions such as resource availability, temperature, pH, etc. may influence the outcomes of interactions among bacterial communities (Grossart et al., 2004; Long et al., 2005; 2013) and the evolution of mutualistic relationships (Flórez et al., 2015). We here explored possible influences of year, breeding attempt and breeding conditions (captivity *vs.* wild) on nestedness estimates of bacterial communities of breeding hoopoes.

Material and Methods

Field work

The fieldwork was performed during the breeding seasons 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 (Martín-Vivaldi et al., 2009). In 2011 hoopoes were also sampled in two captive populations maintained at the Hoya of Guadix in Granada, and in the Finca Experimental la Hoya, in Almería (36º50´N, 2º28´W) since 2008. Breeding pairs of hoopoes were housed in independent cages at least 3m x 2m x 2m installed in the open,

150 scattered and isolated to avoid interactions among pairs. Cages had access to soil and
151 provided with live food (crickets, vitamin-enriched fly larvae) and meat (beef heart) *ad*
152 *libitum*.

153 The hoopoe is a hole-nesting species that mainly breed in open woods or open
154 areas with scattered trees (Martín-Vivaldi et al., 2014a). Hoopoe females usually lay
155 two clutches of 6-8 eggs along the breeding season, between February and July (Martín-
156 Vivaldi et al., 1999).

157

158 Bacterial sampling

159 Incubating females were sampled 14 days after laying the first egg. We wore new latex
160 gloves cleaned with ethanol during the whole sampling process. Incubating females
161 were caught from the nest box, feathers around the gland were separated and washed
162 with ethanol to avoid contamination, and 5 µl of uropygial secretion were collected with
163 a micropipette directly from within the uropygial gland. The secretion was introduced in
164 a sterile 1.5 mL microfuge tube and stored at 4ºC. Afterwards, we sampled the complete
165 bill and belly (brood patch) of the females and the eggshells of the whole clutch. Each
166 sample was collected by cleaning the surfaces with a sterile swab slightly wet with
167 sterile sodium phosphate buffer (0.2 M, pH 7.2). The swabs were preserved in 1.5 mL
168 microfuge tubes with 1.2 ml of buffer at 4ºC. Gloves were cleaned with ethanol after
169 collecting each of the samples of a nest to avoid contamination among samples, and,
170 within 12 hours after collection, all samples were stored at -20ºC until the molecular
171 analyses.

172

173 Laboratory work

Given the viscosity of the uropygial secretion, bacterial DNA from these samples was extracted with a commercial kit (The FavorPrep™ Blood Genomic DNA Extraction Kit). Bacterial DNA from swabs kept in phosphate buffer was extracted by following Chelex-based DNA isolation protocol, proposed by Martín-Platero et al. (2010). The use of different DNA isolation methodologies is not a problem for our goals. Higher DNA isolation yields will not produce higher richness detection with the analysis method applied (ARISA, see below). ARISA (Automated rRNA Intergenetic Spacer Region) capture most abundant populations with low power detecting the long tail of low abundant ones. Thus, bacterial communities were characterized following the well-established ARISA protocol (Fisher & Triplett, 1999). Briefly, we amplified the 16S/23S intergenic spacer region by using the primer pair ITSF and ITSReub consisted of 5′-GTCGTAACAAGGTAGCCGTA-3′ (forward primer sequence) and 5′-GCCAAGGCATCCACC-3′ labeled fluorescently with 6-FAM (reverse primer sequence) (Cardinale et al., 2004). PCR amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 2.5x5 PRIME MasterMix including 1.5mM Magnesium, 200 mM dNTPs, 1.25 U Taq polymerase (5 PRIME, Hamburg, Germany), 0.2 mM of primers and 5µl of diluted DNA 1:10. PCR reactions were carried out in Eppendorf Mastercycler nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 °C for 2 min, followed by 30 cycles with denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by means of automated fluorescent capillary electrophoresis on 3130 Genetic Analyzer and electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems).

199 These analyses were realized in the ING unity (Genetic Information) of CIC (Scientific
200 Instrumentation Center) of the University of Granada.

201 Resulting fragment lengths were analyzed with Peak Scanner v 1.0 (Applied
202 Biosystems) by the "Microsat G5" method. We considered peaks with values of relative
203 fluorescence intensity higher than 0.09% and fragments above a threshold of 50
204 fluorescence units, ranging between 100 and 1,000 bp (base pairs). Operational
205 Taxonomic Units (OTUs) were established by calculating the best binning frame of
206 different fragment lengths considering a window size (WS) of 4bp and a distance
207 between two consecutive binning frames (Sh) of 0.1. This exercise was carried out in
208 "R" environment (http://cran.r-project.org/, R.2.12.2 (R Development Core Team,
209 2010)) following scripts by Ramette (2009) at https://www.mpi-
210 bremen.de/en/Software_2.html. We identified 145 OTUs that appeared with different
211 frequencies in different hoopoes bacterial communities.

212

213 Sample sizes and statistical analyses

214 We collected 468 bacterial samples from 81 females, but we failed to amplify bacterial
215 DNA of 21 samples from uropygial gland, bill, brood patch or eggshells coming from
216 10 females. We, thus, considered 71 individual females with complete information of
217 bacterial communities of the secretion, bill, brood patch, and eggshells. Of these
218 females, 20 were sampled twice, 18 during the same season (i.e. two consecutive
219 breeding attempts) and 2 during their first breeding attempt of the two study years. Two
220 more females were sampled three times; one of them during consecutive breeding
221 events in 2011, and the other one was sampled once during the first breeding attempt of
222 2010 and twice during 2011. The remaining 49 females were only sampled during their
223 first breeding attempt. We performed 27 samplings in 2010 and 78 in 2011.

224

225 Nestedness estimations

226 The network between OTUs and sampled bacterial communities was built with the
227 "cca" method of the "plotweb" function in the library Bipartite (Dormann et al., 2008)
228 of the statistical software R.2.12.2 (R Development Core Team, 2010). As index of
229 nestedness, for each female and sampling event, we calculated the metric based on
230 overlap and decreasing fill (NODF) (Almeida-Neto et al., 2008; Almeida-Neto &
231 Ulrich, 2011) as implemented in the user-friendly web interface NeD
232 (http://ecosoft.alwaysdata.net/) by Strona et al. (2014). NODF can be estimated for
233 columns and rows and does not depend on number of rows and columns considered
234 (Almeida-Neto et al., 2008). NODF for columns would therefore inform of nestedness
235 of communities among sampling places, while NODF for rows will determine whether
236 the rarest OTUs are present in the sampling place that also have the most common
237 (Almeida-Neto et al., 2008). NODF is dependent on the arrangement of columns and
238 rows which allow testing hypothesis about the cause of nestedness (i.e. direction of
239 colonization) by ordering columns and rows according to criteria representing different
240 hypotheses (Almeida-Neto et al., 2008; Ulrich et al., 2009; Almeida-Neto & Ulrich,
241 2011; Traveset et al., 2014). To test our hypothesis we thus arranged columns following
242 the predicted colonization sequence from the uropygial gland through the bill and brood
243 patch to the eggshell and estimated NODF of columns, while rows (OTUs identity)
244 were arranged from those detected in all locations to those detected in only one or none.
245 We thus organized presence absence matrices for each sampling event (individual
246 females during a single reproductive event and study year) as including all bacterial
247 strains (OTUs) detected in samples from secretion, beak, brood patch or eggshells.
248 Locations of bacterial communities were in columns ordered following the expected

direction of nestedness (secretion, beak, brood patch or eggshells). OTUs identities were therefore organized as rows (ESM 1).

The significance of NODF of columns (hereafter NODF) values was assessed against 999 randomization using the Fixed row total – Equiprobable column totals (EF) null model that maintain observed row totals but allow column totals to vary randomly. This null model retains species occurrence frequencies per row but allow species richness per site (column totals) to vary randomly and equiprobably (Gotelli, 2000), which adjust to the hypothesis tested. NeD (Strona et al., 2014) computes Z-values as

$$Z = {(NIr - \overline{NIs})}\Big/{\sigma(\overline{NIs})}$$

Where NIr is the NODF index of the matrix under examination, $\overline{NIs}$ is the average value of the set of index values for the null matrices generated by the program and $\sigma(\overline{NIs})$ is the standard deviation. Z-values > 1.64 indicate statistical significance at P = 0.05.

We estimated NODF and Z-values with matrices built for each individual sampling considering the four kinds of bacterial communities, but also excluding community of brood patches because hoopoes may directly smear uropygial secretion on the eggshells with the bill. In all cases communities were arranged according to the hypothesis tested. We later estimated average effect size of nestedness (i.e. NODF index) of bacterial communities of hoopoes and of Z-values, and tested for possible effects of breeding attempt, study year and captivity on the strength of communities' nestedness. Statistical significance of average NODF values was inferred from the 95% CI of Z-values (i.e. whether or not it includes the threshold value of 1.64).

Statistical models

Captive populations were only sampled in 2011 and thus the effect of study year on nestedness of bacterial communities of hoopoes was explored only considering samples from the wild population. The statistical General Linear Model (GLM) included the NODF values as dependent variable, year, breeding attempt and their interaction as fixed effects, and female identity nested within study year and its interaction with breeding attempt as random factors. Similarly, for exploring the effect of captivity on NODF values, we only used information from 2011, the only study year with samples from captive and wild nests. In this case the GLM model included breeding condition (captivity *vs.* wild), breeding attempt and their interaction as fixed effects, and female identity nested within breeding condition and its interaction with breeding attempt as the random factors. These models were reduced removing terms one by one starting with that with the largest associated p-value, up to p-values smaller than 0.1. Residuals of performed models did follow a Gaussian distribution. GLM analyses were performed in Statistica 10.0 (Statsoft Inc., 2011).

Since the bacterial community of the secretion may access eggshells directly from the bill (e.g. Path: Secretion-Bill- Egg; hereafter SBE), or indirectly throughout the contact of bill with the brood patch (e.g. Path: Secretion-Bill-Brood-Egg; hereafter SBPE) (Martín-Vivaldi et al., 2014b), we performed the above analyses for NODF values estimated for SBE and SBPE bacterial meta-communities (NODF's values and information of individual samples are shown in ESM 2).

Results

We identified 145 different OTUs in the bacterial communities of hoopoes. 124 of these OTUs were detected in the uropygial secretion, 101 in the bill, 96 in the brood patch

296 and 95 in the eggshell bacterial communities (Fig 1). The OTU richness observed per
297 sampled nest ranged from 11 to 60 (N = 97, Mean (SE) = 33 (1.1), Mode = 40).

298 On average, sampled bacterial communities of hoopoes were nested from the
299 uropygial gland to the eggshells (Fig. 1) independently of considering or not the
300 bacterial community of brood patch in the expected hierarchy of communities (Fig 2).
301 Nestedness of bacterial communities ordered in the opposite direction (from the eggs to
302 the uropygial secretion) were far from statistical significance being Z estimates close to
303 zero (NODF: mean = 14.12, CI: 12.25-17.27, Z-NODF, mean: -0.06, CI: -0.34-0.23)
304 (Egg –Beak – Gland: NODF: mean = 9.37, CI: 6.93-11.80, Z-NODF, mean: -0.33, CI: -
305 0.58- -0.07).

306 Nestedness of hoopoe's bacterial communities did significantly vary between
307 study years (Table 1), being stronger in 2011 than in 2010 (Fig 2). Moreover, whether
308 or not the sampled nests were from captivity or from wild populations did not affect
309 nestedness strength (Table 1). Further, NODF estimates for second breeding attempt
310 tended to be higher than those for first clutches (Table 1). In addition, the effect of
311 female identity did not reach statistical significance (Table 1) in any of the statistical
312 models indicating that within-females variance is not significantly lower than the
313 variance among nests of different females.

314 Finally, NODF estimates for groups of bacterial communities including or not
315 that of brood patch provide similar results, suggesting that eggshell bacterial community
316 was equally nested in that of the brood patch than in the bacterial community of bill. All
317 these results suggest that bacterial community of hoopoe eggshell is nested within that
318 of the brood patch and/or bill; and that these bacterial communities are nested within
319 that of symbiotic bacteria in the uropygial gland (Fig 1). These results therefore support
320 the hypothetical pathway of bacteria from the uropygial gland to the egg surface.

Discussion

Our results show a general nested pattern of bacterial communities of hoopoes from the uropygial gland to the eggshell, which is consistent across all individual females. The level of nestedness of hoopoes' bacterial communities varied between study years and reproductive events, indicating environmental influences on the estimates. These results therefore show that bacterial communities of eggshells and body parts of female hoopoes are nested within the community in the uropygial gland. Below we discuss this interpretation and the importance of estimating nestedness of bacterial communities for understanding mechanisms (i.e. structure of bacterial communities) and inferring causality of similarities among bacterial communities of hoopoes that could be extended to other mutualistic systems.

Hoopoes harbor antibiotic producing bacteria in their uropygial gland that prevent feather degradation (Ruiz-Rodríguez et al., 2009) and trans-shell contamination of embryos (Soler et al., 2008; Martín-Vivaldi et al., 2014b). Previous explorations of the bacterial community hosted in the uropygial gland of adult females and nestling hoopoes was performed by mean of traditional culture techniques and mainly detected few species of the Genus *Enterococcus* (Soler et al., 2008; Ruiz-Rodríguez et al., 2012; 2013; 2014). Modern molecular techniques allowed detecting a more complex bacterial community in the uropygial secretion of females with 145 different OTUs (fragment size of the 16S/23S intergenetic space region varying between 103 and 999 bp). Bacterial community of the uropygial secretion was even more diverse than those of the beak, brood patch and eggshells (see results) (Martínez-García et al., 2015; Rodríguez-Ruano et al., 2015). The higher diversity of the uropygial community, together with the known antimicrobial activity of secretion (Soler et al., 2008; Martín-Vivaldi et al.,

2010) and of some of their bacterial symbionts (mainly enteroccocci (OTU307 and OTU407 for Enterococcus faecalis), Martín-Platero et al., 2006; Ruiz-Rodríguez et al., 2012; 2013) opened the possibility of explaining the detected evidence of nestedness among bacterial communities at places that directly or indirectly became in contact with the uropygial secretion (i.e. beak, feathers, brood patch, and eggshells). It is possible that the antimicrobial activity of uropygial secretion kills non-resistant bacterial strains at these locations, whereas most of the bacteria in the uropygial secretion will colonize beak, feathers, brood patch, and eggshells. Because of the detected pattern of nestedness, but also because of differences in environmental conditions experienced by bacteria in the uropygial gland and on other sampled locations, bacterial communities of hoopoe's bill could include resistant bacteria to the antimicrobials of the uropygial secretion (migrants or residents) plus those from the uropygial secretion that were able to grow in aerobic conditions by using secretion or food remains or keratin for growth. Similarly, bacterial communities of brood patch and eggshell could include resistant bacteria and those from the uropygial secretion that resist beak environmental conditions.

Environmental factors may also affect composition of bacterial communities. It is known for instance that resource availability and temperature influence antagonistic activity of different bacterial strains (Prasad et al., 2011; Rypien et al., 2010) and, thus, abiotic and biotic factors might drive the outcomes of interactions among bacterial communities. We have detected significant variation in nestedness of hoopoes bacterial communities in relation to year and breeding attempt, and thus the distribution patterns of multiple bacterial strains within host different habitats (i.e. nestedness) may be partially explained by associated changes in environmental conditions affecting for instance within-communities antagonistic activity. Particularly interesting is the effect

371 of year since nestedness among hoopoes microbiomes were only detected in 2011, the
372 year with the highest diversity of bacteria in the uropygial secretion (Martínez-García et
373 al., 2015) suggesting that a more diverse microbiota of the uropygial secretion is better
374 able to influence eggshell microbiome. In previous work, we have also detected strong
375 environmental effects on the acquisition of enterococci bacterial symbionts (Ruiz-
376 Rodríguez et al., 2014) that highlight a possible effect of the environment determining
377 bacterial community of the uropygial secretion and, thus, characteristics of the
378 symbiotic relationship between hoopoes and bacteria.

379 An alternative non-ecological explanation worth discussing here is the
380 possibility that the detected nestedness was the consequence of considering dead or
381 non-active bacteria in locations others than the uropygial gland. The molecular
382 techniques we used detect both active and dead bacteria and, therefore, characterized
383 communities may include inactive OTUs from the uropygial secretion that may be
384 randomly dragged towards the eggshells. Simply because of random processes, bacteria
385 from the secretion that do not resist environmental conditions at the bill of hoopoes, will
386 also be transported and thereby detected by molecular methods in samples from the
387 brood patch and eggshells. Obviously, because dead bacteria at the bill will pass to the
388 eggshell, they will be detected at lower rates in samples from the eggshells than in those
389 from the beak or the brood patch. Besides, the ARISA approach detects just the
390 dominant members of the community making unlikely the detection of the so called
391 rare-biosphere or low abundant bacteria such as those in a dormant state. Although we
392 cannot completely reject this possibility, using traditional culture techniques, we have
393 previously found a positive relationship between densities of symbiotic bacteria (i.e.
394 enterococci) on the eggshells and in the secretion of hoopoes (Martín-Vivaldi et al.,

2014b) indicating that, at least, some of the bacteria in the uropygial secretion colonize the eggshell.

The meta-community approach used here has as far as we know never been used to characterize the association between mutualistic communities protecting hosts and those including potential pathogens. From an ecological perspective, symbionts that are adaptive for hosts and for instance protect embryos from pathogenic infections are in fact influencing or determining bacterial communities of egg covers. The beneficial effects may be achieved by either/both, (i) directing antimicrobial chemicals from symbionts to the eggshells and/or, (ii) transporting symbionts to the egg covers where they grow and protect embryos. The former possibility would result in a microbial community of resistant microbes, whereas the later would be detected by nested patterns of communities. Interestingly, it may be even possible that some bacteria producing antibiotics within the hosts (i.e. glands) were not able to grow outside, but their chemical products facilitated colonization of eggs cover by other symbionts. We still have very limited knowledge of mechanisms of microbial symbiont protecting hosts. The characterization of relationships (i.e. nestedness) between communities including pathogenic and/or symbiotic microorganisms, and the detection of geographical or temporal changes in species composition and/or interaction in the context of network (Poisot et al., 2011; 2012; 2014), or within classical meta-community theory (Costello et al., 2012; Pillai et al., 2014), will definitely help to understand mechanisms and evolution of host-microbial mutualisms functioning.

Our results show a hierarchical relationship between bacterial community in the uropygial gland of hoopoes and that of the eggshell, where symbionts and/or their antibiotic chemicals act preventing trans-shell bacterial colonization (Martín-Vivaldi et al., 2014b). Therefore, some bacterial strains from the uropygial secretion that are

present in the eggshells may directly straiten pathogens joining the bacterial community. Although this possibility should be further tested, the meta-community approach used here allows us to infer the direction of bacterial colonization, which is the basic prediction of the hypothesis of symbiotic bacteria functioning on the eggshells of hoopoes. The mutualistic relationship between hoopoes and bacteria may have evolved favoring bacteria with antimicrobial properties that are able to reach eggshell after colonizing bills and brood patch. The meta-community approach used here allows us to infer that it may be the case. We hope these results encourage further research in this and other host-microbial mutualistic systems.

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Table 1: Results from General Linear Models explaining variation in nestedness index (NODF) and of statistics reflecting the strength of nestedness of every considered matrix (Z-values) in relation to study year, whether or not the study nest was in captivity or in natural conditions and breeding attempt (i.e. first or second clutches). Since the population in captivity was only studied in a single year, the effects of year and of captivity were explored in different models. Female identity nested within year or captivity was included in the model as random factor (R) to account for the within females nest design of the data set. Interaction between the fixed (F) factors was included in the statistical model, whereas that between breeding attempt and the random factors is the error term of the model. Reduced final models are also shown. Effects associated with p-values < 0.1 are highlighted in bold.

		Effects of years and breeding attempt						Effects of captivity and breeding attempt					
		NODFindex			Z-values			NODFindex			Z-values		
		df	F	P	df	F	p	df	F	p	df	F	p
SBPE													
Year (1) / Captivity (1)	**F**	**1,48.0**	**4.27**	**0.044**	**1,38.6**	**3.01**	**0.091**	1,64.2	0.85	0.360	1,62.3	0.43	0.514
Breedingattempt (2)	F	1,4.0	0.39	0.564	1,4.0	0.16	0.710	1,18	1.05	0.320	1,18	0.51	0.486
(1) x (2)	F	1,4.0	0.65	0.465	1,4.0	0.13	0.738	1,18	0.61	0.445	1,18	2.94	0.104
Female id (Year) (3)	R	46,4.0	1.23	0.477	46,4.0	0.66	0.789	47,18	0.83	0.703	47,18	1.07	0.451
(2) X (3) (error term)	R	4,0.0			4,00			18			18		
Reducedmodels													
Year (1)	**F**	**1,52**	**4.04**	**0.049**	**1,52**	**4.93**	**0.031**						
Breedingattempt								**1,67**	**3.98**	**0.05**	**1,67**	**4.23**	**0.044**
SBE													
Year (1) / Captivity (1)	**F**	**1,49.7**	**7.23**	**0.010**	**1,49.2**	**5.95**	**0.018**	1,59.6	0.51	0.477	1,61.6	0.02	0.877
Breedingattempt (2)	F	1,4.0	0.89	0.398	1,4.0	2.36	0.199	**1,18.0**	**4.39**	**0.051**	**1,18.0**	**3.28**	**0.087**
(1) x (2)	F	1,4.0	0.68	0.456	1,4.0	0.05	0.833	1,18.0	0.00	0.993	1,18.0	0.05	0.826
Female id (Year) (3)	R	46,4.0	1.72	0.32	46,4.0	1.48	0.387	47,18.0	1.46	0.193	47,18.0	1.16	0.374
(2) X (3) (Error Term)	R	4,0.0			4,0.0			18,0.0			18,0.0		
Reducedmodel													
Year (1)	**F**	**1,52**	**6.31**	**0.015**	**1,52**	**6.36**	**0.015**						
Breedingattempt								**1,67**	**2.84**	**0.096**	**1,67**	**3.76**	**0.057**

Fig 1: Simple heatmap showing nestedness of the matrix data showing prevalence of each OUT in bacterial samples from the uropygial secretion, bill, brood patch and eggshell of hoopoes.Heatmap were built by pooling bacterial communities of all individuals together and, therefore, has not analytical value, but of visualization of the hierarchized bacterial communities of hoopoes. OTUs were arranged minimizing the number of crossing, which facilitates visualization of overlap of other communities with that of the uropigial secretion.

Fig 2: Mean ± 95% CI of nestedness index (NODF) of bacterial communities of uropygial secretion, bill, brood patch and eggshells (SBPE, circles) of hoopoes, and of those of the secretion, bill and eggshell (SBE, squares). We provide values considering all samples together, but also for different years (only wild nests considered), different breeding attempts (only 2011 nests considered), and for captivity and wild hoopoe populations (only 2011 nests considered). Dotted lines represent the threshold value (1.64) for statistical significance of nestedness.

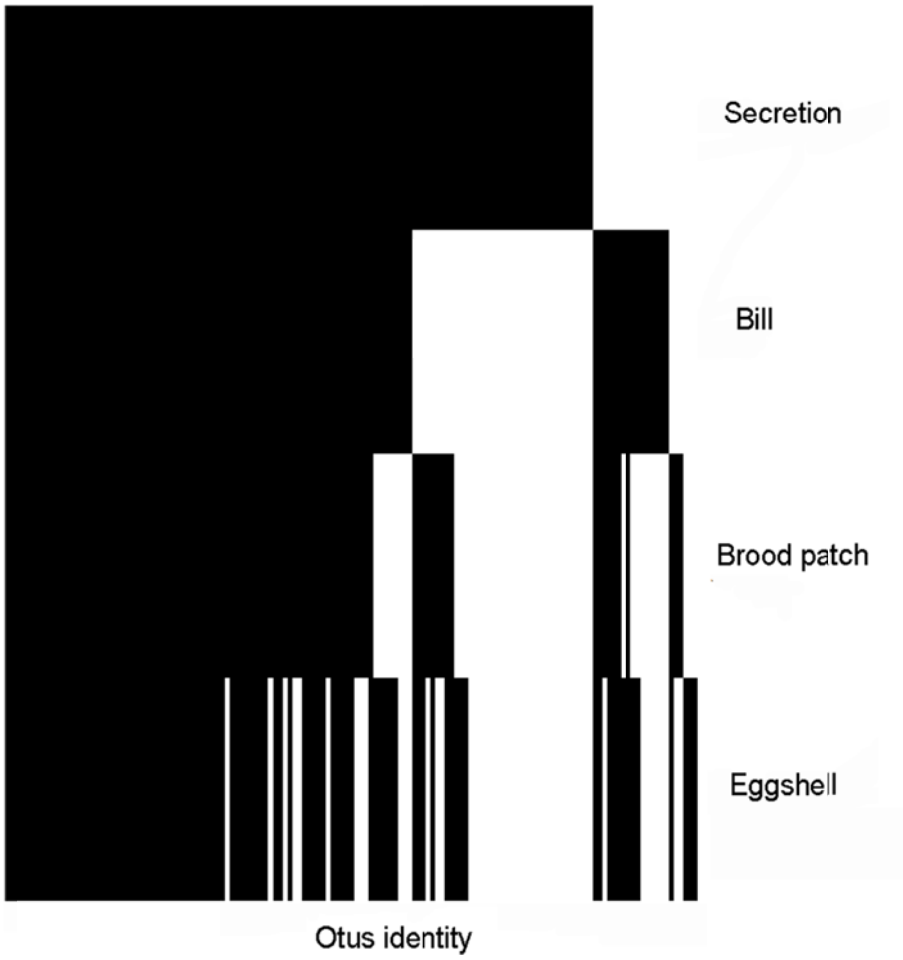
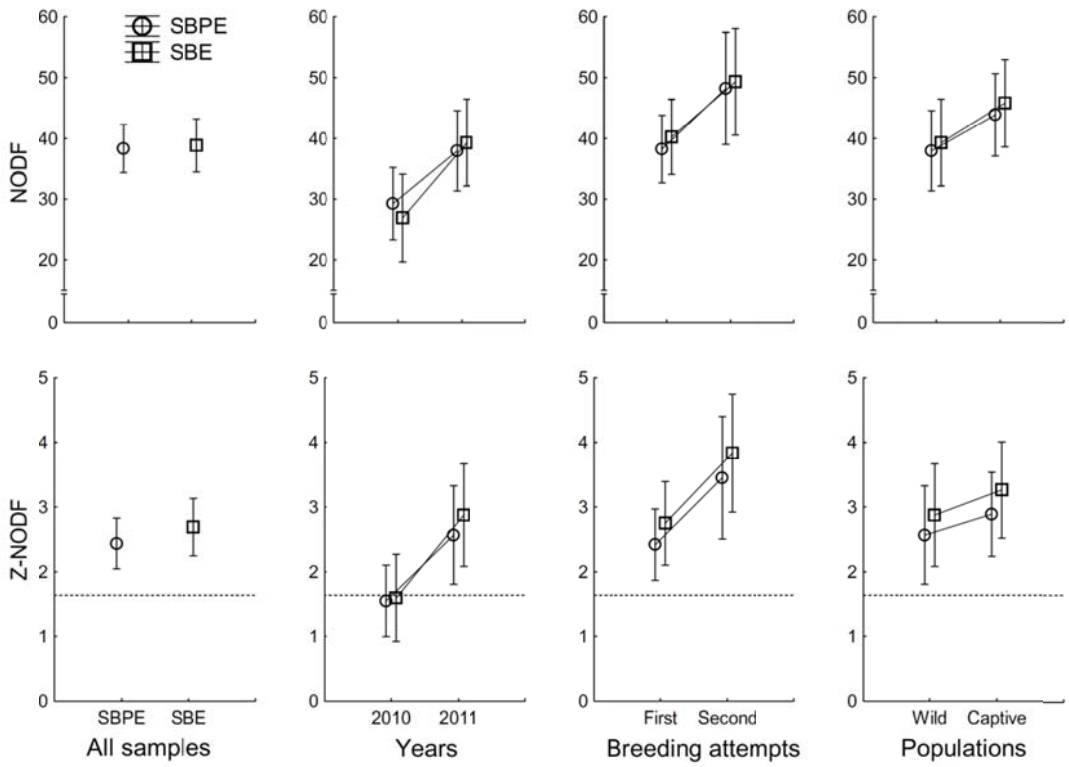

Secretion
Bill
Brood patch
Eggshell
Otus identity

Fig 2


SBPE
SBE
NODF
Z-NODF
SBPE
SBE
All samples
2010
2011
Years
First
Second
Breeding attempts
Wild
Captive
Populations

2