The Linnean Society of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2016, **118**, 763–773. With 2 figures.

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

JUAN J. SOLER[1*†], ÁNGELA MARTÍNEZ-GARCÍA[1†], SONIA M. RODRÍGUEZ-RUANO[2], MANUEL MARTÍNEZ-BUENO[2], ANTONIO M. MARTÍN-PLATERO[2], JUAN M. PERALTA-SÁNCHEZ[2] and MANUEL MARTÍN-VIVALDI[3]

[1]*Estación Experimental de Zonas Áridas (CSIC), E-04120, Almería, Spain*
[2]*Departamento de Microbiogía, Universidad de Granada, E-18071, Granada, Spain*
[3]*Departamento de Zoología, Universidad de Granada, E-18071, Granada, Spain*

Received 28 November 2015; revised 15 December 2015; accepted for publication 15 December 2015

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 the nestedness of communities, but this approach has rarely been applied to communities of microbial symbionts. We used this approach here, and estimated the 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 (Automated rRNA Intergenetic Spacer Region) and studied the 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 events influencing the 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. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 763–773.

KEYWORDS: bacteria meta-community – birds – ecological network – mutualistic bacteria – preening – symbiosis – uropygial secretion.

INTRODUCTION

Host species may receive from their microbial symbionts a multitude of benefits (mutualistic) and costs (parasitic). 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

*Corresponding author. E-mail: jsoler@eeza.csic.es
†These authors contributed equally to this work.

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, Bradley & Klironomos, 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, Hay & Fenical, 1989; Barbieri *et al.*, 1997, 2001; Currie *et al.*, 1999; Kaltenpoth *et al.*, 2005; Cardoza, Klepzig & Raffa, 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, 2014b; Soler *et al.*, 2014). 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 detecting some of these bacteria 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, Almeida & Gotelli, 2009; Ulrich & Almeida-Neto, 2012; Traveset, Kueffer & Daehler, 2014). The nestedness concept originated in the context of explaining insular biotas as result of colonization by a source pool of species from the 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 the 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 the 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 (36°50′N, 2°28′W) since 2008. Breeding pairs of hoopoes were housed in independent cages (at least 3 × 2 × 2 m) installed in the open, scattered and isolated to avoid interactions among pairs. Cages had access to soil and birds were provided with live food (crickets, vitamin-enriched fly larvae) and meat (beef heart) *ad libitum*.

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

Bacterial sampling

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

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 (Automated rRNA Intergenetic Spacer Region), see below). ARISA 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′ labelled fluorescently with 6-FAM (reverse primer sequence) (Cardinale *et al.*, 2004). PCR amplifications were performed in 50 μL reaction volumes containing ultrapure H_2O, 2.5× 5 PRIME MasterMix including 1.5 mM magnesium, 200 mM dNTPs, 1.25 U *Taq* polymerase (5 PRIME, Hamburg, Germany), 0.2 mM of primers and 5 μL 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). These analyses were realized in the ING unity (Genetic Information) of CIC (Scientific Instrumentation Center) of the University of Granada.

Resulting fragment lengths were analyzed with Peak Scanner v 1.0 (Applied Biosystems) by the 'Microsat G5' method. We considered peaks with values of relative fluorescence intensity higher than 0.09% and fragments above a threshold of 50

fluorescence units, ranging between 100 and 1000 bp (base pairs). Operational Taxonomic Units (OTUs) were established by calculating the best binning frame of different fragment lengths considering a window size (WS) of 4 bp and a distance between two consecutive binning frames (Sh) of 0.1. This exercise was carried out in 'R' environment [http://cran.r-project.org/, R.2.12.2 (R Development Core Team, 2010)] following scripts by Ramette (2009) at https://www.mpi-bremen.de/en/Software_2.html. We identified 145 OTUs that appeared with different frequencies in different hoopoes bacterial communities.

Sample sizes and statistical analyses

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

Nestedness estimations

The network between OTUs and sampled bacterial communities was built with the 'cca' method of the 'plotweb' function in the library Bipartite (Dormann, Gruber & Fruend, 2008) of the statistical software R.2.12.2 (R Development Core Team, 2010). As an index of nestedness, for each female and sampling event, we calculated the metric based on overlap and decreasing fill (NODF) (Almeida-Neto *et al.*, 2008; Almeida-Neto & Ulrich, 2011) as implemented in the user-friendly web interface NeD (http://ecosoft.alwaysdata.net/) by Strona *et al.* (2014). NODF can be estimated for columns and rows and does not depend on number of rows and columns considered (Almeida-Neto *et al.*, 2008). NODF for columns would therefore inform of nestedness of communities among sampling places, while NODF for rows will determine whether the rarest OTUs are present in the sampling place that also have the most common (Almeida-Neto *et al.*, 2008). NODF is dependent on the arrangement of columns and rows which allow testing hypothesis about the cause of nestedness (i.e. direction of colonization) by ordering columns and rows according to criteria representing different hypotheses (Almeida-Neto *et al.*, 2008; Ulrich *et al.*, 2009; Almeida-Neto & Ulrich, 2011; Traveset *et al.*, 2014). To test our hypothesis we thus arranged columns following the predicted colonization sequence from the uropygial gland through the bill and brood patch to the eggshell and estimated NODF of columns, while rows (OTUs identity) were arranged from those detected in all locations to those detected in only one or none. We thus organized the presence-absence matrices for each sampling event (individual females during a single reproductive event and study year) as including all bacterial strains (OTUs) detected in samples from secretion, beak, brood patch or eggshells. 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 (Supporting information, Data S1).

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

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

where NIr is the NODF index of the matrix under examination, $\overline{\mathrm{NIs}}$ is the average value of the set of index values for the null matrices generated by the program and $\sigma(\overline{\mathrm{NIs}})$ 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).

As 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 (NODFs values and information of individual samples are shown in Supporting information, Data S2).

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

On average, sampled bacterial communities of hoopoes were nested from the uropygial gland to the eggshells (Fig. 1) independently of considering or not the bacterial community of brood patch in the expected hierarchy of communities (Fig. 2). Nestedness of bacterial communities ordered in the opposite direction (from the eggs to the uropygial secretion) were far from statistical significance being Z estimates close to zero (NODF: mean = 14.12, CI: 12.25 to 17.27, Z-NODF, mean: −0.06, CI: −0.34 to 0.23)

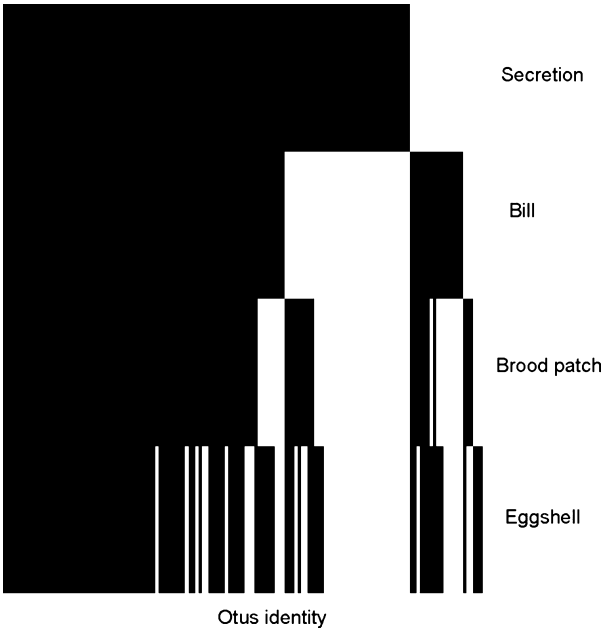

Figure 1. Simple heatmap showing the nestedness of the matrix data showing prevalence of each OTU in bacterial samples from the uropygial secretion, bill, brood patch and eggshell of hoopoes. Heatmaps were built by pooling bacterial communities of all individuals together and, therefore, has not analytical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

(Egg –Beak – Gland: NODF: mean = 9.37, CI: 6.93–11.80, Z-NODF, mean: −0.33, CI: −0.58 to −0.07).

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

Finally, NODF estimates for groups of bacterial communities including or not that of brood patch provide similar results, suggesting that eggshell bacterial community was equally nested in that of the brood patch than in the bacterial community of bill. All these results suggest that bacterial community of hoopoe eggshell is nested within that of the brood patch and/or bill; and that these bacterial communities

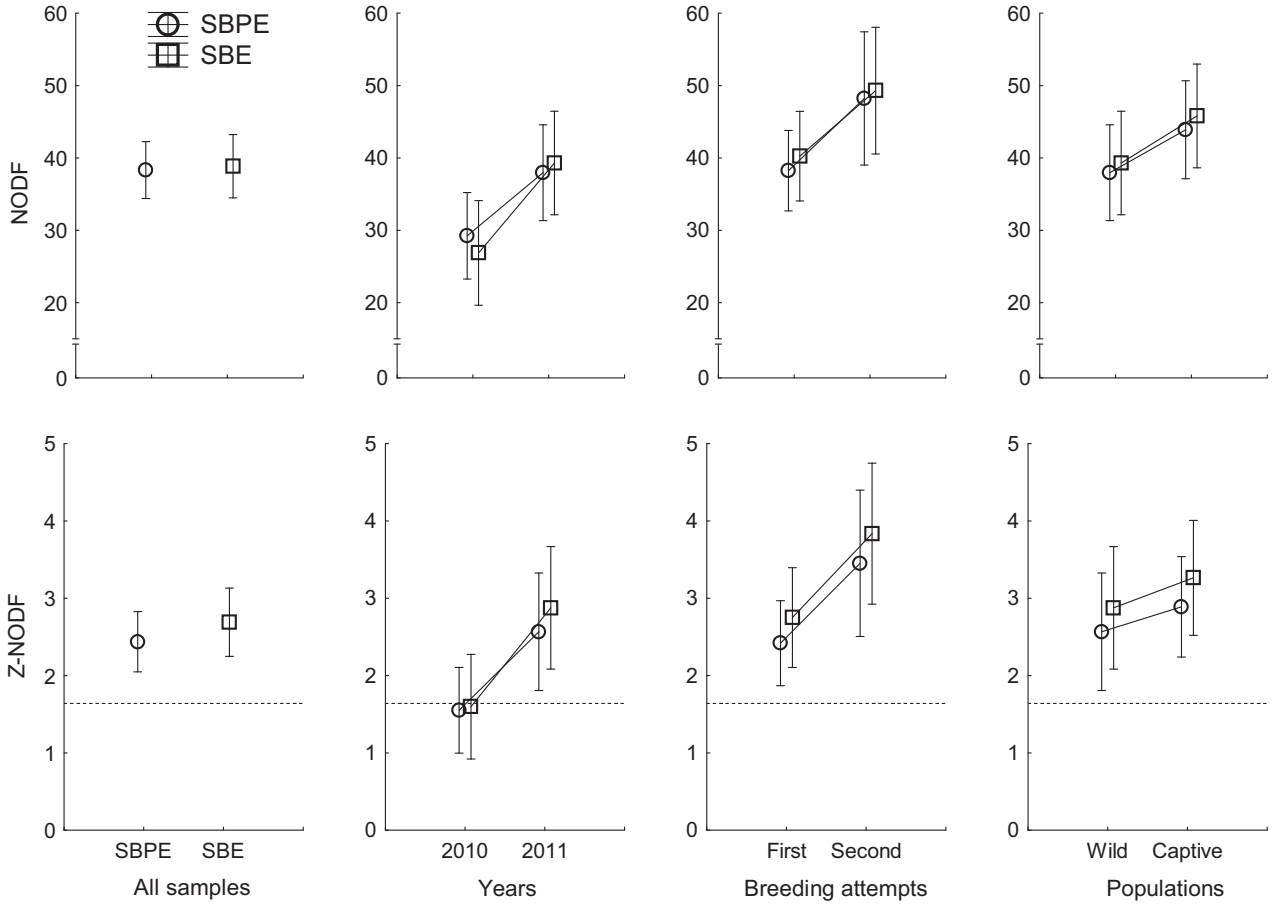

Figure 2. Mean ± 95% CI of nestedness index (NODF) of bacterial communities of uropygial secretion, bill, brood patch and eggshells (SBPE, circles) of hoopoes, and of those of the secretion, bill and eggshell (SBE, squares). We provide values considering all samples together, but also for different years (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.

are nested within that of symbiotic bacteria in the uropygial gland (Fig. 1). These results therefore support 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 harbour 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

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)

		Effects of years and breeding attempt						Effects of captivity and breeding attempt					
		NODF index			*Z*-values			NODF index			*Z*-values		
		d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*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
Breeding attempt (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) × (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) × (3) (error term)	R	4,0.0			4,00			18			18		
Reduced model													
Year (1)	**F**	**1,52**	**4.04**	**0.049**	**1,52**	**4.93**	**0.031**						
Breeding attempt								**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
Breeding attempt (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) × (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) × (3) (error term)	R	4,0.0			4,0.0			18,0.0			18,0.0		
Reduced model													
Year (1)	**F**	**1,52**	**6.31**	**0.015**	**1,52**	**6.36**	**0.015**						
Breeding attempt								**1,67**	**2.84**	**0.096**	**1,67**	**3.76**	**0.057**

As 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.

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 enterococci (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 (Rypien, Ward & Azam, 2010; Prasad *et al.*, 2011) 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

(i.e. nestedness) within host different habitats (i.e. body parts) may be partially explained by associated changes in environmental conditions affecting for instance within-communities antagonistic activity. Particularly interesting is the effect of year since nestedness among hoopoes microbiomes were only detected in 2011, the year with the highest diversity of bacteria in the uropygial secretion (Martínez-García *et al.*, 2015) suggesting that a more diverse microbiota of the uropygial secretion is better able to influence eggshell microbiome. In previous work, we have also detected strong environmental effects on the acquisition of enterococci bacterial symbionts (Ruiz-Rodríguez *et al.*, 2014) that highlight a possible effect of the environment determining bacterial community of the uropygial secretion and, thus, characteristics of the symbiotic relationship between hoopoes and bacteria.

An alternative non-ecological explanation worth discussing here is the possibility that the detected nestedness was the consequence of considering dead or non-active bacteria in locations others than the uropygial gland. The molecular techniques we used detect both active and dead bacteria and, therefore, characterized communities may include inactive OTUs from the uropygial secretion that may be randomly dragged towards the eggshells. Simply because of random processes, bacteria from the secretion that do not resist environmental conditions at the bill of hoopoes, will also be transported and thereby detected by molecular methods in samples from the brood patch and eggshells. Obviously, because dead bacteria at the bill will pass to the eggshell, they will be detected at lower rates in samples from the eggshells than in those from the beak or the brood patch. Besides, the ARISA approach detects just the dominant members of the community making unlikely the detection of the so-called rare biosphere or low abundant bacteria such as those in a dormant state. Although we cannot completely reject this possibility, using traditional culture techniques, we have previously found a positive relationship between densities of symbiotic bacteria (i.e. 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: (1) directing antimicrobial chemicals from symbionts to the eggshells; and/or (2) 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; Poisot, Stouffer & Gravel, 2015), or within classical meta-community theory (Costello *et al.*, 2012; Pillai, Gouhier & Vollmer, 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 straighten 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 favouring 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.

ACKNOWLEDGEMENTS

We thank Estefanía López Hernández and Olga Corona Forero for help in laboratory work; Ana Belén García, Jonathan Romero, Manuel Soto, Magdalena Ruiz-Rodríguez and Jorge Doña for help in caring for captive hoopoes. Laura Arco, Emilio Pagani and Tomás Perez Contreras helped with the field work. Giovanni Strona provided advice and helped us with the NODF calculations. The manuscript benefits

from comments on a previous version by José María Gómez Reyes Miguel Angel Rodriguez-Gironés, Magdalena Ruiz-Rodríguez and Eva Valdivia. We also thank three anonymous reviewers for their helpful comments. Support by funding was provided by Spanish Ministerio de Economía y Competitividad, European funds (FEDER) (CGL2013-48193-C3-1-P, CGL2013-48193-C3-2-P) and Junta de Andalucía (P09-RNM-4557). AMG had a PhD grant from the Junta de Andalucía (P09-RNM-4557).

AUTHORS' CONTRIBUTION

JJS and MM-V designed the study with considerable assistance from MM-B. AM-G and SRR performed all molecular analyses and binning with considerable assistance of AMM-P. AM-G and JMPS performed most of the field work with assistance from MM-V and JJS. JJS performed all statistical analyses and wrote and first version with help from MM-V; all other authors substantially contributed to the final version.

REFERENCES

Aguirre-von-Wobeser E, Soberon-Chavez G, Eguiarte LE, Ponce-Soto GY, Vazquez-Rosas-Landa M, Souza V. 2014. Two-role model of an interaction network of free-living gamma-proteobacteria from an oligotrophic environment. *Environmental Microbiology* **16:** 1366–1377.

Almeida-Neto M, Ulrich W. 2011. A straightforward computational approach for measuring nestedness using quantitative matrices. *Environmental Modelling & Software* **26:** 173–178.

Almeida-Neto M, Guimaraes P, Guimaraes PR, Loyola RD, Ulrich W. 2008. A consistent metric for nestedness analysis in ecological systems: reconciling concept and measurement. *Oikos* **117:** 1227–1239.

Archie EA, Theis KR. 2011. Animal behaviour meets microbial ecology. *Animal Behaviour* **82:** 425–436.

Banning J, Weddle A, Wahl G III, Simon M, Lauer A, Walters R, Harris R. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. *Oecologia* **156:** 423–429.

Barbieri E, Barry K, Child A, Wainwright N. 1997. Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of *Loligo pealei* (Cephalopoda: Loliginidae). *Biological Bulletin* **193:** 275–276.

Barbieri E, Paster BJ, Hughes D, Zurek L, Moser DP, Teske A, Sogin ML. 2001. Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: Loliginidae). *Environmental Microbiology* **3:** 151–167.

Bascompte J, Jordano P, Melian CJ, Olesen JM. 2003. The nested assembly of plant-animal mutualistic networks. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 9383–9387.

Bright M, Bulgheresi S. 2010. A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology* **8:** 218–230.

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia A, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Applied and Environmental Microbiology* **70:** 6147–6156.

Cardoza YJ, Klepzig KD, Raffa KF. 2006. Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology* **31:** 636–645.

Chagnon PL, Bradley RL, Klironomos JN. 2012. Using ecological network theory to evaluate the causes and consequences of arbuscular mycorrhizal community structure. *New Phytologist* **194:** 307–312.

Cordero OX, Wildschutte H, Kirkup B, Proehl S, Ngo L, Hussain F, Le Roux F, Mincer T, Polz MF. 2012. Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science* **337:** 1228–1231.

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. 2012. The application of ecological theory toward an understanding of the human microbiome. *Science* **336:** 1255–1262.

Currie CR, Scott JA, Summerbell RC, Malloch D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398:** 701–704.

Dormann CF, Gruber B, Fruend J. 2008. Introducing the Bipartite package: analysing ecological networks. *R News* **8:** 8–11.

Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. 2012. Animal behavior and the microbiome. *Science* **338:** 198–199.

Fisher MM, Triplett EW. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology* **65:** 4630–4636.

Flórez LV, Biedermann PHW, Engl T, Kaltenpoth M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports* **32:** 904–936.

Fortuna MA, Stouffer DB, Olesen JM, Jordano P, Mouillot D, Krasnov BR, Poulin R, Bascompte J. 2010. Nestedness versus modularity in ecological networks: two sides of the same coin? *Journal of Animal Ecology* **79:** 811–817.

Gil-Turnes MS, Hay ME, Fenical W. 1989. Symbiotic marine-bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246:** 116–118.

Gotelli NJ. 2000. Null model analysis of species co-occurence patterns. *Ecology* **81:** 2606–2621.

Grossart HP, Schlingloff A, Bernhard M, Simon M, Brinkhoff T. 2004. Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microbiology Ecology* **47:** 387–396.

Jacquemyn H, Brys R, Waud M, Busschaert P, Lievens B. 2015. Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytologist* **216:** 1127–1134.

Kaltenpoth M, Gottler W, Herzner G, Strohm E. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* **15:** 475–479.

Long RA, Azam F. 2001. Antagonistic interactions among marine pelagic bacteria. *Applied and Environmental Microbiology* **67:** 4975–4983.

Long RA, Rowley DC, Zamora E, Liu J, Bartlett DH, Azam F. 2005. Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*. *Applied and Environmental Microbiology* **71:** 8531–8536.

Long RA, Eveillard D, Franco SLM, Reeves E, Pinckney JL. 2013. Antagonistic interactions between heterotrophic bacteria as a potential regulator of community structure of hypersaline microbial mats. *FEMS Microbiology Ecology* **83:** 74–81.

Martínez-García A, Soler JJ, Rodríguez-Ruano SM, Martínez-Bueno M, Martín-Platero AM, Juárez-García N, Martín-Vivaldi M. 2015. Preening as a vehicle for key bacteria in hoopoes. *Microbial Ecology* **70:** 1024–1033.

Martín-Platero AM, Valdivia E, Ruiz-Rodríguez M, Soler JJ, Martín-Vivaldi M, Maqueda M, Martínez-Bueno M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe *Upupa epops*. *Applied and Environmental Microbiology* **72:** 4245–4249.

Martín-Platero AM, Peralta-Sánchez JM, Soler JJ, Martínez-Bueno M. 2010. Chelex-based DNA isolation procedure for the identification of microbial communities of eggshell surfaces. *Analytical Biochemistry* **397:** 253–255.

Martín-Vivaldi M, Palomino JJ, Soler M, Soler JJ. 1999. Determinants of reproductive success in the Hoopoe *Upupa epops*, a hole-nesting non-passerine bird with asynchronous hatching. *Bird Study* **46:** 205–216.

Martín-Vivaldi M, Ruiz-Rodríguez M, Soler JJ, Peralta-Sanchez JM, Mendez M, Valdivia E, Martin-Platero AM, Martínez-Bueno M. 2009. Seasonal, sexual and developmental differences in hoopoe *Upupa epops* preen gland morphology and secretions: evidence for a role of bacteria. *Journal of Avian Biology* **40:** 191–205.

Martín-Vivaldi M, Peña A, Peralta-Sánchez JM, Sánchez L, Ananou S, Ruiz-Rodríguez M, Soler JJ. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society of London B, Biological Sciences* **277:** 123–130.

Martín-Vivaldi M, Doña J, Romero-Masegosa J, Soto-Cárdenas M. 2014a. Abubilla – *Upupa epops*. In: Salvador A, Morales MB, eds. *Enciclopedia Virtual de los Vertebrados Españoles*. Madrid: Museo Nacional de Ciencias Naturales, http://www.vertebradosibericos.org/aves/upuepo.html

Martín-Vivaldi M, Soler JJ, Peralta-Sánchez JM, Arco L, Martín-Platero AM, Martínez-Bueno M, Ruiz-Rodríguez M, Valdivia E. 2014b. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. *Journal of Animal Ecology* **83:** 1289–1301.

McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 2013. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America* **110:** 3229–3236.

Montesinos-Navarro A, Segarra-Moragues JG, Valiente-Banuet A, Verdú M. 2012. The network structure of plant-arbuscular mycorrhizal fungi. *New Phytologist* **194:** 536–547.

Pillai P, Gouhier TC, Vollmer SV. 2014. The cryptic role of biodiversity in the emergence of host-microbial mutualisms. *Ecology Letters* **17:** 1437–1446.

Poisot T, Lepennetier G, Martinez E, Ramsayer J, Hochberg ME. 2011. Resource availability affects the structure of a natural bacteria-bacteriophage community. *Biology Letters* **7:** 201–204.

Poisot T, Canard E, Mouillot D, Mouquet N, Gravel D. 2012. The dissimilarity of species interaction networks. *Ecology Letters* **15:** 1353–1361.

Poisot T, Stouffer DB, Gravel D. 2015. Beyond species: why ecological interaction networks vary through space and time. *Oikos* **124:** 243–251.

Prasad S, Manasa P, Buddhi S, Singh SM, Shivaji S. 2011. Antagonistic interaction networks among bacteria from a cold soil environment. *FEMS Microbiology Ecology* **78:** 376–385.

R Development Core Team. 2010. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Ramette A. 2009. Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Applied and Environmental Microbiology* **75:** 2495–2505.

Rodríguez-Ruano SM, Martín-Vivaldi M, Martín-Platero AM, López-López JP, Peralta-Sánchez JM, Ruiz-Rodríguez M, Soler JJ, Valdivia E, Martínez-Bueno M. 2015. The Hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. *PLoS ONE* **10:** e0139734.

Ruiz-Rodríguez M, Valdivia E, Soler JJ, Martin-Vivaldi M, Martin-Platero AM, Martinez-Bueno M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology* **212:** 3621–3626.

Ruiz-Rodríguez M, Valdivia E, Martín-Vivaldi M, Martín-Platero AM, Martínez-Bueno M, Méndez M, Peralta-Sánchez JM, Soler JJ. 2012. Antimicrobial activity and genetic profile of enterococci isolated from hoopoes uropygial gland. *PLoS ONE* **7:** e41843.

Ruiz-Rodríguez M, Martinez-Bueno M, Martin-Vivaldi M, Valdivia E, Soler JJ. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal sym-

bionts isolated from the hoopoe's uropygial gland. *FEMS Microbiology Ecology* **85:** 495–502.

Ruiz-Rodríguez M, Soler JJ, Martín-Vivaldi M, Martín-Platero AM, Méndez M, Peralta-Sánchez JM, Ananou S, Valdivia E, Martínez-Bueno M. 2014. Environmental factors shape the community of symbionts in the uropygial gland of hoopoes more than genetic factors. *Applied and Environmental Microbiology* **80:** 6714–6723.

Rypien KL, Ward JR, Azam F. 2010. Antagonistic interactions among coral-associated bacteria. *Environmental Microbiology* **12:** 28–39.

Scheuring I, Yu DW. 2012. How to assemble a beneficial microbiome in three easy steps. *Ecology Letters* **15:** 1300–1307.

Scott JJ, Oh DC, Yuceer M, Klepzig KD, Clardy J, Currie CR. 2008. Bacterial protection of beetle-fungus mutualism. *Science* **322:** 63.

Soler JJ, Martín-Vivaldi M, Ruiz-Rodríguez M, Valdivia E, Martín-Platero AM, Martínez-Bueno M, Peralta-Sánchez JM, Méndez M. 2008. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. *Functional Ecology* **22:** 864–871.

Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Arco L, Juárez-García-Pelayo N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. *Naturwissenschaften* **101:** 697–705.

Statsoft Inc. 2011. *STATISTICA (data analysis software system), version 10*. Available at: www.statsoft.com

Strona G, Galli P, Seveso D, Montano S, Fattorini S. 2014. Nestedness for Dummies (NeD): a user-friendly web interface for exploratory nestedness analysis. *Journal of Statistical Software* **59:** 1–9.

Traveset A, Kueffer C, Daehler CC. 2014. Global and regional nested patterns of non- native invasive floras on tropical islands. *Journal of Biogeography* **41:** 823–832.

Ulrich W, Almeida-Neto M. 2012. On the meanings of nestedness: back to the basics. *Ecography* **35:** 865–871.

Ulrich W, Almeida M, Gotelli NJ. 2009. A consumer's guide to nestedness analysis. *Oikos* **118:** 3–17.