Acquisition of uropygial gland microbiome by hoopoe nestlings

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Keywords: byproduct cooperation, coevolution, mutualism, preen gland, symbiotic bacteria

Word count: 6724 without references

Running headline: Uropygial microbiome acquisition

Acknowledgements. Jonathan Romero, Manuel Soto, Jorge Doña, Ana Belén García, Alicia Astasio, Elisa García, Rosa María Morales and Marcos Gómez helped in caring for captive hoopoes. Sonia Rodríguez-Ruano performed the DNA extraction of the nestling secretions. Juan Manuel Peralta and two anonymous referees revised a previous version of the MS. Support by funding was provided by Spanish Ministerio de Ciencia e Innovación, European funds (FEDER) (CGL2009-14006, CGL2010- 19233-C03-01, CGL2010-19233-C03-03, CGL2013-48193-C3-1-P, CGL2013-48193-C3-2-P) and Junta de Andalucía (P09- RNM-4557). AM-G had a predoctoral grant from the Junta de Andalucía (P09-RNM-4557).

1

2 **Abstract**

3 Mutualistic symbioses between animals and bacteria depend on acquisition of appropriate
4 symbionts while avoiding exploitation by non-beneficial microbes. The mode of acquisition of
5 symbionts would determine, not only the probability of encountering, but also evolutionary
6 outcomes of mutualistic counterparts. The microbiome inhabiting the uropygial gland of the
7 European hoopoe (*Upupa epops*) includes a variety of bacterial strains, some of them providing
8 antimicrobial benefits. Here, the mode of acquisition and stability of this microbiome is analyzed
9 by means of Automated rRNA Intergenic Spacer Analysis and two different experiments. The first
10 experiment impeded mothers´ access to their glands, thus avoiding direct transmission of
11 microorganisms from female to offspring secretions. The second experiment explored the stability
12 of the microbiomes by inoculating glands with secretions from alien nests. The first experiment
13 provoked a reduction in similarity of microbiomes of mother and nestlings. Interestingly, some
14 bacterial strains were more often detected when females had not access to their glands, suggesting
15 antagonistic effects among bacteria from different sources. The second experiment caused an
16 increase in richness of the microbiome of receivers in terms of prevalence of Operational
17 Taxonomic Units (OTUs) that reduced differences in microbiomes of donors and receivers. That
18 occurred because OTUs that were present in donors but not in receivers incorporated to the
19 microbiome of the latter, which provoked that cross-inoculated nestlings got similar final
20 microbiomes that included the most prevalent OTUs. The results are therefore consistent with a
21 central role of vertical transmission in bacterial acquisition by nestling hoopoes, and support the
22 idea that the typical composition of the hoopoe gland microbiome is reached by the incorporation
23 of some bacteria during the nestling period. This scenario suggests the existence of a coevolved
24 core microbiome composed by a mix of specialized vertically transmitted strains and facultative
25 symbionts able to coexist with them. The implications of this mixed mode of transmission for the
26 evolution of the mutualism are discussed.

Introduction

27 The life of every animal is conditioned by interactions with an extremely high variety of ubiquitous
28 bacteria that are able of colonizing and exploiting any nutritional resource in any chemical form [1].
29 Many of those potential partners are enemies, while some others do not affect the host, or even
30 provide benefits. Evolutionary processes should therefore select for strategies that reduce
31 encounters with parasitic microorganisms; and increase the probability of recruitment of the
32 beneficial ones [2]. Benefits of the association with bacteria can have multiple forms: from nutritive
33 resources to defensive products [3] or inactivation of toxic residues [4]. Hosts provide beneficial
34 microorganisms with physical support and resources for growth, which could also be exploited by
35 undesired parasitic-symbiont partners (e.g. [5]). Even though the risk of exploitation of host
36 resources by non-mutualistic symbionts is considered a destabilizing force of mutualistic
37 relationships (reviewed in [6]), mutualisms with bacteria are ubiquitous and remain during
38 evolutionary time [4] indicating the existence of mechanisms preventing parasitic exploitation.

39 One of the main questions in evolutionary ecology is to disentangle mechanisms explaining
40 mutualistic coevolutionary relationships while preventing the intromission of the surrounding
41 potential exploiters. Several studies have modelled the stability of mutualisms and reviewed the
42 current evidence for these systems to be stable [7-11]. In this sense, vertical transmission from
43 parents would reduce the risk of exploitation and provide descendants with an adequate pool of
44 collaborators [2,12-15]. However, hosts that acquire their mutualistic symbionts horizontally are
45 more compromised by parasites' exploitation because they need to recruit appropriated symbionts
46 from the surroundings, which depend upon availability [16,17]. Indeed, they need to collect
47 beneficial strains while avoiding colonization by parasites ("partner choice" [18]). Such selection
48 procedures involve recognition, attachment, regulation of immune responses, and control of
49 nutrient release for the symbiont [17,19,20]. Horizontal transmission may also imply some
50 advantages since it allows a plastic selection of cooperators and the best option may differ for
51 different environmental conditions (e.g. [21]). For instance, bacterial symbionts producing more
52 antimicrobials that benefit hosts can be established automatically within the symbiont community

53 of hosts ("byproduct cooperation" [4,22]) and impede colonization of parasitic symbionts.
54 Therefore, even complex communities resulting from horizontal transmission reach stability, as
55 shown by the microbiome of rumen of mammals that has demonstrated high inertia and resilience
56 after perturbations, including the inoculation of alien communities [23]. Some models have even
57 suggested the possibility that mixed communities, including both vertically and horizontally
58 acquired strains, would further guarantee the stability of the association [24]. The scenarios
59 described above, therefore, indicate that knowing the mode of acquisition of symbionts is essential
60 for understanding functioning, stability and evolution of mutualistic associations.
61 An interesting model for exploring the importance of mechanisms of symbiont acquisition is
62 the system formed by the hole-nester European hoopoe (*Upupa epops*) and the bacteria living in its
63 uropygial gland [25-27]. The uropygial secretion of Upupiformes (hoopoes and their relative
64 woodhoopoes) is special (dark and odorous) due to the presence of symbiotic bacteria in the gland
65 [28,29]. In the case of the hoopoes, their symbionts change the composition of the oil produced by
66 the bird, generate metabolites with antimicrobial properties [30-33], and positively affect hatching
67 success [27] when the female voluntarily impregnates their eggshell during incubation [34,35]. The
68 special secretions with bacteria are only maintained during the stay within the nest-hole of females
69 and nestlings [29] and, therefore, must be acquired every breeding season. Results of previous
70 Automated rRNA Intergenic Spacer Analysis (ARISA) showed that both females and nestlings host
71 a bacterial community formed by a group of eight highly prevalent strains accompanied by a long
72 list of Operational Taxonomic Units (OTUs) in a range of frequencies of appearance from 50% to
73 10% [36]. Moreover, cross-fostering experiments suggested that nestlings are able to acquire new
74 symbiont strains when moved to a different nest [36,37]. In this way, the stability of the bacterial
75 community could depend on the possibility of encountering new strains and, thus, vertical and
76 horizontal acquisition of symbionts may explain microbiomes of nestlings [36,37]. However, the
77 factors affecting the dynamic of colonization of the gland by bacterial symbionts are not known,
78 and differences in competitive ability of different bacterial strains reaching the uropygial gland are
79 supposed to affect their success colonizing and growing in the uropygial secretion [38]. This

80 hypothesis can be experimentally tested by inoculating glands with secretions harboring bacteria
81 coming from different nest environments [16,39]. Such experiment will also allow identifying
82 particular bacterial strains able to colonize uropygial glands where they were not present.

83 In this context, glands of nestlings close to fledge were inoculated with secretion from alien
84 nestlings that acquired their microbiome in different nests. Assuming some level of vertical
85 transmission of symbionts, to increase differences among donor and receptor of inocula, cross-
86 inoculations were performed between nestlings from nests with mothers that had full or
87 experimentally restricted access to their uropygial gland. Impeding bird access to their glands by
88 covering them has previously been used with success to test effects of uropygial secretions in
89 several studies [34,35,40,41]. We have used here such an approach as a reversible alternative to the
90 extirpation of glands (e.g. [42]). In this way, two crossed experimental treatments were performed:
91 (1) restriction of gland access for females and (2) cross-inoculation of nestling secretions. Thus,
92 only a fraction of experimentally inoculated nestlings had the possibility of acquiring bacterial
93 strains from their mothers' glands. This experimental approach also allow testing the influence of
94 vertical transmission (i.e. female treatment) on the effect of experimental inoculation of nestling
95 uropygial glands.

96 With this experimental design, the following hypotheses and predictions were tested: (a) there
97 is vertical transmission of bacteria directly from female to offspring glands if the composition of
98 microbiomes of nestlings is affected by experimental covering of female glands. (b) If hoopoes
99 harbor a particular co-evolved microbial assemblage in secretions, the cross-inoculation experiment
100 should result in the colonization of glands by complementary bacterial strains, commonly detected
101 in hoopoes. This should particularly be the case in nests with experimental females where the
102 vertical transmission of commonly detected strains to uropigial gland of nestlings is restricted. This
103 scenario also predicts an increase in microbiome similarity of secretions after the inoculation
104 experiment. (c) On the other hand, if different communities are the result of different optimal
105 microbiomes for different individuals (see [23]), there should be evidence of stability (i.e., no
106 effects of cross-inoculation on similarity) and a limited probability of colonization by new bacterial

107 strains. (d) Finally if different strains differ in the ability to colonize and establishing in the
108 microbiome of the uropygial gland, we should find that the effects of experimental restriction of
109 vertical transmission or the inoculation experiment on particular microorganisms, will depend on
110 these abilities (i.e. identity).

111

112 **Material and Methods**

113 *Study area*

114 The study was performed during the 2010 and 2012 breeding seasons, in a captive population of
115 hoopoes maintained since 2008. The captive pairs were distributed in three localities with
116 appropriate facilities in southern Spain; the Faculty of Sciences of the Granada University
117 (37°18′N, 3°61′W, Granada province), the Hoya of Guadix (37°31′N, 3°12′W, Granada province),
118 and the Finca Experimental la Hoya of EEZA-CSIC (36º84´N, 2º47´W, Almería province). All
119 females and nestlings were ringed with numbered aluminum rings for individual recognition.
120 Breeding pairs were housed in independent cages of at least 3m x 2m x 2m installed in the open,
121 scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages
122 had access to soil and live food (crickets, fly larvae and meat (beef heart)) *ad libitum*. Each pair had
123 a nest-box made in cork (internal height * width * depth: 35 * 18 * 21 cm, bottom-to-hole height:
124 24 cm, entrance diameter 5.5 cm) with a two cm layer of thin pine bark pieces on the bottom. Cages
125 were visited and inspected daily from mid-February to the end of July.

126 Several studies have found that microbiomes of wild and captive populations of animals
127 differ (reviewed in [43]). We have previously shown that microbiomes of wild hoopoes are slighter
128 richer than those of captive hoopoes [44,45]. Such differences, however, only affected to a small
129 fraction of the microbiome bacterial strains and, thus, we believe that our experiments in captivity
130 allow to infer valid conclusions on the way hoopoes acquire their uropygial gland microbiome in
131 the wild.

132

133 *Experimental design*

134 Two experimental approaches were used; one to restrict the vertical transmission of bacterial
135 communities from females to offspring, and the other to detect possible changes in the microbiome
136 composition of the uropygial secretion of nestlings along their stay in the nest after inoculation with
137 alien secretions.

138 In Experiment 1, females' access to the uropygial gland was manipulated from the start of
139 laying until they finished brooding (Fig 1a, see [34] for details of the method for covering female
140 glands). Thus, experimental females could not use the uropygial secretion to cover eggs [34,35] or
141 to preen nestlings or themselves [36], and the direct or indirect transmission of bacteria from female
142 gland to those of their nestlings was impeded. Two groups of control birds were established.
143 Control I females were provided with a similar structure as experimental females, but not covering
144 the gland entrance and thus not preventing normal access to secretions. A third group of breeding
145 females (control II) were visited and handled at the same rate as those of experimental and control I
146 groups. Females were assigned to a particular treatment in sequence by laying date, alternating
147 experimental with one of the two control treatments, which were alternatively selected to get a
148 similar sample size between experimental females and both control groups combined. Treatments
149 were balanced within sub-populations. In order to test the effect of the experiment on the
150 transmission of symbiotic bacteria from mother to offspring, the microbiomes of their uropygial
151 glands were compared. Female glands were sampled on the day of start of laying, before
152 manipulation, and those of nestlings on the 16th day of the nestling period (Fig. 1a).

153 Experiment 2 was performed with 16 days old chicks of mothers that were subject to the
154 Experiment 1. After sampling secretions, half of the nestlings in each brood (Controls) were
155 inoculated with 3 µl of their own secretion with a sterile micropipette within the papilla of the
156 gland, while the other half of the brood (Experimentals) were inoculated with 3 µl of secretion
157 proceeding from nestlings of a different nest. Except for the few cases in which wild broods were

158 used as donors of secretions, mothers of donor nestlings were under different experimental
159 treatment than those of receivers. The 3 µl of secretion used for inoculation were pipetted from a
160 sterile microfuge tube where the available secretion of each nestling was previously homogenized.
161 Nests were paired by hatching date so that secretions were interchanged between nestlings of
162 similar ages. Experimental and control treatments were assigned to nestlings in each brood
163 alternating along the size hierarchy. The oldest nestlings of the two broods within a duplica were
164 assigned the same treatments, but alternated between successive pairs of nests. To estimate the
165 change in microbiomes caused by the experiment, nestlings were sampled again after seven days
166 (Fig. 1b).

167 We only considered breeding attempts from captive females for which initial and final
168 secretions of at least one control and one experimental nestling were obtained. Only one nestling
169 per treatment (the oldest surviving on day 23) was included per nest. Complete information (initial
170 and final samples of one control and one experimental nestling) was obtained for 18 nests, but the
171 information on the communities of females and donors only for 17 and 15 nests respectively. For
172 this reason, sample sizes differ among performed analyses. All broods were from different females,
173 except for two of the same female in 2012 that received two different treatments (experimental and
174 control II) which have been considered as independent information in the analyses.

175 *Sampling protocol*

176 Incubating females and nestlings were caught by hand and, after sampling uropygial secretions (see
177 [27] for sampling method), were released again within the nest box. The secretion was transferred
178 to a sterile microfuge tube, homogenized with a micropipette and stored at 4º C until used for
179 inoculation in the following 2 hours. Other two aliquots of 5 µl were separated in different sterile
180 microfuge tubes, one for estimation of the load of mesophilic bacteria by culture methods, and
181 other was frozen for the molecular analysis of the microbiome composition.

182 *Bacterial load*

183 To estimate the load of cultivable bacteria, secretions were cultured in Petri dishes with Tryptic Soy
184 Agar (TSA) medium. Plates were inoculated, within the same day of sampling, with 5 µl of serial
185 dilutions of the secretions in sterile Phosphate Buffer Saline (PBS), and cultures were incubated
186 aerobically at 37 °C for 24 h. Bacterial load was expressed as number of colony-forming units
187 (CFUs) per µl of secretion.

188 *Molecular analyses*

189 Bacterial DNA was extracted from uropygial secretions with a commercial KIT (The FavorPrep™
190 Blood Genomic DNA Extraction Kit, Favorgen). Automated rRNA Intergenic Spacer Analysis
191 (ARISA) was used to characterize the composition of bacterial communities (see Supplemental
192 Material-I for details). The purpose here is not to describe the composition of hoopoe uropygial
193 microbiomes, but explore influence of mothers (i.e. vertical transmission) and of later colonization
194 (i.e. inoculation) on the microbiome of nestling hoopoes, with particular emphasis on bacteria of
195 known beneficial effects (i.e. enterococci). Thus, the use of ARISA is appropriate here.
196 Furthermore, particular OTUs from ARISA have previously been suggested to interact with the life
197 history characteristics of hoopoes [36,46,47], which will allow interpretation of results of particular
198 OTUs in the performed experiment in relation to what is already known of their role in these
199 communities. Given that it is possible that different fragment lengths came from identical bacterial
200 strains or species, except when indicated we use them as Operational Taxonomic Units (OTUs) of
201 unknown taxonomic affiliation. The generated presence-absence matrix was used in all analyses.

202 The hoopoe nestlings involved in the inoculation experiment harbored a total of 82 OTUs,
203 49 of which were present only in up to three individuals before the experimental inoculation of their
204 glands. For the calculation of richness only the 33 OTUs present in at least four of those samples
205 were considered [47]. Moreover, to understand the dynamic of the main assemblages naturally
206 encountered in hoopoes, and for analyses considering differences in composition among bacterial
207 communities, only those with at least 50% prevalence were considered (for this calculation we only
208 included samples of females and the first available for nestlings of control females). There were 16

OTUs fulfilling this criterion, which are named by the length of their ITS fragment (OTUs 182, 242, 254, 278, 306, 310, 326, 330, 346, 350, 406, 422, 466, 474, 534 and 566), which hereafter are referred as the "core microbiome" of secretions. The main effects of both experiments on microbiome composition did not change if we use the 33 OTUs with prevalence >3 instead.

Statistical analyses

Three different estimates have been used characterizing the microbiome of the uropygial secretion of nestlings: richness (number of OTUs), composition (matrices of similarities among microbiomes using Jaccard index) and cultivable bacterial load (CFUs/μl). Both richness and bacterial load approached normal distributions after log transformation (Kolmogorov-Smirnov tests $p > 0.2$) and were thus used in General Linear Models (GLMs hereafter).

To detect possible opposed effects of experiments on different components of the microbial communities, sample positions in the multiscale Jaccard distance space were decomposed in two or three first axes of Principal Coordinates Analyses (PCo). The coordinates (i.e. scores) of each sample were used as dependent variables and the statistical tests performed with Primer7.

Before the inoculation treatments, bacterial load, richness and composition of microbiomes of the secretion of nestlings in nests of females under the two control treatments did not differ significantly (GLMs, after controlling for nest identity nested within experimental treatment as random factor, bacterial load: $F(1, 9) = 0.001$, $p = 0.97$; richness: $F(1, 9) = 1.44$, $p = 0.269$; microbiome composition PCo1: $F(1, 9) = 0.019$, $p = 0.894$; PCo2: $F(1, 9) = 0.958$, $p = 0.360$). Thus, data of the broods of control-I and control-II females were pooled in a single group of control nests in subsequent analyses.

The effect of female treatment on similarity between nestling and female microbiomes, as well as the effect of the inoculation experiment on microbiomes of initial and final nestling samples were explored in repeated measures ANOVAs. The fixe effect of treatment was included as

234 between-factor (Experimental/Control female for Experiment 1; Cross-inoculated/Control nestlings
235 for Experiment 2), and type of individual (i.e., nestling or female) and time of sampling,
236 respectively, as the within fixed factors. For analyses related to Experiment 1, the repeated factor
237 tests for differences between females and nestlings in PCo scores. For those related to Experiment
238 2, the repeated factor compared PCo scores between the initial and final samples (those obtained
239 one week after inoculation) of nestlings.

240 To estimate the dispersion of samples within the groups of cross-inoculated and control
241 nestlings in the PCo space, differences in the deviations from the median of each group were
242 calculated with PermDisp. We have also calculated average similarity of samples within groups and
243 probability of occurrence of the most frequent OTUs with Simper analyses. These analyses let
244 testing how much did microbiomes of nestlings within each experimental group converged after
245 inoculation. All these tools have been used in Primer7.

246 Bacterial loads of secretion of 16 days old nestlings did not differ between years (GLM after
247 controlling for nest identity nested within year (GLM, $F(1,17) = 2.58$, $p = 0.126$), nor did PCo1
248 scores (GLM, $F(1,17) = 1.32$, $p = 0.268$), although the effect of year on PCo2 scores approached
249 significance (GLM, $F(1,17) = 3.63$, $p = 0.076$). Since results for PCo scores are qualitatively
250 identical independent of inclusion of year as an additional independent factor, only results of
251 models that do not include year identity are shown. However, richness of bacterial community of
252 nestlings at this age were lower in 2012 than in 2010 (GLM, $F(1,17) = 6.18$, $p = 0.024$) and, thus,
253 year identity was included in the analyses explaining variation in richness.

254 Neither richness, nor bacterial load (CFUs/μl), or PCo scores differed among subpopulations
255 (GLMs, all $p>0.05$) and treatments were balanced within them. Thus, subpopulation identity was
256 not included in the analyses. Moreover, nestlings sharing the same nest have identical values for
257 female related variables (e.g. bacterial load in CFUs/ μl) and, to account for non-independence of

258 information of nestlings within the same nest, the degrees of freedom were adjusted to number of
259 sampled nests.

260 Finally, for analyses exploring similar association for different OTUs, the false-discovery-rate
261 (FDR) correction was applied to establish the appropriate *q* values, which were the calculated *p*
262 values after the FDR correction [48].

263 **Results**

264

265 <u>Bacterial richness</u>

266 The hoopoe nestlings involved in the inoculation experiment on average harbored 13.4 ± 6.0
267 (SD) OTUs per sample (N = 72 samples, including together those obtained before and a week after
268 the inoculation treatment). Females had less OTUs than nestlings (using only nestling samples
269 before inoculation; GLM controlling for the random effect of nest identity, comparison female-
270 nestling: $F(1,34) = 17.57$, $p < 0.001$; females: mean ± SD = 6.29 ± 2.59, N = 17; nestlings: mean ±
271 SD = 12.56 ± 5.78, N = 36; nest identity: $F(17, 34) = 1.30$, $p = 0.248$).

272 Manipulation of female access to their glands did not significantly affect the number of OTUs
273 present in nestlings' glands (GLM controlling for year, $F(1,14) = 0.22$, $p = 0.646$). Moreover,
274 richness of nestlings' gland communities did not depend on that of their mothers at the beginning of
275 incubation (GLM controlling for year, $F(1,14) = 1.24$, $p = 0.284$).

276 The inoculation experiment produced a significant increase in the number of OTUs present in
277 experimental nestlings in comparison with their control brothers that were inoculated their own
278 secretion (Table 1, Fig. 2).

279 The detected effect of the inoculation experiment on community richness resulted from an
280 increase in the prevalence of most OTUs in experimental nestlings, while such prevalence remained
281 stable in control nestlings (Fig. 3, Repeated measures ANOVA for the prevalence of each OTU,
282 interaction between type of nestling and within factor: ($F(1,64) = 22.9$, $p < 0.0001$).

283

284 Microbiome composition

285

286 The composition of nestlings' core microbiomes differed from those of their mothers
287 (repeated measures MANOVA with PCo1 and PCo2 scores: R1, Wilks = 0.194, $F (2, 15) = 64.4$, p
288 < 0.0001). In addition, microbiomes were more similar between control females and their nestlings
289 than between experimental females and their nestlings (repeated measures MANOVA: R1* female
290 treatment, Wilks = 0.781, $F (2, 15) = 4.33$, $p = 0.033$). Mostly, these effects were due to differences
291 related to PCo2 (Table 2, Fig. 4a). Scores of this PCo explained 19.1 % of total variance of the
292 microbiome composition of samples (Fig. 4b).

293 The inoculation experiment also affected the composition of the microbiome present in
294 nestling glands (repeated measures MANOVA with PCo1, PCo2 and PCo3 scores: R1 (paired
295 comparison before-after inoculation), Wilks = 0.645, $F (3, 32) = 5.88$, $p = 0.003$). The microbiomes
296 of experimental nestlings (cross-inoculated with secretions from a different nest) experienced larger
297 changes than those of control nestlings (repeated measures MANOVA: R1* nestling treatment,
298 Wilks = 0.781, $F (3, 32) = 4.44$, $p = 0.010$). The effects of the inoculation experiment were due to
299 differences in PCo1 and PCo2 scores (Table 3, Fig. 5). Moreover, final among-individuals variation
300 was higher for control (deviations from the median of each group mean(SE) = 46.7(3.8)) than for
301 experimental (mean(SE) = 20.2(5.0)) nestlings (PermDisp, $F (1, 34) = 17.70$, $p < 0.001$). The
302 microbiome composition of the final samples of cross-inoculated nestlings showed a higher level of
303 similarity among them (Simper, average similarity = 77.46 %, with 10 OTUs of the core
304 microbiome with a probability of presence > 80%) than among those of control nestlings (average
305 similarity 44.84 %, only 4 OTUs with a probability of occurrence > 60 %). Indeed, samples of
306 cross-inoculated nestlings appeared close to each other in the PCo space (Fig. 5), in the area that
307 correlates with a higher number of OTUs (Fig. 4 in Supplemental material-II).

308 For the subsample of nests with information on the microbiome of donors and receivers,
309 differences in their PCo1 scores positively correlated with the changes in microbiome composition
310 caused by the inoculation experiment (only experimental nestlings, Linear Regression $F(1, 13) =$

311 15.17, p = 0.0018, R^2 = 0.539). This relationship did not reach statistical significance for PCo2
312 scores (F(1, 13) = 4.56, p = 0.052, R^2 = 0.260).
313
314 Cultivable bacterial density
315 Bacterial density of nestling secretions at 16 days of age was positively correlated with that of
316 the secretions of their mothers at the beginning of incubation (Table 4, Fig. 6). Interestingly, this
317 association occurs in nests of control but not in those of experimental females with covered
318 uropygial glands (Table 4, Fig. 6b). In addition, we found that nestlings of experimental females
319 harbored in their secretions more cultivable bacteria than those of females with access to their
320 glands (Fig. 6a).
321 The density of cultivable bacteria in the secretions of nestlings one week after the inoculation
322 experiment did not differ from their initial bacterial density (Repeated measures ANOVA, R1:
323 F(1,33) = 0.06, p = 0.816). It was the case for nestlings inoculated with either own or alien
324 secretions (R1* Nestling type: F(1,33) = 0.15, p = 0.702).
325
326 Experimental effects on particular OTUs
327
328 Six OTUs of nestling secretions were significantly affected by the experiments. The covering of
329 female glands affected the presence of four OTUs in the secretion of nestlings. In nestlings of
330 mothers that had not access to their uropygial gland, prevalence of OTUs 346 and 466 decreased,
331 while that of OTUs 306 and 406 increased (Table 5). Moreover, the presence of the OTU566 in
332 nestlings was not affected by female manipulation, but was positively associated with its detection
333 in their mothers, either experimental or control (Table 5). For all these OTUs, but also for most in
334 the core microbiome, the effect of the inoculation experiment depended on microbiome
335 composition of donors and receivers. Whenever an OTU was present in the donor and not in the
336 receiver, the latter incorporated to the nestling secretion. In addition, there was an association
337 between the cultivable bacterial density in secretions and the presence of particular OTUs in their

338 microbiome. It was higher for secretions harboring OTU306, while presence of OTUs 242, 346 and
339 566 was negatively related to cultivable bacterial density (summary of results in Table 5, analyses
340 in Supplemental Material-II).
341

342 **Discussion**

343
344 Experimental results support for the first time the hypothesis that the microbiome hosted in the
345 uropygial gland of nestling hoopoes is in part transmitted vertically from mother to offspring, but
346 also that nestling microbiomes maintain their ability to incorporate new strains during the entire
347 nesting period. These results have important consequences for the understanding of the dynamic
348 and evolution of the relationships between hoopoes and their uropygial gland symbionts.
349 The detected effects of covering female glands support the influence of female uropygial
350 secretion conforming the microbiome of that of their offspring. Differences between nestlings and
351 mothers in microbiome composition and cultivable bacterial density were significantly larger for
352 nests of females with impeded access to their glands. Previous studies performing cross-fostering
353 experiments already showed the existence of genetic effects (i.e. nest of origin) on the composition
354 of the microbiome of hoopoe nestlings [36,37]. However, similarities due to nest of origin might be
355 due to direct transmission from females to offspring or common acquisition by mothers and
356 nestlings, and cross-fostering experiments of nestlings do not allow to distinguish between these
357 two possibilities. Results presented here definitely show that vertical transmission is in fact
358 responsible of the previously detected effect of nest of origin, at least partially. This is an important
359 result, because vertical transmission of those strains assures that part of the microbial community
360 that nestlings harbor in their glands come from an individual (their mother), which has already been
361 successful in surviving and breeding, thus transferring an optimized microbiota adapted to their
362 particular environment [2,13,14]. It is well established that the evolution of vertically transmitted
363 symbionts selects for characteristics that benefit both hosts and symbionts [15]. Moreover, when
364 hosts benefits are mediated by antibiotic production, as it occurs in hoopoes [27,31-34], the vertical

365 transmission of one antibiotic producing strain would constraint the recruitment of non-resistant
366 ones and favor recruitment of other antibiotic producers [24]. The existence of several bacterial
367 strains co-transmitted vertically from mother to offspring could also imply the evolution of
368 tolerance, co-operation or resource dependence among them [49]. Vertical transmission also affects
369 the evolution of the symbiotic relationship among counterparts [2,15]. Whenever symbionts
370 complete their life cycle within a host species, and their fitness become close related to that of their
371 host, the coevolutionary process provokes symbiont specialization on hosts as well as genetic
372 barriers preventing gene flow among close relatives living in different hosts. Thus, similarly to
373 what has been described in other systems [2], such process would lead to separated evolution
374 among isolated populations of hosts, therefore predicting the existence of different microbial
375 symbiont strains in different hoopoe populations, subspecies or species. However, particularities of
376 the hoopoe-bacteria system may affect specialization and speciation processes of hoopoe
377 symbionts. For instance, secretion of females drastically change during the non-breeding season
378 (see below), which may imply either adaptations of the symbionts to resist that period, specialized
379 body reservoirs in females, or ability to change of microhabitat within the female body. Future
380 studies should explore such possibilities.

381 Interestingly, there were clear inter-nest differences in the microbiomes harbored by
382 nestlings. This could be caused by a selective adaptive acquisition of bacteria [23], but also might
383 be the consequence of differences in availability in nest environments. In accordance with the latter
384 possibility, the experimental inoculation of secretions from a different nest provoked marked
385 changes in richness and composition of microbiomes of nestlings' secretions after a particular
386 community had been established along two thirds of their nestling cycle. Detected changes in the
387 microbiome of nestlings due to inoculation were mainly explained by acquisition of strains present
388 in the donor individual that were absent in the receiver secretion. Thus, after the experimental
389 inoculations, communities of experimental nestlings from different nests became more similar to
390 each other than those of control nestlings. These results suggest that there is a group of OTUs that
391 are commonly found in hoopoe nestlings secretions and that conform to the typical microbiome. A

392 similar effect was found for enterococci strains differing in bacteriocin production that varied in
393 their presence among hoopoe nests [38].
394
395 The experimental approaches and results allow discussing the origin of particular OTUs of
396 nestling secretions. When females could not use their secretions, many nestlings failed in harboring
397 OTU346 and OTU466, suggesting female secretion is the main source of these two OTUs.
398 Moreover, although detection of OTU566 in offspring was positively associated with its presence in
399 females, the manipulation of female access to glands did not affect its acquisition by nestlings.
400 Therefore, either, environmental conditions shared by nestlings and mother are responsible for the
401 recruitment of this OTU in their uropygial secretions, or there is transmission from female to
402 offspring by other via different of the female gland. We know from previous work [47] that
403 prevalence of OTU566 in glands and cloacae of females as well as on the eggshells are very similar
404 (55%, 45%, 35% respectively). Something similar occurs with OTU306, which is especially
405 common in female cloaca and on the eggshells (70% and 57% respectively, [47]) in comparison
406 with prevalence in female uropygial gland (25%, 35% in the present study). OTU306 (together with
407 OTU406) characterize the microbiomes of offspring of females without access to their glands. All
408 this evidence suggests that the cloaca of mothers is the most probable source of these bacteria (at
409 least of the OTU566 and OTU306) for nestlings. We know that *Enterococcus faecalis,* by far the
410 most prevalent cultivable bacteria species growing from hoopoe secretions [27,32,38], produces an
411 ARISA peak at 306 (Antonio Martín-Platero, pers. comm.). Interestingly, OTU306 was the only
412 OTU that was positively related to density of cultivable bacteria in the secretion of nestling
413 hoopoes. Previous studies have also shown that *E. faecalis,* and some others *Enterococcus* strains
414 isolated from hoopoe secretions, produce bacteriocins with an ample antimicrobial spectrum
415 [30,32,38] and that their abundance in the secretion of female hoopoes is positively related to
416 hatching success [34]. Thus, detecting the possible source of this OTU for hoopoe secretions is of
417 particular interest and, casting light on this subject, the experimental results suggest that hoopoes
418 recruit it from the cloacae to uropygial glands.

419 Several results suggest that colonization of nestlings´ secretions by particular OTUs depends
420 on the presence of other bacteria. First, the experimental covering of female glands resulted in
421 nestling microbiomes with increased prevalence of OTU306 and OTU406 and this suggests that
422 particular OTUs transmitted vertically prevent their establishment in the uropygium of nestlings.

423 Second, this experiment also affected the mother-offspring relationship in the density of
424 cultivable bacteria. The abundance of cultivable bacteria in mother and offspring was related only
425 for control females, while when the experiment impeded vertical transmission (experimental
426 females), nestlings harbored greater loads of cultivable bacteria, and those loads were not predicted
427 by that in mother glands. This suggests that the bacteria growing from nestlings´ secretions of
428 control females are a subset of those growing from nestlings´secretions in which vertical
429 transmission has been impeded. Probably they are those with the potential to live in hoopoe
430 secretions in presence of OTUs normally transmitted from female glands, while nestlings from
431 experimental females harbor also less competitive strains able to grow in this experimental scenario
432 of reduced competence. Given that OTU306 is the main predictor of cultivable bacteria load in
433 secretions, this result can be interpreted in terms of abundance of OTU306. Since OTU306
434 probably represents mainly *Enterococcus faecalis* (see above), this possibility implies the existence
435 of mechanisms selecting for particular Enterococci strains (i.e. those with higher antimicrobial
436 potential).

437 Only three bird species are known to host symbiotic bacteria inside the uropygial gland in
438 healthy individuals: the red-billed woodhoopoe (*Phoeniculus purpureus*), the European hoopoe and
439 the turkey (*Meleagris gallopavo,* [50]). While in the turkey the influence of the symbionts
440 (*Corynebacterium uropygiale*) has not yet been studied, in both Upupiform species, symbionts are
441 responsible of several properties of the secretions including their defensive function [29,51,52]. In
442 the red-billed woodhoopoe, all individuals maintain the symbiosis in the uropygial gland
443 throughout the year, what has driven the evolution of a specific *Enterococcus* symbiont (*E.*
444 *phoeniculicula*, [28]), although the whole community has never been studied by molecular methods
445 in this system. In the case of European hoopoes, the association with bacteria in the gland is not

446 continuous, but cyclic [27,29]. This probably has prevented to some extent the specialization of the
447 symbiotic relationship [53,54]. Thus, the enterococci found in hoopoes are common in several
448 environments [32] and from the results in the present study they probably are recruited from the
449 cloaca. The microbiome of the uropygial secretion of hoopoes is more complex than that usually
450 established in animal glands specialized in hosting symbionts (e.g. single actinobacteria species
451 protecting fungus growing ants' gardens [55,56]). It includes a combination of 8 to 27 more
452 frequent OTUs accompanied by up to 124 scarce OTUs with reduced prevalence [44], and results
453 here suggest different sources for them. A few are inherited from mother to nestling gland, others
454 may come either from female or nestling cloaca, and many others can apparently be obtained from
455 environmental sources accessible within the nest-hole [36,37,45].

456 Despite such apparently complex microbiome, a more or less stable combination of OTUs is
457 typical (core microbiome), and results suggest that the competitive ability of strains vertically
458 transmitted from mothers gland differentially promote or restrict the establishment of others that are
459 able of colonizing the nestling gland from other sources (see [24]). Previously, it was shown that
460 the prevalence of different enterococci strains in the glands was related to their ability to produce
461 antimicrobial bacteriocins [38]. All these results suggest that a mutualism based in byproduct
462 cooperation [4,22] determine the microbial composition of hoopoe uropygial gland microbiome.
463

464 Summarizing, hoopoe nestling uropygial glands harbor core microbiomes that to some degree
465 vary in their composition, but, when experimentally put in contact, converge to the same
466 combination of OTUs. The experiments further demonstrate the importance of vertical transmission
467 determining the core microbiome of nestling glands, and suggest the existence of cloaca or gut
468 reservoirs for particular OTUs. Further advance in the understanding of evolution of this system
469 should address the determination of the taxonomic affiliation of these most common OTUs, in
470 order to study their particular function in the symbiotic community, and finding the inter-annual
471 reservoir for the vertically transmitted symbionts.
472

473 **Ethical approval**

474 We performed the study following relevant Spanish national (Decreto 105/2011, April 19) and
475 regional guidelines. The ethics committee of the Spanish National Research Council (CSIC)
476 approved the protocol, and the Consejería de Medio Ambiente de la Junta de Andalucía, Spain,
477 provided all necessary permits for nest and nestling manipulations (Ref: SGYB/FOA/AFR/CFS) as
478 good as the establishment and maintenance of the captive breeding population (Resolution of April
479 14 2008).

480

481 **Conflict of Interest**

482 The authors declare that they have no conflict of interest.

483

484 **Data accessibility**

485 Data used in this paper can be found in the CSIC Institutional Repository. (Accession numbers still
486 not available)

487

488 **Authors' contributions**

489 MM-V, JJS, AMG, MRR and MMB conceived the ideas and designed methodology; AMG, LA
490 and NJG-P collected the data with considerable help from MM-V and JJS; MM-V and JJS analyzed
491 the data; MM-V led the writing of the manuscript with considerable help by JJS. All authors
492 contributed critically to the drafts and gave final approval for publication.

493

494 **References**

495

496 1. Moran NA (2002) The ubiquitous and varied role of infection in the lives of animals and plants.
497 American Naturalist 160:S1-S8
498 2. Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. Nature
499 Reviews Microbiology 8 (3):218-230

500 3. Florez LV, Biedermann PHW, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals
501 with prokaryotic and eukaryotic microorganisms. Natural Product Reports 32 (7):904-936
502 4. Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis.
503 Proceedings of the National Academy of Sciences of the United States of America 108:10800-
504 10807
505 5. Morales J, Kokkori S, Weidauer D, Chapman J, Goltsman E, Rokhsar D, Grossman AR, Nowack
506 ECM (2016) Development of a toolbox to dissect host-endosymbiont interactions and protein
507 trafficking in the trypanosomatid *Angomonas deanei*. BMC Evolutionary Biology 16 (1):247
508 6. Bronstein JL (2001) The exploitation of mutualisms. Ecology Letters 4 (3):277-287
509 7. Johnstone RA, Bshary R (2002) From parasitism to mutualism: partner control in asymmetric
510 interactions. Ecology Letters 5 (5):634-639
511 8. Ferriere R, Gauduchon M, Bronstein JL (2007) Evolution and persistence of obligate mutualists
512 and exploiters: competition for partners and evolutionary immunization. Ecology Letters 10
513 (2):115-126
514 9. Doebeli M, Hauert C (2005) Models of cooperation based on the Prisoner's Dilemma and the
515 Snowdrift game. Ecology Letters 8 (7):748-766
516 10. Foster KR, Wenseleers T (2006) A general model for the evolution of mutualisms. Journal of
517 Evolutionary Biology 19 (4):1283-1293
518 11. Lee CT (2015) Inherent demographic stability in mutualist-resource-exploiter interactions.
519 American Naturalist 185 (4):551-561
520 12. Shapiro JW, Turner PE (2014) The impact of transmission mode on the evolution of benefits
521 provided by microbial symbionts. Ecology and Evolution 4 (17):3350-3361
522 13. Ewald PW (1987) Transmission modes and evolution of the parasitism-mutualism continuum.
523 Annals of the New York Academy of Sciences 503:295-306
524 14. Douglas AE (1998) Host benefit and the evolution of specialization in symbiosis. Heredity
525 81:599-603

526 15. Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms:
527 exploring the paths between conflict and cooperation. Trends in Ecology & Evolution 14 (2):49-
528 53
529 16. Simonsen AK, Stinchcombe JR (2014) Standing genetic variation in host preference for
530 mutualist microbial symbionts. Proceedings of the Royal Society B-Biological Sciences 281
531 (1797)
532 17. Schwartzman JA, Koch E, Heath-Heckman EAC, Zhou L, Kremer N, McFall-Ngai MJ, Ruby
533 EG (2015) The chemistry of negotiation: Rhythmic, glycan-driven acidification in a symbiotic
534 conversation. Proceedings of the National Academy of Sciences of the United States of America
535 112 (2):566-571
536 18. Bull JJ, Rice WR (1991) Distinguishing mechanisms for the evolution of cooperation. Journal
537 of Theoretical Biology 149 (1):63-74
538 19. Chaston J, Goodrich-Blair H (2010) Common trends in mutualism revealed by model
539 associations between invertebrates and bacteria. FEMS Microbiology Reviews 34 (1):41-58
540 20. Heil M, Barajas-Barron A, Orona-Tamayo D, Wielsch N, Svatos A (2014) Partner manipulation
541 stabilises a horizontally transmitted mutualism. Ecology Letters 17 (2):185-192
542 21. Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial
543 mutualists. Proceedings of the Royal Society B-Biological Sciences 274 (1620):1905-1912
544 22. Sachs JL, Mueller UG, Wilcox TP, Bull JJ (2004) The evolution of cooperation. Quarterly
545 Review of Biology 79 (2):135-160
546 23. Weimer PJ (2015) Redundancy, resilience, and host specificity of the ruminal microbiota:
547 implications for engineering improved ruminal fermentations. Frontiers in Microbiology 10 (6):
548 296. doi: 10.3389/fmicb.2015.00296
549 24. Scheuring I, Yu DW (2012) How to assemble a beneficial microbiome in three easy steps.
550 Ecology Letters 15 (11):1300-1307
551 25. Rodríguez-Ruano SM, Martín-Vivaldi M, Martín-Platero AM, López-López JP, Peralta-
552 Sánchez JM, Ruiz-Rodríguez M, Soler JJ, Valdivia E, Martínez-Bueno M (2015) The hoopoe's

553 uropygial gland hosts a bacterial community influenced by the living conditions of the bird.
554 PLoS One 10 (10)
555 26. Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Ruiz-Rodríguez M (2010) Antibiotic-
556 producing bacteria as a possible defence of birds against pathogenic microorganisms. Open
557 Ornithology Journal 3:93-100
558 27. Soler JJ, Martín-Vivaldi M, Ruiz-Rodríguez M, Valdivia E, Martín-Platero AM, Martínez-
559 Bueno M, Peralta-Sánchez JM, Méndez M (2008) Symbiotic association between hoopoes and
560 antibiotic-producing bacteria that live in their uropygial gland. Functional Ecology 22 (5):864-
561 871
562 28. Law-Brown J, Meyers PR (2003) *Enterococcus phoeniculicola* sp nov., a novel member of the
563 enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe, *Phoeniculus*
564 *purpureus* International Journal of Systematic and Evolutionary Microbiology 53:683-685
565 29. Martín-Vivaldi M, Ruiz-Rodríguez M, Soler JJ, Peralta-Sánchez JM, Méndez M, Valdivia E,
566 Martín-Platero AM, Martínez-Bueno M (2009) Seasonal, sexual and developmental differences
567 in hoopoe preen gland morphology and secretions. Evidence for a role of bacteria. Journal of
568 Avian Biology 40:191-205
569 30. Martín-Platero AM, Valdivia E, Ruiz-Rodríguez M, Soler JJ, Martín-Vivaldi M, Maqueda M,
570 Martínez-Bueno M (2006) Characterization of antimicrobial substances produced by
571 *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe *Upupa epops*
572 Applied and Environmental Microbiology 72:4245-4249
573 31. Martín-Vivaldi M, Peña A, Peralta-Sánchez JM, Sánchez L, Ananou S, Ruiz-Rodríguez M,
574 Soler JJ (2010) Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic
575 bacteria. Proceedings of the Royal Society of London, Series B Biological Sciences 277
576 (1678):123-130
577 32. Ruiz-Rodríguez M, Valdivia E, Martín-Vivaldi M, Martín-Platero AM, Martínez-Bueno M,
578 Méndez M, Peralta-Sánchez JM, Soler JJ (2012) Antimicrobial activity and genetic profile of
579 enteroccoci isolated from hoopoes uropygial gland. PLoS One 7 e41843: 41841-41846

580 33. Ruiz-Rodríguez M, Valdivia E, Soler JJ, Martín-Vivaldi M, Martín-Platero AM, Martínez-
581 Bueno M (2009) Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather
582 degradation. Journal of Experimental Biology 212 (22):3621-3626
583 34. Martín-Vivaldi M, Soler JJ, Peralta-Sánchez JM, Arco L, Martín-Platero AM, Martínez-Bueno
584 M, Ruiz-Rodríguez M, Valdivia E (2014) Special structures of hoopoe eggshells enhance the
585 adhesion of symbiont-carrying uropygial secretion that increase hatching success. Journal of
586 Animal Ecology 83 (6):1289-1301
587 35. Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Arco L, Juárez-García-Pelayo N (2014)
588 Hoopoes color their eggs with antimicrobial uropygial secretions. Naturwissenschaften 101
589 (9):697-705
590 36. Martínez-García A, Martín-Vivaldi M, Ruiz-Rodríguez M, Martínez-Bueno M, Arco L,
591 Rodríguez-Ruano S, Peralta-Sánchez JM, Soler JJ (2016) The microbiome of the uropygial
592 secretion in hoopoes is shaped along the nesting phase. Microbial Ecology 72 (1):252-261 doi:
593 10.1007/s00248-016-0765-1
594 37. Ruiz-Rodríguez M, Soler JJ, Martín-Vivaldi M, Martín-Platero AM, Méndez M, Peralta-
595 Sánchez JM, Ananou S, Valdivia E, Martínez-Bueno M (2014) Environmental factors shape the
596 community of symbionts in the hoopoe uropygial gland more than genetic factors. Applied and
597 Environmental Microbiology 80 (21):6714-6723
598 38. Ruiz-Rodríguez M, Martínez-Bueno M, Martín-Vivaldi M, Valdivia E, Soler JJ (2013)
599 Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated
600 from the hoopoe's uropygial gland. FEMS Microbiology Ecology 85 (3):495-502
601 39. Hammond AC (1995) *Leucaena toxicosis* and its control in ruminants. Journal of Animal
602 Science 73 (5):1487-1492
603 40. Giraudeau M, Czirják GÁ, Duval C, Bretagnolle V, Gutierrez C, Guillon N, Heeb P (2013)
604 Effect of preen oil on plumage bacteria: An experimental test with the mallard. Behavioural
605 Processes 92:1-5 doi: 10.1016/j.beproc.2012.08.001

606 41. Giraudeau M, Duval C, Guillon N, Bretagnolle V, Gutierrez C, Heeb P (2010) Effects of access
607 to preen gland secretions on mallard plumage. Naturwissenschaften 97 (6):577-581.
608 doi:10.1007/s00114-010-0673-z
609 42. Czirják GÁ, Pap PL, Vágási CI, Giraudeau M, Mureşan C, Mirleau P, Heeb P (2013) Preen
610 gland removal increases plumage bacterial load but not that of feather-degrading bacteria.
611 Naturwissenschaften 100 (2):145-151. doi:10.1007/s00114-012-1005-2
612 43. Hird SM (2017) Evolutionary biology needs wild microbiomes. Frontiers in Microbiology
613 8:725
614 44. Martínez-García A, Soler JJ, Rodríguez-Ruano SM, Martínez-Bueno M, Martín-Platero AM,
615 Juárez-García-Pelayo N, Martín-Vivaldi M (2015) Preening as a vehicle for key bacteria in
616 hoopoes. Microbial Ecology 70 (4):1024-1033
617 45. Rodríguez-Ruano S. (2015) Diversidad bacteriana en la glándula uropigial de la abubilla:
618 Dinámica estacional y beneficios asociados. PhD Thesis, Universidad de Granada.
619 46. Soler JJ, Martínez-García A, Rodríguez-Ruano S, Martínez-Bueno M, Martín-Platero AM,
620 Peralta-Sánchez JM, Martín-Vivaldi M (2016) Nestedness of hoopoes' bacterial communities:
621 symbionts from the uropygial gland to the eggshell. Biological Journal of the Linnean Society
622 118 (4):763–773 doi: 10.1111/bij.12772
623 47. Martínez-García A, Martín-Vivaldi M, Rodríguez-Ruano S, Peralta-Sánchez JM, Valdivia E,
624 Soler Juan J (2016) Nest bacterial environment affects microbiome of hoopoe eggshells, but not
625 that of the uropygial secretion PLoS One 11 (7). doi:10.1371/journal.pone.0158158
626 48. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful
627 approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological)
628 57 (1):289-300.
629 49. Vautrin E, Vavre, F (2009) Interactions between vertically transmitted symbionts: cooperation
630 or conflict? Trends in Microbiology 17 (3):95–99
631

632 50. Braun MS, Zimmermann S, Danner M, Rashid Ho, Wink M (2016) *Corynebacterium*
633 *uropygiale* sp. nov., isolated from the preen gland of Turkeys (*Meleagris gallopavo*). Systematic
634 and Applied Microbiology 39 (2):88-92
635 51. Law-Brown J (2001) Chemical defence in the red-billed woodhoopoe, *Phoeniculus purpureus*.
636 University of Cape Town, Cape Town.
637 52. Burger BV, Reiter B, Borzyk O, Du Plessis MA (2004) Avian exocrine secretions. I. Chemical
638 characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe,
639 *Phoeniculus purpureus*. Journal of Chemical Ecology 30 (8):1603-1611
640 53. Moran NA, Wernegreen JJ (2000) Lifestyle evolution in symbiotic bacteria: insights from
641 genomics. Trends in Ecology & Evolution 15 (8):321-326
642 54. Mandel MJ (2010) Models and approaches to dissect host-symbiont specificity. Trends in
643 Microbiology 18 (11):504-511
644 55. Cafaro MJ, Poulsen M, Little AEF, Price SL, Gerardo NM, Wong B, Stuart AE, Larget B,
645 Abbot P, Currie CR (2011) Specificity in the symbiotic association between fungus-growing
646 ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B-Biological
647 Sciences 278 (1713):1814-1822
648 56. Andersen SB, Hansen LH, Sapountzis P, Sorensen SJ, Boomsma JJ (2013) Specificity and
649 stability of the *Acromyrmex-Pseudonocardia* symbiosis. Molecular Ecology 22 (16):4307-4321
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651

Table 1. Results of a Repeated Measures ANOVA analyzing the effect of the inoculation of uropygial secretion, collected from their own gland or from a nestling of a different nest (nestling type), on the number of Operational Taxonomic Units (OTUs) detected in nestling secretions before and one week after inoculation (R: repeated measures). The model also included the effect of year and the random effect of nest identity nested within study year. Significant effects are marked in bold.

		df	F	p
R1	Fixed	1	3.27	0.088
R1 x Nestling Type	Fixed	1	7.46	**0.011**
R1 x Year	Fixed	1	0.07	0.796
R1 x Nest (Year)	Random	16	1.45	0.227
Error		17		

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Table 2. Effects of the covering of female glands (Female treatment) on the similarities between mothers and their nestlings (repeated measure R1) in the composition of the core microbiome of their uropygial secretions. Females' samples were collected before the experiment covering their glands and those of nestlings when they were 16 days old. Each repeated factor (R1) compares PCo scores of nestling and female. Results for R1 did not qualitatively change when not including the interaction with female treatment in the model. DFs adjusted to the number of nests. Significant p-values are in bold.

		df	F	p
PCo1				
R1	Fixed	1	4.04	0.062
R1 x Female treatment	Fixed	1	1.55	0.223
Error		16		
PCo2				
R1	Fixed	1	112.85	**<0.0001**
R1 x Female treatment	Fixed	1	5.65	**0.030**
Error		16		

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Table 3. Results from Repeated measures ANOVAs exploring the effects of the experimental inoculation of nestlings glands with own uropygial secretion or that of a foreign nestling (nestling type) on the change of the microbiome composition after the inoculation experiment (repeated measure R1). Each repeated factor (R1) compares PCo scores of nestlings before and after inoculation. Results for R1 did not change when not including the interaction with type of nestling in the model. Significant p-values are in bold.

		df	F	p
PCo1				
R1	Fixed	1	11.46	**0.002**
R1 x Nestling type	Fixed	1	4.92	**0.033**
Error		34		
PCo2				
R1	Fixed	1	1.61	0.214
R1 x Nestling type	Fixed	1	5.02	**0.032**
Error		34		
PCo3				
R1	Fixed	1	5.87	**0.021**
R1 x Nestling type	Fixed	1	3.90	0.056
Error		34		

660

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662

Table 4. Results of a General Linear Model (GLM) exploring the association between density of cultivable bacteria in nestling secretions (dependent variable, (cfu/μl)) and that of females. Whether or not uropygial gland of females was experimentally covered (female treatment) as well as its interaction with the bacterial density of female secretion were included in the model (1*2). The main effects remained statistically significant when removing the interaction. Degrees of freedom were adjusted to number of nests. Significant p-values are in bold.

	df	F	p
1) Female treatment	1	8.99	**0.011**
2) Log(CFUs/μl) female secretion	1	5.30	**0.038**
3) 1 * 2	1	3.91	0.069
	12		

663

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Table 5. Summary of effects for the Operational Taxonomic Units (OTUs) with significant contributions to properties of the microbiome of hoopoe nestling secretions (see analyses in Supplemental Material-II).

OTU	Affected by covering female gland	Related with presence in female gland	Effect of presence in donor	Relation with TSA growing	Probable source*
242			Yes	Negative	External
306	Positive		Yes	Positive	Gut
346	Negative		Yes	Negative	Female gland
406	Positive		Yes		External/Gut
466	Negative	Yes**	Yes		Female gland
566		Yes	Yes	Negative	Gut

666 * See explanations in Discussion
667 ** Present in all females except in one experimental
668
669
670
671

672 FIGURE CAPTIONS

673

674 **Fig. 1** Schematic representation of the design and steps used for the two experiments. a)
675 Experiment 1: manipulation of female access to the uropygial gland, to test the direct transmission
676 of bacteria from female to offspring glands. Green arrows represent the hypothesized vertical
677 transmission of bacteria. b) Experiment 2: cross-inoculation of secretions among nestlings from
678 different nests, to test the stability of microbiomes and strain ability in colonizing a gland

679

680 **Fig. 2** Effect of the experiment inoculating uropygial glands of hoopoe nestlings with secretions
681 from the same nestling (control) or from a nestling of a different nest (experimental) on richness of
682 the bacterial community hosted in their gland

683

684 **Fig. 3** Prevalence of Operational Taxonomic Units (OTUs) found in uropygial secretions of
685 nestling hoopoes before (black bars) and a week after (white bars) the inoculation of their glands
686 with (a) their own secretion or (b) the secretion from a nestling of a different nest

687

688 **Fig. 4** (a) Influence of manipulating females´ access to their uropygial gland on the differences
689 between hoopoe females and their nestlings in PCo 2 scores. Means and 95% confidence intervals
690 calculated for the number of nests are presented. (b) Differences in the composition of the hoopoe
691 uropygial gland core microbiome, among control, experimental females and their nestlings, as
692 reflected for PCo1 and PCo2

693

694 **Fig. 5** PCo plot representing, by their proximity, the resemblance in composition of the microbiome
695 among samples of uropygial secretions from nestlings before (initial) and a week after (final) the
696 inoculation experiment. Control nestlings were inoculated their own secretion, while another
697 nestling from each nest (cross-inoculated) was inoculated with secretion taken from a different
698 brood

699

700 **Fig. 6** Effects of manipulating female´s access to their uropygial gland on (a) the abundance of
701 cultivable bacteria in nestling secretions and (b) the relationship between the bacterial growth from
702 female and nestling secretions

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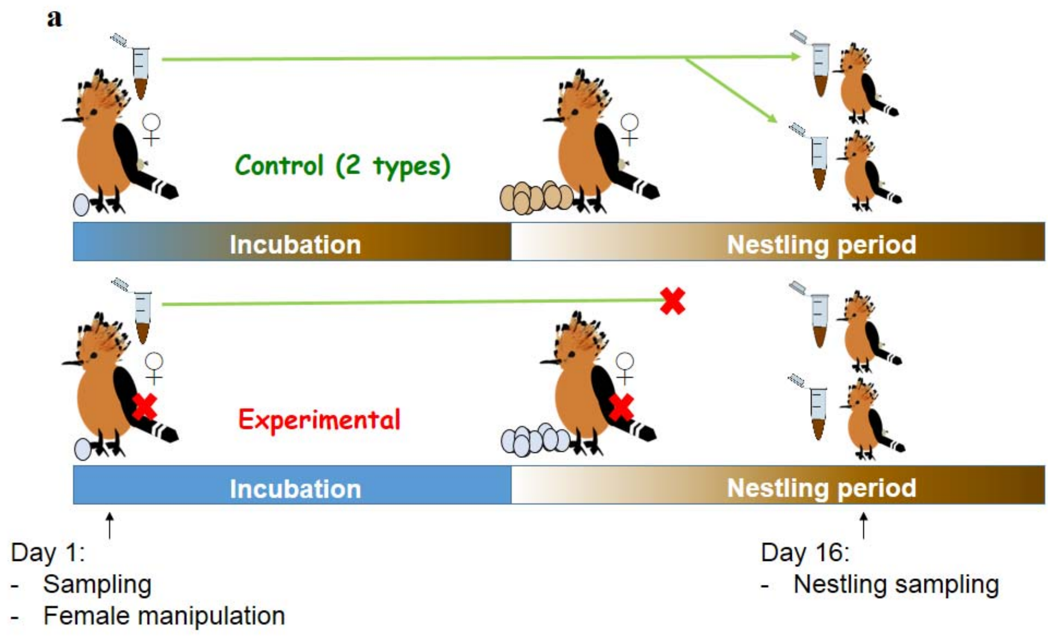
705 **Fig 1**



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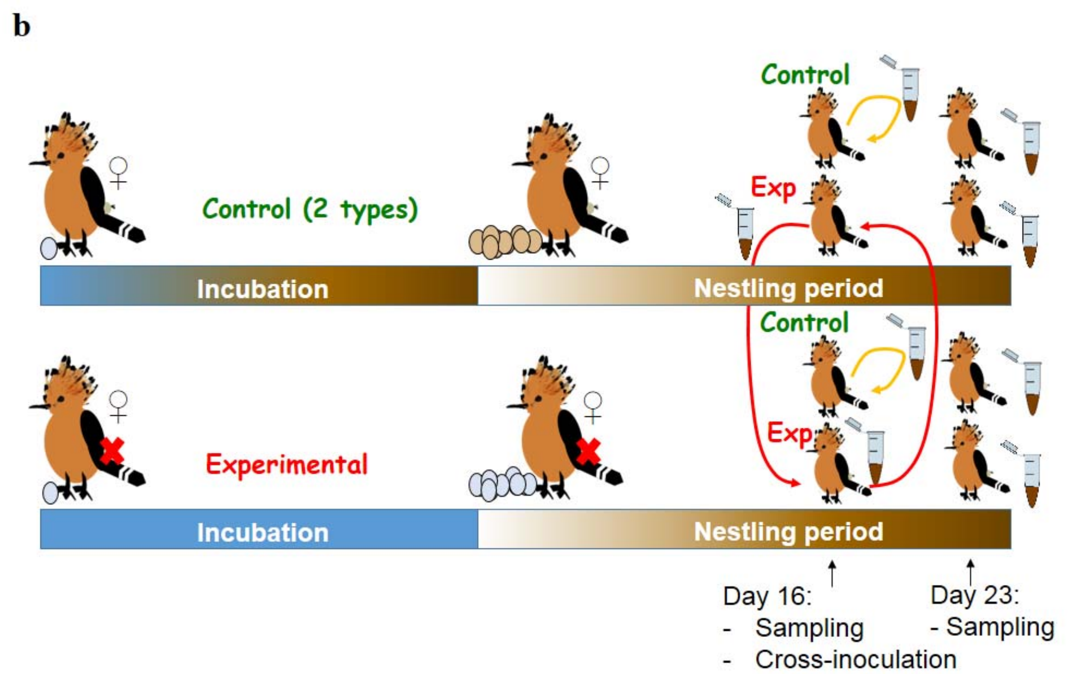

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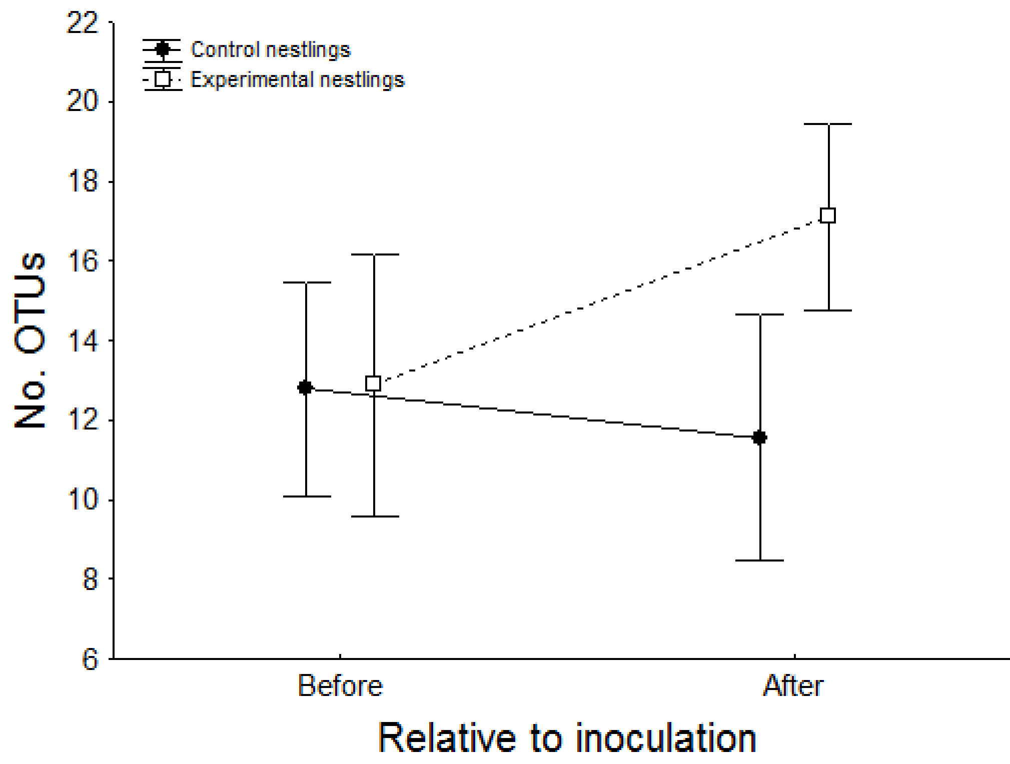
710 **Fig. 2**



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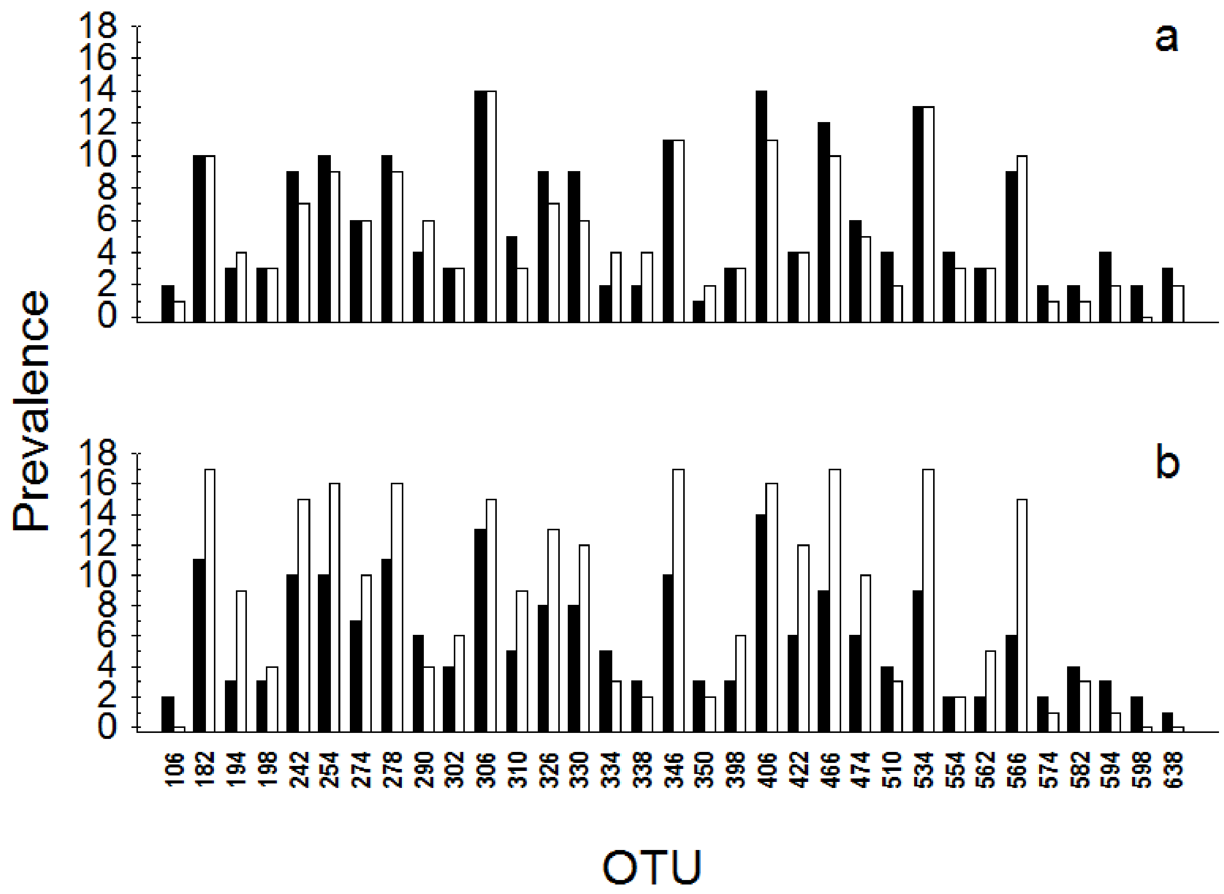
714 **Fig 3.**



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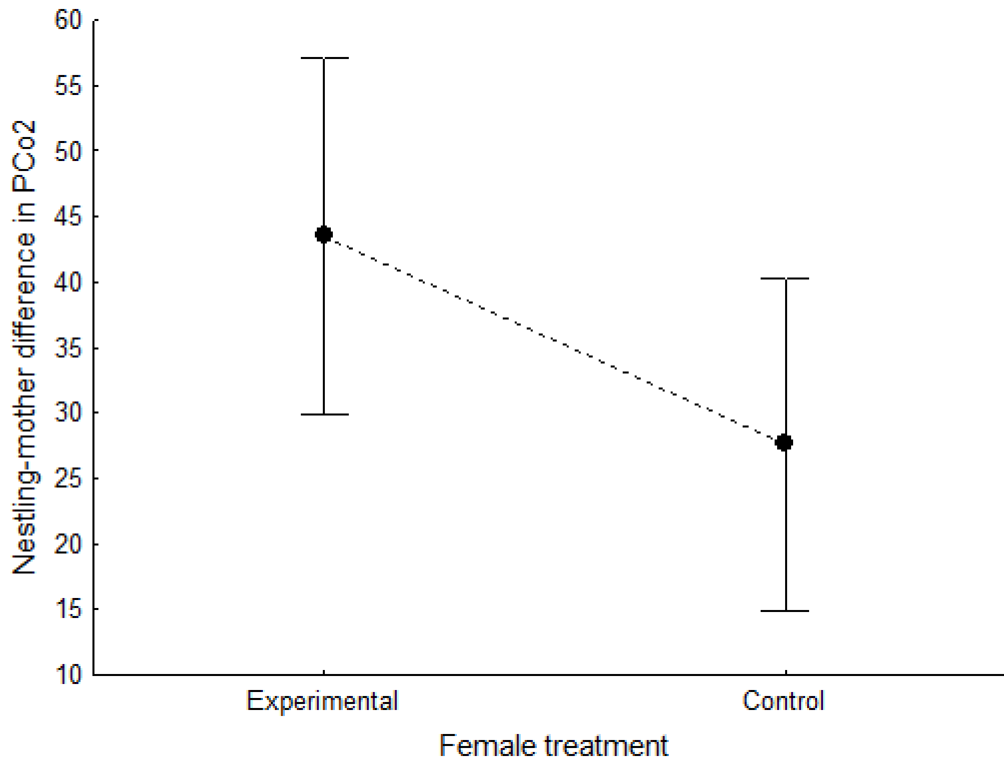
718 **Fig. 4a**



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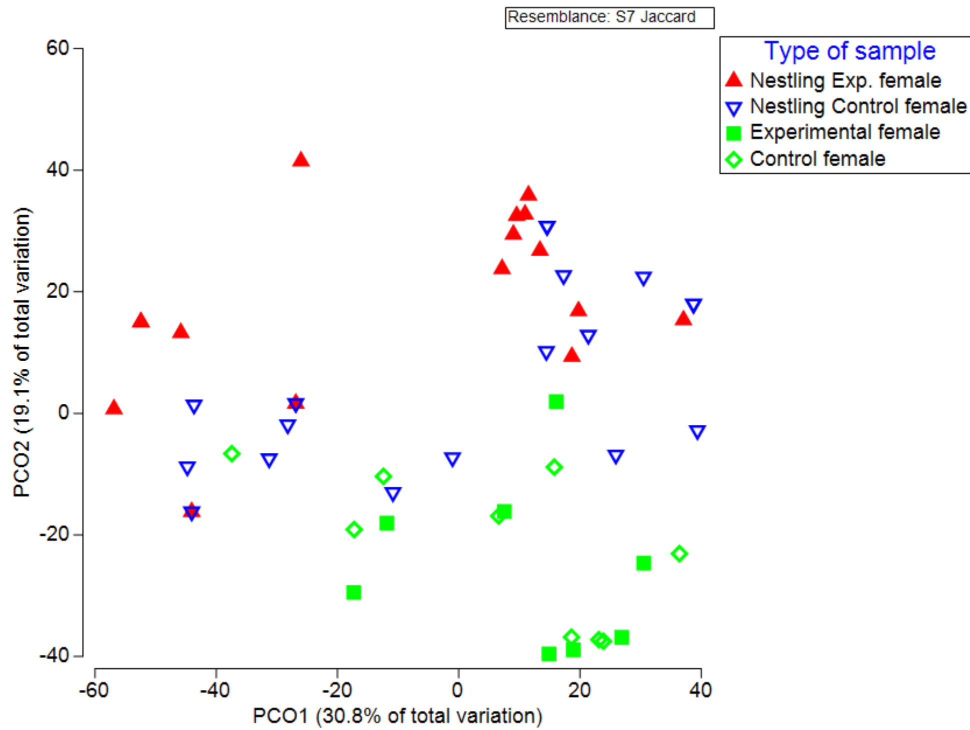
720 **Fig 4b**



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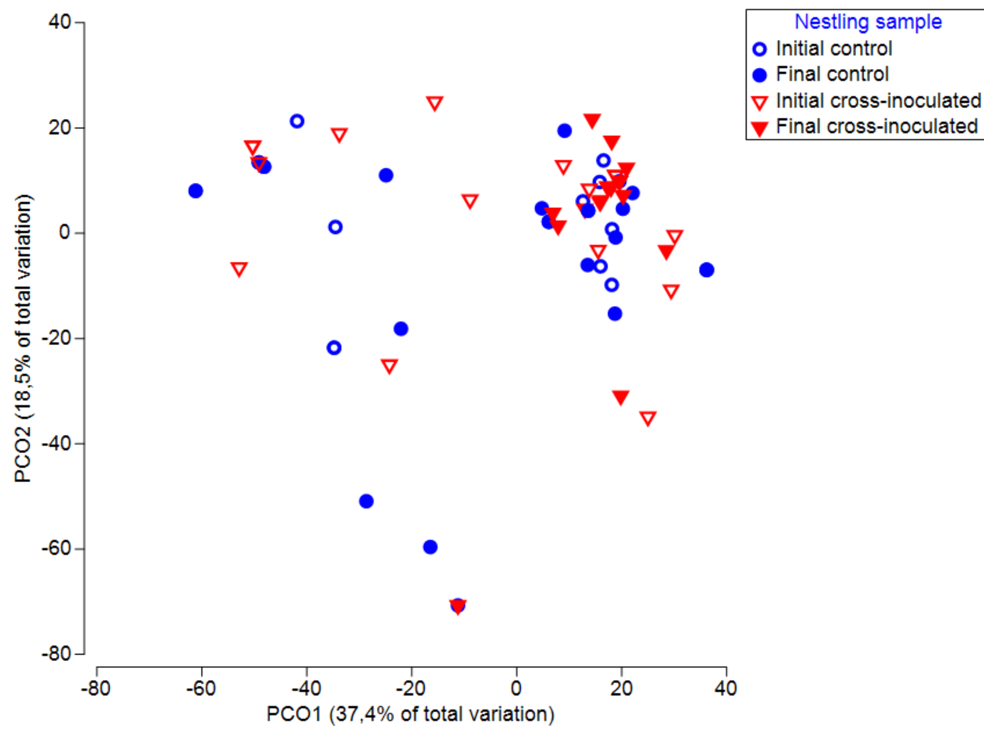
725 **Fig 5**



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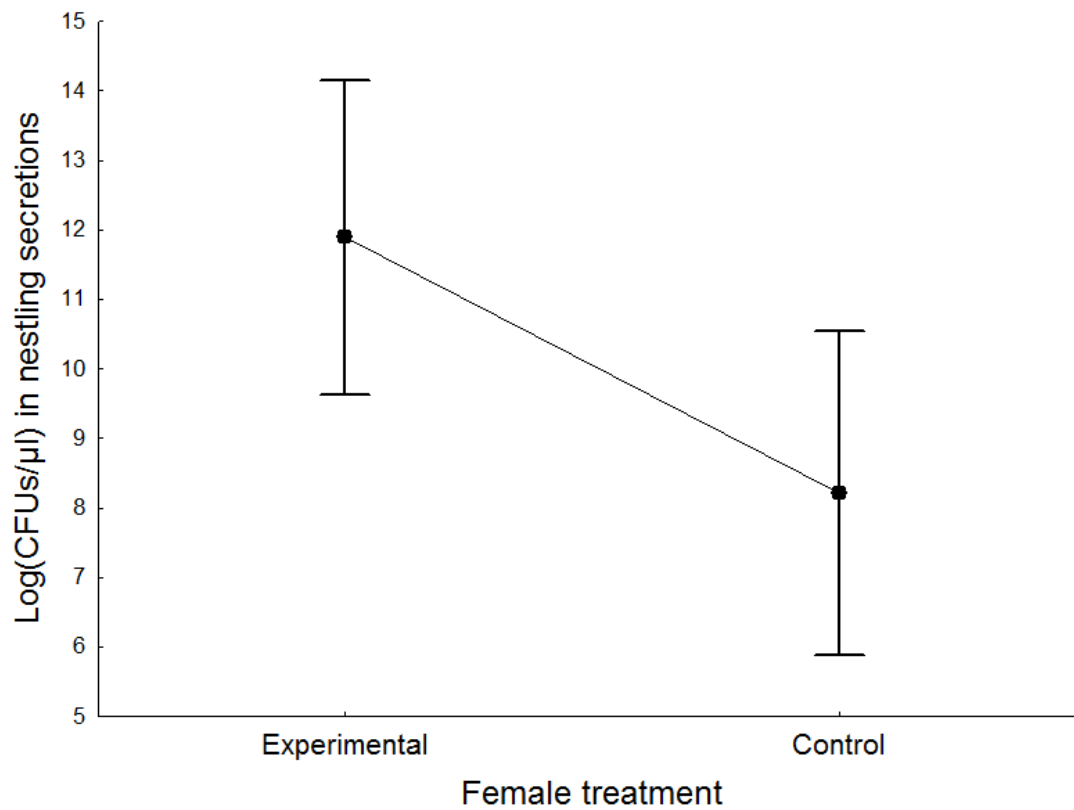
729 **Fig. 6a**



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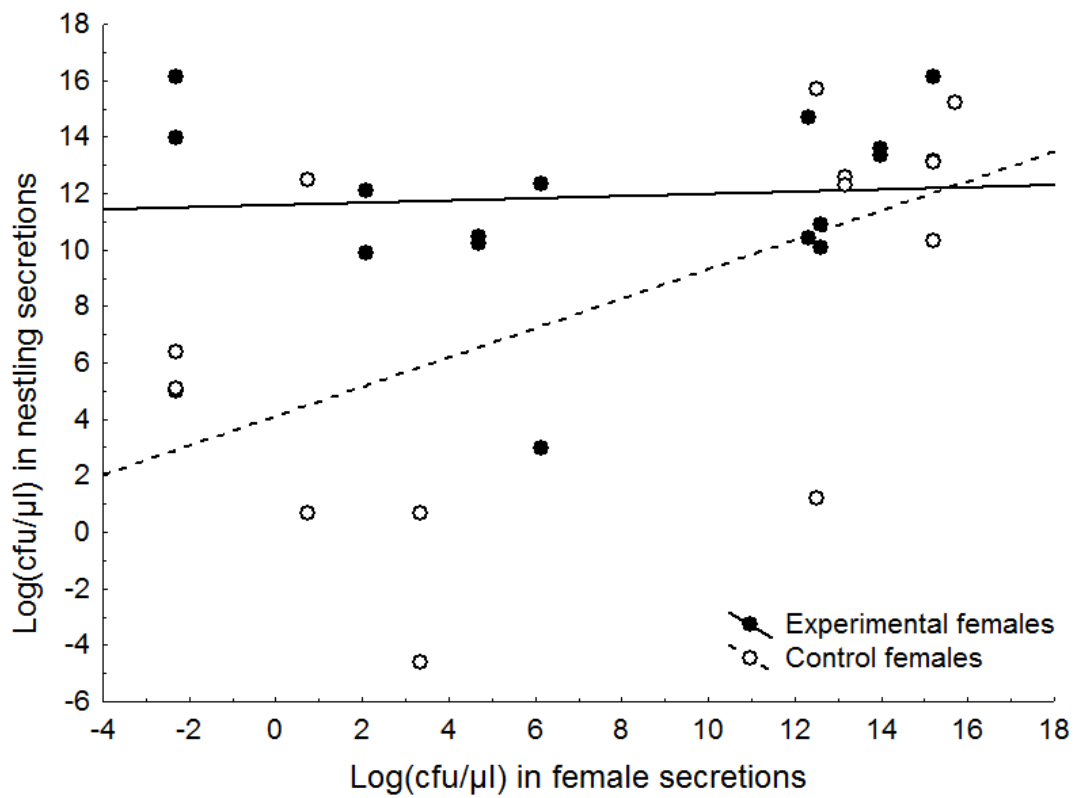
731 **Fig 6b**



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735 **Acquisition of uropygial gland microbiome by hoopoe nestlings**

736 *Microbial Ecology*

737 M. Martín-Vivaldi[a,b]*, J. J. Soler, Ángela Martínez-García, L. Arco, N. Juárez-García-Pelayo, M. Ruiz-

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742

743 **SUPPLEMENTAL MATERIAL -I**. ARISA methodology

744

745 ARISA (Automated rRNA Intergenic Spacer Analysis, Fisher & Triplett 1999) amplifies an intergenic

746 transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. The ITS was amplified using

747 the primer pair ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITSReub (5´-

748 GCCAAGGCATCCACC-3´) (Cardinale *et al.* 2004). The primer ITSReub was labelled fluorescently with

749 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H2O, 1x 5 PRIME

750 MasterMix including 1.5 mM Magnesium, 200 mM dNTPs, 1.25 U Taq polymerase (5 PRIME, Hamburg,

751 Germany), 0.2 mM of primers and 5µl of diluted DNA 1:10. PCRs were carried out in Eppendorf

752 Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation

753 at 94 °C 2 min, followed by 30 cycles with denaturation at 94 °C 45 s, annealing at 52 °C 45 s and

754 extension at 72 °C 1 min, with a final extension at 72 °C 5 min. Amplified PCR products were diluted 1:10

755 and denatured by heating in formamide. Fragment lengths were determined by mean of automated

756 fluorescent capillary electrophoresis on 3130 Genetic Analyzer. Electropherogram peak values were

757 calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size

758 Standard (both Applied Biosystems).

759 Peak Scanner v 1.0 (Applied Biosystems) was used to determine fragment lengths identifying
760 different bacterial Operational Taxonomic Units (OTUs) within each sample. Scripts in R-environment
761 [http://cran.r-project.org/]) available at http://www.ecology-research.com, were used for binning DNA
762 fragment lengths from different samples. Binning exercise was performed by establishing a window size of
763 4 pair of bases and a distance of two consecutive binning frames (i.e. shift) of 0.1. Only peaks with values
764 of relative intensity of fluorescence larger than 0.09% and fragments above a threshold of 50 fluorescence
765 units that ranged between 100 and 1,000 bp (Ramette 2009) have been considered. Molecular
766 fingerprinting techniques are highly reproducible, robust, and have been proven useful for comparative
767 analysis of microbial community structure (Loisel *et al.* 2006; Bent & Forney 2008).

768 **References**

769 Bent, S.J. & Forney, L.J. (2008). The tragedy of the uncommon: understanding limitations in the analysis
770 of microbial diversity. ISME Journal, 2, 689–695. doi: 10.1038/ismej.2008.44

771 Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A., Rizzi, A., Zanardini, E., Sorlini, C., Corselli,
772 C. & Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal
773 intergenic spacer analysis of complex bacterial communities. Applied and Environmental
774 Microbiology, 70, 6147–6156. doi: 10.1128/AEM.70.10.6147-6156.2004

775 Fisher, M.M. & Triplett, E.W. (1999). Automated approach for ribosomal intergenic spacer analysis of
776 microbial diversity and its application to freshwater bacterial communities. Applied
777 Environmental Microbiology, 65, 4630–4636.

778 Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenès, J.P. & Godon, J.J. (2006). Denaturing
779 gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular
780 fingerprintings revisited by simulation and used as a tool to measure microbial diversity.
781 Environmental Microbiology, 8, 720–731. doi: 10.1111/j.1462-2920.2005.00950.x

782 Ramette, A. (2009). Quantitative community fingerprinting methods for estimating the abundance of
783 operational taxonomic units in natural microbial communities. Applied and Environmental
784 Microbiolology, 75, 2495–2505. doi: 10.1128/AEM.02409-08

785

786 **Acquisition of uropygial gland microbiome by hoopoe nestlings**

787 *Microbial Ecology*

788 M. Martín-Vivaldi[a,b]*, J. J. Soler, Ángela Martínez-García, L. Arco, N. Juárez-García-Pelayo, M. Ruiz-

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792 b) Estación Experimental de Zonas Áridas (CSIC) E-04120 Almería, Spain.
793
794 **SUPPLEMENTAL MATERIAL-II.** Importance of particular OTUs
795
796 In order to know which of the OTUs detected in secretions by ARISA analyses were responsible of the

797 detected experimental effects on cultivable bacterial load and composition of bacterial communities, we

798 explored the association of the presence of each OTU of the core microbiome with these effects.

799

800 **Relationship with cultivable bacterial load**

801 The best GLZ models (those with lower AIC values differing in less than two unities) explaining bacterial

802 load of secretions (considering all secretions from nestlings and females) included combinations of eight

803 OTUs (Table 1) with positive and negative associations. The more clear association was detected for the

804 OTU306 (Fig. 1) suggesting that it is the main component of the microbiome of hoopoe uropygial secretions

805 able of growing in TSA medium in aerobic conditions. The detection of both positive and negative

806 associations between presence of some OTUs and cultivable bacterial density may be the consequence of

807 direct antagonistic effects or competence between both groups of OTUs.

Table 1. Best subsets of OTUs present in hoopoe uropygial secretions explaining bacterial growth in TSA general medium in a GLZ model with logit link function and a normal distribution.

Model		OTUs										df		AIC

1		242	254	306	346							4		578,1685
2		242		306	346		422		534	566		6		578,8353
3		242	254	306	346		422		534	566		7		578,8357
4		242	254	306	346					566		5		578,9262
5		242	254	306	346	406						5		579,0754
6		242	254	306	346				534	566		6		579,1720
7				306	346		422		534	566		5		579,2048
8		242	254	306	346			466				5		579,4899
9				306	346				534	566		4		579,5372
10		242		306			422		534	566		5		579,5399

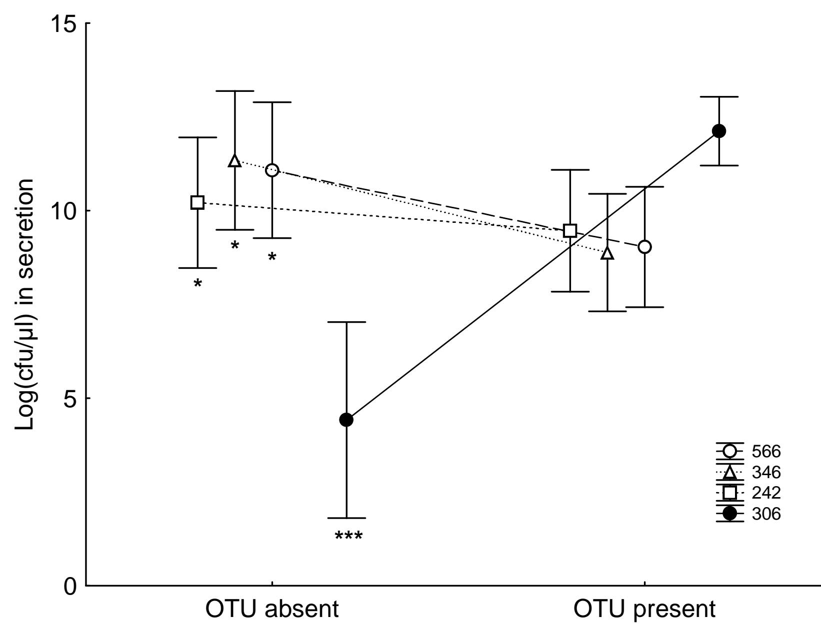

Figure 1. Relationship between the presence of OTUs in hoopoe secretions and their cultivable bacterial load. Only the OTUs from the best subsets in table 2 with a significant Wald value in the whole effects model (* p<0,05, *** p< 0,001) are presented. Whiskers show marginal means and 95% confident intervals

820 **Influence of female microbiome on nestling microbiome**

821

822 The effect of covering female glands on the similarity of the microbiomes of nestlings and females was

823 specially associated to the presence in secretions of three OTUs. Two of them correlated to the space

824 occupied by the microbiomes of nestlings of experimental females (OTU306 and OTU406, Fig. 2) and

825 another correlated to the position of the microbiomes of nestlings of control females (OTU466, Fig. 2). In

826 accordance with this interpretation, the presence of these three OTUs in nestlings was affected by

827 manipulation of female access to its gland (Table 2). In addition, prevalence of the OTU346 in nestlings was

828 significantly associated with female experimental treatments when considering only females with the OTU

829 in their glands (GLZ, Wald= 4.78, $p = 0.029$, $q = 0.046$). For two additional OTUs (OTU466 and OTU346)

830 the manipulation of female access to their glands caused a reduction in the percentage of nestlings that

831 harbored the OTU (Fig. 3). On the other hand, two OTUs (306 and 406) were more frequent in nestlings

832 from experimental than from control females (Fig. 3).

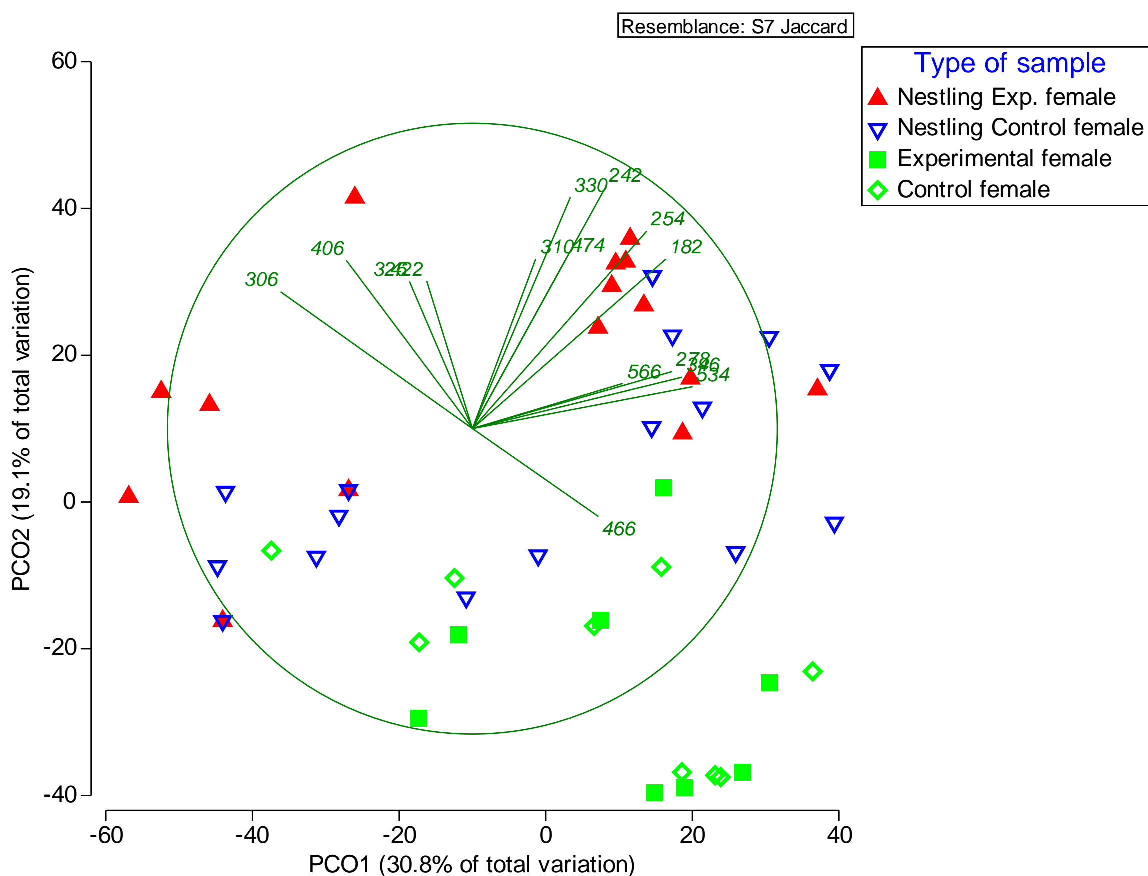

Figure 2. PCO plot showing the resemblance between samples of the experiment manipulating female access

to its gland. The influence of each of the OTUs of the core microbiome is represented by the green lines being their length the value of the Spearman correlation coefficient.

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Table 2. Influence of (1) the presence of an OTU in female secretions and (2) the experimental manipulation of female access to its gland (female treatment), on the occurrence of the same OTU in nestlings uropygial gland secretions. The table shows the results only for the four OTUs of the core microbiome with a significant effect of these factors. The best models according to the AIC criterion for each OTU in a GLZ design with both factors as predictors are presented. We applied the false discovery rate correction for multiple tests (the 14 tests for the OTUs of the core microbiome present in females) to calculate the q-values (adjusted p-values). The factors of each model with a significant effect are indicated in bold.

OTU	Model	OTU presence in female	Female treatment	df	AIC	L.Ratio Chi^2/Wald	p	q-value
306	1	+	**+**	2	35,49	9,81	0,007	**0.030**
	2		**+**	1	36,21	7,09	0,008	**0.034**
406	1		**+**	1	35,54	5.56	0,018	**0.046**
	2	+	+	2	37,33	5.77	0,056	0.143
466*			**+**	1	-	4,86	0,028	**0.046**
566	1	**+**		1	30,55	20,11	<0,00001	**<0.0001**
	3	**+**	+	2	32,47	20,19	<0,0001	**<0.001**

836 * Given that OTU466 was present in all control females, we excluded the factor "OTU presence in female" from this analysis

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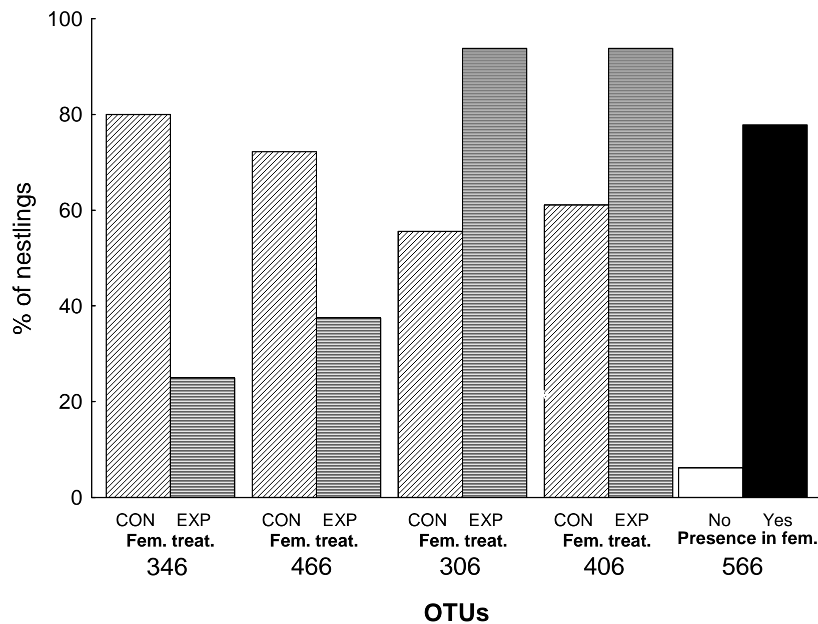

839

840 Figure 3. Influence of the experimental covering of female glands (Fem. treat.) and the presence of an OTU in females at the
841 beginning of incubation (Presence in fem.) on its occurrence in their nestlings at 16 days of age. Graphs show only the effect for the
842 five OTUs for which female manipulation or its presence in females significantly influenced presence in nestling secretions. CON =
843 Control females. EXP = Experimental females. For OTU346, only the nests with its presence in the female (9 females and 18
844 nestlings) are considered (see text), for the remaining OTUs all nests with available information on the microbiome of female
845 secretion are included (17 females and 34 nestlings). The OTU566 was present in nine females and absent in eight females.

846

847

848 Occurrence of the OTU566 in the microbiome of nestlings and their mothers were positively associated (Fig.

849 3) independently of female experimental treatment (Wald = 0.077, p = 0.782). This result suggests

850 independent acquisition of nestlings and females within the nest environment, not dependent on transmission

851 to nestlings from the mother's gland after female manipulation.

852

853 **Microbiome enrichment by cross-inoculation**

854

855 Most cross-inoculated nestlings acquired new OTUs. Indeed, the final samples of cross-inoculated nestlings

856 clearly congregated in the corner of the PCO space correlating with a greater number of OTUs (Fig. 4). The

857 two OTUS whose prevalence in nestlings was reduced by the experiment covering female glands (OTU346

858 and OTU466) were closely associated with this section of the PCO space (Fig. 4) suggesting that they are

859 among the components of the community that explain the detected experimental effects of cross-inoculation.

860 Moreover, for 10 out of the 14 OTUs of the core microbiome present in females (all except OTU278,
861 OTU310, OTU330, OTU350 and OTU474), there was a significant relationship of the initial differences
862 between receiver and donor and the change in harboring a particular OTU after inoculation (Pearson Chi-
863 squares, after FDR correction for multiple tests, all $q < 0.05$). For these ten OTUs, in 92.1 % of the cases in
864 which they were present in a donor and not in the receiver, the inoculated nestling incorporated the OTU to
865 its microbiome.

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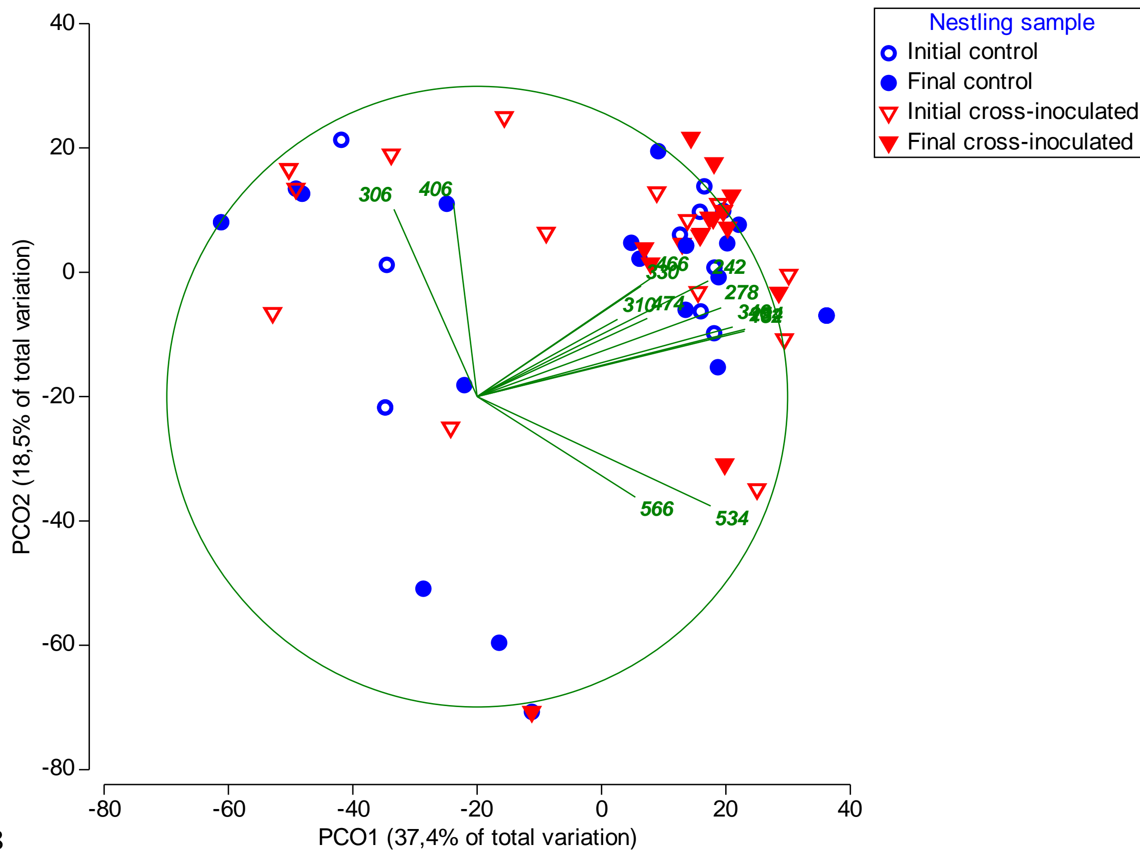

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869 Figure 4. PCO plot showing the resemblance between initial and final samples of nestlings for the two groups of the inoculation
870 experiment. The vectors of the correlations of all OTUs with PCO axes are drawn in green.

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