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## The gut microbiota of brood parasite and host nestlings reared within the same environment: Disentangling genetic and environmental effects.

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1 **The gut microbiota of brood parasite and host nestlings reared within**  
2 **the same environment: Disentangling genetic and environmental**  
3 **effects.**

4  
5 Running title: Gut microbiota of parasites and hosts

6  
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## 30 **Summary**

31 Gut microbiota are essential for host health and survival, but we are still far  
32 from understanding the processes involved in shaping their composition and  
33 evolution. Controlled experimental work under lab conditions as well as  
34 human studies pointed at environmental factors (i.e., diet) as the main  
35 determinant of the microbiota with little evidence of genetic effects, while  
36 comparative interspecific studies detected significant phylogenetic effects.  
37 Different species, however, also differ in diet, feeding behavior and  
38 environmental characteristics of habitats, all of which also vary  
39 interspecifically, and, therefore, can potentially explain most of the detected  
40 phylogenetic patterns. Here, we take advantage of the reproductive strategy  
41 of avian brood-parasites and investigate gut microbiotas (esophageal (food  
42 and saliva) and intestinal) of great spotted cuckoo (*Clamator glandarius*) and  
43 magpie (*Pica pica*) nestlings that grow in the same nests. We also estimated  
44 diet received by each nestling and explored its association with gut  
45 microbiota characteristics. Although esophageal microbiota of magpies and  
46 great spotted cuckoos raised within the same environment (nest) did not vary,  
47 the microbiota of cloacal samples showed clear interspecific differences.  
48 Moreover, diet of great spotted cuckoo and magpie nestlings explained the  
49 microbiota composition of esophageal samples, but not of cloaca samples.  
50 These results strongly suggest a genetic component determining the  
51 intestinal microbiota of host and parasitic bird species, indicating that  
52 interspecific differences in gut morphology and physiology are responsible  
53 for such interspecific differences.

54

55 **Key words:** Brood parasitism, Diet, Gut microbiota, Genetic factors, Great  
56 spotted cuckoo, Magpie

## 57 **Introduction**

58 The study of microbiotas associated with different environments, including  
59 animal hosts, has become one of the most fruitful areas of biological research  
60 during the last decade [1-3]. Reasons include that microorganisms should  
61 have profound impact on animal life and evolution, particularly those in  
62 close contact with animals, as is the case of gut microbiota [4, 5]. Gut  
63 microbiota shares intricate relationships with their hosts and are essential for  
64 host health and survival [6, 7]. For instance, it plays a key role in the  
65 development of a healthy immune system [8, 9], brain physiology and  
66 behavior [10], host protection from pathogenic infections [11], nutrient  
67 absorption [12] and provisioning of hosts with essential or otherwise  
68 inaccessible nutrients from food [13]. Despite the importance of gut  
69 microbiotas for animal life, we are still far from a general understanding of  
70 the processes involved in shaping their composition and evolution [14].

71       Controlled experimental work under lab conditions with animal  
72 models, as well as human studies, clearly detected strong environmental  
73 components determining gut microbiota with little evidence of genetic  
74 effects [15, 16]. Diet is, perhaps, the main environmental factor since  
75 experimental modifications of diets resulted in drastic changes in gut  
76 microbiota [17-19]. Social and physical environments are also important as  
77 both experimentally cohoused mice [20, 21] and cohabiting unrelated  
78 humans [22, 23] showed convergent gut microbiota composition. Studies of  
79 wild animals have also reported important variation in gut microbiotas in  
80 relation to, for instance, seasonal and habitat differences [24, 25]. Effects of  
81 genetic factors determining gut microbiota at the intraspecific level are  
82 considered rather weak [18, 26, 27]. Conversely, most evidence of genetic  
83 factors influencing gut microbiota comes from comparative interspecific  
84 studies showing consistent species-specific differences [15, 28, 29] or even  
85 phylogenetic covariation between animals and their gut microbial

86 communities [30-32]. Different species, however, also differ in diet, feeding  
87 behavior and environmental characteristics of inhabited areas, and, therefore,  
88 most of the phylogenetic patterns may also be driven by environmental  
89 factors that vary interspecifically, making environmental factors co-vary  
90 with phylogenetic differences [32, 33]. This makes it difficult to disentangle  
91 the effects of environmental and genetic (i.e. phylogenetic) factors on gut  
92 microbiota.

93 To cope with this problem, Knowles et al. [15] explored the effect of  
94 habitat (i.e., location) and common evolutionary history (i.e., species  
95 identity) in species of three widespread mammal genera, some of them living  
96 in sympatry with each other. This approach allowed them to statistically  
97 separate the effects of habitat and species, concluding that the latter was  
98 dominant over the former factor [15]. Interestingly, interspecific differences  
99 in gut microbiota positively covaried with dietary differences, and, thus, the  
100 detected genetic component may also reflect the species-specific dietary  
101 niche of hosts. However, gut morphology and physiological characteristics  
102 of hosts are usually closely related to dietary niche [33-35]. Consequently,  
103 effects of gut microbiota that are *a priori* attributable to dietary niche might  
104 be over-interpreted, and should be controlled not only for host phylogeny but  
105 also for gut morphology [28]. Thus, the genetic effect of gut microbiota of  
106 animals due to anatomical or physiological characteristics should be  
107 determined after controlling for the effect of diet, sociality, and physical  
108 environment. Avian brood parasitism model systems bring us a golden  
109 opportunity to dissect this relationship [36].

110 Brood parasitism is a form of reproductive strategy by which parasites  
111 lay their eggs in the nests of other species so that when their eggs hatch in  
112 the host's nest, the host adults subsequently rear their offspring [37]. Brood  
113 parasites and their hosts are usually phylogenetically distant species and,  
114 during growth, nestlings of both species receive a similar diet in identical

115 social and physical environments [38]. In addition, brood parasite and host  
116 adults may differ in their dietary niche and consequently in gut morphology  
117 and physiology. In this case, interspecific differences in gut microbiota of  
118 cuckoo and host nestlings would be hardly explained by environmental  
119 factors [36]. The brood parasite – host system formed by the great spotted  
120 cuckoo (*Clamator glandarius*) (hereafter cuckoo) and their magpie (*Pica*  
121 *pica*) host fits all these characteristics. They are phylogenetically distant  
122 species [39], brood parasite and host nestlings may develop together within  
123 the same nest and are fed with a similar diet by adult magpies [40, 41].  
124 Moreover, while adult magpies have an omnivorous diet [42], adult great  
125 spotted cuckoos mainly feed on hairy caterpillars [38], and, consequently,  
126 morphology of their guts differs even at the fledgling stage [43]. We already  
127 know that the gut microbiota of cuckoo nestlings differ from that of host  
128 nestlings [36, 43] and is intermediate between those of cuckoo adults and  
129 magpie nestlings [36]. However, the cuckoo and magpie nestlings used for  
130 comparison in these previous studies did not share their nests with nestlings  
131 of the other species. Thus, it is possible that differences in diet and/or in  
132 transmitted microbiota from adult magpies to nestlings were partially  
133 responsible for the detected interspecific differences, and, as a result, the  
134 genetic component of gut microbiota may have been over-interpreted.

135 To demonstrate further differences in the genetic component of the gut  
136 microbiota of cuckoo and magpie nestlings, we here experimentally moved  
137 cuckoo eggs among magpie nests so that the cuckoo eggs hatched a few days  
138 later than magpie eggs in these nests. This procedure dramatically increased  
139 the likelihood of nestlings of both species surviving until the fledging stage  
140 while sharing identical social and physical environments [41]. Moreover, we  
141 collected bacterial samples from the cloaca and from the nestlings' beak  
142 (esophagus), which included those of saliva and food received from magpie  
143 adults. These samples allowed us to explore for the first time the possibility

144 that interspecific differences detected in these esophageal microbiotas  
145 explained previously detected differences in cloacal microbial communities.  
146 Within this framework, a genetic component determining interspecific  
147 differences in the gut microbiota will be identified if esophageal microbiotas  
148 of cuckoo and host siblings do not vary, but clear differences in cloacal  
149 microbiota are detected. This prediction assumes that cuckoos and magpies  
150 that share the same nests are fed a similar diet that bestows similar food  
151 associated microbiotas. Here, we tested these predictions and assumptions of  
152 genetic component explaining interspecific differences in gut microbiotas of  
153 magpies and great spotted cuckoos. Bacterial groups responsible for  
154 interspecific differences in gut microbiotas of magpies and great spotted  
155 cuckoo have been described elsewhere [36], and, thus, here we concentrate  
156 on exploring the associations between esophageal and intestinal bacterial  
157 communities, as well as the association between microbiotas and diet.

158

## 159 **Materials and Methods**

160

### 161 *Study area and species*

162 The study was performed in the Hoya de Guadix (37°18' N, 3°11' W),  
163 southern Spain; a high-altitude plateau where magpie nests are frequently  
164 parasitized by great spotted cuckoos [44]. The vegetation is sparse with oak  
165 (*Quercus ilex*), almond (*Prunus dulcis*) and pine (*Pinus halepensis* and *Pinus*  
166 *pinaster*) trees where a relatively high density of magpie nests is present.

167 Magpies lay one egg per day until they complete a typical clutch size  
168 of six or more eggs. Normally, magpies start to incubate when the 4<sup>th</sup> egg is  
169 laid, and their eggs hatch after 21 days of incubation. The great spotted  
170 cuckoo is a non-evictor cuckoo, and it can share a nest with foster siblings.  
171 Nonetheless, the incubation period of cuckoo eggs is 5-6 days shorter than  
172 that of magpie eggs [41]. Thus, in the case that brood parasitism occurs

173 before magpie incubation starts, cuckoo eggs hatch some days before those  
174 of magpies, which confers a great advantage to cuckoo nestlings when  
175 competing for food with foster siblings during the nestling period [40, 45].  
176 In most cases, magpie nestlings are outcompeted by cuckoo nestlings unless  
177 brood parasitism occurs after magpies finish laying their eggs [46]. The  
178 nestling periods also differ according to species, with cuckoos spending 16-  
179 18 days and magpies 21-23 days in the nest [47].

180

### 181 *Field work*

182 At the beginning of April 2018, we searched intensively on a weekly basis  
183 for new magpie nests in the study area, which allowed us to detect new  
184 magpie nests during the egg laying stage. In the case of brood parasitism,  
185 surveillance of magpie nests allowed us to envisage hatching time of magpie  
186 and cuckoo eggs. To maximize the number of nests where great spotted  
187 cuckoo and magpie nestlings developed together, we translocated cuckoo  
188 and magpie eggs among nests to adjust expected hatching day of magpie  
189 chicks to be two days in advance of cuckoo eggs (Fig. 1). We did so in eight  
190 magpie nests from which both great spotted cuckoos and magpies  
191 successfully fledged.

192         When magpie and cuckoo nestlings in experimental nests were about  
193 12 and 10 days old respectively, we collected esophageal samples, i.e. the  
194 food and the saliva they received from adult parents, by means of neck-collar  
195 trials [48] (Fig. 1). Briefly, we used plastic zip ties of three millimeters width  
196 and of different color for nestling recognition. These neck-collars were  
197 carefully placed around nestlings' necks in such a way that it allowed normal  
198 respiration, but ingestion of food provided by adult magpies was prevented.  
199 Once neck-collars were adjusted, we cut the remaining length of zip ties and  
200 returned the chicks to the nest. After approximately two hours, we returned  
201 to the experimental nest, collected food samples from nestlings' esophagus,



202 and carefully cut the zip ties with scissors. Since adult magpies transported  
203 food to the nests in their gular cavity and regurgitated food to begging  
204 nestlings, nestlings received not only food, but also saliva from their parents.  
205 Therefore, the liquid part of the collected samples consisted of a mixture of  
206 adult magpie and nestling saliva. Samples were immediately transferred into  
207 500  $\mu$ L of lysis buffer (50 mM Tris-HCl, 0,5 % SDS, 2 mM EDTA, 100 mM  
208 NaCl) and kept at 4°C in a portable fridge until arrival at the laboratory a few  
209 hours later, where the samples were frozen at -20°C for further analysis.  
210 Collar trials were only performed in experimental nests (number of nests =  
211 8, number magpie nestlings sampled = 17, number of cuckoo nestling  
212 sampled = 8).

213 Cloacal samples were collected from magpie and cuckoo nestlings  
214 close to the time of abandoning their nests (15-16 and 18-19 days old  
215 cuckoos and magpies, respectively). Only the two heavier host nestlings and  
216 all cuckoo nestlings found in magpie nests were sampled. These samples  
217 were collected by injecting and repipetting 500  $\mu$ L of sterile phosphate buffer  
218 (0.1 M  $\text{Na}_2\text{HPO}_4$  and 0.1 M  $\text{NaH}_2\text{PO}_4$ , pH 7.4) in nestlings' cloaca using  
219 sterile tips and an automatic micropipette. Collected samples were stored in  
220 500  $\mu$ L of lysis buffer (50 mM Tris-HCl, 0,5 % SDS, 2 mM EDTA, 100 mM  
221 NaCl), and kept at 4°C in a portable fridge until the portable fridge was  
222 transported back to the laboratory a few hours later. Samples were then  
223 stored at -20 °C until further analysis.

224

### 225 *Sample preparation*

226 For food item identifications, samples were laid out on a petri dish where  
227 food items were separated from each other. Since we were interested in  
228 characterizing bacterial communities of food, to avoid contamination,  
229 manipulation of prey items was conducted under aseptic condition, with  
230 previously sterilized tweezers while wearing gloves previously washed with

231 ethanol. Once food items were separated from each other, we took photos  
232 that were later used for identification. Whenever possible, invertebrate  
233 animal food was classified to the family level via visual inspection (ESM-  
234 Figure 1). Detected vegetable food items were in all cases green barley grain.  
235 Identification of some small pieces of food items was not possible and they  
236 were labelled as ‘unidentified’.

237 Esophageal samples contained solid tissues in suspension with buffer  
238 lysis and saliva from adult and nestling birds. The liquid content (hereafter  
239 saliva) were separated from the solid one and kept in different 2 mL  
240 microfuge tubes. Collar samples containing solid tissues (hereafter,  
241 esophageal samples) and cloaca samples were first mechanically  
242 homogenized using zirconia ceramic beads.

243 Briefly, 2 mL microcentrifuge tube filled with zirconia (100 mg)  
244 ceramic beads of approximately 2 mm depth were added with 800  $\mu$ L of  
245 cloacal samples; or with 100 mg of previously mixed esophageal samples  
246 adding solution 2 (Xtrem biotech®) to make up 800  $\mu$ L. The tubes were then  
247 vortexed for three minutes for mechanical digestion of solid matters. Tubes  
248 containing saliva sample was centrifuged in 18,000 rpm for two minutes and  
249 the precipitate was suspended in 700  $\mu$ L of lysis solution. Afterward, the  
250 tubes containing the three types of samples were added with 10  $\mu$ L of  
251 proteinase K (10 mg/mL) and incubated in thermal blocks in two rounds of  
252 incubation (55°C for 15 min, and 90°C for eight min). The tubes were then  
253 cooled at room temperature before being centrifuged at 14,000 rpm for five  
254 minutes. 700  $\mu$ L of supernatants were transferred to new 1.5 mL  
255 microcentrifuge tubes and 250  $\mu$ L of solution 3 (Xtrem biotech®) was added  
256 into each tube followed by vortex for 30 seconds. The tubes were then  
257 incubated in ice for 10 minutes and centrifuged in 14,000 rpm for five  
258 minutes. 700  $\mu$ L of supernatant from each tube was then transferred to new  
259 microcentrifuge tubes. 650  $\mu$ L of binding buffer (20 mM HCl-Tris pH 8, 4

260 M Guanidinium isothiocyanate, 1 mM DTT) and 50  $\mu$ L of silica beads (Sigma  
261 S-5631) dissolved in 6 M Guanidinium Chloride [49] was added to each tube  
262 followed by agitation by inversion. The tubes were then centrifuged in  
263 10,000 rpm for one minute. The supernatant was discarded by vacuum  
264 suction and the precipitate was then resuspended in 500  $\mu$ L of binding buffer  
265 for two minutes. The tubes were centrifuged again in 10,000 rpm for one  
266 minute and the supernatant was discarded via vacuum suction. 1000  $\mu$ L of  
267 wash buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM NaCl, 50 %  
268 ethanol) was added to suspend the precipitate for two minutes and the tubes  
269 were then centrifuged in 10,000 rpm for one minute. The supernatant was  
270 discarded, and the pellet was dried in vacuum using a drying machine. For  
271 DNA elution, 200  $\mu$ L of MilliQ water were added and the tubes were  
272 incubated in 55 °C for five minutes. Finally, the tubes were centrifuged in  
273 18,000 rpm for 2 minutes. This step was repeated once to increase the purity  
274 of DNA from the tube. The supernatant was collected for further analysis.

275

#### 276 *DNA amplification for high throughput sequencing*

277 We amplified the region spanning from V6 to V8 of the bacterial gene 16S  
278 RNA following procedure described in Ruiz-Rodríguez et al. [36]. First  
279 PCR was carried out using two sets of primers, B969F\_bar1 and BA1406R  
280 and B969F\_bar2 and BA1406R. PCR was performed in 25  $\mu$ L reactions  
281 containing 2.5  $\mu$ L of 3 mM of each primer, 2.5  $\mu$ L of PCR water, 12.5  $\mu$ L of  
282 iProof HF Master Mix (BioRad®) and 5  $\mu$ L of diluted DNA 1:10. PCR  
283 conditions included 98 °C for one minute, followed by 25 cycles of 98 °C  
284 for 10 seconds, 52 °C for 20 seconds and 72 °C for 15 seconds, and a final  
285 elongation step at 72 °C for 5 minutes. PCR products were purified using  
286 HigherPurity™ DNA Purification SPRI Magnetic Beads (Canvax®)  
287 according to user manual and eluted in 40  $\mu$ L of distilled water. Successful  
288 amplification was checked by electrophoresis in 1 % agarose.

289 Second PCR was applied to add the ‘barcodes’ (Nextera XT barcodes)  
290 designed to tag individual samples. These barcodes contain sequences that  
291 overlap with the sequences of the primers used in the first PCR [50]. PCR  
292 was performed in 25  $\mu\text{L}$  reactions containing 3.3  $\mu\text{L}$  of 3 mM of each primer,  
293 0.9  $\mu\text{L}$  of PCR water, 12.5  $\mu\text{L}$  of iProof HF Master Mix (BioRad®) and 5  
294  $\mu\text{L}$  of purified PCR product from first PCR. This PCR was conducted by  
295 98°C for one minute, followed by 8 cycles of 98°C for 10 seconds, 55°C for  
296 20 seconds and 72°C for 15 seconds, and a final elongation step at 72°C for  
297 five minutes. Purification steps from the first PCR were repeated and eluted  
298 in 40  $\mu\text{L}$  of distilled water and electrophoresis in 1 % agarose were  
299 performed to determine successful amplification.

300 Concentration of DNA of PCR product was measured using Qubit®  
301 3.0 Fluorometer. The sample with the lowest concentration of DNA was  
302 chosen as the baseline concentration and the concentration of each sample  
303 was adjusted to be equimolar to this baseline concentration before all the  
304 samples were pooled. High throughput sequencing was performed in a single  
305 cartridge in Illumina MiSeq platform (López Neyra Institute, Granada,  
306 Spain).

307

### 308 *Sequence analyses*

309 Sequence data were analyzed with QIIME 2 v2018.8 (Quantitative Insights  
310 Into Microbial Ecology) [51, 52]. Primer trimming and sequence quality  
311 filtering were performed using default parameters. Paired sequences were  
312 joined following default parameters in QIIME2. We then used Deblur, a sub-  
313 Operational Taxonomic Unit (sOTU) to further remove sequencing errors  
314 and to create the sub-OTU table [53] with a fragment length set at 380 bp.  
315 Afterwards, sequences were aligned and followed by building a de novo  
316 phylogenetic tree following Janssen et al. [54]. Taxonomy assignment was  
317 performed based on Greengenes 13\_08 with a similarity of 99% [55]. Lastly,

318 non-bacterial, chloroplastidial and mitochondrial sequences were removed  
319 from the sub-OTU table as well as non-taxonomically assigned sequences at  
320 the phylum level.

321 Sequences from next-generation sequencing techniques may include  
322 reagent contaminants due to DNA extraction, amplification, and library  
323 preparation kits. This may influence characterization of bacterial  
324 communities, mainly of those with very small microbial biomass [56, 57].  
325 However, we analyzed cloacal and esophageal samples, which contained  
326 relatively high bacterial biomass. More importantly, we were not interested  
327 on characterizing microbiotas, but exploring expected differences between  
328 microbiotas that had been characterized with identical protocols and, thus,  
329 included identical reagent contaminants if any. Thus, reagent contaminants  
330 would negligibly influence our results.

331

### 332 *Statistical analyses and sample sizes*

333 We examined the effect of genetic and non-genetic components in the  
334 determination of gut microbiota by exploring the influence of species  
335 identity and gut site in alpha and beta microbial diversity [58]. Alpha  
336 diversity is the diversity within a sample, while beta diversity measures the  
337 between samples differences.

338 Alpha diversity of gut microbiotas was explored by means of  
339 Shannon's diversity index, which considers the number of detected OTUs  
340 and abundance of each of them [59]. Interspecific differences were explored  
341 in General Linear Mixed Models (GLMM) by full crossing the two  
342 independent variables, i.e. species identity (magpie and cuckoo) and gut  
343 region (saliva, food and cloaca) as the independent factors, and nest identity  
344 as random factors. The model also included interactions between fixed and  
345 random factors to account for the repeated measurement nature (i.e. within  
346 nest comparisons) of our data sets. Residuals were visually inspected for

347 normality and homoscedasticity, justifying the use of parametric tests. Alpha  
348 diversity statistical tests were performed in Statistica 10.0.

349 Beta diversity matrices were calculated using weighted and  
350 unweighted UniFrac distance [60, 61] based on a rarefied OTU table at 4000  
351 sequence per sample. Principal Coordinate Analysis (PCoA) plot was  
352 generated using these UniFrac distance matrices. While weighted Unifrac  
353 weighs sequence according to the number of sequence found per OTU in a  
354 given sample, unweighted UniFrac simply counts the absence or presence of  
355 an OTUs in a given sample. In other words, for any given sample, weighted  
356 UniFrac gives more importance to the most abundant bacteria while  
357 unweighted UniFrac gives more importance to bacteria found in minority.  
358 Both weighted and unweighted UniFrac distance matrices were then used in  
359 non-parametric multivariate statistical test, PERMANOVA, implemented in  
360 PRIMER-7 (Anderson, 2001) with 9,999 permutations. Briefly, the models  
361 testing for interspecific and gut location differences included these two  
362 factors as fixed effects, and nest identity nested within the interaction of  
363 fixed factors as random effects. To explore interspecific differences of  
364 microbiota associated to the cloaca, saliva and food of magpies and great  
365 spotted cuckoos, we ran three separate models that included species and nest  
366 identity as the fixed and the random factors, respectively. Similarly, to  
367 explore within species differences in microbiota due to gut locations, we ran  
368 two sets of models, one for great spotted cuckoos and another for magpies  
369 that included gut location and nest identity as fixed and random factors,  
370 respectively. Post-hoc comparisons between pairs of gut locations were  
371 explored in separate models with two-level fixed factor (i.e. pairwise). By  
372 including nest identities as random factor, all these PERMANOVAs  
373 assumed within-nests comparisons and, thus, only considered nests with the  
374 two species and the three gut locations, which drastically reduces degrees of  
375 freedom. However, because the effects of fixed factors rarely depended on

376 nest identity (see Results), to increase statistical power, we also ran models  
377 that did not include the random factor and showed results in Electronic  
378 Supplementary Material (ESM, Table 2).

379         The associations between diet and microbiotas were explored by  
380 means of Mantel tests in the R environment (Version 3.6.1.). Matrices of  
381 dissimilarities of diet were calculated based on Bray Curtis dissimilarity  
382 index. Matrices of dissimilarities of species identity were built with 0's  
383 (when the pairwise of individual was of the same species) and 1's (when the  
384 pairwise of individual was of different species). As matrices of similarities  
385 of bacterial communities, we used both weighted and unweighted UniFrac  
386 distance matrices. Afterward, by using the R package 'ecodist' version 1.2.3  
387 [62], we performed autocorrelation analyses (Mantel tests with 9,999  
388 permutations), with matrices of bacterial communities at different locations  
389 as dependent variables, and matrices of species identity and diet as  
390 explanatory variables. We estimated the strength of the correlation (Beta  
391 values), and whether these values differed significantly from zero (P values).  
392 Unfortunately, nestlings were not individually marked at the time of  
393 sampling their diet and, thus, we were not able to match diet with cloaca  
394 samples at the level of the individual. To solve this problem, we randomly  
395 matched cloaca and diet samples within the same nests and species. We did  
396 so four times and, because results did not vary qualitatively, we used average  
397 estimates and associated p-values.

398         We sampled microbiota of 33 magpie and 22 great spotted cuckoo  
399 nestlings in 18 magpie nests. In ten of these magpie nests, great spotted and  
400 magpie nestlings grew together, in six only magpies, and in two nests only  
401 cuckoos developed. As a rule, only two magpies and all cuckoo nestlings  
402 were sampled per nest, the cloaca was always sampled, but saliva and food  
403 were only sampled in 8 nests containing cuckoo and magpie nestlings.  
404 Bacterial DNA amplification and sequencing failed for some samples and,

405 thus, samples sizes shown in the ESM (Table 1) differed among nests,  
406 species, and sample location.

407

## 408 **Results**

409 Gut microbiota of great spotted cuckoo nestlings were not significantly more  
410 diverse than those of the magpie host nest-mates, independently of gut  
411 location (GLMM,  $F = 0.18$ , d.f. = 1, 11.8,  $P = 0.68$ ). Moreover,  
412 independently of species (GLMM, interaction between species and gut  
413 location,  $F = 0.03$ , d.f. = 2, 10.6,  $P = 0.97$ ), cloacal microbiota tends to be  
414 more diverse than the microbiota of saliva or food (GLMM,  $F = 3.28$ , d.f. =  
415 2, 10.6,  $P = 0.064$ , Fig. 2).

416 In beta diversity analyses, we found that microbiota of nestlings reared  
417 in magpie nests depended on species identity (magpie *vs.* great spotted  
418 cuckoo) and gut location (cloaca, saliva and food) (Species x Gut location  
419 comparisons in Table 1,  $P < 0.001$ , and ESM-Table 2). The effect of species  
420 identity was, therefore, due to the microbiota detected when comparing  
421 cloacal samples, since microbiota of esophageal samples (saliva and food)  
422 did not differ interspecifically (see species comparisons in Table 1).  
423 Accordingly, principal coordinate analyses on both unweighted and  
424 weighted UniFrac distances showed clear clustering of cloacal samples ( $P <$   
425  $0.012$ ), but those of saliva ( $P > 0.5$ ) or food samples ( $P > 0.5$ ) of both species  
426 were distributed randomly between species (Table 1, ESM-Table 2, Fig.  
427 3). Intraspecific differences due to gut microbiota locations reached statistical  
428 significance in both great spotted cuckoo ( $P < 0.003$ ) and magpie (only for  
429 weighted UniFrac distances,  $P = 0.004$ ) nestlings (Table 1, ESM-Table 2,  
430 Fig. 4). These differences were mainly due to those between cloacal  
431 microbiota and microbiota of esophageal (both saliva and food) samples  
432 (Table 1, ESM-Table 2), which were more apparent in cuckoo than in magpie  
433 samples (Fig. 4).



434 Finally, diet collected from great spotted cuckoos and their magpie  
435 foster siblings explained differences in gut microbiota at different locations  
436 after controlling for the effect of species identity (Table 2). The strength of  
437 the positive associations, however, varied depending on the location and the  
438 diversity index used to estimate distance matrices of microbiota. In terms of  
439 unweighted UniFrac differences, diet of great spotted cuckoo and magpie  
440 nestlings explained microbiota composition of esophageal samples (Saliva:  
441  $P = 0.049$ ; Food:  $P = 0.001$ ), but not of cloaca samples ( $P = 0.32$ ).

442

### 443 **Discussion**

444 Although esophageal microbiota of magpies and great spotted cuckoos  
445 raised within the same environment (nest) did not vary significantly, the  
446 microbiota of cloacal samples showed clear interspecific differences.  
447 Analyses were performed by comparing microbiotas of great spotted  
448 cuckoos with those of their magpie foster siblings, with which they shared  
449 environmental conditions and parental care from the same adults. Thus,  
450 interspecific differences can only be interpreted as being the result of genetic  
451 factors determining gut microbiota. Moreover, diet of great spotted cuckoo  
452 and magpie nestlings explained microbiota composition of esophageal  
453 samples, but not of cloaca samples, pointing out the well-known role of diet  
454 determining the gut microbiota. Below, we discuss the importance of these  
455 results, possible caveats of the statistical inferences, and the advantages of  
456 using parasitism as a model system to disentangle genetic and environmental  
457 factors determining gut microbiota, and to explore mechanisms underlining  
458 interspecific differences as well as functional consequences.

459 Given the importance of gut microbiota for animal life, including brain  
460 physiology and behavior of hosts [10], determining the importance of  
461 environmental and genetic factors explaining the gut microbiota of animals  
462 is a major topic not only in microbiology, but also in ecology and evolution

463 [3]. Most support for a genetic component of gut microbiota in animals came  
464 from comparative studies where species-specific differences and  
465 phylogenetic relatedness influences were considered as evidence of genetic  
466 factors determining gastro-intestinal microbiota [30, 32, 35]. However,  
467 different species also varied in environmental conditions that they  
468 experience and their lifestyles, including those related to habitat  
469 characteristics, foraging behavior and diet; all of which being well-known  
470 environmental factors determining gut microbiota of animals.

471 To overcome such problems when exploring genetic influences,  
472 several recently published papers carried out comparative analyses while  
473 controlling for interspecific differences in diet composition [28, 32], habitat  
474 exploitation [15, 36], and/or host physiology [32]. Briefly, Amato et al. [28]  
475 compared the gut microbiota of 18 species of wild non-human primates that  
476 represented a range of gut morphological specialization. They concluded that  
477 the influence of host phylogeny was much stronger than that of host dietary  
478 niche, mainly because of the importance of host physiology as a determinant  
479 of gut microbiota. The rationale of the study is that the represented species  
480 do not share identical environment and, thus, authors statistically controlled  
481 for host geographic locations and actual dietary intake. In contrast, Knowles  
482 et al. [15] explored the microbiota and diet of sympatric small mammal  
483 species across multiple habitats, which allowed for statistically disentangling  
484 environmental (habitat and diet) and genetic (species identity) components  
485 of gut microbiota. They also found that diet shapes microbiota but due to  
486 species-specific diet, they concluded in favor of a large influence of genetic  
487 factors explaining gut microbiota of small mammals. However, another  
488 study of artiodactyls that also explored the gut microbiota of pairs of  
489 sympatric species across the Americas failed to detect phylogenetic effects  
490 explaining gut microbiota, although each geographic area displayed a unique  
491 gut microbiota composition [63]. Finally, Ruiz-Rodríguez et al. [36] took

492 advantage of the brood parasitism model-system and detected that gut  
493 microbiota of magpie nestlings differed from that of great spotted cuckoo  
494 nestlings although both species were reared in different nests of the same  
495 species. Here we went one step further and experimentally forced cuckoos  
496 and magpies to develop within the same nests, so that they share the same  
497 parents under identical environmental conditions. Our results confirm  
498 interspecific differences in cloacal microbiota, therefore, suggesting that  
499 previous findings were not due to magpies and great spotted cuckoos  
500 developing in different magpie nests.

501         Several non-exclusive possibilities might explain interspecific  
502 differences after controlling for environmental conditions. The first one is  
503 related to the existence of processes of host-symbiont co-diversification due  
504 to vertical inheritance of microbiota; a hypothesis predicting that gut  
505 microbiota diversification should increase with phylogenetic distances for  
506 which some studies found support [15, 64, 65]. However, vertical  
507 transmission of gut microbiota cannot explain the detected interspecific  
508 differences between magpies and cuckoos because brood parasitic nestlings  
509 do not have contact with cuckoo adults until nest independence [66].

510         Another possibility is that closely related species, or individuals of the  
511 same species, are more likely to share genetic or behavioral mechanisms that  
512 allow horizontal acquisition of similar bacteria from the environment [5].  
513 This mechanism includes dietary preferences, which cannot explain the  
514 detected interspecific differences in cloacal microbiotas of cuckoos and  
515 magpies because of our results and previous research [40] showed that  
516 magpies and great spotted cuckoos received a similar diet from magpie  
517 adults. Moreover, the microbiota of food collected from esophageal samples  
518 did not differ between species and, thus, external environmental conditions  
519 related to diet cannot explain interspecific differences in the intestinal  
520 microbiota of these two species. Interestingly, and in agreement with this

521 inference, among-individual differences in diet were positively related to  
522 differences in esophageal microbiota, but among individual differences in  
523 diet did not associate with differences in intestinal microbiotas of these  
524 species. These results, on the one hand, point to the importance of diet  
525 determining esophageal microbiotas, and, on the other, suggest that similar  
526 diets might result in significantly different intestinal microbiota when  
527 comparing different species. Therefore, these results strongly suggest a  
528 genetic component of intestinal microbiota that is independent of external  
529 environmental factors including diet.

530         The interspecific differences should therefore be explained by  
531 anatomical or physiological characteristics that influence gut microbiota, and  
532 that differ between magpies and great spotted cuckoos. Innate and adaptive  
533 immune system, gut morphology and mucus characteristics can differentially  
534 select components of the intestinal microbiota [28, 30, 67-69]. We know that  
535 great spotted cuckoos and magpies differ in their immune system [70]; that  
536 cellular immunity of magpies and body condition of great spotted cuckoos  
537 predicted cloacal microbiota [71]; and that, different from magpies, the  
538 intestine morphology of cuckoos is shorter and contains a relative large  
539 caecum [43]. These interspecific morphological and physiological  
540 differences are therefore likely explanations for the detected interspecific  
541 differences in the intestinal microbiota that deserve further investigation.  
542 Future studies could, for instance, explore the effect of the immune system  
543 by experimentally enhancing cellular immune response [72], and exploring  
544 differential interspecific effects in intestinal microbiotas of magpies and of  
545 great spotted cuckoos developing under identical environmental conditions.  
546 The study system would also allow exploring the consequences of having  
547 different intestinal microbiota but feeding similar diets in terms of, for  
548 instance, nutrient absorption capacity. Intestinal digestibility of great spotted  
549 cuckoo nestlings is less efficient than that of magpie host nestlings [73], so

550 this might be related to the detected differences in their intestinal microbiota.  
551 The association between intestinal microbiotas and nutrients in the feces of  
552 cuckoo and host nestlings growing in the same nest would allow detecting  
553 bacteria responsible for the interspecific differences in nutrient absorption.  
554 Shotgun metagenomics and multi-omic methodologies applied to the  
555 cuckoo-host system will allow us to understand the causes and consequences  
556 of the detected interspecific differences, and, thus, exploring genetic  
557 mechanisms behind the genetic effect of microbiota while controlling for  
558 environmental conditions.

559 To summarize, our results strongly suggest a genetic component  
560 determining the intestinal microbiota of the study species while controlling  
561 for the effect of several environmental components including both abiotic  
562 and biotic conditions related to nest of rearing as parental identity, behavior  
563 and diet. The fact that interspecific differences in esophageal microbiota  
564 were not strong strengthens the possibility that interspecific differences in  
565 gut morphology and physiology are responsible for the observed differences  
566 in gut microbiota. Similar to laboratory experiments, the reproductive  
567 strategy of brood parasitism allows researchers to locate genetically  
568 unrelated individuals under identical environmental conditions and diet,  
569 where exploration of the influence of the genetic component on gut  
570 microbiota under natural conditions is made possible. This approach escapes  
571 from the undesired effects of captivity in gut microbiota [28, 74, 75], and,  
572 thus, brood parasitism should be considered a model system for exploring  
573 genetic and environmental factors affecting gut microbiota of animals as well  
574 as mechanisms and related consequences.

575

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794

795 Figure legends

796

797 Figure 1: Time schedule scheme and fieldwork protocol followed to  
798 synchronizing great spotted cuckoo and magpie eggs hatching within the  
799 same magpie nest. Incubation period of great spotted cuckoo eggs is on  
800 average four days shorter than that of magpie eggs. Magpie females lay one  
801 egg per day and start to incubate with the fourth egg. Thus, cuckoo eggs  
802 detected in magpie nests before incubation started were moved (black arrows  
803 connected two nests) to magpie nests where the expected hatching date of  
804 host eggs will occur in advance of or the same day as the experimental  
805 cuckoo egg. Date of collecting esophageal (saliva and food) and cloacal  
806 samples are shown in red and purple colors, respectively.

807

808 Figure 2: Shannon index values of alpha diversity of microbiota of cloaca,  
809 food and saliva samples of great spotted cuckoo and magpie nestlings.  
810 Values are weighted means  $\pm$  Standard Errors (Boxes), 95% CI (Whiskers).  
811 We also show raw data collected from great spotted cuckoo and magpie  
812 nestlings.

813

814 Figure 3: Principal coordinate analyses on both unweighted and weighted  
815 UniFrac distances of microbiotas of great spotted cuckoo (Cuckoos) and  
816 Magpie nestlings from cloaca and esophageal (saliva and food) samples. We  
817 show percentage of variance explained by the two first axes.

818

819 Figure 4: Principal coordinate analyses on both unweighted and weighted  
820 UniFrac distances of microbiotas of great spotted cuckoo (Cuckoos) and  
821 magpie nestlings from cloaca and esophageal (saliva and food) samples. We  
822 show percentage of variance explained by the two first axes.

823

824 Table 1: Number of magpie and great spotted cuckoo nestlings with cloaca,  
 825 saliva and prey sampled in different nests. Total collected samples (Tot S)  
 826 from cloaca, saliva and prey of both species are also shown. Nests with  
 827 samples from great spotted cuckoos and magpies are in bold font.  
 828 PERHAPS MOVE TO APPENDIX

Nests ID	Magpies				Great spotted cuckoos				TOT S
	Cloaca	Saliva	Prey	Tot S	Cloaca	Saliva	Prey	Tot S	
<b>G34</b>	2	2	2	6	2	1	1	4	10
<b>G04</b>	3	3	3	9	3	2	2	7	16
<b>G02</b>	2	3	3	8	1	1	1	3	11
G26B	2	0	0	2	0	0	0	0	2
G21B	1	0	0	1	0	0	0	0	1
G22	2	0	0	2	0	0	0	0	2
<b>G30</b>	2	0	0	2	1	0	0	1	3
<b>G60</b>	1	0	0	1	1	0	0	1	2
<b>G35C</b>	2	2	3	7	2	1	1	4	11
G09	2	0	0	2	0	0	0	0	2
G07	2	0	0	2	0	0	0	0	2
G10	2	0	0	2	0	0	0	0	2
G51	0	0	0	0	2	0	0	2	2
G60C	0	0	0	0	1	0	0	1	1
<b>G33B</b>	2	2	2	6	1	1	1	3	9
<b>G53</b>	2	3	3	8	0	1	1	2	10
<b>G32</b>	2	2	3	7	1	0	0	1	8
<b>G60B</b>	1	0	1	2	1	1	1	3	5
TOT	30	17	20	67	16	8	8	32	99

829



830 Table 2: Results from PERMANOVAs testing for the effects of species  
 831 identity on the microbiota of cloaca and esophageal (saliva and prey)  
 832 samples of great spotted cuckoos and magpies that shared identical  
 833 environmental conditions (i.e. nest identity) during development.

834

		Unweighted UniFrac			Weighted UniFrac		
		Pseudo-	df	P	Pseudo-	df	P
		F			F		
<b>Species x gut location comparisons</b>							
<b>Species</b>	<b>Fixed</b>	<b>1.66</b>	<b>1, 50</b>	<b>0.0076</b>	<b>3.49</b>	<b>1, 50</b>	<b>0.0045</b>
<b>Gut loc</b>	<b>Fixed</b>	<b>2.36</b>	<b>2, 50</b>	<b>0.0001</b>	<b>7.79</b>	<b>2, 50</b>	<b>0.0001</b>
<b>Sp x G</b>	<b>Fixed</b>	<b>1.45</b>	<b>2, 50</b>	<b>0.0074</b>	<b>2.31</b>	<b>2, 50</b>	<b>0.0098</b>
<b>Nest (Sp*G)</b>	<b>Rnd</b>	<b>1.17</b>	<b>50, 43</b>	<b>0.0001</b>	<b>1.21</b>	<b>50, 43</b>	<b>0.0521</b>
<b>Species comparisons</b>							
<b><u>Cloacal samples</u></b>							
<b>Species</b>	<b>Fixed</b>	<b>2.85</b>	<b>1, 8</b>	<b>0.0109</b>	<b>4.96</b>	<b>1, 8</b>	<b>0.0111</b>
Nests	Rnd	1.12	17, 8	0.0826	1.3	17, 8	0.1242
Nest*Species	Rnd	1.17	8, 19	0.1052	1.88	8, 19	0.0093
<b><u>Esophageal samples</u></b>							
<b><i>Saliva</i></b>							
Species	Fixed	0.95	1, 6	0.5315	0.56	1, 6	0.6126
Nests	Rnd	1.12	7, 6	0.1669	0.51	7, 6	0.9837
Nest*Species	Rnd	0.83	6, 13	0.9308	0.87	6, 13	0.6346
<b><i>Prey</i></b>							
Species	Fixed	0.88	1, 5	0.5857	0.21	1, 5	0.9397
<b>Nests</b>	<b>Rnd</b>	<b>1.61</b>	<b>7, 5</b>	<b>0.0009</b>	<b>1.56</b>	<b>7, 5</b>	<b>0.0617</b>
Nest*Species	Rnd	1.01	5, 11	0.4836	0.97	5, 11	0.4859
<b>Gut location comparisons</b>							
<b><i>Great spotted cuckoos</i></b>							
<b>locations</b>	<b>Fixed</b>	<b>2.03</b>	<b>2, 11</b>	<b>0.0026</b>	<b>3.84</b>	<b>2, 11</b>	<b>0.0012</b>
Nests	Rnd	1.46	11, 11	0.0157	1.25	11, 11	0.2788
Nest*loc	Rnd	1.12	11, 7	0.2847	0.84	11, 7	0.7161
<b>Loc (Cl - Sa)</b>	<b>Fixed</b>	<b>1.97</b>	<b>1, 11</b>	<b>0.0102</b>	4.90	<b>1, 11</b>	0.0037
Nests	Rnd	1.30	11, 6	0.0132	1.42	11, 6	0.0899
Nest*loc	Rnd	1.21	5, 6	0.0772	1.10	5, 6	0.3688

<b>Loc (Cl - Pr)</b>	<b>Fixed</b>	<b>2.51</b>	<b>1, 11</b>	<b>0.0017</b>	<b>4.02</b>	<b>1, 11</b>	<b>0.0086</b>
Nests	Rnd	<b>1.35</b>	<b>11, 6</b>	<b>0.0055</b>	1.36	<b>11, 6</b>	0.1376
Nest*loc	Rnd	<b>1.36</b>	<b>5, 6</b>	<b>0.0126</b>	1.40	<b>5, 6</b>	0.1531
Loc (Sa - Pr)	Fixed	1.28	1, 6	0.1251	0.96	1, 6	0.4424
Nests	Rnd	<b>1.47</b>	<b>6, 6</b>	<b>0.0273</b>	0.58	6, 6	0.8534
Nest*loc	Rnd	0.77	6, 2	0.9464	0.23	6, 2	0.9969

*Magpies*

Locations	Fixed	1.29	2, 13	0.1469	<b>3.03</b>	<b>2, 13</b>	<b>0.0040</b>
Nests	Rnd	<b>1.23</b>	<b>15, 13</b>	<b>0.0028</b>	1.13	15, 13	0.2504
Nest*Loc	Rnd	1.14	13, 36	0.0575	1.23	13, 36	0.1118
<u>Loc (Cl - Sa)</u>	Fixed	<b>1.55</b>	<b>1, 7</b>	<b>0.0228</b>	<b>5.03</b>	<b>1, 7</b>	<b>0.0054</b>
Nests	Rnd	1.04	15, 7	0.2667	0.98	15, 7	0.5259
Nest*loc	Rnd	<b>1.12</b>	<b>7, 26</b>	<b>0.0911</b>	1.15	7, 26	0.2613
<u>Loc (Cl - Pr)</u>	Fixed	1.34	1, 6	0.1092	<b>2.63</b>	<b>1, 6</b>	<b>0.0463</b>
Nests	Rnd	<b>1.22</b>	<b>15, 6</b>	<b>0.0097</b>	1.21	15, 6	0.1552
Nest*loc	Rnd	<b>1.31</b>	<b>6, 24</b>	<b>0.0100</b>	<b>1.71</b>	<b>6, 24</b>	<b>0.0164</b>
<u>Loc (Sa - Pr)</u>	Fixed	0.87	1, 6	0.7303	0.66	1, 6	0.7049
Nests	Rnd	1.60	7, 6	0.0001	<b>1.42</b>	<b>7, 6</b>	<b>0.0653</b>
Nest*loc	Rnd	0.93	6, 22	0.7914	0.90	6, 22	0.6292

835

836

837 Table 3: Results from Mantel tests exploring the association between  
 838 distance matrices of prey collected (i.e. diet) from great spotted cuckoo and  
 839 magpie nestlings and their gut microbiotas (saliva, prey and cloaca)  
 840 estimated as weighted and unweighted distance matrices. Matrices of  
 841 species identity (same or different species) were also included in the  
 842 models to control the expected association for the effect of species identity.

843

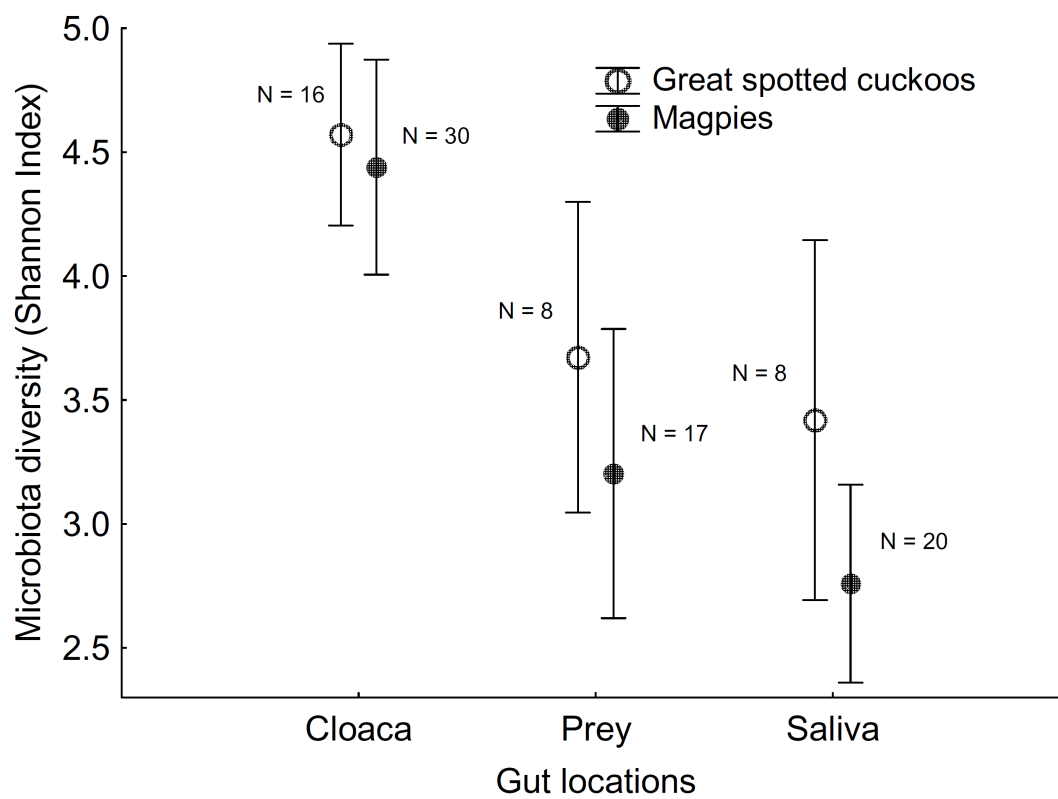
		Unweighted UniFrac		Weighted UniFrac	
		Beta	P	Beta	P
<b><i>Cloacal samples</i></b>					
	<b>Species</b>	<b>0.039</b>	<b>0.035</b>	<b>0.141</b>	<b>0.002</b>
	Diet	0.045	0.322	0.087	0.337
<b><i>Esophageal samples</i></b>					
<i>Saliva</i>					
	Species	0.002	0.881	0.004	0.927
	Diet	<b>0.060</b>	<b>0.049</b>	0.131	0.225
<i>Prey</i>					
	Species	<b>-0.024</b>	<b>0.046</b>	-0.006	0.869
	Diet	<b>0.131</b>	<b>0.001</b>	0.040	0.665

844

845

846 Figure 1: Shannon index values of alfa-diversity of microbiota of cloaca,  
847 prey and saliva samples of great spotted cuckoos and magpie nestlings.  
848 Values are weighted means  $\pm$  Standard Errors.

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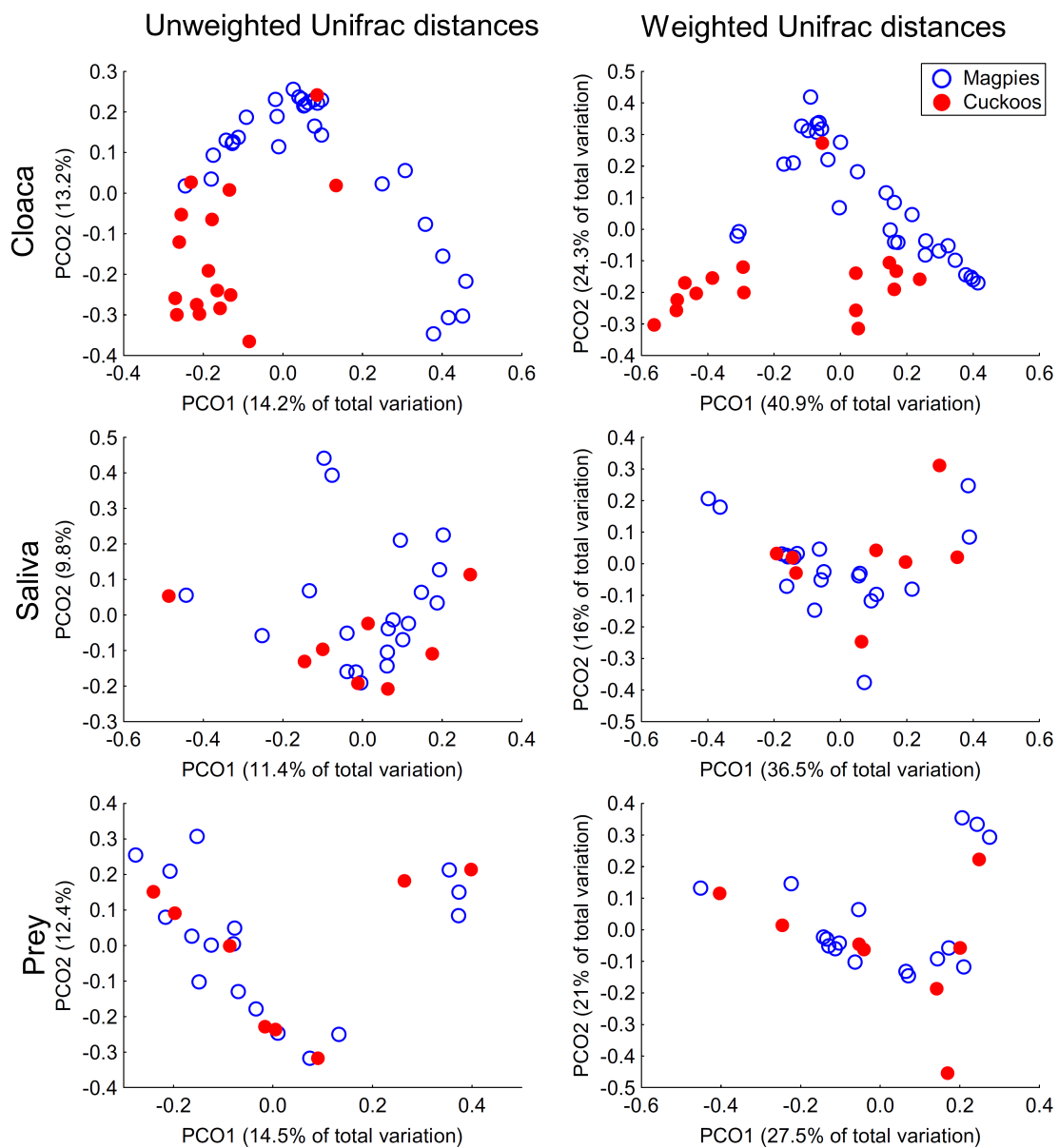
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854 Fig 2: Principal coordinate analyses on both unweighted and weighted  
 855 UniFrac distances of microbiotas of great spotted cuckoo (Cuckoos) and  
 856 Magpie nestlings from cloaca and esophageal (saliva and prey) samples. We  
 857 show percentage of variance explained by the two first axes as well as the  
 858 coordinates of each sample on them.

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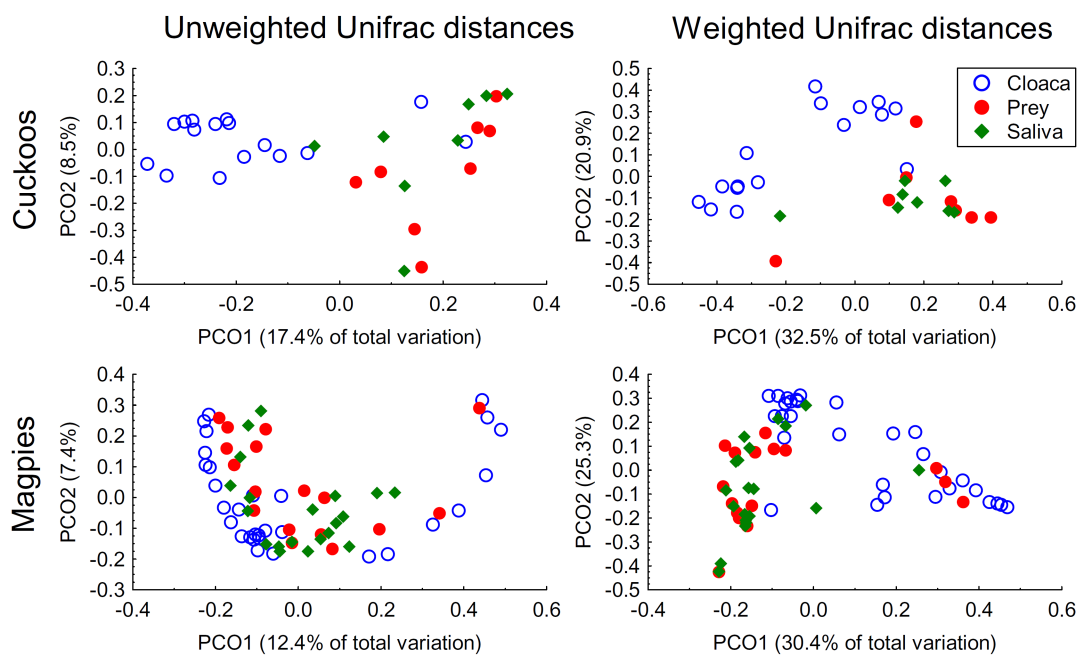
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862

863 Figure 3: Principal coordinate analyses on both unweighted and weighted  
 864 UniFrac distances of microbiotas of great spotted cuckoo (Cuckoos) and  
 865 Magpie nestlings from cloaca and esophageal (saliva and prey) samples. We  
 866 show percentage of variance explained by the two first axes as well as the  
 867 coordinates of each sample on them.

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870

871

872 Suppl Material

873

		Unweighted UniFrac			Weighted UniFrac		
		Pseudo- F	df	P	Pseudo- F	df	P
<b>Species x gut location comparisons</b>							
<b>Species</b>	<b>Fixed</b>	<b>1.86</b>	<b>1, 93</b>	<b>0.0025</b>	<b>4.23</b>	<b>1, 93</b>	<b>0.0008</b>
<b>Gut loc</b>	<b>Fixed</b>	<b>2.69</b>	<b>2, 93</b>	<b>0.0001</b>	<b>8.23</b>	<b>2, 93</b>	<b>0.0001</b>
<b>Sp x G</b>	<b>Fixed</b>	<b>1.63</b>	<b>2, 93</b>	<b>0.0008</b>	<b>2.07</b>	<b>2, 93</b>	<b>0.0113</b>
<b>Species comparisons</b>							
<b><u>Cloacal samples</u></b>							
<b>Species</b>	<b>Fixed</b>	<b>4.60</b>	<b>1, 44</b>	<b>0.0001</b>	<b>9.32</b>	<b>1, 44</b>	<b>0.0001</b>
<b><u>Esophageal samples</u></b>							
<i>Saliva</i>							
Species	Fixed	0.86	1, 26	0.8006	0.93	1, 26	0.4488
<i>Prey</i>							
Species	Fixed	0.70	1, 3	0.9665	0.479	1, 3	0.9029
<b>Gut location comparisons</b>							
<b><u>Great spotted cuckoos</u></b>							
<b>Gut loc</b>	<b>Fixed</b>	<b>2.45</b>	<b>2, 29</b>	<b>0.0001</b>	<b>4.25</b>	<b>2, 29</b>	<b>0.0001</b>
<i>Pos-hocs</i>							
<b>Gloc (CI - Sa)</b>	<b>Fixed</b>	<b>2.75</b>	<b>1, 22</b>	<b>0.0005</b>	<b>5.43</b>	<b>1, 22</b>	<b>0.0002</b>
<b>Gloc (CI - Pr)</b>	<b>Fixed</b>	<b>3.44</b>	<b>1, 22</b>	<b>0.0002</b>	<b>5.56</b>	<b>1, 22</b>	<b>0.0003</b>
<b>Gloc (Sa - Pr)</b>	<b>Fixed</b>	<b>0.89</b>	<b>1, 14</b>	<b>0.6344</b>	<b>0.45</b>	<b>1, 14</b>	<b>0.8166</b>
<b><u>Magpies</u></b>							
<b>Gut loc</b>	<b>Fixed</b>	<b>1.55</b>	<b>2, 64</b>	<b>0.0041</b>	<b>5.96</b>	<b>2, 64</b>	<b>0.0001</b>
<i>Pos-hocs</i>							
<b>Gloc (CI - Sa)</b>	<b>Fixed</b>	<b>1.86</b>	<b>1, 48</b>	<b>0.0046</b>	<b>9.52</b>	<b>1, 48</b>	<b>0.0001</b>
<b>Gloc (CI - Pr)</b>	<b>Fixed</b>	<b>1.80</b>	<b>1, 45</b>	<b>0.0104</b>	<b>6.29</b>	<b>1, 45</b>	<b>0.0003</b>
<b>Gloc (Sa - Pr)</b>	<b>Fixed</b>	<b>0.79</b>	<b>1, 35</b>	<b>0.8968</b>	<b>0.60</b>	<b>1, 35</b>	<b>0.7480</b>

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