



HAL
open science

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

Chop Yan Lee, Juan Manuel Peralta-Sánchez, Manuel Martínez- Bueno, Anders Pape Møller, Miguel Rabelo-Ruiz, Carmen Zamora-Muñoz, Juan José Soler

► To cite this version:

Chop Yan Lee, Juan Manuel Peralta-Sánchez, Manuel Martínez- Bueno, Anders Pape Møller, Miguel Rabelo-Ruiz, et al.. The gut microbiota of brood parasite and host nestlings reared within the same environment: Disentangling genetic and environmental effects.. The International Society of Microbiological Ecology Journal, 2020, 14 (11), pp.2691-2702. 10.1038/s41396-020-0719-y . hal-03024917

HAL Id: hal-03024917

<https://cnrs.hal.science/hal-03024917>

Submitted on 26 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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
7 **Chop Yan Lee^{1*}, Juan Manuel Peralta-Sánchez^{1*}, Manuel Martínez-**
8 **Bueno^{1,2}, Anders Pape Møller^{4,5}, Miguel Rabelo-Ruiz ¹, Carmen**
9 **Zamora-Muñoz^{2,3} and Juan José Soler^{2,6,*}**

10
11 ¹ Departamento de Microbiología, Universidad de Granada, 18071-
12 Granada, Spain.

13 ² Unidad asociada (CSIC): Coevolución: cucos, hospedadores y bacterias
14 simbiotes. Universidad de Granada, 18071-Granada, Spain.

15 ³ Departamento de Zoología, Universidad de Granada, 18071-Granada,
16 Spain.

17 ⁴ Ecologie Systématique Evolution, Université Paris-Sud, CNRS,
18 AgroParisTech, Université Paris-Saclay, Orsay Cedex, France.

19 ⁵ Ministry of Education Key Laboratory for Biodiversity Science and
20 Ecological Engineering, College of Life Sciences, Beijing Normal
21 University, Beijing, 100875, China.

22 ⁶ Departamento de Ecología Funcional y Evolutiva, Estación Experimental
23 de Zonas Áridas (CSIC), Almería. Spain.

24
25 * These authors contributed equally to this work.

26
27 Corresponding author: Juan J Soler,
28 Ctra. Sacramento S/N, La Cañada de San Urbano, 04120-Almería, Spain.
29 Tel. 34 950 281045, e-mail: jsoler@eeza.csic.es

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 4th 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 μ 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 μ L of sterile phosphate buffer
218 (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 , pH 7.4) in nestlings' cloaca using
219 sterile tips and an automatic micropipette. Collected samples were stored in
220 500 μ 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 μ L of
245 cloacal samples; or with 100 mg of previously mixed esophageal samples
246 adding solution 2 (Xtrem biotech®) to make up 800 μ 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 μ L of lysis solution. Afterward, the
250 tubes containing the three types of samples were added with 10 μ 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 μ L of supernatants were transferred to new 1.5 mL
255 microcentrifuge tubes and 250 μ 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 μ L of supernatant from each tube was then transferred to new
259 microcentrifuge tubes. 650 μ L of binding buffer (20 mM HCl-Tris pH 8, 4

260 M Guanidinium isothiocyanate, 1 mM DTT) and 50 μ 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 μ 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 μ 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 μ 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 μ L reactions
281 containing 2.5 μ L of 3 mM of each primer, 2.5 μ L of PCR water, 12.5 μ L of
282 iProof HF Master Mix (BioRad®) and 5 μ 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 μ 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 μ L reactions containing 3.3 μ L of 3 mM of each primer,
293 0.9 μ L of PCR water, 12.5 μ L of iProof HF Master Mix (BioRad®) and 5
294 μ 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 μ 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

576 **Acknowledgments:**

577 We thank Manuel Martin-Vivaldi Martínez and Jarkko Rutila for discussion
578 on the interest of exploring genetic and environmental factors of gut

579 microbiotas of brood parasitic and host nestlings. The research group
580 benefits from facilities, including an apartment, provided by the city hall of
581 Guadix where a small lab to quickly process the samples was installed.
582 Financial support was provided by the Spanish Ministerio de Ciencia,
583 Innovación y Universidades and European (FEDER) funds (CGL2017-
584 83103-P). All procedures were conducted under licence from the
585 Environmental Department of the Regional Government of Andalucía, Spain
586 (reference SGYB/FOA/AFR). All applicable international, national, and/or
587 institutional guidelines for the care and use of animals were followed.

588

589

590

References

591

592 [1] Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, Prill RJ,
593 Tripathi A, Gibbons SM, Ackermann G, et al. A communal catalogue reveals Earth's
594 multiscale microbial diversity. *Nature* 2017; **551**: 457-463.

595 [2] Parfrey LW, Moreau CS & Russell JA. Introduction: The host-associated
596 microbiome: Pattern, process and function. *Mol Ecol* 2018; **27**: 1749-1765.

597 [3] Moran NA, Ochman H & Hammer TJ. Evolutionary and ecological consequences of
598 gut microbial communities. *Annu Rev Ecol Evol Syst* 2019; **50**: 451-475.

599 [4] McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas
600 AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, et al. Animals in a bacterial world, a new
601 imperative for the life sciences. *Proc Natl Acad Sci USA* 2013; **110**: 3229-3236.

602 [5] Colston TJ & Jackson CR. Microbiome evolution along divergent branches of the
603 vertebrate tree of life: what is known and unknown. *Mol Ecol* 2016; **25**: 3776-3800.

604 [6] O'Hara AM & Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; **7**:
605 688-693.

- 606 [7] Browne HP, Neville BA, Forster SC & Lawley TD. Transmission of the gut
607 microbiota: spreading of health. *Nat Rev Microbiol* 2017; **15**: 531.
- 608 [8] Mao K, Baptista AP, Tamoutounour S, Zhuang L, Bouladoux N, Martins AJ, Huang
609 Y, Gerner MY, Belkaid Y & Germain RN. Innate and adaptive lymphocytes sequentially
610 shape the gut microbiota and lipid metabolism. *Nature* 2018; **554**: 255-259.
- 611 [9] Belkaid Y & Hand TW. Role of the microbiota in immunity and inflammation. *Cell*
612 2014; **157**: 121-141.
- 613 [10] Sherwin E, Bordenstein SR, Quinn JL, Dinan TG & Cryan JF. Microbiota and the
614 social brain. *Science* 2019; **366**: eaar2016.
- 615 [11] Buffie CG & Pamer EG. Microbiota-mediated colonization resistance against
616 intestinal pathogens. *Nat Rev Immunol* 2013; **13**: 790.
- 617 [12] Clemente JC, Ursell LK, Parfrey LW & Knight R. The impact of the gut microbiota
618 on human health: an integrative view. *Cell* 2012; **148**: 1258-1270.
- 619 [13] Rosenbaum M, Knight R & Leibel RL. The gut microbiota in human energy
620 homeostasis and obesity. *Trends Endocrinol Metabol* 2015; **26**: 493-501.
- 621 [14] Foster KR, Schluter J, Coyte KZ & Rakoff-Nahoum S. The evolution of the host
622 microbiome as an ecosystem on a leash. *Nature* 2017; **548**: 43.
- 623 [15] Knowles SCL, Eccles RM & Baltrūnaitė L. Species identity dominates over
624 environment in shaping the microbiota of small mammals. *Ecol Lett* 2019; **22**: 826-837.
- 625 [16] Henderson G, Cox F, Ganesh S, Jonker A, Young W, Janssen PH & Global Rumen
626 Census C. Rumen microbial community composition varies with diet and host, but a core
627 microbiome is found across a wide geographical range. *Sci Rep* 2015; **5**.
- 628 [17] Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS &
629 Sonnenburg JL. Diet-induced extinctions in the gut microbiota compound over
630 generations. *Nature* 2016; **529**: 212.

- 631 [18] Carmody Rachel N, Gerber Georg K, Luevano Jesus M, Jr., Gatti Daniel M, Somes
632 L, Svenson Karen L & Turnbaugh Peter J. Diet Dominates Host Genotype in Shaping the
633 Murine Gut Microbiota. *Cell Host & Microbe* 2015; **17**: 72-84.
- 634 [19] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling
635 AV, Devlin AS, Varma Y, Fischbach MA, et al. Diet rapidly and reproducibly alters the
636 human gut microbiome. *Nature* 2014; **505**: 559-563.
- 637 [20] Seedorf H, Griffin Nicholas W, Ridaura Vanessa K, Reyes A, Cheng J, Rey
638 Federico E, Smith Michelle I, Simon Gabriel M, Scheffrahn Rudolf H, Woebken D, et al.
639 Bacteria from Diverse Habitats Colonize and Compete in the Mouse Gut. *Cell* 2014; **159**:
640 253-266.
- 641 [21] Hildebrand F, Nguyen TLA, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P,
642 Liston A & Raes J. Inflammation-associated enterotypes, host genotype, cage and inter-
643 individual effects drive gut microbiota variation in common laboratory mice. *Genome*
644 *Biol* 2013; **14**: R4.
- 645 [22] Schloss PD, Iverson KD, Petrosino JF & Schloss SJ. The dynamics of a family's gut
646 microbiota reveal variations on a theme. *Microbiome* 2014; **2**: 25.
- 647 [23] Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D,
648 Caporaso JG, Knights D, Clemente JC, Nakielny S, et al. Cohabiting family members
649 share microbiota with one another and with their dogs. *eLife* 2013; **2**: e00458.
- 650 [24] Maurice CF, Cl Knowles S, Ladau J, Pollard KS, Fenton A, Pedersen AB &
651 Turnbaugh PJ. Marked seasonal variation in the wild mouse gut microbiota. *ISME J* 2015;
652 **9**: 2423-2434.
- 653 [25] Ren T, Boutin S, Humphries MM, Dantzer B, Gorrell JC, Coltman DW, McAdam
654 AG & Wu M. Seasonal, spatial, and maternal effects on gut microbiome in wild red
655 squirrels. *Microbiome* 2017; **5**: 163.

- 656 [26] Wang J, Chen L, Zhao N, Xu X, Xu Y & Zhu B. Of genes and microbes: solving the
657 intricacies in host genomes. *Protein & Cell* 2018; **9**: 446-461.
- 658 [27] Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, Costea
659 PI, Godneva A, Kalka IN, Bar N, et al. Environment dominates over host genetics in
660 shaping human gut microbiota. *Nature* 2018; **555**: 210.
- 661 [28] Amato KR, G. Sanders J, Song SJ, Nute M, Metcalf JL, Thompson LR, Morton JT,
662 Amir A, J. McKenzie V, Humphrey G, et al. Evolutionary trends in host physiology
663 outweigh dietary niche in structuring primate gut microbiomes. *ISME J* 2019; **13**: 576-
664 587.
- 665 [29] Nishida AH & Ochman H. Rates of gut microbiome divergence in mammals. *Mol*
666 *Ecol* 2018; **27**: 1884-1897.
- 667 [30] Brooks AW, Kohl KD, Brucker RM, van Opstal EJ & Bordenstein SR.
668 Phylosymbiosis: Relationships and functional effects of microbial communities across
669 host evolutionary history. *PLoS Biol* 2016; **14**: e2000225.
- 670 [31] Ochman H, Worobey M, Kuo C-H, Ndjango J-BN, Peeters M, Hahn BH &
671 Hugenholtz P. Evolutionary Relationships of Wild Hominids Recapitulated by Gut
672 Microbial Communities. *PLoS Biol* 2010; **8**: e1000546.
- 673 [32] Kartzinel TR, Hsing JC, Musili PM, Brown BRP & Pringle RM. Covariation of diet
674 and gut microbiome in African megafauna. *Proc Natl Acad Sci USA* 2019; **116**: 23588-
675 23593.
- 676 [33] Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L,
677 Henrissat B, Knight R & Gordon JI. Diet drives convergence in gut microbiome functions
678 across mammalian phylogeny and within humans. *Science* 2011; **332**: 970-974.

- 679 [34] Delsuc F, Metcalf JL, Wegener Parfrey L, Song SJ, González A & Knight R.
680 Convergence of gut microbiomes in myrmecophagous mammals. *Mol Ecol* 2014; **23**:
681 1301-1317.
- 682 [35] Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel
683 ML, Tucker TA, Schrenzel MD, Knight R, et al. Evolution of mammals and their gut
684 microbes. *Science* 2008; **320**: 1647-1651.
- 685 [36] Ruiz-Rodríguez M, Martín-Vivaldi M, Martínez-Bueno M & Soler JJ. Gut
686 microbiota of great spotted cuckoo nestlings is a mixture of those of their foster magpie
687 siblings and of cuckoo adults. *Genes* 2018; **9**: 381.
- 688 [37] Davies NB. Cuckoo adaptations: trickery and tuning. *J. Zool.* 2011; **284**: 1-14.
- 689 [38] Payne RB. *The cuckoos*. New York, Oxford University Press, 2005.
- 690 [39] Prum RO, Berv JS, Dornburg A, Field DJ, Townsend JP, Lemmon EM & Lemmon
691 AR. A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA
692 sequencing. *Nature* 2015; **526**: 569.
- 693 [40] Soler M, Martínez JG, Soler JJ & Møller AP. Preferential allocation of food by
694 magpie *Pica pica* to great spotted cuckoo *Clamator glandarius* chicks. *Behav Ecol*
695 *Sociobiol* 1995; **37**: 7-13.
- 696 [41] Soler JJ, Martínez JG, Soler M & Møller AP. Coevolutionary interactions in a host-
697 parasite system. *Ecol Lett* 2001; **4**: 470-476.
- 698 [42] Birkhead TR. *The Magpies. The ecology and behaviour of black-billed and yellow-*
699 *billed magpies*. London, T & A D Poyser, 1991.
- 700 [43] Ruiz-Rodríguez M, Lucas FS, Heeb P & Soler JJ. Differences in intestinal microbiota
701 between avian brood parasites and their hosts. *Biol J Linn Soc* 2009; **96**: 406-414.

- 702 [44] Soler JJ, Martin-Galvez D, De Neve L & Soler M. Brood parasitism correlates with
703 the strength of spatial autocorrelation of life history and defensive traits in Magpies.
704 *Ecology* 2013; **94**: 1338-1346.
- 705 [45] Moreno-Rueda G, Soler M, Soler JJ, Martínez JG & Pérez-Contreras T. Rules of
706 food allocation between nestlings of the black-billed magpie *Pica pica*, a species showing
707 brood reduction. *Ardeola* 2007; **54**: 15-25.
- 708 [46] Soler M, Soler JJ & Martínez JG. Duration of sympatry and coevolution between the
709 great spotted cuckoo (*Clamator glandarius*) and its primary host, the magpie (*Pica pica*).
710 In: SI Rothstein & SK Robinson (eds.). *Parasitic Birds and their hosts, studies in*
711 *coevolution*. Oxford University Press, Oxford, 1998, pp. 113-128.
- 712 [47] Soler M & Soler JJ. Growth and development of great spotted cuckoos and their
713 magpie host. *Condor* 1991; **93**: 49-54.
- 714 [48] Martín-Gálvez D, Pérez-Contreras T, Soler M & Soler JJ. Benefits associated with
715 escalated begging behaviour of black-billed magpie nestlings overcompensate the
716 associated energetic costs. *J Exp Biol* 2011; **214**: 1463-1472.
- 717 [49] Hoss M & Paabo S. DNA extraction from Pleistocene bones by a silica-based
718 purification method. *Nucleic Acids Research* 1993; **21**: 3913-3914.
- 719 [50] Hamady M, Walker J, Harris J, Gold N & Knight R. Hamady M, Walker JJ, Harris
720 JK, Gold NJ, Knight R.. Error-correcting barcoded primers for pyrosequencing hundreds
721 of samples in multiplex. *Nat Meth* 2008; **5**: 235-237.
- 722 [51] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK,
723 Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-
724 throughput community sequencing data. *Nat Meth* 2010; **7**: 335.
- 725 [52] Bolyen E & Rideout JR & Dillon MR & Bokulich NA & Abnet CC & Al-Ghalith
726 GA & Alexander H & Alm EJ & Arumugam M & Asnicar F, et al. Reproducible,

- 727 interactive, scalable and extensible microbiome data science using QIIME 2. *Nat*
728 *Biotechnol* 2019; **37**: 852-857.
- 729 [53] Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech Xu Z,
730 Kightley EP, Thompson LR, Hyde ER, Gonzalez A, et al. Deblur Rapidly Resolves
731 Single-Nucleotide Community Sequence Patterns. *mSystems* 2017; **2**: e00191-00116.
- 732 [54] Janssen S, McDonald D, Gonzalez A, Navas-Molina JA, Jiang L, Xu ZZ, Winker K,
733 Kado DM, Orwoll E, Manary M, et al. Phylogenetic Placement of Exact Amplicon
734 Sequences Improves Associations with Clinical Information. *mSystems* 2018; **3**: e00021-
735 00018.
- 736 [55] DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T,
737 Dalevi D, Hu P & Andersen GL. Greengenes, a chimera-checked 16S rRNA gene
738 database and workbench compatible with ARB. *Appl Environ Microbiol* 2006; **72**: 5069-
739 5072.
- 740 [56] Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P,
741 Parkhill J, Loman NJ & Walker AW. Reagent and laboratory contamination can critically
742 impact sequence-based microbiome analyses. *BMC Biology* 2014; **12**: 87.
- 743 [57] de Goffau MC, Lager S, Salter SJ, Wagner J, Kronbichler A, Charnock-Jones DS,
744 Peacock SJ, Smith GCS & Parkhill J. Recognizing the reagent microbiome. *Nature*
745 *Microbiology* 2018; **3**: 851-853.
- 746 [58] Whittaker RH. Evolution and measurement of species diversity. *Taxon* 1972; **21**:
747 213-251.
- 748 [59] Shannon CE. A mathematical theory of communication. *Bell Labs Tech J* 1948; **27**:
749 379-423.
- 750 [60] Lozupone C & Knight R. UniFrac: a new phylogenetic method for comparing
751 microbial communities. *Appl Environ Microbiol* 2005; **71**: 8228-8235.

- 752 [61] Lozupone CA, Hamady M, Kelley ST & Knight R. Quantitative and qualitative β
753 diversity measures lead to different insights into factors that structure microbial
754 communities. *Appl Environ Microbiol* 2007; **73**: 1576-1585.
- 755 [62] Goslee SC & Urban DL. The ecodist package for dissimilarity-based analysis of
756 ecological data. *J Stat Softw* 2007; **22**: i07.
- 757 [63] Moeller A, Suzuki T, Lin D, Lacey E, Wasser S & Nachman M. Dispersal limitation
758 promotes the diversification of the mammalian gut microbiota. *Proc Natl Acad Sci USA*
759 2017; **114**: 201700122.
- 760 [64] Moeller AH, Caro-Quintero A, Mjungu D, Georgiev AV, Lonsdorf EV, Muller MN,
761 Pusey AE, Peeters M, Hahn BH & Ochman H. Cospeciation of gut microbiota with
762 hominids. *Science* 2016; **353**: 380-382.
- 763 [65] Groussin M, Mazel F, Sanders JG, Smillie CS, Lavergne S, Thuiller W & Alm EJ.
764 Unraveling the processes shaping mammalian gut microbiomes over evolutionary time.
765 *Nat Commun* 2017; **8**: 14319.
- 766 [66] Soler M & Soler JJ. Innate versus learned recognition of conspecifics in great spotted
767 cuckoos *Clamator glandarius*. *Anim Cogn* 1999; **2**: 97-102.
- 768 [67] Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, Conner ME,
769 Earl AM, Knight R, Bjorkman PJ, et al. Gut microbiota utilize immunoglobulin A for
770 mucosal colonization. *Science* 2018.
- 771 [68] Thaïss CA, Zmora N, Levy M & Elinav E. The microbiome and innate immunity.
772 *Nature* 2016; **535**: 65.
- 773 [69] Sicard J-F, Le Bihan G, Vogeleer P, Jacques M & Harel J. Interactions of intestinal
774 bacteria with components of the intestinal mucus. *Front Cellular Infection Microbiol*
775 2017; **7**.

776 [70] Soler JJ, Møller AP, Soler M & Martínez JG. Interactions between a brood parasite
777 and its host in relation to parasitism and immune defence. *Evol Ecol Res* 1999; **1**: 189-
778 210.

779 [71] Ruiz-Rodríguez M, Soler JJ, Lucas FS, Heeb P, Palacios M, Martín-Gálvez D, De
780 Neve L, Pérez-Contreras T, Martínez JG & Soler M. Bacterial diversity at the cloaca
781 relates to an immune response in magpie *Pica pica* and to body condition of great spotted
782 cuckoo *Clamator glandarius* nestlings. *J Avian Biol* 2009; **40**: 42-48.

783 [72] Soler JJ, De Neve L, Pérez-Contreras T, Soler M & Sorci G. Trade-off between
784 immunocompetence and growth in magpies: an experimental study. *Proc R Soc Lond B*
785 *Biol Sci* 2003; **270**: 241-248.

786 [73] Soler M, Rubio LA, Perez-Contreras T, Ontanilla J & De Neve L. Intestinal
787 digestibility of great spotted cuckoo nestlings is less efficient than that of magpie host
788 nestlings. *Biol J Linn Soc* 2017; **122**: 675-680.

789 [74] Clayton JB, Vangay P, Huang H, Ward T, Hillmann BM, Al-Ghalith GA, Travis
790 DA, Long HT, Tuan BV, Minh VV, et al. Captivity humanizes the primate microbiome.
791 *Proc Natl Acad Sci USA* 2016; **113**: 10376-10381.

792 [75] Kohl K, Skopec M & Dearing MD. Captivity results in disparate loss of gut microbial
793 diversity in closely related hosts. *Cons. Physiol.* 2014; **2**: cou009.

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	
G34	2	2	2	6	2	1	1	4	10
G04	3	3	3	9	3	2	2	7	16
G02	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
G30	2	0	0	2	1	0	0	1	3
G60	1	0	0	1	1	0	0	1	2
G35C	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
G33B	2	2	2	6	1	1	1	3	9
G53	2	3	3	8	0	1	1	2	10
G32	2	2	3	7	1	0	0	1	8
G60B	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		
Species x gut location comparisons							
Species	Fixed	1.66	1, 50	0.0076	3.49	1, 50	0.0045
Gut loc	Fixed	2.36	2, 50	0.0001	7.79	2, 50	0.0001
Sp x G	Fixed	1.45	2, 50	0.0074	2.31	2, 50	0.0098
Nest (Sp*G)	Rnd	1.17	50, 43	0.0001	1.21	50, 43	0.0521
Species comparisons							
<u>Cloacal samples</u>							
Species	Fixed	2.85	1, 8	0.0109	4.96	1, 8	0.0111
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
<u>Esophageal samples</u>							
Saliva							
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
Prey							
Species	Fixed	0.88	1, 5	0.5857	0.21	1, 5	0.9397
Nests	Rnd	1.61	7, 5	0.0009	1.56	7, 5	0.0617
Nest*Species	Rnd	1.01	5, 11	0.4836	0.97	5, 11	0.4859
Gut location comparisons							
<u>Great spotted cuckoos</u>							
locations	Fixed	2.03	2, 11	0.0026	3.84	2, 11	0.0012
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
Loc (Cl - Sa)	Fixed	1.97	1, 11	0.0102	4.90	1, 11	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

Loc (Cl - Pr)	Fixed	2.51	1, 11	0.0017	4.02	1, 11	0.0086
Nests	Rnd	1.35	11, 6	0.0055	1.36	11, 6	0.1376
Nest*loc	Rnd	1.36	5, 6	0.0126	1.40	5, 6	0.1531
Loc (Sa - Pr)	Fixed	1.28	1, 6	0.1251	0.96	1, 6	0.4424
Nests	Rnd	1.47	6, 6	0.0273	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	3.03	2, 13	0.0040
Nests	Rnd	1.23	15, 13	0.0028	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	1.55	1, 7	0.0228	5.03	1, 7	0.0054
Nests	Rnd	1.04	15, 7	0.2667	0.98	15, 7	0.5259
Nest*loc	Rnd	1.12	7, 26	0.0911	1.15	7, 26	0.2613
<u>Loc (Cl - Pr)</u>	Fixed	1.34	1, 6	0.1092	2.63	1, 6	0.0463
Nests	Rnd	1.22	15, 6	0.0097	1.21	15, 6	0.1552
Nest*loc	Rnd	1.31	6, 24	0.0100	1.71	6, 24	0.0164
<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	1.42	7, 6	0.0653
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

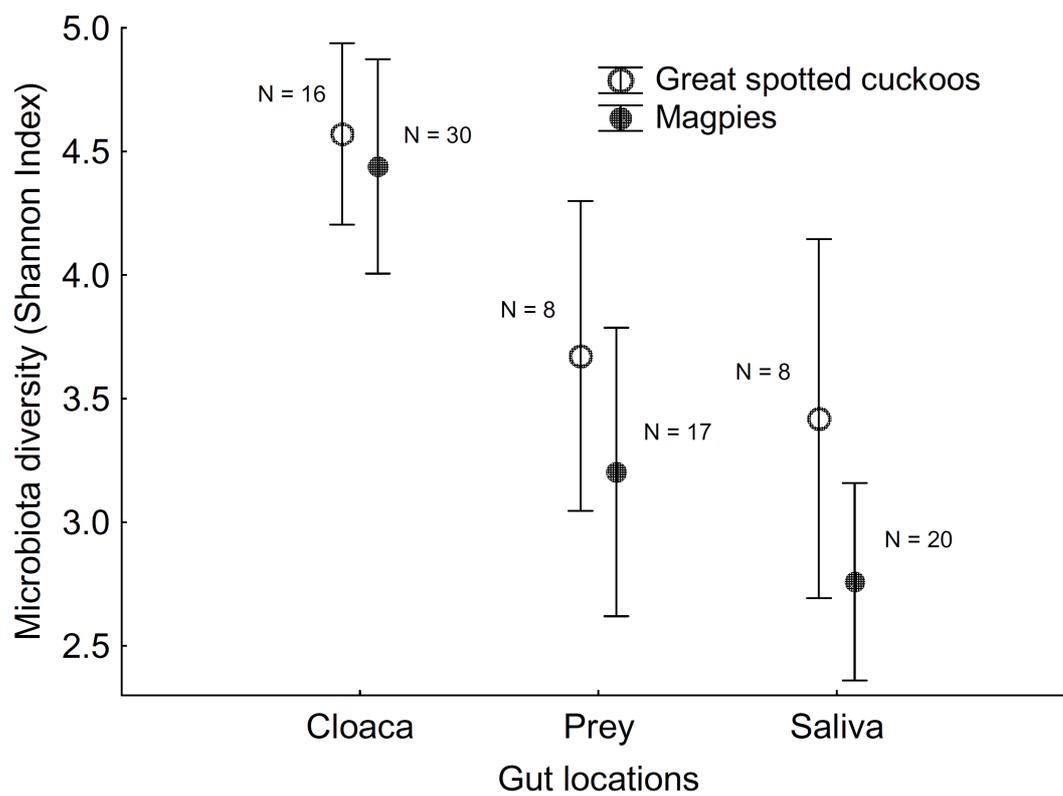
		Unweighted UniFrac		Weighted UniFrac	
		Beta	P	Beta	P
<i>Cloacal samples</i>					
	Species	0.039	0.035	0.141	0.002
	Diet	0.045	0.322	0.087	0.337
<i>Esophageal samples</i>					
<i>Saliva</i>					
	Species	0.002	0.881	0.004	0.927
	Diet	0.060	0.049	0.131	0.225
<i>Prey</i>					
	Species	-0.024	0.046	-0.006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

849



850

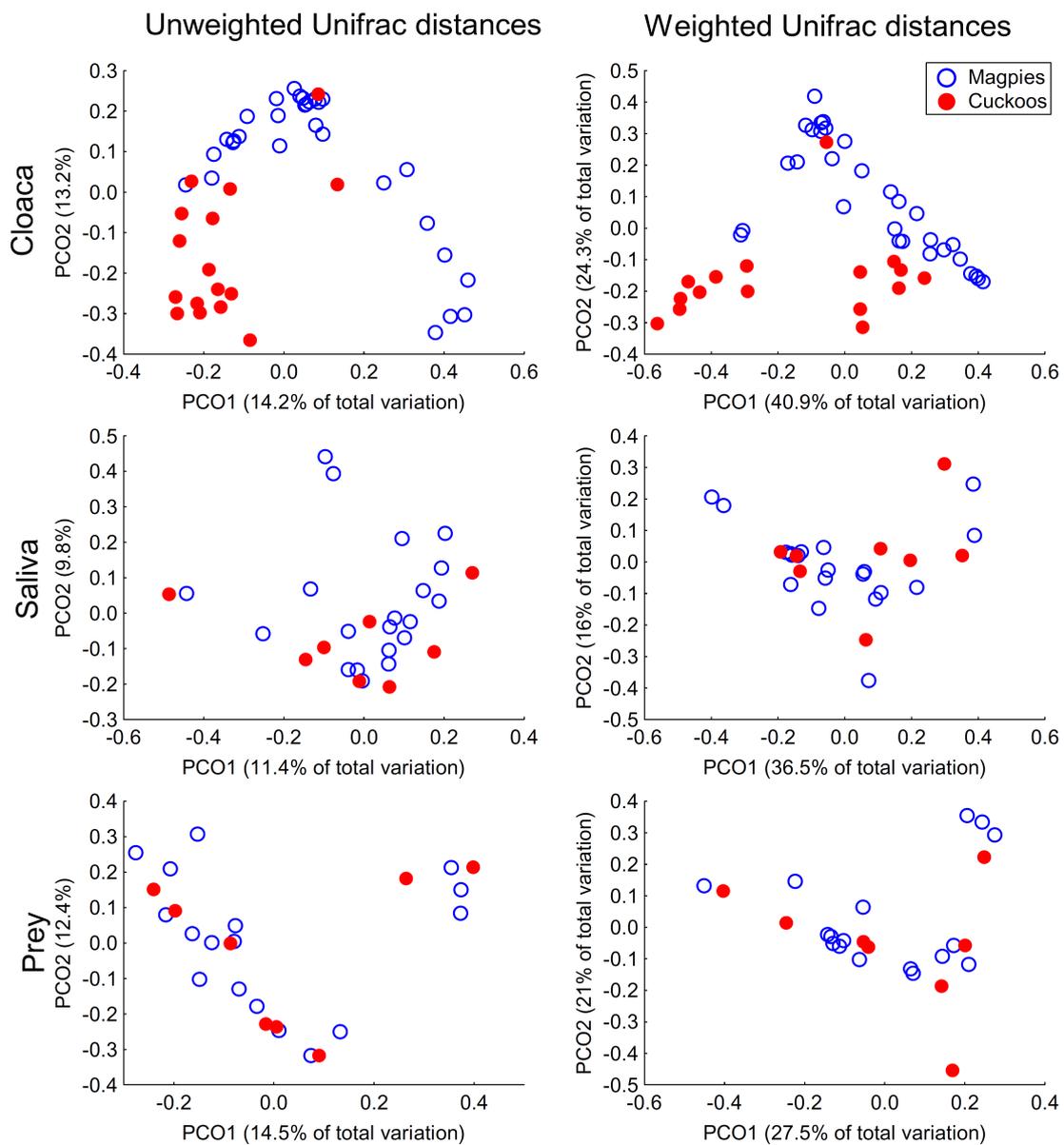
851

852

853

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.

859



860

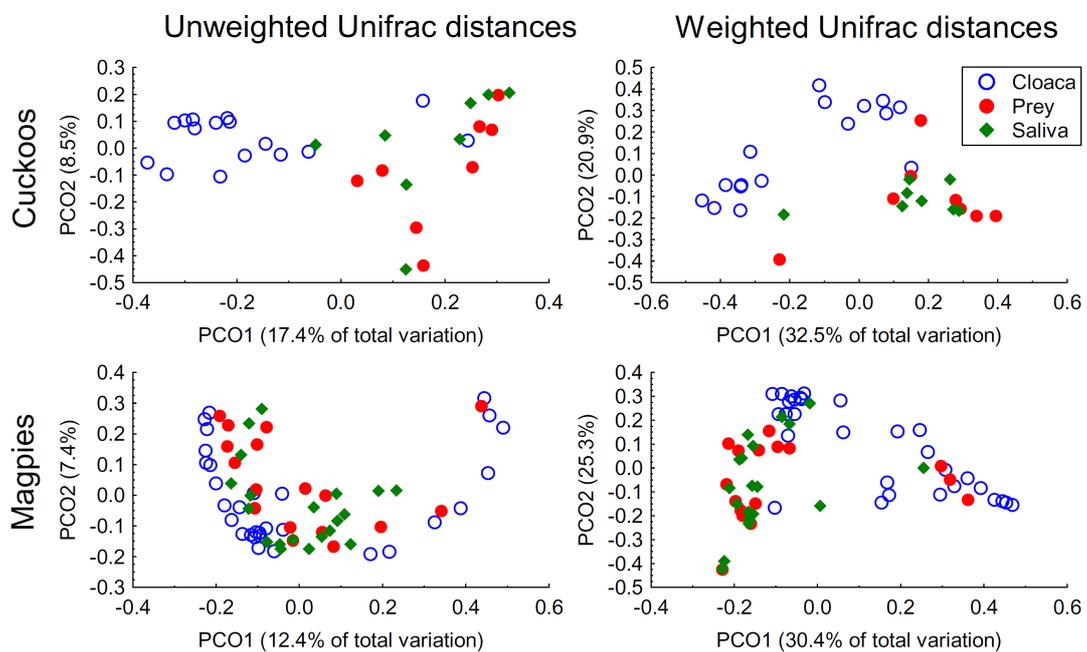
861

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.

868

869



870

871

872 Suppl Material

873

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

874