bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

1 # The scent of symbiosis: gut bacteria
2 # affect social interactions in
3 # leafcutting ants

4

5 Serafino Teseo[1*], Jelle S. van Zweden[2*], Luigi Pontieri[2], Pepijn W. Kooij[2], Søren
6 J. Sørensen[3], Tom Wenseleers[4], Michael Poulsen[2], Jacobus J. Boomsma[2] &
7 Panagiotis Sapountzis[2]

8

9 [1]Unité de Biologie Fonctionnelle et Adaptative (BFA) UMR8251 - CNRS -
10 Université Diderot, Sorbonne Paris Cité, 4 Rue Marie Andrée Lagroua Weill
11 Hallé, 75013, Paris, France.
12 [2]Centre for Social Evolution, Section for Ecology and Evolution, Department of
13 Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen
14 East, Denmark.
15 [3]Section of Microbiology, Department of Biology, University of Copenhagen,
16 Universitetsparken 15, 2100 Copenhagen East, Denmark.
17 [4]Laboratory of Socioecology and Social Evolution, Department of Biology, KU
18 Leuven, Naamsestraat 59, 3000, Leuven, Belgium

19

20 [*] These authors contributed equally

21

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

22 **Abstract**

23 Animal gut microbiota affect host physiology and behaviour. In eusocial
24 Hymenoptera, where colony-level integrity is preserved via a nestmate
25 discrimination system based on cuticular hydrocarbon mixtures, microorganismal
26 effects may influence social dynamics. Although nestmate recognition has
27 undergone a thorough exploration during the last four decades, few studies have
28 investigated the putative role of gut microbes. Here we integrate metagenomic,
29 chemical and behavioural approaches to test whether gut microbes affect
30 nestmate recognition in *Acromyrmex echinatior* leaf-cutting ants. Treating
31 workers with a sterile diet or with antibiotics resulted in a substantial alteration of
32 their gut microbial communities. In pairwise social interactions, untreated vs.
33 antibiotic-treated nestmates behaved more aggressively than other nestmate
34 and non-nestmate pairs, suggesting that the suppression of microbes indirectly
35 alters chemical social cues and triggers aggressive behaviour. Chemical
36 analyses on treated individuals revealed a decrease in the abundance of two
37 metapleural gland antifungal compounds, and we confirmed the correspondence
38 between aggression levels and chemical profile differences. Feeding microbiota-
39 remodelled ants with conspecific faecal droplets partially restored the original
40 bacterial communities. Furthermore, non-nestmates fed with faecal droplets from
41 different colonies were unusually aggressive compared to pairs fed with faecal
42 droplets from the same colony. This suggests that chemicals derived from
43 microbial strains may shape nestmate recognition, opening novel questions
44 about the role of microorganisms in the evolution of social behaviour.

45

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

46 **Keywords**: gut microbiota, *Acromyrmex echinatior*, social evolution, cuticular
47 hydrocarbons

48 Introduction

49 In the evolution of mutualistic relationships between metazoans and prokaryotes,
50 animals have co-opted the metabolic versatility of microbes to upgrade their
51 physiology, while microorganisms have found favourable environments in animal
52 bodies. As part of the physiology of their animal hosts, symbiotic microbes are
53 also involved in their behavioural processes (1–5). Many insightful discoveries
54 about the physiological and behavioural effects of symbiotic microorganisms
55 stem from the study of germ-free or germ-remodelled animals. Research
56 comparing germ-free mice to their untreated counterparts has revealed microbial
57 gut symbionts to affect anxiety-like behaviour (6–8) and social interactions (9).
58 Similarly, a flourishing corpus of *Drosophila* studies suggests that gut microbes
59 mediate a plethora of physiological/behavioural processes, including specific
60 appetites for proteins (10), mate choice and mating dynamics (11–14) and the
61 recognition of kin and familiar individuals (15).
62

63 This increasing awareness about the behavioural role of microbes opens
64 questions about how microorganisms may affect the behavioural ecology of
65 animals with radical social adaptations, such as the eusocial insects. These live
66 in family groups with a permanent division of reproductive labour, worker castes
67 and sophisticated chemical communication (16). Eusocial insect colonies exhibit
68 complex behaviours resulting from the cooperative interactions of individuals,
69 such as foraging, nursing and nest construction, as well as policing and colony
70 defence against unrelated intruders. Across taxa, group-level integrity is ensured

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

71 by a chemical-based nestmate recognition system, which relies on signals
72 encoded in long-chain hydrocarbons forming a waxy layer on the insect cuticle
73 (cuticular hydrocarbons, or CHC).
74 Our current knowledge about the microbial effects on eusocial insect CHC-
75 mediated social behaviour is limited and inconsistent. *Reticulitermes speratus*
76 termites fed with bacteria extracted from individuals of unrelated colonies are
77 attacked by nestmates, and antibiotic-mediated manipulation of bacterial
78 communities affects nestmate recognition behaviour (17). In *Pogonomyrmex*
79 *barbatus* harvester ants, individuals with experimentally-augmented cuticular
80 microbiomes are rejected by nestmates more than controls, whereas antibiotic-
81 treated individuals are not. This suggests that cuticle-dwelling microbes influence
82 nestmate recognition dynamics (18). Contrarily, however, topical antibiotic
83 administration on *Acromyrmex subterraneus* leafcutter ants does not affect
84 cuticular hydrocarbon profiles (19). Similarly, a study on *Camponotus* carpenter
85 ants revealed a negative correlation between the levels of the bacterial gut
86 symbiont *Blochmannia* and CHC quantities, whereas relative CHC proportions
87 were not affected (20). Finally, antibiotic administration affects interspecific but
88 not intraspecific social interactions in the Argentine ant *Linepithema humile*,
89 suggesting gut microbiota not to be involved in nestmate recognition in this ant
90 species (21).
91
92 Despites providing insights into the role of symbiotic microbes in CHC-mediated
93 nestmate recognition, these studies either investigate microbial effects through
94 behavioural tests, without correlating CHC measures (17,21), or compare CHC
95 profiles between antibiotic-treated and control individuals without behavioural
96 tests (18–20). To gain more insights into the interplay between gut microbiota

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

97 and chemical-based social interactions, we here seek to implement an
98 integrative analysis of CHC, gut bacterial communities and nestmate recognition
99 behaviour. We remodelled the previously-characterized gut microbiota of
100 *Acromyrmex echinatior* leafcutter ants (22,23) to investigate the link between gut
101 bacterial communities and socially relevant chemical signals. Our hypothesis
102 was that remodelling of the ant gut microbiota would result in a chemical profile
103 shift with measurable effects on social interactions. Therefore, in a first set of
104 experiments, we either mildly suppressed or completely eliminated the native gut
105 microbiota of *A. echinatior* using respectively a sterile sucrose diet or antibiotics
106 (Round 1). We then characterized the effects on the ant gut microbial
107 communities and chemical profiles, and used dyadic aggression trials to evaluate
108 treatment-dependent changes of gut microbial communities with chemical
109 profiles and behaviour. In a second set of experiments, we partially restored the
110 original ant gut microbial communities with the aim to rescue the chemical and
111 behavioural effects obtained with the experimental diet and the antibiotics
112 (Round 2). To achieve this, we fed experimentally-treated individuals with faecal
113 droplets of untreated conspecifics and again analysed their microbial
114 communities, chemical profiles and social interactions.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

115 **Material and methods**

116 *Experimental design*

117 We used workers from four *A. echinatior* colonies (Ae150, Ae322, Ae153 and
118 Ae331, hereafter named respectively A, B, C and D) with an already
119 characterized gut microbiota (24,25) for the experiments (Figure 1). Colonies
120 were collected in Gamboa, Republic of Panama in 2003-2010, and kept at 25˚C,
121 70% RH and 12:12 L:D photoperiod. Individuals were taken from fungus gardens
122 (375 individuals/colony, N=1500) and placed in groups of 15 in sterile Petri
123 dishes (Ø 90×15mm; 25 dishes/colony, N=100) including a food container. For
124 each colony, we randomly assigned workers to two treatment groups: 1) ants
125 kept on a sterile 10% sucrose solution diet (150 individuals/colony); 2) ants kept
126 on the same sterile 10% sucrose solution supplemented with 1mg/ml of the
127 antibiotic tetracycline (225 individuals/colony). After two weeks, 522 individuals
128 were tested in a first series of experiments (Round 1) including aggression
129 assays and analyses of CHC (GC-MS, see below) and gut microbiota (qPCR
130 and MiSeq, see below). The remaining individuals underwent the experiments in
131 Round 2, where we attempted to restore their original microbiota by feeding ants
132 on a sterile 10% sucrose solution supplemented with faecal droplets (0.033
133 droplets/μl) obtained by squeezing abdomens of untreated workers from the
134 source colonies. Colony B and D workers received nestmate droplets, whereas A
135 and C workers received non-nestmate droplets (from colonies B and D,
136 respectively, Figure 1). After one week, individuals were tested in aggression
137 assays and used for CHC and microbiota analyses. Throughout the experiments,
138 we monitored survival to determine the effects of our treatments on ant mortality
139 (details in the Supplementary information, Figure S1).

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

140 Finally, we set up an independent smaller-scale experiment in which we
141 compared the microbial communities of ant guts, heads and thoraxes before and
142 after the experimental diet treatment (details in the Supplementary Information,
143 Figure S2).
144

145 *Aggression assays*

146 We tested microbiota-remodelled and untreated ants against nestmates and
147 non-nestmates from the original colonies, in both experimental rounds (3-5
148 replicates/combination, total=260 tests; Figure 1, Data set S1). Assays consisted
149 of dyadic encounters in Petri dishes with clean filter paper on the bottom. For two
150 minutes after first contact, an observer (blind with respect to ant treatment and
151 original colony) used the software Etholog 2.25 (26) to quantify the frequency
152 and duration of biting, mandible opening, antennation and absence of contact
153 (Figure S3). For statistical analyses, we excluded the mandible opening
154 behaviour (usually considered as aggressive) because pooling it with biting
155 produced similar results (Supplementary Information). In addition, because
156 keeping ants in the same Petri dishes for three weeks resulted in a significant
157 effect on aggression (Binomial GLMM with 'Petri dish' as fixed and 'colony' as
158 random variables, $z=3.21$, $Df=141$, $p=0.001$), we considered only interactions
159 between nestmates kept in different Petri dishes.

160 We analysed data in R using the packages *lme4*, *car* and *multcomp* (27–29),
161 fitting generalized linear mixed models (GLMMs). Classifying biting as
162 aggressive behaviour and antennal contact as non-aggressive allowed measures
163 to be analysed as a single binomial response variable. For Round 1, the initial
164 model included biting/antennation frequencies as response variable, whereas

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

165 nestmate, diet treatment and their interaction were included as fixed factors. The
166 colony origin of experimental individuals was included as random effect term.
167 The model used for Round 2 included the same response variable as Round 1
168 model, and 'faecal droplets' was included as an additional fixed factor (two
169 levels: from nestmates or non-nestmates). We tested the significance of fixed
170 effects using the *car* function 'Anova'. Where needed, we conducted post hoc
171 planned contrasts between groups of interest using the *multcomp* function 'glht',
172 correcting alpha values with false discovery rate (FDR).
173

174 *DNA extractions*

175 Four to eight workers per treatment per colony used in the aggression assays
176 were ice anesthetized and individually dissected in sterile Phosphate Buffered
177 Saline (PBS). Pooled crop, midgut, hindgut, Malpighian tubules and fat body
178 cells were stored at -20°C until DNA extractions, and immediately homogenized
179 after thawing in 200µl ATL buffer supplemented with 20µl proteinase K (Qiagen)
180 using sterile pestles. Subsequently, Ø0.45 mm glass beads were added and
181 tubes vortexed for 30s, after which the samples were incubated at 56°C
182 overnight under constant agitation. DNA was extracted using the Qiagen Blood
183 and Tissue kit, and all samples were eluted in 100µl AE elution buffer.
184

185 *16S rRNA qPCR analyses*

186 The gut microbiota of *A. echinatior* normally consists of five predominant OTUs
187 (Operational Taxonomic Units, representing a cluster of bacterial 16S rRNA
188 sequences of ≥97% similarity) (22,25,30). These belong to the genus *Wolbachia*

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

189 (*wolAcro1,* including two strains: *w*SinvictaA and *w*SinvictaB) and the orders
190 Entomoplasmatales (class: Mollicutes, OTUs *EntAcro1*, *EntAcro2* and
191 *EntAcro10*) and Rhizobiales (class: Alpha-Proteobacteria, OTU *RhiAcro1*
192 (25,31–33)). In order to monitor how the dietary treatment affected the
193 communities, we screened individual worker guts with qPCR (detailed methods
194 in Supplementary Information, text and Table S1) on the five most abundant
195 OTUs (Data set S2, procedures described in (25)), with Cycle threshold (Ct)
196 mean of replicated samples used as a measure of amplicon abundance. The
197 elongation factor 1 alpha (EF-1α) was used as a reference gene (34). Each run
198 included two negative controls with no added template for each gene used. Data
199 were ordinated using an unscaled principal coordinate analysis (PCoA) and inter-
200 sample distances were again calculated using Canberra, Hellinger and Bray-
201 Curtis methods. We estimated the difference in variation among groups using the
202 HOMOVA command (with Bonferroni-adjusted alpha-values) implemented in
203 mothur (35). For analysis, we initially used a standard curve with PCR products
204 in tenfold dilution series of known concentration (fold change method) to
205 calculate the PCR efficiency using the REST software (36). Data were imported
206 in R and expressed as $\Delta\Delta C_T$ values, i.e. as the fold change relative to the EF-1α
207 control gene (37), always using zero as reference. We used linear mixed models
208 (LMM) with the $\Delta\Delta C_T$ values as response variable, 'diet treatment' (i.e.,
209 untreated, sugar-treated or tetracycline-treated) and 'experimental round' (i.e.
210 Round 1 or 2) as fixed variables, and 'colony' (A, B, C or D) as random variable.
211 Tukey post hoc tests were performed to evaluate significant differences between
212 groups. We used GLMs to pair aggression data with distances calculated using
213 ΔC_T values fold change differences, or with absolute copy numbers calculated

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

214 using the qPCR data. All correlations were performed in R using the lme4,
215 vegan, effects, Rmisc and ggplot2 packages (27,38–41).
216

217 *16S rRNA MiSeq analyses*

218 To investigate whether novel OTUs appeared with the dietary treatment, we
219 screened ant worker guts individually with 16S rRNA MiSeq sequencing.
220 Amplicons were generated using the 515F/806R primers targeting the 16S rDNA
221 V4 region (42), purified using the Agencourt AMPure XP (Beckman Coulter) and
222 quantified using Quant-iT dsDNA High-Sensitivity Assay Kit and Qubit
223 fluorometer (Invitrogen) to allow for dilution and mixing in equal concentrations
224 before sequencing. Sequencing (for details see the Supplementary Information)
225 took place in the Section of Microbiology at the University of Copenhagen using
226 an Illumina MiSeq. Data [Genbank: SAMN04261407 - SAMN04261536 and
227 SAMN05362797 - SAMN05362832] were analysed using mothur (35). Details on
228 the mothur procedure are given in the Supplementary Information. After
229 filtering/processing the sequencing data and clustering at 97%, rarefaction tables
230 were constructed using pseudo-replicate OTU datasets containing 1-272000
231 sequences with 1000 iterations/pseudo-replicate, and the resulting curves were
232 visualized in Microsoft Excel 2013. The final OTU Table was rarefied at 5000
233 reads after manual inspection of the rarefaction curves, which reduced the
234 number of OTUs to 1500. We used the MiSeq data to calculate Canberra and
235 Bray-Curtis distances, after which we used Non-metric MultiDimensional Scaling
236 (NMDS) to ordinate and visualize the effects. We used GLMs to pair aggression
237 data with Bray-Curtis or Canberra distances calculated from the 16S rRNA

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

238 MiSeq data. Correlations were performed in R v3.2.3 using the lme4, vegan,
239 effects, Rmisc and ggplot2 packages (27,38–41).

240

241 *Cuticular hydrocarbon analyses*

242 CHCs were extracted by immersing the dissected heads and thoraces of
243 aggression test individuals first in 150 µl HPLC-grade hexane, and then in 150 µl
244 HPLC-grade chloroform (chemicals from Sigma-Aldrich, Belgium), both for 10
245 min under continuous agitation. The two extracts were mixed, and the solvent
246 evaporated at room temperature in a laminar flow cupboard. The dry extract was
247 then dissolved in 30µl hexane, of which 3µl were injected in a Shimadzu QP2010
248 Ultra GC-MS (splitless injector mode). Details on the GC-MS settings are given
249 in the Supplementary Information. Our initial integration analysis of GC-MS runs
250 detected 137 peaks, of which we selected 73 that had a relative abundance
251 larger than 0.1% (Data set S3). Peak areas of cuticular compounds were
252 integrated using R v3.1.0 (using package *xcms*, script available upon request)
253 and normalized using a Z-transformation (43). To compare odour profiles among
254 different rounds, we used linear mixed models (LMM) with the relative
255 abundance of each compound as the response variable, 'diet treatment' and
256 'experimental round' as fixed variables, and 'colony' as a random variable. We
257 conducted linear hypotheses using the *multcomp* R package (44) function glht to
258 evaluate differences between diet treatments in the same Rounds, and between
259 the same diet treatments across Rounds. All p-values were corrected for false
260 discovery rate (FDR) given that we had conducted 73 separate tests per
261 contrast. We used a coinertia analysis (45) to check for correlations between
262 CHCs (transformed to logarithmic data) and qPCR measures (ΔCt values) of the

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

263 six most abundant bacterial taxa (see details about these taxa below). In short,
264 we generated two independent data matrices (either using the individual profiles
265 or the pairwise differences for each trial), performed PCA analyses and paired
266 them using the coinertia analysis using a Monte-Carlo test with 10000
267 permutations.
268

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

269 **Results**

270 *Survival analysis*

271 During Round 1, mortality increased in tetracycline treated workers (Cox
272 proportional hazard model, $p < 0.001$), similar to what had been observed in a
273 previous study (25). However, this effect disappeared in Round 2, when all ants
274 were fed on faecal droplets (Figure S1), suggesting that the harmful effect of
275 tetracycline lasted only as long as it was administered to the ants.

276

277 *Aggression tests (Round 1)*

278 In Round 1, where ants were isolated from their original colonies and fed on
279 sterile sucrose diets with/without antibiotics (Figure 1), we found a significant
280 nestmate*treatment interaction (χ^2=6.9803, df=2, p=0.0305). The diet treatment
281 had a significant effect on aggression, whereas being non-nestmates did not
282 (diet treatment: χ^2=29.62, df=4, p<0.001; nestmates vs. non-nestmates: χ^2=1.64,
283 df=1, p=0.18; N=260 dyadic aggression assays). This result was mostly due to
284 the high biting frequency between tetracycline-treated ants and their untreated
285 former nestmates taken from fungus gardens, which was significantly higher
286 from all other nestmate trials (all p<0.05, Figure 2A, Table S2). For tetracycline-
287 treated and sucrose-treated ants, aggression levels between non-nestmates
288 were not significantly different from those observed in nestmate trials
289 (tetracycline-treated: z=0.365, p=0.715, Figure 2A; sucrose-treated: z=1.284,
290 p=0.287). Contrarily, in the sucrose- vs. tetracycline-treated groups, non-
291 nestmate trials showed higher aggression than nestmate trials (z=2.375,
292 p=0.045).

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

293

294 *Aggression tests (Round 2)*

295 During Round 2, across sucrose-treated pairs, tetracycline-treated pairs and
296 sucrose- vs tetracycline treated pairs, aggression was low in nestmate
297 encounters fed with nestmate faecal droplets, higher in non-nestmate encounters
298 fed with nestmate faecal droplets and maximal in non-nestmate encounters fed
299 with non-nestmate faecal droplets (Figure 2b). In particular, the highest
300 aggression levels appeared in encounters between non-nestmate sucrose-
301 treated ants fed with faecal droplets from different colonies (z=2.05, df=85,
302 p=0.041; Figure 2b, Table S2). Tetracycline-treated pairs showed instead low
303 aggression (Figure 2b), regardless of whether they were fed on the same or
304 different faecal droplets. Sucrose-treated vs tetracycline-treated ants exhibited
305 low aggression levels, similar to tetracycline-treated pairs. We found a significant
306 effect of the interaction faecal droplet*treatment (χ^2=7.57, df=2, p<0.05), but not
307 diet treatment*nestmate (χ^2=3.36, df=2, p=0.18; Figure 2b, Table S2). All three
308 main effects were significant (faecal droplets: χ^2=7.99, df=1, p<0.05; diet
309 treatment: χ^2=36.97 df=4, p<0.001; Nestmate: χ^2=5.2437 df=1, p<0.05).

310

311 *Gut bacterial communities changes (Round 1)*

312 We examined the changes of the gut bacterial communities using both 16S
313 rRNA MiSeq amplicon sequencing and 16S rRNA qPCR to measure the levels of
314 the five most abundant OTUs (Data set S2; (22,25,30). Microbiomes of
315 tetracycline-treated individuals were most strongly affected, showing the lowest
316 variance, whereas untreated individuals collected from fungus gardens showed

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

317 the highest variance (HOMOVA, p<0.001; Figure S4). While gut bacterial
318 communities of ants reared on fungus gardens or sucrose differed significantly
319 from those of tetracycline-reared ants (p<0.001 and p=0.013, respectively;
320 Figure S4), their communities were different, even though not significantly, from
321 each other (p=0.053). Furthermore, diet had the strongest effect on the
322 differences between bacterial communities of treatment groups (PERMANOVA,
323 $F_{2,53}$=9.162, p<0.001), while colony origin did not (PERMANOVA, $F_{3,53}$=1.758,
324 p=0.931). When examining each of the abundant OTUs individually, *EntAcro1*
325 and *RhiAcro1* decreased in tetracycline- and moderately in sucrose-treated
326 individuals, while *w*SinvictaB was largely unaffected by diet (Figures 3, S5a).
327 *w*SinvictaA (present only in colony C, Data set S2), *EntAcro2* and *EntAcro10*
328 (present respectively in 9 and 20 of 37 tested untreated workers taken from
329 fungus gardens; Data set S2), increased slightly in sucrose- and decreased in
330 tetracycline-treated ants, but these effects were not significant. The head, thorax
331 and gut microbial community comparisons showed that the most marked change
332 were of the gut bacterial communities (detailed results in the Supplementary
333 Information; Figure S5).
334

335 *Gut bacterial communities changes (Round 2)*

336 The NMDS ordination showed that the gut bacterial communities of tetracycline-
337 treated individuals before and after faecal droplet feeding were not significantly
338 different. Contrarily, the gut bacterial communities of sugar-treated individuals
339 were closer to those of untreated workers (from original fungus gardens) after
340 faecal droplet feeding (Round 1), suggesting a shift towards the original
341 communities (Figure S5). Interestingly, the gut bacterial samples of sugar-treated

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

342 individuals exhibited a clear separation depending on which faecal droplets
343 (nestmates or non-nestmates) they were fed on (PERMANOVA, $F_{1,43}$=23.17,
344 p<0.001 and $F_{1,43}$=12.10, p<0.001, respectively and $F_{1,43}$=5.04, p=0.004 for their
345 interaction), whereas there was no such effect in the tetracycline-treated group
346 (Figure S5). Tetracycline-treated ants undergoing faecal droplet feeding showed
347 an increase of all OTUs but *EntAcro2* and *RhiAcro1*, whose levels were further
348 reduced; sucrose-treated ants showed an increase of all gut bacterial taxa
349 examined (Figure 3), but only changes in *EntAcro1*, *EntAcro2*, *EntAcro10* and
350 *w*SinvictaB (respectively: t=-2.69, p=0.008; t=-2.02, p=0.044; t=-5.32, p<0.001;
351 t=-5.95, p<0.001) were significant.
352

353 *Regression of bacterial changes and aggression*

354 To identify which of the OTUs best explains aggression, and since the high
355 variation both in aggression and gut microbiota composition precluded us from
356 comparing mean effects, we regressed the observed aggression between
357 nestmate test pairs on differences in abundance of their gut bacteria (Figure 4).
358 Foremost of all, this analysis confirmed that more aggression was observed
359 when nestmate pairs differed more in their gut microbial community (1500 MiSeq
360 OTUs, using Bray-Curtis distances between pairs, binomial GLMM with
361 'distance' and 'round' as fixed variables and 'colony' as random variable, z=4.91,
362 p<0.001). For individual taxa we used the more accurate qPCR data focusing on
363 the main five taxa, regressing the observed aggression against their ΔΔCt values
364 for each of the six gut bacterial OTUs (Figure 4). This analysis showed that
365 aggression was not positively affected by either of the two *Wolbachia* strains
366 (Figure 4; *wSinvictaA*: z=-0.66, p=0.509; *wSinvictaB*: z=-3.75, p<0.001), or

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

367 *EntAcro2* (z=-0.872, p=0.383), but showed significant positive effects for
368 *EntAcro1* (z=2.10, p=0.035), *EntAcro10* (z=6.09, p<0.001) and *RhiAcro1*
369 (z=5.85, p<0.001). However, only differences in abundance of *RhiAcro1* had a
370 strong effect on aggression in both rounds of the experiment (Figure 4; Data set
371 S4; Round 1: z=3.25, p<0.001; Round 2: z=4.17, p<0.001), suggesting that
372 Rhizobiales are a driving force in the recognition of nestmates (see Data set S4;
373 similar results were obtained when both biting and mandible opening were
374 treated as aggressive behaviors).

375

376 *Effects of diet on CHC profiles*

377 Compared to untreated individuals from original fungus gardens, sucrose- and
378 tetracycline-treated individuals of Round 1 exhibited a strong reduction of 4-oxo-
379 octanoic and 4-oxo-decanoic acids (LMMs with 'diet' and 'round' as fixed
380 variables and 'colony' as random variable, FDR-corrected p-values for multiple
381 comparisons; 4-oxo-octanoic acid: untreated *vs.* sucrose-treated, t=15.20,
382 p<0.001, untreated *vs*. tetracycline-treated, t=15.01, p<0.001; 4-oxo-decanoic
383 acid: untreated *vs*. sucrose-treated, t=-13.10, p<0.001, untreated *vs*. tetracycline-
384 treated, t=-13.66, p<0.001). However, changes to these compounds were
385 significant in all pairwise comparisons of all treatment groups, suggesting that it
386 is more related to the isolation than the removal of bacteria (Data set S4). On the
387 other hand, changes in n-C36 and n-C40 were only significant in pairwise
388 comparisons of ants treated with antibiotics and ants reared on their original
389 colonies.

390

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

391 Considering all experimental individuals of Rounds 1 and 2, we found a
392 significant correspondence between chemical profiles (CHC) and qPCR data
393 (RV=0.143, P<0.001; Figure S6). The OTUs *EntAcro1* and *RhiAcro1* co-varied
394 the most with the two metapleural gland acids. There was a significant
395 correspondence between chemical profiles and qPCR gut bacterial abundances
396 also when the acids from the metapleural gland were excluded (RV=0.141,
397 p<0.001). Contrarily, we found no significant correspondences when instead of
398 using the individual CHC and qPCR profiles, we used each aggression pair as a
399 single sample and for input we used the odour differences and ΔΔCt values in
400 aggression pairs (RV=0.122, p=0.707). This suggested that bacterial
401 abundances can explain some of the changes in odour that occurred in the diet
402 treatments (because there is a highly significant correlation between the two data
403 sets), but not to such an extent that they accurately reflect odour differences
404 between individuals that may lead to (a lack of) recognition.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

405 ## Discussion

406 *Experimental treatment affects gut microbial communities*

407 The gut microbial communities of tetracycline-treated ants underwent the most
408 substantial deviation from those of untreated nestmates (two taxa decreased
409 strongly, three slightly and one was not affected; Figure 3), whereas those of
410 sucrose-treated ants exhibited a relatively milder shift (three taxa decreased
411 slightly, two increased slightly and one was not affected). These results show
412 that while the antibiotic suppressed most of the ant microbial community, the
413 sterile sucrose diet affected only mildly the various taxa, altering their relative
414 abundance.

415

416 Feeding workers with conspecific faecal droplets only partially restored the
417 original gut microbial communities of sucrose- and tetracycline-treated ants,
418 suggesting that the elimination of strains such as *RhiAcro1* and *EntAcro2* was
419 irreversible. This may imply that some bacteria need to be established during
420 larval development or early adult life and cannot be reintroduced later. In
421 addition, new OTUs emerged (i.e., *EntAcro10*) which may have prevented the
422 original OTUs from tissue re-colonization (cf., 46,47). We also cannot exclude
423 that the tetracycline effect lasted for some time even after its administration was
424 suspended (one week before the preparation of the corresponding microbial
425 DNA samples), potentially interfering with the faecal droplet-mediated microbial
426 gut re-colonization.

427

428 Although we provide evidence for which bacterial taxa are most affected by our
429 experimental treatments, amplicon sequencing often does not allow

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

430 distinguishing bacterial strains with identical 16S sequences (33,48,49). Thus,
431 we cannot exclude the effect of gut bacteria on behaviour to be driven by the
432 interaction of multiple bacterial strains with different metabolic potential, and
433 further research should implement methods that allow considering this.
434

435 *Microbiota remodelling affects ant cuticular chemical profiles*

436 Gut bacteria play essential metabolic roles in several ant species, such as the
437 production of metabolites that are absent in host diets or help cover energy
438 needs (50–53), and bacterial removal may thus impair metabolism functions and
439 ant wellbeing. Social withdrawal of unhealthy ant workers has been
440 demonstrated for at least two ant species (54,55), and it is conceivable that the
441 ostracism of the sick ants is driven by changes in CHCs (or other volatile
442 compounds important in communication). We found consistent and significant
443 decreases of two metapleural gland acids (4-oxo-octanoic and 4-oxo-decanoic)
444 (56,57) in sucrose-treated ants, and reductions in these compounds and two
445 linear alkanes (n-c36 and n-c40) in tetracycline-treated individuals. These
446 chemical profile differences may have been caused by symbiotic gut bacteria
447 affecting CHC biosynthesis, either directly by contributing to the CHC pool, or
448 indirectly by affecting host metabolism and therefore host CHC production. This
449 inference is further supported by the fact that CHCs are synthesized in the
450 oenocytes (12), which are heavily colonized by *EntAcro1* bacteria (25) and are
451 the centre of the intermediate metabolism in ants (58).
452

453 Tetracycline treatment may not only have affected the gut microbes, but also
454 those living on the ant cuticle (such as the Actinobacteria *Pseudonocardia*

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

455 (24,34)). Such effects may result from the direct actions of the antibiotic or from
456 indirect effects, such as changes in host metabolism or production of nutrients
457 sustaining cuticular bacterial growth. In *Drosophila*, cuticular microbes have been
458 hypothesized to affect chemical profiles by using CHC as a carbon source or a
459 substrate for degradative enzymes (12), and previous research on
460 *Pogonomyrmex* ants showed that topical antibiotic administration can alter CHC
461 profiles, confirming a possible role of surface microbes in CHC profile
462 determination (18). However, the same kind of antibiotic treatment applied on
463 *Acromyrmex subterraneus* did not affect CHC profiles (19). Work on *Drosophila*
464 suggests that the innate immune response may also mediate CHC profiles (59),
465 and innate immune responses to bacteria affects CHC profiles in honey bees
466 (60). Thus, multiple factors are conceivably involved and likely interact to form
467 worker CHC profiles, and disentangling this complex of host-symbiont
468 interactions and contributions will require extensive further work.
469 Although we provide correlational evidence for our dietary treatments to act on
470 gut bacteria, which may indirectly affect metapleural gland acids and cuticular
471 hydrocarbons via the metabolism, we cannot exclude other types of effects.
472 Across insect taxa, symbiotic bacteria produce a plethora of volatile
473 semiochemicals (61,62), and studies on *Drosophila* suggest compounds deriving
474 from the metabolism of gut microbes to mediate interactions between individuals
475 (15,63). Eusocial Hymenoptera may have integrated the bacterial metabolism in
476 their chemical-based social dynamics, and ants may thus rely on a social
477 communication system based on chemicals produced by its commensal bacteria.
478 This would allow discriminating against unfamiliar odours from different bacterial
479 communities and would be complementary to the well-studied CHC-mediated
480 nestmate recognition system. Accordingly, we cannot exclude that the altered

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

481 behaviour observed in our aggression tests may at least partially depend on
482 volatile semiochemicals from bacterial metabolism. In future experiments,
483 nestmate recognition assays should be implemented in which microbiota-
484 remodelled interacting individuals are prevented from relying on non-volatile
485 cuticular social cues.
486

487 *Microbiota remodelling affects social interactions*

488 Encounters between antibiotic-treated and untreated nestmates produced the
489 highest aggression levels, suggesting that the antibiotic treatment affects
490 chemical cues that are relevant for social interactions. Aggression due to diet
491 treatment was clearly higher than aggression due to nestmate status and the
492 treatment effect to aggression was directly dependant to the gut bacterial titre:
493 the diet effect on aggression was always clear in sucrose-treated ants but tend to
494 diminish (contrasts were less clear) in tetracycline-treated ants (Figure 2A:
495 nestmate (NM) and non-nestmate (nNM) aggression levels of Tetracycline vs
496 Tetracycline treated ants were almost identical). This further suggested that the
497 gut bacterial communities (or certain taxa) are having an active role in nestmate
498 recognition which possibly disappears when these bacterial symbionts get
499 eliminated.
500

501 *The impact of partial restoration of the gut microbiota on social interactions*

502 After faecal droplets administration, nestmate dyadic encounters revealed
503 aggression levels only moderately (and non-significantly) higher than those of
504 Round 1. While this moderate increase in aggression may be an effect of the

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

505 relatively longer separation of the experimental ant groups (three instead than
506 two weeks in different petri dishes), the lack of significant differences between
507 rounds is consistent with only partial restoration of microbial gut communities. In
508 non-nestmate aggression assays, pairs of tetracycline-treated and tetracycline-
509 vs. sucrose-treated ants again showed low aggression. In contrast, aggression
510 was high in sucrose-treated pairs when interacting individuals were fed with
511 faecal droplets from different colonies. This implies that, although bacterial
512 communities were only partially restored, colony-specific faecal droplets did
513 affect the cues determining behavioural outcomes of the social interactions. This
514 partial restoration also rules out the possibility of changes in behaviour due a
515 direct effect of tetracycline, which is known to impair mitochondrial function and
516 thus potentially could induce confounding effects (64).
517

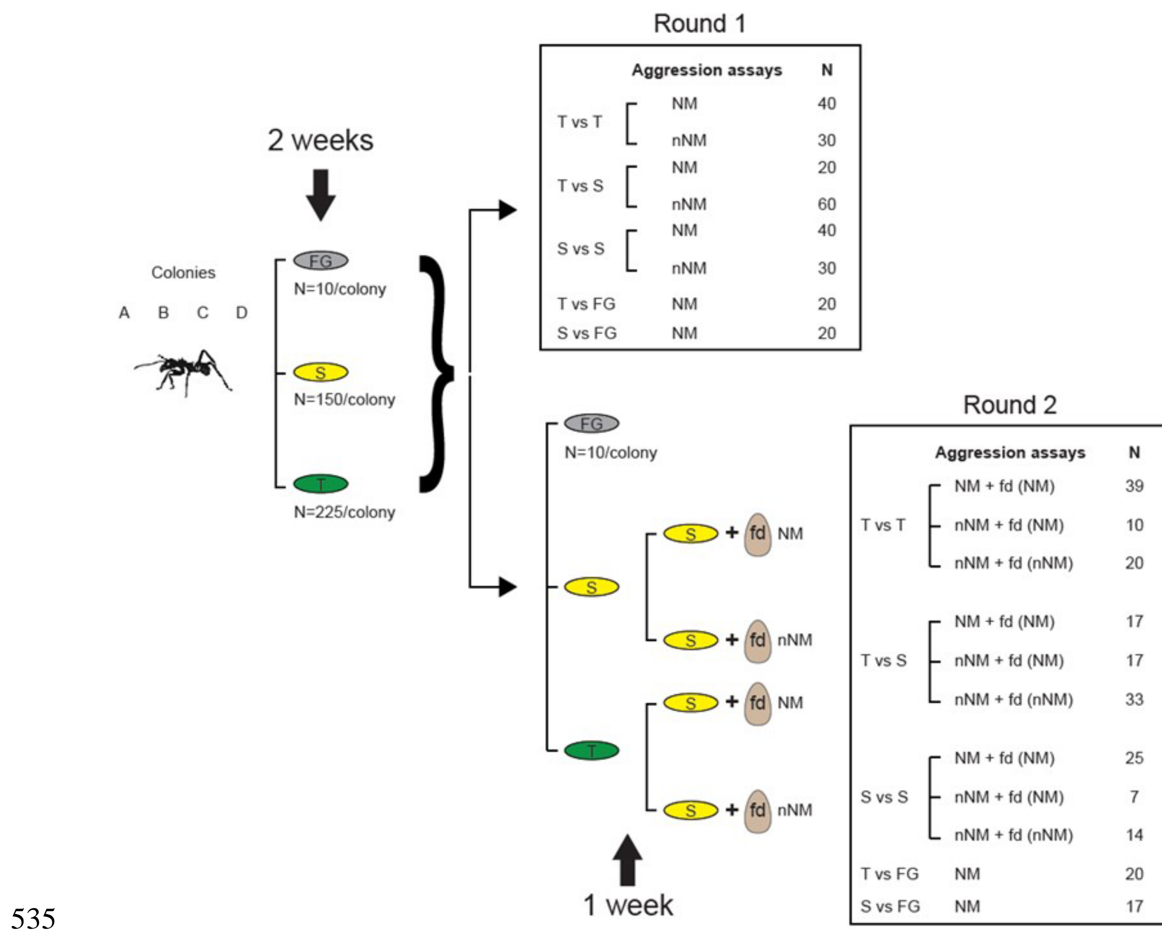
518 ## Conclusions

519 In this study, we seek to take an integrative approach to explore the role of gut
520 microbial symbionts in the social dynamics of a eusocial organism. Remodelling
521 of the ant gut microbiota produced effects on both the chemicals ants display as
522 socially-relevant recognition signals and their resulting behaviours. Our findings
523 suggest that the observed effects mostly depend on two bacterial taxa, the
524 previously identified major gut symbionts of leafcutting ants *EntAcro1* and
525 *RhiAcro1*. Further research will be needed to address the mechanisms
526 underlying the link between these symbionts and behavioural modifications.
527 Either the altered microbial communities may result in chemical profiles that
528 cause individuals to look more like non-nestmates, or microbiota-remodelled ants
529 may be recognized as sick, and aggression towards such individuals could serve

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

530 to prevent the spread of infections to optimize colony health and efficiency.
531 Regardless, our findings provide evidence that gut bacterial symbionts may be
532 involved in kin recognition by contributing to shape ant CHC signatures, with
533 implications for our understanding of social insect-symbiont evolution.

534 # Figures



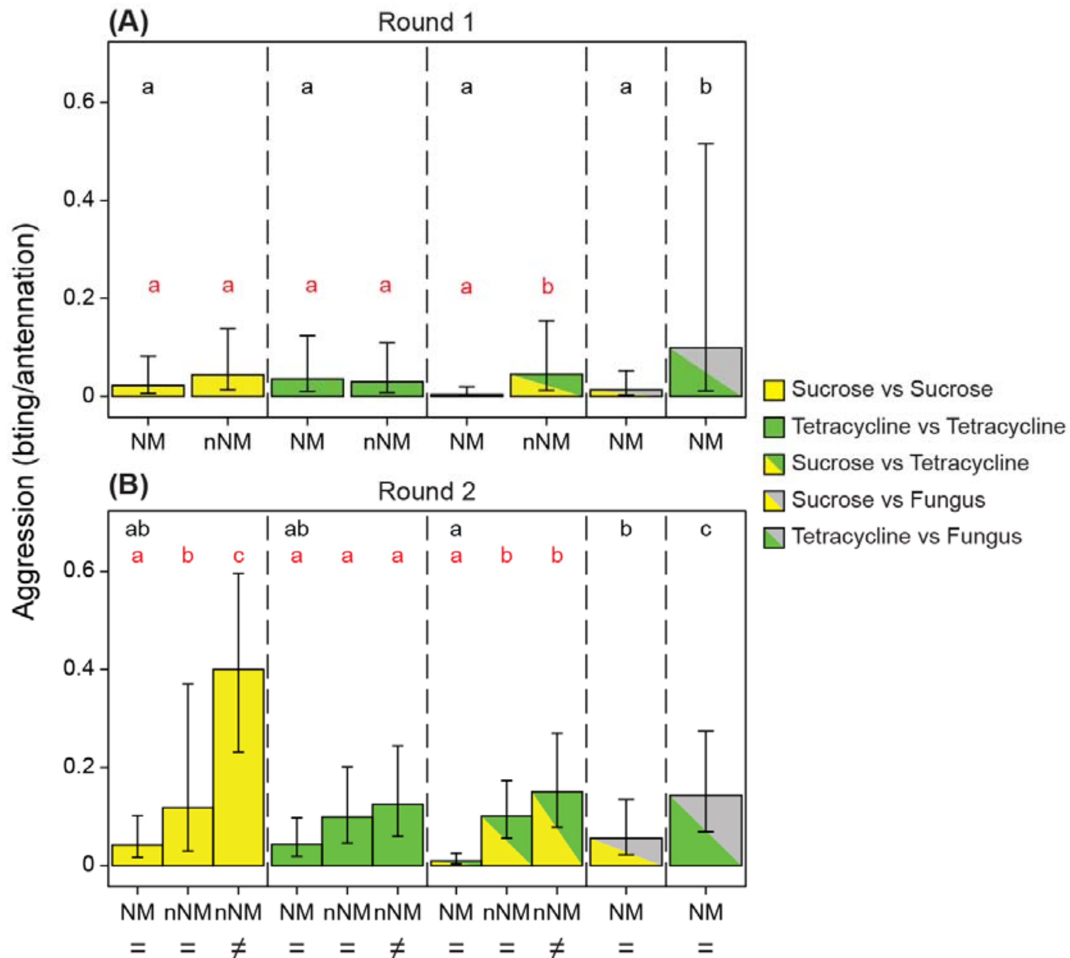
535

536 **Figure 1. Experimental design.** Ants were collected from fungus gardens (FG)
537 and kept for two weeks on a sucrose (S) or Tetracycline (T) diet (Round 1). After
538 two weeks we performed aggression trials among the three treatment groups.
539 Individual guts were then dissected for gut bacterial characterization (16S-qPCR
540 and 16S-MiSeq), whereas thoraxes and heads were used for chemical

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

541 extractions and subsequent GC-MS analyses. In Round 2, the remaining
542 individuals were fed for one week on faecal droplets (fd) either from their original
543 nestmates (NM) or non-nestmates (nNM) from a different colony. These ants
544 were then tested in aggression trials similar to Round 1 and collected for gut
545 microbiota characterization and GC-MS analyses.
546

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



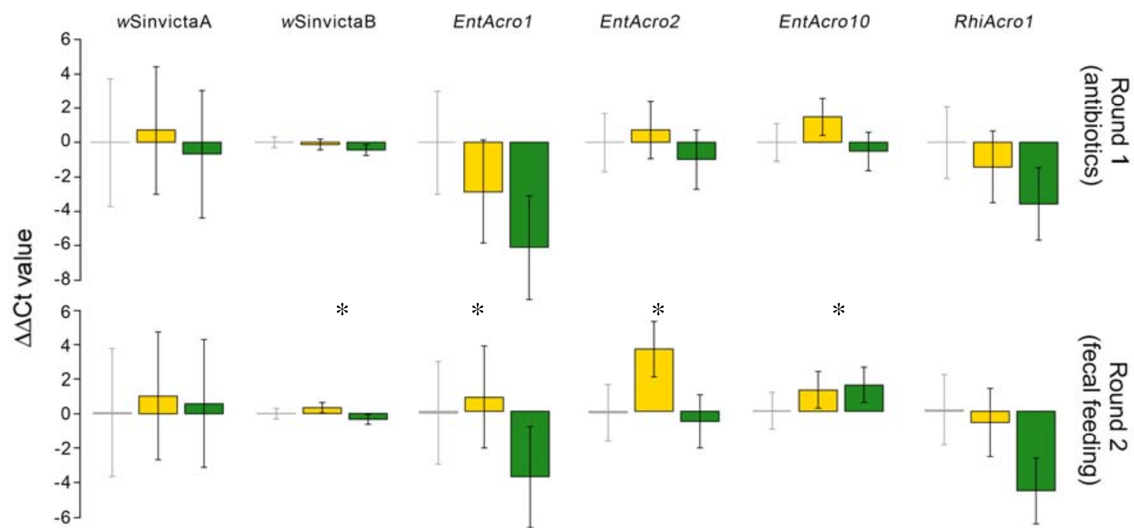
547
548 **Figure 2. Effects of sucrose and tetracycline treatment on aggression.**
549 Mean biting/antennation ratio, with upper and lower confidence limits as error
550 bars. **A.** Round 1. Tetracycline-treated vs. untreated nestmate ant pairs show the
551 highest aggression levels, surpassing non-nestmate trials. **B.** Round 2.
552 Encounters between individuals fed on faecal droplets. In nestmate trials,
553 tetracycline-treated vs. untreated individuals still show the highest aggression.
554 Letters indicate significance in pairwise comparisons (different letters =
555 statistically significant differences). Red font indicates comparisons within
556 treatment category (e.g. sucrose vs sucrose, tetracycline vs tetracycline)
557 whereas black font shows comparisons across treatment categories (e.g. trials
558 between sucrose-treated nestmates compared to trials between tetracycline-

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

559 treated nestmates). In non-nestmate encounters, sucrose-treated individuals fed
560 with faecal droplets from different colonies (≠) show the highest aggression
561 levels, whereas non-nestmates fed with faecal droplets from the same colonies
562 (=) exhibit low aggression. NM = nestmates; nNM = non-nestmates; FD= Faecal
563 droplets. See Table S2 for details on statistical comparisons among treatment
564 groups.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

565



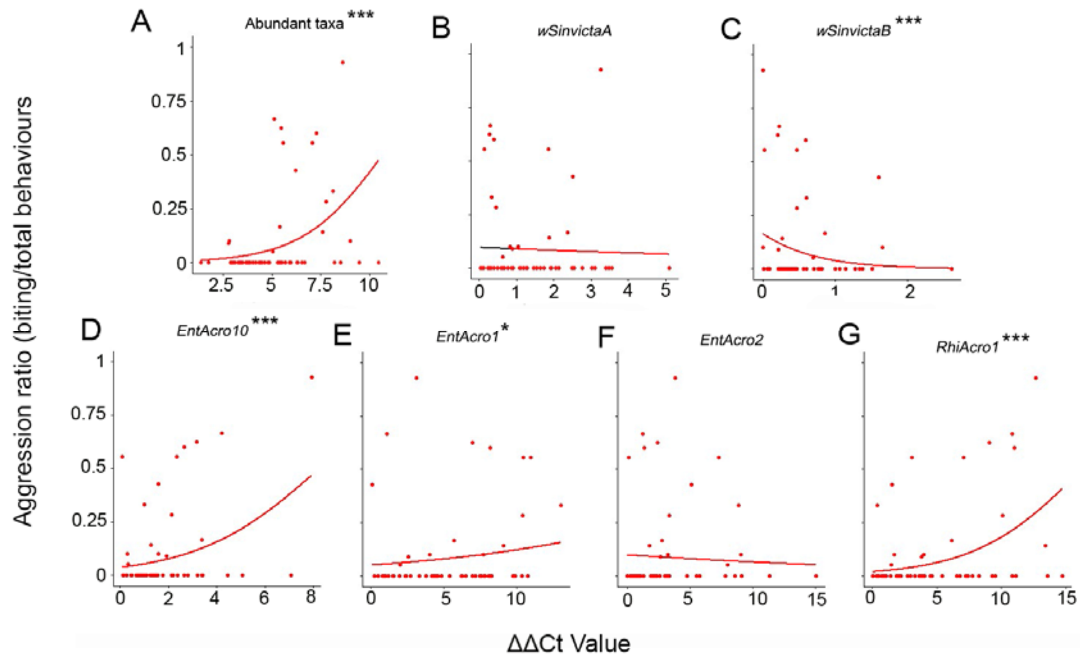
566 **Figure 3. A. qPCR bacterial titres fold change of the six most abundant**
567 **bacteria after experimental Rounds 1 and 2.** Fold change is normalized to ant
568 EF-1α copies, and the microbial strain levels of untreated conspecifics (ants
569 reared on FG) are used as reference level (y=0). Scale bars represent standard
570 errors. Asterisks indicate significance levels, $p<0.05$. Yellow: sucrose-treated;
571 green bars: tetracycline-reared ants.

572

573

574

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



575

576 **Figure 4. A. Association between aggression level and differences in the**
577 **abundant gut bacteria using GLMMs**. The relationship between biting
578 frequency and bacterial abundances measured using the qPCR fold change (Ct
579 distance) for both rounds for all six or for individual bacterial taxa. Curves
580 represent predicted values from the models. Asterisks indicate significance
581 levels: * p<0.05, ** p<0.01, and *** p<0.001.

582

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

583 **References**

584 1. Archie EA, Theis KR. Animal behaviour meets microbial ecology. Anim Behav.
585 2011 Sep 1;82(3):425–36.

586 2. Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. Microbiology.
587 Animal behavior and the microbiome. Science. 2012 Oct 12;338(6104):198–9.

588 3. Dance A. Microbes take charge. Proc Natl Acad Sci U S A. 2014 Feb
589 11;111(6):2051–3.

590 4. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut
591 microbiota on brain and behaviour. Nat Rev Neurosci. 2012 Oct;13(10):701–12.

592 5. Archie EA, Tung J. Social behavior and the microbiome. Curr Opin Behav Sci.
593 2015 Dec 1;6(Supplement C):28–34.

594 6. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al.
595 The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic
596 system in a sex-dependent manner. Mol Psychiatry. 2013 Jun;18(6):666–73.

597 7. Heijtz RD, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, et al. Normal
598 gut microbiota modulates brain development and behavior. Proc Natl Acad Sci. 2011 Feb
599 1;108:3047–52.

600 8. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior
601 and central neurochemical change in germ-free mice. Neurogastroenterol Motil Off J Eur
602 Gastrointest Motil Soc. 2011 Mar;23(3):255–64, e119.

603 9. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-
604 Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and
605 Synaptic Deficits in Offspring. Cell. 2016 Jun 16;165(7):1762–75.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

606 10. Leitão-Gonçalves R, Carvalho-Santos Z, Francisco AP, Fioreze GT, Anjos M,
607 Baltazar C, et al. Commensal bacteria and essential amino acids control food choice
608 behavior and reproduction. PLOS Biol. 2017 Apr 25;15(4):e2000862.

609 11. Sharon G, Segal D, Ringo JM, Hefetz A, Zilber-Rosenberg I, Rosenberg E.
610 Commensal bacteria play a role in mating preference of Drosophila melanogaster. Proc
611 Natl Acad Sci U S A. 2010 Nov 16;107(46):20051–6.

612 12. Ringo J, Sharon G, Segal D. Bacteria-induced sexual isolation in Drosophila. Fly
613 (Austin). 2011 Dec;5(4):310–5.

614 13. Arbuthnott D, Levin TC, Promislow DEL. The impacts of Wolbachia and the
615 microbiome on mate choice in Drosophila melanogaster. J Evol Biol. 2016
616 Feb;29(2):461–8.

617 14. Morimoto J, Simpson SJ, Ponton F. Direct and trans-generational effects of male
618 and female gut microbiota in Drosophila melanogaster. Biol Lett. 2017 Jul;13(7).

619 15. Lizé A, McKay R, Lewis Z. Kin recognition in Drosophila: the importance of
620 ecology and gut microbiota. ISME J. 2014 Feb;8(2):469–77.

621 16. Gadau J, Fewell J. Organization of Insect Societies: From Genome to
622 Sociocomplexity. Harvard University Press; 2009. 638 p.

623 17. Matsuura K. Nestmate recognition mediated by intestinal bacteria in a termite,
624 Reticulitermes speratus. Oikos. 2001 Jan 1;92(1):20–6.

625 18. Dosmann A, Bahet N, Gordon DM. Experimental modulation of external
626 microbiome affects nestmate recognition in harvester ants (Pogonomyrmex barbatus).
627 PeerJ. 2016 Jan 25;4:e1566.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

628 19. de Souza DJ, Lenoir A, Kasuya MCM, Ribeiro MMR, Devers S, Couceiro J da C,
629 et al. Ectosymbionts and immunity in the leaf-cutting ant Acromyrmex subterraneus
630 subterraneus. Brain Behav Immun. 2013 Feb 1;28(Supplement C):182–7.

631 20. José de Souza D, Devers S, Lenoir A. Blochmannia endosymbionts and their
632 host, the ant Camponotus fellah: Cuticular hydrocarbons and melanization. C R Biol.
633 2011 Oct 1;334(10):737–41.

634 21. Lester PJ, Sébastien A, Suarez AV, Barbieri RF, Gruber MAM. Symbiotic
635 bacterial communities in ants are modified by invasion pathway bottlenecks and alter
636 host behavior. Ecology. 2017 Mar 1;98(3):861–74.

637 22. Van Borm S, Billen J, Boomsma JJ. The diversity of microorganisms associated
638 with *Acromyrmex* leafcutter ants. BMC Evol Biol [Internet]. 2002 May 3;2. Available
639 from: ://000207645900001

640 23. Sapountzis P, Zhukova M, Hansen LH, Sørensen SJ, Schiøtt M, Boomsma JJ.
641 *Acromyrmex* leaf-cutting ants have simple gut microbiota with nitrogen-fixing potential.
642 Appl Environ Microbiol. 2015 Aug 15;81(16):5527–37.

643 24. Andersen SB, Hansen LH, Sapountzis P, Sørensen SJ, Boomsma JJ. Specificity
644 and stability of the Acromyrmex–Pseudonocardia symbiosis. Mol Ecol. 2013 Aug
645 1;22(16):4307–21.

646 25. Sapountzis P, Zhukova M, Hansen LH, Sørensen SJ, Schiøtt M, Boomsma JJ.
647 Acromyrmex Leaf-Cutting Ants Have Simple Gut Microbiota with Nitrogen-Fixing
648 Potential. Appl Environ Microbiol. 2015 Aug 15;81(16):5527–37.

649 26. Ottoni EB. EthoLog 2.2: a tool for the transcription and timing of behavior
650 observation sessions. Behav Res Methods Instrum Comput J Psychon Soc Inc. 2000
651 Aug;32(3):446–9.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

652 27. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models
653 Using **lme4**. J Stat Softw [Internet]. 2015 [cited 2017 Sep 20];67(1). Available from:
654 http://www.jstatsoft.org/v67/i01/

655 28. Fox J, Weisberg S. An R Companion to Applied Regression.
656 http://socserv.socsci.mcmaster.ca/jfox/Books/Companion. 2011;

657 29. multcomp citation info [Internet]. [cited 2017 Sep 29]. Available from:
658 https://cran.r-project.org/web/packages/multcomp/citation.html

659 30. Zhukova M, Sapountzis P, Schiøtt M, Boomsma JJ. Diversity and Transmission
660 of Gut Bacteria in *Atta* and *Acromyrmex* Leaf-Cutting Ants during Development. Front
661 Microbiol [Internet]. 2017 [cited 2017 Oct 10];8. Available from:
662 https://www.frontiersin.org/articles/10.3389/fmicb.2017.01942/full

663 31. Ahrens ME, Shoemaker D. Evolutionary history of Wolbachia infections in the
664 fire ant Solenopsis invicta. BMC Evol Biol. 2005 May 31;5:35.

665 32. Nygaard S, Zhang G, Schiøtt M, Li C, Wurm Y, Hu H, et al. The genome of the
666 leaf-cutting ant Acromyrmex echinatior suggests key adaptations to advanced social life
667 and fungus farming. Genome Res. 2011 Jan 8;21(8):1339–48.

668 33. Andersen SB, Boye M, Nash DR, Boomsma JJ. Dynamic *Wolbachia* prevalence
669 in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. J Evol Biol. 2012
670 Jul;25(7):1340–50.

671 34. Andersen SB, Yek SH, Nash DR, Boomsma JJ. Interaction specificity between
672 leaf-cutting ants and vertically transmitted Pseudonocardia bacteria. BMC Evol Biol.
673 2015 Feb 25;15:27.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

674 35. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al.
675 Introducing mothur: Open-Source, Platform-Independent, Community-Supported
676 Software for Describing and Comparing Microbial Communities. Appl Environ
677 Microbiol. 2009 Jan 12;75(23):7537–41.

678 36. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©)
679 for group-wise comparison and statistical analysis of relative expression results in real-
680 time PCR. Nucleic Acids Res. 2002 May 1;30(9):e36.

681 37. Pfaffl MW. Quantification strategies in real-time PCR. A–Z Quant PCR. 2004
682 Jan 1;89–113.

683 38. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al.
684 vegan: Community Ecology Package [Internet]. 2017 [cited 2017 Nov 19]. Available
685 from: https://cran.r-project.org/web/packages/vegan/index.html

686 39. Fox J, Weisberg S, Friendly M, Hong J, Andersen R, Firth D, et al. effects: Effect
687 Displays for Linear, Generalized Linear, and Other Models [Internet]. 2017 [cited 2017
688 Nov 19]. Available from: https://cran.r-project.org/web/packages/effects/index.html

689 40. Hope RM. Rmisc: Rmisc: Ryan Miscellaneous [Internet]. 2013 [cited 2017 Nov
690 19]. Available from: https://cran.r-project.org/web/packages/Rmisc/index.html

691 41. Wickham H, Chang W, RStudio. ggplot2: Create Elegant Data Visualisations
692 Using the Grammar of Graphics [Internet]. 2016 [cited 2017 Nov 19]. Available from:
693 https://cran.r-project.org/web/packages/ggplot2/index.html

694 42. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al.
695 Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq
696 platforms. ISME J. 2012 Aug;6(8):1621–4.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

697 43. Aitchison J (John). The statistical analysis of compositional data [Internet].
698 Chapman and Hall; 1986 [cited 2017 Sep 20]. Available from:
699 http://www.bcin.ca/Interface/openbcin.cgi?submit=submit&Chinkey=123671

700 44. Hothorn T, Bretz F, Westfall P. Simultaneous Inference in General Parametric
701 Models. Biom J. 2008 Jun 1;50(3):346–63.

702 45. Dolédec S, Chessel D. Co-inertia analysis: an alternative method for studying
703 species–environment relationships. Freshw Biol. 1994 Jun 1;31(3):277–94.

704 46. Salem H, Florez L, Gerardo N, Kaltenpoth M. An out-of-body experience: the
705 extracellular dimension for the transmission of mutualistic bacteria in insects. Proc R Soc
706 B Biol Sci [Internet]. 2015 Apr 7 [cited 2016 Jul 25];282(1804). Available from:
707 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4375872/

708 47. Anderson KE, Rodrigues PAP, Mott BM, Maes P, Corby-Harris V. Ecological
709 Succession in the Honey Bee Gut: Shift in Lactobacillus Strain Dominance During Early
710 Adult Development. Microb Ecol. 2015 Dec 19;71(4):1008–19.

711 48. Engel P, Stepanauskas R, Moran NA. Hidden diversity in honey bee gut
712 symbionts detected by single-cell genomics. PLoS Genet. 2014 Sep;10(9):e1004596.

713 49. Kuo C-H. Scrambled and not-so-tiny genomes of fungal endosymbionts. Proc
714 Natl Acad Sci U S A. 2015 Jun 23;112(25):7622–3.

715 50. Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce
716 NE. Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants.
717 Proc Natl Acad Sci. 2009 Dec 15;106(50):21236–41.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

718 51. de Souza DJ, Bezier A, Depoix D, Drezen JM, Lenoir A. Blochmannia
719 endosymbionts improve colony growth and immune defence in the ant Camponotus
720 fellah. BMC Microbiol. 2009;9:29.

721 52. Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ, et al.
722 Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*.
723 BMC Biol. 2007;5:48.

724 53. Hu Y, Sanders JG, Łukasik P, D'Amelio CL, Millar JS, Vann DR, et al. Nitrogen
725 conservation, conserved: 46 million years of N-recycling by the core symbionts of turtle
726 ants. bioRxiv. 2017 Sep 7;185314.

727 54. Heinze J, Walter B. Moribund Ants Leave Their Nests to Die in Social Isolation.
728 Curr Biol. 2010 Feb 9;20(3):249–52.

729 55. Bos N, Lefèvre T, Jensen AB, D'ettorre P. Sick ants become unsociable. J Evol
730 Biol. 2012 Feb 1;25(2):342–51.

731 56. Ortius-Lechner D, Maile R, Morgan ED, Boomsma JJ. Metapleural Gland
732 Secretion of the Leaf-cutter Ant Acromyrmex octospinosus: New Compounds and Their
733 Functional Significance. J Chem Ecol. 2000 Jul 1;26(7):1667–83.

734 57. Larsen J, Fouks B, Bos N, d'Ettorre P, Nehring V. Variation in nestmate
735 recognition ability among polymorphic leaf-cutting ant workers. J Insect Physiol. 2014
736 Nov 1;70:59–66.

737 58. Arrese EL, Soulages JL. Insect fat body: energy, metabolism, and regulation.
738 Annu Rev Entomol. 2010;55:207–25.

739 59. Ryu J-H, Ha E-M, Lee W-J. Innate immunity and gut–microbe mutualism in
740 Drosophila. Dev Comp Immunol. 2010 Apr 1;34(4):369–76.

bioRxiv preprint doi: https://doi.org/10.1101/335521; this version posted May 31, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

741 60. Richard F-J, Aubert A, Grozinger CM. Modulation of social interactions by
742 immune stimulation in honey bee, Apis mellifera, workers. BMC Biol. 2008 Nov
743 17;6:50.

744 61. Davis TS, Crippen TL, Hofstetter RW, Tomberlin JK. Microbial volatile
745 emissions as insect semiochemicals. J Chem Ecol. 2013 Jul;39(7):840–59.

746 62. Ezenwa VO, Williams AE. Microbes and animal olfactory communication:
747 Where do we go from here? BioEssays News Rev Mol Cell Dev Biol. 2014
748 Sep;36(9):847–54.

749 63. Venu I, Durisko Z, Xu J, Dukas R. Social attraction mediated by fruit flies'
750 microbiome. J Exp Biol. 2014 Apr 15;217(8):1346–52.

751 64. Moullan N, Mouchiroud L, Wang X, Ryu D, Williams EG, Mottis A, et al.
752 Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for
753 Caution in Biomedical Research. Cell Rep. 2015 Mar 10;

754

755