Gut microbes contribute to variation in foraging intensity in the honey bee, *Apis mellifera*.

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Competing Interests

The authors declare no competing interests.

Abstract

Gut microbiomes are increasingly recognized for mediating diverse biological aspects of their hosts, including complex behavioral phenotypes. While many studies have reported that experimental disruptions to the gut microbiome result in atypical host behavior, studies that address how gut microbes contribute to adaptive behavioral trait variation are rare. Eusocial insects represent a powerful model to test this, due to their simple microbiomes and complex division of labor characterized by colony-level variation in behavioral phenotypes. While previous studies report correlational differences in gut microbiome associated with division of labor, here, we provide evidence that gut microbes play a causal role in defining differences in foraging behavior between honey bees. Gut microbial community structure consistently differed between hive-based nurse bees and bees that leave the hive to forage for floral resources. These differences were associated with variation in the abundance of individual microbes, including *Bifidobacterium asteroides, Bombilactobacillus mellis,* and *Lactobacillus melliventris*. Manipulations of colony demography and individual foraging experience suggested that differences in microbiome composition were associated with task experience. Moreover, single microbe inoculations with *B. asteroides*, *B. mellis,* and *L. melliventris* caused changes in foraging intensity. These results demonstrate that gut microbes contribute to division of labor in a social insect, and support a role of gut microbes in modulating host behavioral phenotypic variation.

Introduction

Gut microbiomes are emerging as important drivers and modulators of host phenotype [1], with evidence supporting the role of microbiomes in host digestion and nutrition, immune health, development, and, more recently, behavior [2–7]. Over the past decade, the “microbiota-gut-brain axis” [7], which describes bidirectional interactions between the gut microbiome and host brain and behavior, has developed as an increasingly important field of study with implications for

45 understanding the ecology and evolution of host-microbe interactions and animal behaviors.
46 However, current studies of the microbiota-gut-brain axis across animal taxa are largely
47 correlational, and causal studies exploring the relationship between gut microbes and host
48 behavior are rare [7].

49 The functional relationship between hosts and microbiomes varies across host traits. In
50 many cases, this relationship is obligatory, where the microbiome is necessary for normal
51 functioning of the host [7–10]. Recently, it has been proposed that gut microbiomes play a
52 facultative role in host phenotype, such that gut microbiomes contribute to phenotypic variation
53 between individuals [11, 12]. Results in support of a facultative relationship come from studies
54 across taxonomic groups. These include variations in non-behavioral and behavioral phenotypes
55 [11, 12], such as behaviors associated with aging and senescence [13–18] and severity of
56 neurodevelopmental disorders [19–21], as well as adaptive variations in behavioral traits both
57 within [22, 23] and between species [24]. Eusocial insects represent a powerful model for
58 understanding how gut microbes contribute to adaptive behavioral trait variation due to their
59 relatively simple and stable gut microbiomes and their complex division of labor [25–27]. This
60 division of labor is characterized by polyphenism between reproductive and non-reproductive
61 individuals, as well as colony-level behavioral trait variation between non-reproductive individuals
62 performing different tasks [25, 28]. In eusocial insects, gut microbes have been shown to
63 contribute to natural variation in memory [22] and social interactions [29]. Likewise, studies
64 indicate an association between gut microbiome and division of labor in eusocial insects [25, 30–
65 33]. However, whether gut microbes play a causal role in any aspect of eusocial insect division of
66 labor remains unknown.

67 We use the European honey bee, *Apis mellifera,* to investigate the causal relationship
68 between the gut microbiome and division of labor. Honey bees are a highly eusocial insect with
69 well-characterized and tractable social behaviors and gut microbiomes. In typical honey bee
70 colonies, sterile worker bees exhibit age-related division of labor, which is based on a pattern of

individual behavioral maturation. Typically, adult worker bees perform brood care ("nursing") and other activities in the hive during the first 1-3 weeks of adult life and then transition to foraging behaviors outside of the hive for the final 1-2 weeks of their life [28, 34]. However, honey bee division of labor is flexible and responsive to changing colony needs, and thus does not exclusively rely on the typical age patterns of behavioral maturation [35–39]. In addition, honey bee foragers exhibit considerable individual variation in preference for nectar or pollen [40, 41], exploratory behavior [42], and general foraging intensity [43]. Therefore, honey bee division of labor involves individual variation in both behavioral maturation and task performance [44].

The honey bee gut microbial community, most of which resides in the hindgut, is well-characterized and relatively simple, composed of ~10-20 different species of facultatively anaerobic and microaerophilic host-adapted bacteria within the taxonomic groups of Actinomycetes (*Bifidobacterium*), Lactobacillaceae (*Bombilactobacillus* Firm-4 and *Lactobacillus* Firm-5), Gammaproteobacteria (*Gilliamella* and *Friscella*), Alphaproteobacteria (*Bartonella* and *Bombella*), and Betaproteobacteria (*Snodgrassella*) [27]. Furthermore, the individual members of the honey bee gut microbiome are consistently present, but differ in abundance across different individuals and populations of honey bees [27]. Of particular interest, the composition of honey bee gut microbial communities can be experimentally manipulated. All species can be cultured [45] and used to inoculate young bees [29, 46, 47], who must acquire their microbiome from older bees or hive materials [48]. Furthermore, previous studies indicate an association between the gut microbiome and various aspects of division of labor in the honey bee [30–32, 49, 50]. Due to these attributes of the honey bee gut microbiome, it is a great model for understanding the causal effects of gut microbes on complex host behaviors. We took advantage of these behavioral and microbiome features to determine whether gut microbes play a causal role in honey bee division of labor, with a specific focus on behavioral maturation and variation in foraging intensity.

Materials and Methods

Animal Husbandry, treatments, and collections

Honey bee colonies were managed using standard beekeeping techniques at the University of Illinois Bee Research Facility in Urbana, IL. Honey bees in this area are a genetic mixture of subspecies, primarily *Apis mellifera ligustica* and *carnica* subspecies. For collection of nurses and foragers, nurses were identified as those actively feeding brood on a brood frame, and foragers were identified as those returning to the hive with pollen loads on their hind legs or having a distended abdomen due to nectar loads [37, 51]. To reduce genetic variation between workers in the colonies used for the single-cohort colony, big-back colony and single microbe inoculation studies (described below), we used bees derived from queens that were each instrumentally inseminated with sperm from a different single drone (SDI) (queen rearing and inseminations were performed by Sue Cobey, Honey Bee Insemination Service, Washington State University and Dr. Osman Kaftanoglu, Apimaye USA). Sample size for 16S rRNA sequencing studies was 10 bees per group as in [52]. All bees used in 16S rRNA sequencing analyses were washed once with 12.5% bleach in water and twice with double deionized water and flash frozen. All samples were stored at -80°C until further analysis.

Single- and double-cohort colonies

Single-cohort colonies (SCC) were established in the summer of 2020 as previously described [36, 37, 51]. Honeycomb frames with pupal brood were taken from one or more source colonies derived from the above-mentioned queens and placed in a 34°C incubator at 60-65% relative humidity. About 1000 newly eclosed bees (<24 hours old) were gently brushed from these frames <24 hours later and placed in a small five-frame hive box with a new, unrelated mated queen, one honey frame, an empty honeycomb frame, and three new frames with wax-covered plastic foundation upon which bees can build new wax honeycomb. SCCs were kept outdoors, and bees

121 were collected as typical-age nurses and precocious foragers at approximately one week of age
122 (7-9 days of age, variation due to rainy weather), and as over-age nurses and typical-age foragers
123 at three weeks of age. To obtain over-age nurses, frames containing brood were removed and
124 replaced with empty comb two weeks after queen introduction to ensure that a new cohort of bees
125 did not eclose before collections. For the SCC depicted in Fig. 2 and Supplementary Fig. 1, bees
126 from four distinct SDI source colonies (unrelated queens and drones) were used to construct a
127 single SCC. Four SDI source colonies were used to obtain the necessary 1000 newly eclosed
128 bees, and allowed us to test for the role of source colony in gut microbiome development
129 (Supplementary Fig. 1). For each collection (Day 9 and Day 21), 10 bees of each source colony
130 were collected for each behavioral task group. On Day 9, nurses were only able to be collected
131 for two source colonies. For the SCC depicted in Supplementary Fig. 4, bees from a single SDI
132 source colony were used, and groups of 10 bees were collected at each collection (Day 7 and
133 Day 21) for each behavioral task group.

134 Double-cohort colonies (DCC) were used to assess the effects of different inoculation
135 treatments (see below) on foraging behaviors in a common colony environment. DCCs were
136 established similarly to SCCs with a new, mated queen, 1 plastic honeycomb frame provisioned
137 with honey and pollen paste, 1 empty plastic honeycomb frame, ~200 7-day old barcoded
138 treatment bees (100 of bees from a single microbe inoculated treatment group and 100 from a
139 corresponding microbiota-depleted group) and ~800 newly eclosed unbarcoded “background”
140 bees (mixed colony backgrounds), whose addition contributed to accelerated behavioral
141 maturation of the older cohort [51, 53]. In the presence of the older cohort, younger “background”
142 bees are not expected to contribute to the foraging force [54] and did not throughout the course
143 of the experiment.

144

145 **Big-back colonies**

146 Big-back colonies were established as previously described [36, 51, 53] and kept outdoors in the
147 summer of 2021. For each colony, ~800-900 newly eclosed bees from a single SDI source colony
148 were used; half with a paint mark on the thorax, and half with a plastic tag attached to the thorax
149 (~3 mm diameter, ~1 mm thick; "big-back" bees). Four days later, ~400 newly eclosed bees from
150 ~10 mixed source colonies (headed by naturally mated queens) were introduced to increase the
151 proportion of precocious foragers in the first cohort [36, 51, 53]. The entrance to the hive was
152 blocked by a piece of Plexiglas with holes in it to prevent the big-back bees from leaving the hive,
153 but to allow paint-marked bees to come and go freely. We observed that guard bees patrolled the
154 hive entrance on the inside of the Plexiglas piece.

155 Bees were collected at 10 days of age: returning active foragers and nurses collected as
156 described above, and big-back/inactive forager bees collected as they were attempting to leave
157 the hive via the holes in the plastic, indicating they too were in the behavioral state of foraging but
158 had never left the hive. 10 bees per group were collected from all three colonies, with the
159 exception that only seven nurses from the focal group in colony 2 were found and collected.

160

161 **Gut microbiome DNA extraction, 16S rRNA sequencing and analysis**

162 Frozen honey bee guts were dissected under sterile conditions on dry ice. Combined mid- and
163 hind- guts of individual bees were homogenized by maceration with a disposable sterile pestle
164 (VWR, Radnor, PA, USA). The homogenate was added to a PowerSoil Pro Bead Solution tube
165 (Qiagen, Germantown, MD, USA), and DNA was extracted using a DNeasy PowerSoil Pro DNA
166 isolation kit (Qiagen), following manufacturer's instructions. The hypervariable V4 region of the
167 16S rRNA gene was amplified by PCR in triplicates, with a negative control (no DNA), using
168 Platinum Hot Start PCR Master Mix (Invitrogen, Waltham, MA, USA), primers and barcodes
169 designed in [55] with a final concentration 0.25 μM, and the following cycling conditions: 94° 3
170 min, 35x[94° 45s/50° 60s/72° 90s], 72° 10 min. Before sequencing, PCR products were visualized

171 on agarose gels to confirm negative controls did not have amplification, while all samples had
172 expected amplification. All samples met these criteria, indicating no contamination. Samples were
173 pooled based upon concentrations and sequenced on an Illumina MiSeq with 2x250bp paired-
174 end reads. The samples were split among three sequencing runs: #1: SCCs (Dec 2020), #2: Big-
175 back colonies (July 2021), #3: Typical colony nurses and foragers, single microbe inoculated bees
176 (Dec 2021). Reads from these sequencing runs can be found in NCBI's Sequence Read Archive
177 PRJNA958253. Sequences for typical colonies from naturally mated queens were retained from
178 [49].

179 Samples were demultiplexed using QIIME2, and paired end reads were truncated at the
180 first base with a quality score of <Q3 using DADA2 [56]. Paired-end reads were merged and
181 amplicon sequence variants (ASV) were identified using DADA2 [57]. Chimeric ASVs were
182 removed and the remaining ASVs were taxonomically classified using the BEExact database [58].
183 ASVs that were taxonomically identified as a bee specific genus by the BEExact database, but
184 were unclassified at the species level, were subsequently classified to species level if possible,
185 using NCBI megaBLAST. To do this, representative 16S rRNA gene V4 sequences for each ASV
186 obtained from the QIIME2 rep-seqs.qza object were BLASTed against the entire NCBI nucleotide
187 database, and unclassified ASVs were called as the first identified full species with the lowest E-
188 score, if query cover > 80% and percent identity > 92%. Prior to statistical analyses, ASVs that
189 were identified as mitochondrial or chloroplast were removed from the data. Mitochondrial and
190 chloroplast reads were retained for visualization of single microbe inoculated and microbiota-
191 depleted bee gut microbiomes.

192 For sequencing run #1, 3,220,034 (1,610,017 pairs) total sequence reads were obtained,
193 1,431,503 pairs were filtered, merged and identified as non-chimeric (90%), and 627 ASVs were
194 identified. For sequencing run #2, 945,310 (472,655 pairs) total sequence reads were obtained,
195 424,785 pairs were filtered, denoised, merged and identified as non-chimeric (90%), and 110
196 ASVs were identified. For sequencing run #3, 2,538,722 (1,269,361 pairs) total sequence reads

197 were obtained, 1,141,046 pairs were filtered, denoised, merged and identified as non-chimeric
198 (90%), and 832 ASVs were identified.

199 To estimate the abundance of individual honey bee-associated microbial species in each
200 sample, the read counts for all ASVs that matched the same species were combined. We retained
201 the following honey bee-associated species: Acetobacteraceae related to *Commensalibacter*
202 [possibly Alpha-2.1 and Alpha-2.2], *Bartonella spp.*, *Bifidobacterium asteroides*, *Bifidobacterium*
203 *coryneforme* [syn. *Bifidobacterium indicum*], *Bombella apis* [previously *Parasaccharibacter*
204 *apium*], *Frischella perrara*, *Gilliamella apicola*, *Lactobacillus apis*, *Lactobacillus helsingborgensis*,
205 *Lactobacillus kullabergensis*, *Bombilactobacillus mellifer* [previously *Lactobacillus mellifer*],
206 *Bombilactobacillus mellis* [previously *Lactobacillus mellis*], *Lactobacillus melliventris*,
207 *Apilactobacillus kunkeei*, and *Snodgrassella alvi* [27, 59, 60]. We also retained other unclassified
208 *Lactobacillus* species, *Limosilactobacillus spp.*, *Arsenophonus spp.*, *Klebsiella spp.*, and *Mixta*
209 *spp.* because they made up a significant portion of reads (>500 reads in at least 1 sample). In the
210 case of single-microbe inoculated and microbiota-depleted bees (see below), *Enterococcus spp.*,
211 *Staphylococcus spp.,* and Unassigned (not able to be classified below Kingdom of Bacteria) were
212 additionally retained for visualization. All other ASVs, each comprising 0-1% of total reads per
213 sample, and representing microbes that were not classified in the aforementioned groups and/or
214 are not typically associated with honey bees, were combined and labeled as "other." The variation
215 between samples in the abundance of "other" microbes is similar to previously published datasets
216 [29, 31]. To calculate the proportion of each species in each sample, that species' read count was
217 divided by the total read count for each sample. Raw read counts and/or proportion data were
218 used in analyses as described below. For the depiction of single microbe inoculated bee gut
219 microbiomes, "other" ASVs were combined based on genus (or the lowest taxonomic level
220 identified), and those representing >500 reads in at least 1 sample are depicted in a stacked
221 barplot, while all genera are included in supplementary data on Mendeley Data (DOI:
222 10.17632/f2s47y3nhn).

223 Due to the compositional nature of 16S rRNA sequencing data, typical measures of beta
224 diversity and differential abundance analyses have limitations [61–63]. Therefore we followed
225 analyses outlined in [61]. In short, for beta-diversity analyses, we used clr-transformations of raw
226 read counts for each sample in statistical tests and Aitchison distance for visualization (see
227 "Statistical analysis" section below). To estimate the differential relative abundance of individual
228 microbial species between samples through tools that have a compositional foundation (referred
229 to as "relative abundance" throughout the text), we used Analysis of Composition of Microbiomes
230 with Bias Correction (ANCOM-BC) [62, 64, 65] on raw read counts. This test relies on the total
231 sample read count and relative abundance of individual microbes in each sample to estimate the
232 true abundance of individual microbes in each gut ecosystem while accounting for the
233 compositional nature of sequencing data [64].

234 To estimate absolute bacterial species abundances in individual samples ("absolute
235 abundance"), we quantified the bacterial load in each sample using quantitative PCR (qPCR), as
236 in [29, 30]. We performed standard curves using serial dilutions of plasmids (TOPO pCR2.1
237 (Invitrogen)) containing the target sequence (10^8-10^3 copies per 3 μl), which were calculated from
238 the molecular weight of the plasmid and the DNA concentration of the plasmid. Primer efficiencies
239 were measured using: $E = 10^{(-1/slope)}$ [66], and the copy number of each target in 3 μl of DNA
240 sample was calculated from the sample's C_t score, primer efficiency, and standard curve using: n
241 $= E^{(intercept-Ct)}$x(DNA extraction elution volume/3) [29, 30]. These values were calculated for both
242 the 16S rRNA gene and the *Actin* gene. To account for differences in DNA extraction efficiency,
243 we calculated a normalized number of 16S rRNA gene copies by dividing the number of 16S
244 rRNA gene copies by that sample's number of *Actin* gene copies and multiplying this by the
245 median number of *Actin* copies for that experiment [29, 30]. To calculate the absolute abundance
246 of each microbial species in each sample, the relative abundance (proportion) of each species in
247 each sample was multiplied by the normalized number of 16S rRNA gene copies in that sample

248 [29, 30]. We then took the $\log_{10}$ value of each of these numbers and used these in further
249 analyses, replacing 0s with 1s before we did so.

250 PCRs were performed in triplicate using 10 μl reactions of 0.5 μM primers targeting the
251 16S rRNA gene (F: AGGATTAGATACCCTGGTAGTCC, R: YCGTACTCCCCAGGCGG) or the
252 *A. mellifera Actin* gene (F: TGCCAACACTGTCCTTTCTG, R: AGAATTGACCCACCAATCCA)
253 [46] in 1X *Power*SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) with 3
254 μl of DNA. Each primer set included a no template control. Reactions were performed in 384 well
255 plates using a QuantStudio 6 Flex (Applied Biosystems) with the following cycling conditions: 50°
256 2 min, 95° 10 min, 40x[95° 15 sec/60° 1 min], melt curve: 95° 15 sec, 60° 1 min, 95° 15 sec.
257 Standard curves using serial dilutions of plasmids (TOPO pCR2.1 (Invitrogen)) containing the
258 target sequence (10^8-10^3 copies per 3 μl) were calculated from the molecular weight of the
259 plasmid and the DNA concentration of the plasmid.

260 We found an effect of colony replicate on gut microbial community structure across all
261 experiments, indicating that bees from different colonies have different gut microbial communities
262 and that early environment and/or genetics influences the composition of the gut microbiome.
263 This was the case when comparing bees from different typical colonies (Supplementary Fig. 1A-
264 B), among SCC bees originating from different colony sources (Supplementary Fig. 1C-D), and
265 across bees from different big-back colonies (Supplementary Fig. 1E). Colony differences in
266 honey bee microbiome composition have been previously reported [52].

267

268 **Single microbe inoculations**

269 To identify effects of individual microbes on behavior we treated groups of newly eclosed
270 bees, who emerged under sterile lab conditions, with either an inoculum of a microbe of interest
271 or sterile food in order to produce bees whose microbiomes were composed of a single honey
272 bee-associated microbe (single microbe-inoculated) or no honey bee-associated microbes

273 ("microbiota-depleted," historically referred to in this way since bees lacking a typical honey bee
274 microbiome are not completely microbe-free [46, 67]), respectively. Although single microbe
275 inoculations do not represent a natural microbiome, we chose this approach because it would
276 likely allow for the most control over microbiome composition across replicates. This is because
277 inoculations with multiple microbes may result in variation in microbiome structure between
278 individuals, groups, and/or replicates since honey bee microbiome structure is shaped by both
279 environmental and genetic factors [27, 48, 68]. These experiments occurred in the summer of
280 2021.

281 To obtain bees that lack the dominant honey bee bacterial species in the gut, modified
282 methods from [46, 67] were used. Specifically, tan-colored pupae with dark eyes were gently
283 removed from brood frames from three or four source colonies per trial using ethanol sterilized
284 forceps, placed dorsal side down in sterilized 3D-printed dental grade resin modular pupation
285 plates (Supplementary Fig. 2, design file on Mendeley Data), which were fitted into sterilized
286 Plexiglas cages, and kept in a 34°C incubator with 60-65% relative humidity for 2 days [69].
287 Eclosed bees were moved in groups of 30-40 to sterilized Plexiglas boxes (10 x 10 x 7 cm) [70]
288 in a 32°C incubator at 60-65% relative humidity (5 boxes per treatment for each replicate). Each
289 box was provisioned with a ~1 g sterilized pollen patty and 1.7 mL autoclave (15 minutes)
290 sterilized 25% sucrose with or without inoculum. An equal number of bees from each source
291 colony were added to each box, such that each treatment group (microbiota-depleted or single
292 microbe inoculated) had an equal number of bees of each colony background.

293 For single microbe inoculations, microbes were obtained from the DSMZ Leibniz-Institut
294 in Germany (*B. asteroides* DSM 20089, *B. mellis* DSM 26255, *L. melliventris* DSM 26256) and
295 were streaked on solid media and then cultured under anaerobic conditions at 35°C for 3 nights
296 in de Mann, Rogosa, Sharpe (MRS) (*B. asteroides*) or MRS + 20g/L fructose (*B. mellis and L.*
297 *melliventris*) broth, and glycerol stocks were made and kept at -80°C until future use. Ten days
298 prior to the onset of inoculation experiments, glycerol stocks were streaked on solid MRS (*B.*

299 *asteroides*) or MRS + 20g/L fructose (*B. mellis* and *L. melliventris*) media plates and were grown
300 under anaerobic conditions at 35°C for 3 nights (Oxoid Anaerojar with Anaerogen bags, Thermo
301 Scientific, Waltham, MA, USA). A single colony of bacteria from each plate was cultured under
302 anaerobic conditions (Anaerobic Hungate Culture Tubes with air displaced by CO_2, VWR, Radnor,
303 PA, USA), shaking at 35°C for 3 nights and was then retained at 4°C. Every day, starting 2 days
304 prior to the onset of inoculations, new cultures from this same original stock were prepared by
305 culturing 50 μl of the stock in 5 mL of fresh MRS/MRS + 20g/L fructose broth under anaerobic
306 conditions, shaking at 35°C for 2 nights. These cultures were spun down (3000 rpm for 5 min)
307 and resuspended in 1x PBS to an OD of 1 and 50 μl of a new, fresh culture solution was added
308 to 1.7 mL sterile 25% sucrose water in a new inverted microtube for each treatment box every
309 morning over the course of a 5-day inoculation period. For microbiota-depleted bees, 50 μl of
310 sterile 1x PBS was added to 1.7 mL 25% sucrose water in a new inverted microtube for each
311 treatment box every morning over the course of a 5-day inoculation period. Bees were then fed
312 sterile 50% sucrose water for 2 days following the 5-day inoculation period. Very few bees (1-2
313 per box) died during this inoculation period. Bees were kept in treatment boxes until 7 days old.
314 Unfortunately, we did not keep track of the treatment box each bee came from, rather bees for
315 each treatment were pooled and randomly chosen for 16S rRNA analysis or behavioral assays.
316 Microbe survival in 25% sucrose water was ensured by placing 50 μl of each microbial culture
317 (OD ~1) in 1.5 mL 25% sucrose water overnight and then plating this solution using species
318 specific culturing conditions.
319

320 **Foraging assays and analysis using barcoded bees**

321 To obtain comprehensive records of foraging activity, we used an automated behavioral tracking
322 system ("bCode") that uses a custom matrix barcode, enabling the unique identification of
323 individual bees [71]. After single microbe inoculations, 7-day old inoculated and microbiota-

324 depleted bees were cold-anaesthetized, a unique barcode was affixed to their thorax using super
325 glue, and a spot of colored paint associated with their treatment group was placed on their
326 abdomen. An equal number (~100) of bees from a single microbe inoculated group and a
327 corresponding microbiota-depleted group (same mix of colony backgrounds, same age) were
328 given barcodes from different sets, and were placed together in a DCC. Four colony replicates
329 per microbe treatment were performed. Two experimental days, corresponding with *B. mellis*
330 colony replicate 3 days 1-2 and *L. melliventris* colony replicate 2 days 1-2, experienced bad
331 weather, and thus no foraging occurred on those days.

332 Colonies were kept in a dark, temperature (32°C) and humidity (~50%) controlled building,
333 with access to the outside environment through a plastic tube. To monitor flight activity, an
334 entrance monitor with a video camera (Supplementary Methods) was attached to the hive
335 entrance, on the outside-facing end of this tube (Supplementary Fig. 3). The camera recorded
336 videos of bees entering and leaving the hive from 05:00 to 21:00 daily for a total of six days.
337 Barcodes in these videos were detected as in [71], and detections were filtered to remove tracking
338 errors and misidentifications. A mean of 97.82%, with a range between 96.24%-99.02%, of
339 detections were retained after these filtering steps. We then applied a flight activity detector [72,
340 73] (Supplementary Methods) to the remaining barcode detections to identify passes through the
341 entrance monitor. Computer code for this detector can be found at:
342 https://github.com/gernat/btools.

343 Passes through the entrance monitor determined by the flight activity detector were filtered
344 to remove unused barcodes, whose detections either occurred through detection error or due to
345 bees accidentally entering the wrong experimental colony. A mean of 99.19%, with a range of
346 95.4%-100%, of events were retained after this filtering. Passes were then classified as
347 "incoming," "outgoing," or "other." Since incoming passes had a lower error rate than outgoing
348 passes (Supplementary Table 8), incoming passes alone were used to denote a foraging trip.
349 Individual foraging trips that occurred within 5 min of each other were condensed into a single

event. A bee's first day of foraging was defined as the first day on which it performed at least 4 foraging trips, with at least 50% of these trips occurring during peak foraging hours (11:00-15:00 CST), as per previous studies [43, 74]. These criteria yield similar automated thresholds corresponding to human observations of foraging behaviors [43]. All incoming passes from this first day of foraging were counted as foraging trips, as were all subsequent incoming passes [43]. Likewise, an individual bee was considered a "forager" from this day forward. Due to the large size of these data sets (owing to high dimensional automated behavioral monitoring) the number of foraging trips per bee and treatment group and the number of foragers per treatment group on each experimental day are available on Mendeley Data (DOI: 10.17632/f2s47y3nhn).

To quantify the degree of skew in foraging intensity among all workers, we calculated the Gini coefficient [43, 75] for each experimental colony across all days and each experimental colony on each day, since colony dynamics change with changes in weather and resource availability, and thus likely changed throughout our studies. The values we obtained (Table 2) are similar to those in previous studies [43, 75], indicating differences in foraging intensity between individuals in each colony. To determine which bees performed the majority of the foraging for each experimental colony on each day, we ranked individuals based on the proportion of total foraging trips they performed for the colony on each day. A group of "elite foragers" was defined as the subset of bees (variation, 5-40% of the foragers) performing $\geq$50% of the foraging trips for each colony on each day [43, 75]. Number of elite foragers per treatment group on each day are available on Mendeley Data (DOI: 10.17632/f2s47y3nhn).[74]

Statistical Analysis

All statistical analyses were performed in R (v 4.2.0) [76]. For all analyses, assumptions (e.g., normality, homogeneity of variances) were checked before statistical analysis. The following functions were used in the base statistics package: "shapiro.test" (Shapiro-Wilk Normality Test),

375 “fligner.test” (Fligner-Killeen Test of Homogeneity of Variances), “t.test” (Student’s t-test),
376 “wilcox.test” (Mann-Whitney Rank Sum Test), “aov” (ANOVA), “TukeyHSD” (Tukey’s HSD post
377 hoc test), “Kruskal.test” (Kruskal-Wallis test). Kruskal-Wallis post hoc was performed using
378 “kwAllPairsDunnTest”, PMCMRplus package [77]. Microbiome beta diversity was analyzed using
379 Permutation MANOVAs with 999 permutations (“adonis2”, vegan package [78]) on clr-
380 transformed (“transform”, microbiome package [79]) raw read counts, followed by Pairwise
381 Permutation MANOVAs with 999 permutations and FDR p-value adjustment (“pairwise.adonis”,
382 pairwiseAdonis package [80]), and visualized using Principal Components Analysis (“ordinate”,
383 phyloseq package [81]) with Aitchison distance (“distance”, phyloseq package [81]). The relative
384 abundance of individual microbes was analyzed using raw read counts and ANCOM-BC with FDR
385 adjustment (“ancombc2”, ANCOMBC package [62, 65]) with task as a fixed effect and colony as
386 random effect. Relative abundances were visualized using stacked barplots (“ggplot”, ggplot2
387 package [82]). To compare the absolute abundance of individual microbes, Permutation ANOVAs
388 were used with 999 permutations (“perm.anova,” RVAideMemoire package [83]), and p-values
389 were adjusted for multiple comparisons (“p.adjust” with FDR adjustment). Absolute abundance
390 data were visualized using dotplots (“ggplot,” ggplot2 package [82]). Age at onset of foraging data
391 (using each individual bee’s first day of foraging) were analyzed using a Cox Proportional Hazards
392 model (“coxph,” survival package [84]), stratified by replicate [35, 74], and depicted as survival
393 plots using the cumulative proportion of bees from each treatment group that were identified as
394 foragers on each day. Proportions of foraging trips per individual and last day of foraging data
395 were checked for outliers ("identify_outliers,” rstatix package [85]) and were analyzed as
396 generalized linear mixed-effects models (“glmer,” lme4 package [86]) with log-normal distributions
397 and nAGQ=0, inoculation treatment and day as main factors, and replicate and individual as
398 random factors, followed by “Anova” (car package [87]) to determine main factor significance, and
399 “emmeans” (emmeans package [88]) with Tukey’s p-value adjustment for pairwise comparisons.
400 Linear mixed-effects models (“lmer,” lme4 package [86]) with inoculation treatment and day as

401 main factors, and replicate as random factors, followed by “Anova” (car package [87]) to
402 determine main factor significance, and “emmeans” (emmeans package [88]) with Tukey’s p-
403 value adjustment for pairwise comparisons were used in the remaining behavioral analyses. Gini
404 coefficients were calculated using “Gini” (DescTools package [89]). All behavioral data, except
405 age at onset of foraging, were visualized using boxplots (“ggplot,” ggplot2 package [82]).
406

407 # Results

408 ## Nurses and foragers differ in gut microbial community composition

409 To begin to investigate whether gut microbes influence honey bee division of labor, we compared
410 gut microbial communities in nurse and forager bees, which represent two of the canonical
411 behavioral task groups in honey bee division of labor, as well as two discrete time points in
412 behavioral maturation [28]. Nurses and foragers differ markedly in physiology [37, 90–95],
413 neuroanatomy [96], neurochemistry [42, 97, 98], gene expression [38, 99] and gene regulation
414 [74, 100–102]. Therefore, we reasoned that testing for consistent differences in gut microbial
415 community between these two groups would be a powerful way to test for associations between
416 gut microbiome variation and division of labor.

417 We performed 16S rRNA sequencing on gut samples from nurses and foragers from three
418 unrelated honey bee colonies and reanalyzed, using updated methods [56, 57], a similar dataset
419 from a previously published study that used a different 16S rRNA sequencing method [49]. We
420 found that nurses and foragers differed significantly in gut microbial community structure in both
421 the previously published dataset (Fig. 1A) and our new dataset (Fig. 1B), indicating robust
422 differences associated with division of labor and independent of sequencing method.

423 We next sought to identify individual microbes that differed in abundance between nurse
424 and forager bees to identify candidate microbes specifically associated with division of labor.
425 Since microbes can vary between individuals in relative and/or absolute abundance, we used

426 both of these measures of biological diversity. Both may contribute to ecosystem functioning
427 [103–106], but their relative effects on host behavior are currently unknown [30, 62]. Therefore,
428 we reasoned that using both of these abundance measures would give us a well-rounded sense
429 of individual microbes that are associated with honey bee division of labor. Specifically, we
430 performed a relative abundance analysis (ANCOM-BC [62, 64, 65]) that accounts for the
431 compositional nature of 16S rRNA sequencing data and uses relative abundance measures to
432 estimate the true microbial composition of each gut (hereafter referred to as "relative abundance").
433 We also combined qPCR with 16S rRNA sequencing data analyses [29, 30] to estimate the
434 absolute abundance of individual microbial taxa in each gut ("absolute abundance"). We found
435 that individual gut microbes significantly differed in both relative and absolute abundance between
436 nurses and foragers, while total microbe abundance did not differ between groups (Fig. 1C-D,
437 Supplementary Table 1, Table 1). These results indicate that nurse and forager bees differ
438 significantly in their gut microbial communities. They also identify candidate microbes that may
439 be associated with division of labor.
440

441 **Differences in gut microbial community composition between nurses and foragers**
442 **are dependent upon differences in behavioral state**

443 Under typical colony conditions, nurses and foragers differ in chronological age. However, honey
444 bee division of labor is flexible and responsive to changing colony needs. For example, if a colony
445 experiences a shortage of older bees, some younger individuals will begin to forage at an early
446 age, while a shortage of younger bees will cause bees to continue to act as nurse bees despite
447 advancing chronological age [35–39]. To determine whether the gut microbial differences
448 between nurses and foragers depend on differences in behavioral state and/or age, we exploited
449 this adaptive plasticity to create SCCs, a well-established experimental approach that separates
450 these two factors [36, 37, 51]. In two SCC replicates, we compared gut microbiomes between

451 age-matched nurse and forager bees when they were about one week of age (representing
452 typical-age nurses and precocious foragers) and three weeks of age (representing over-age
453 nurses and typical-age foragers).

454 Age-matched typical-age nurses and precocious foragers did not significantly differ in gut
455 microbial community structure (Fig. 2A, Supplementary Fig. 4A), while age-matched over-age
456 nurses and typical-age foragers did (Fig. 2B, Supplementary Fig. 4B). Similar results were
457 obtained for the abundance of individual microbial taxa: no microbes consistently differed in
458 abundance between typical-age nurses and precocious foragers across the two SCC replicates
459 (Fig. 2C, Fig. 2E, Supplementary Fig. 4C, Supplementary Fig. 4E, Supplementary Table 2,
460 Supplementary Table 3), while four microbes consistently differed in relative abundance and three
461 microbes consistently differed in absolute abundance between over-age nurses and typical-age
462 foragers across the two SCC replicates (Fig. 2D, Fig. 2F, Supplementary Fig. 4D, Supplementary
463 Fig. 4F, Supplementary Table 2, Supplementary Table 3). In addition, the total absolute
464 abundance of microbes differed between age-matched typical-age nurses and precocious
465 foragers in one colony replicate (Fig. 2E, Supplementary Fig. 4E), and between age-matched
466 over-age nurses and typical-age foragers (Fig. 2F, Supplementary Fig. 4F). Overall, these
467 findings indicate that age-dependent differences in behavioral state are associated with
468 differences in gut microbial communities between nurses and foragers.

469 Together, results from the typical colony and three-week old SCC indicate that of the
470 honey bee-associated microbes, *Bombilactobacillus mellis* (previously *Lactobacillus mellis*) and
471 *Lactobacillus melliventris* were consistently (i.e. identified in three or more analyses across the
472 two abundance measures) more abundant in nurses relative to foragers (Table 1), while
473 Acetobacteraceae and *Snodgrassella alvi* were consistently less abundant in nurses relative to
474 foragers (Table 1). In SCCs alone, *Bifidobacterium asteroides* showed consistent lower relative
475 abundance in over-age nurses compared to typical-age foragers (Table 1) and *Gilliamella apicola*
476 (Table 1) showed consistent higher absolute abundance in over-age nurses compared to typical-

477 age foragers. Such robust associations highlight these six microbes as candidate microbes whose
478 abundances may have causal effects on division of labor in honey bees. In addition, *Klebsiella*
479 *spp.*, a taxonomic group not typically considered a honey bee-associate, was more abundant in
480 over-age nurses compared to typical-age foragers (Fig. 1-2, Supplementary Fig. 4, Table 1). This
481 microbial species group has previously been found to be prevalent in nurse bees and, as an
482 environmentally derived potential pathogen to bees, was likely picked up outside of the hive by
483 foragers and disseminated to nurses within the hive [30]. As previously suggested, it may
484 accumulate in the guts of nurses, which are typically heavier and support higher bacterial loads
485 than forager guts [30]. This possibility is consistent with our finding that *Klebsiella* was absent in
486 typical-age nurses and precocious foragers but present in over-age nurses and typical-age
487 foragers, likely indicating that *Klebsiella* was inadvertently introduced to the colony after one
488 week, at which time it began accumulating in the guts of over-age nurses, who have higher
489 bacterial loads than typical-age foragers (Fig. 2, Supplementary Fig. 4).

490 The lack of gut microbial community differences between one-week old SCC nurses and
491 foragers suggests that differences in behavioral maturation rate are not associated with
492 differences in gut microbial community composition. Rather, the observed microbiome differences
493 between over-age nurses and typical-age foragers suggest that associations between gut
494 microbiome and behavioral state depend on worker experience, possibly, as others have
495 suggested, due to task-related differences in environment, diet, and/or metabolic needs [30–32].
496 Since SCCs are composed of bees of a single age cohort, three-week old SCCs are composed
497 of bees that have been performing their respective tasks for prolonged periods (1-2 weeks longer)
498 compared to one-week old SCCs. Therefore, we hypothesized that task experience plays an
499 important role in gut microbial community composition. In the next section, we tested this by
500 manipulating foraging experience since it is known to have strong effects on behavior, brain
501 chemistry, brain structure, and brain gene expression [75, 97, 107, 108].
502

503 **Foraging experience influences gut microbial community structure, while**
504 **behavioral state influences the abundance of most individual microbes**

505 In order to determine the causal effects of behavioral state and foraging experience on honey bee
506 gut microbiome composition, we controlled for behavioral state while manipulating foraging
507 experience. To do this, we created "big-back colonies" in which some foragers were freely able
508 to leave the colony ("active foragers"), while other bees, who appeared at the entrance and
509 showed an inclination to forage, were prevented from ever leaving the hive due to the presence
510 of a thick plastic tag on their backs ("inactive foragers") [51, 53]. We were thus able to compare
511 bees of the same age and behavioral state that differed in foraging experience.

512 We found that nurses and inactive foragers did not differ in gut microbial community
513 structure, while active foragers and inactive foragers did (Fig. 3A). This finding indicates that
514 foraging experience plays an important role in defining differences in gut microbial community
515 structure.

516 When we compared the abundance of individual taxa between age-matched active and
517 inactive foragers, we found that unclassified *Lactobacillus spp.* were higher in relative and
518 absolute abundance in inactive foragers compared to active foragers, *L. apis* was higher in
519 relative abundance in inactive foragers compared to active foragers, and *B. mellis* was lower in
520 relative abundance in inactive foragers compared to active foragers (Fig. 3B-C, Supplementary
521 Table 4). In addition, inactive foragers had a higher absolute abundance of total microbes
522 compared to active foragers (Fig. 3C). This effect seems to be driven by a low absolute total
523 abundance in the active foragers, as the inactive foragers had a median absolute total abundance
524 similar to foragers in typical colonies and three-week old SCCs (Supplementary Tables 1-4).
525 Therefore, while inactive foragers are unable to leave the colony to defecate, differences in
526 microbiome between inactive and active foragers are unlikely to be due to an unhealthy
527 accumulation of bacterial cells in inactive forager guts.

528 The observed difference in *Lactobacillus spp.* abundance between inactive and active
529 foragers matches our earlier finding that this taxonomic group decreases in absolute abundance
530 between nurses and foragers in typical colonies (Fig. 1D), indicating that this microbe group may
531 be associated with task experience. Interestingly, in our earlier analyses *B. mellis* was higher in
532 relative and absolute abundance measures in nurses compared to foragers (Fig. 1, Fig. 2,
533 Supplementary Fig. 4, Table 1). While we did not find an effect on absolute abundance here, it is
534 surprising that *B. mellis* is higher in relative abundance in active foragers compared to inactive
535 foragers. While all of our analyses support a connection between *B. mellis* and division of labor,
536 this relationship may be more complex than originally considered from nurse/forager results
537 alone. The other division of labor associated microbes (Table 1) did not differ in abundance
538 between inactive and active foragers.

539 Overall, these results suggest that the abundance of some microbes is determined by the
540 experience of foraging outside of the hive, while the abundance of most individual microbes is
541 defined by behavioral state. Together with our SCC results, which indicate that differences in the
542 abundance of individual gut microbes may be dependent upon worker experience (Fig. 2D,F,
543 Supplementary Fig. 4D,F), we speculate that honey bee gut microbes and foraging behaviors
544 interact through a feedback model. For example, gut microbes may change in abundance due to
545 foraging experience, possibly incrementally (and thus indiscernibly) at first and building with more
546 experience, and this change in microbiome may then feed back to support host foraging behaviors
547 [32]. In the next section, we test the latter part of this model by assessing the effects of inoculation
548 with individual microbes on host foraging behavior.

549

550 **Inoculation with individual microbes affects group- and individual-level foraging**
551 **intensity**

552 To determine if gut microbes play a causal role in honey bee division of labor, we
553 performed single microbe inoculations with three out of the six individual honey bee microbes
554 robustly associated with division of labor as described above: *B. asteroides*, *B. mellis* and *L.*
555 *melliventris* (Supplementary Fig. 5A-C). In addition to the strong patterns of association reported
556 above, we chose these three microbes due to *B. asteroides*' known effects on host physiology
557 with links to behavior, the high metabolic output associated with taxa in Lactobacillaceae [46],
558 since gut microbial metabolites likely contribute to the microbiota-gut-brain axis [7], and due to
559 these three species' previously published associations with honey bee behavioral task [30–32,
560 109]. Therefore, we reasoned that these three species represented strong candidates to test for
561 causal effects on honey bee division of labor. Using an automated behavioral tracking system
562 [71], we determined the effect of inoculation with these three microbes on two measures of
563 foraging behavior important in division of labor in honey bees: rate of behavioral maturation (age
564 at onset of foraging) and foraging intensity (amount of foraging trips performed by a group or
565 individual) [44]. Our results indicate effects only during the first two days of behavioral monitoring,
566 for reasons that we speculate about in the Discussion.

567 *B. asteroides* inoculated bees did not differ from microbiota-depleted bees in behavioral
568 maturation rate (Fig. 4A) across experimental replicates, but did differ in foraging intensity.
569 Overall, *B. asteroides* inoculated bees performed the majority of the foraging trips for the colony
570 on the first and second days after colony formation (Fig. 4B, Supplementary Table 5). These
571 group-level effects were not due to a difference in number of foragers between inoculated and
572 microbiota-depleted bees (Fig. 4C, Supplementary Table 5). Rather they were at least partly due
573 to a difference in individual-level foraging intensity, as individual *B. asteroides* inoculated foragers
574 performed a majority of the foraging trips for the colony on the first day after colony formation (Fig.
575 4D, Supplementary Table 5). Likewise, *B. asteroides* experimental colonies displayed a degree
576 of skew in foraging intensity among individuals (Table 2) and *B. asteroides* inoculated bees
577 represented a higher proportion of "elite forager" bees, i.e., a small subset of foragers that

578 performed ≥50% of the colony's foraging trips [43, 75], relative to microbiota-depleted bees on
579 the first and second days after colony formation (Fig. 4E, Supplementary Table 5).

580 Similar to *B. asteroides* inoculated bees, *B. mellis* inoculated bees did not differ from
581 microbiota-depleted bees in behavioral maturation rate (Fig. 5A). By contrast, *B. mellis*
582 inoculation did not have an effect on group-level foraging intensity (Fig. 5B, Supplementary Table
583 5) or number of foragers between inoculated and microbiota-depleted bees (Fig. 5C,
584 Supplementary Table 5). Rather, *B. mellis* inoculation influenced individual-level foraging
585 intensity, as *B. mellis* inoculated bees performed a minority of foraging trips for the colony on the
586 first and second days after colony formation (Fig. 5D, Supplementary Table 5). Additionally, *B.*
587 *mellis* inoculation caused a skew in foraging intensity between individual foragers (Table 2), as *B.*
588 *mellis* inoculated bees represented a lower proportion of elite foragers than microbiota-depleted
589 bees on the second day after colony formation (Fig. 5E, Supplementary Table 5).

590 Similar to *B. asteroides* and *B. mellis* inoculated bees, *L. melliventris* inoculated bees did
591 not differ from microbiota-depleted bees in behavioral maturation rate (Fig. 6A). However, *L.*
592 *melliventris* inoculated bees had an opposite effect on foraging intensity from *B. asteroides*
593 inoculated bees: *L. melliventris* inoculated bees performed the minority of foraging trips for the
594 colony on the first and second days after colony formation (Fig. 6B, Supplementary Table 5). This
595 group-level effect was not due to a difference in number of foragers between inoculated and
596 microbiota-depleted bees (Fig. 6C, Supplementary Table 5). Rather, it was at least partly due to
597 a difference in individual-level foraging intensity, as individual *L. melliventris* inoculated foragers
598 performed a minority of the foraging trips for the colony on the second day after colony formation
599 (Fig. 6D, Supplementary Table 5). Likewise, *L. melliventris* colonies displayed a degree of skew
600 in foraging intensity between individual foragers (Table 2), and *L. melliventris* bees represented
601 a lower proportion of elite foragers than microbiota-depleted bees on the second day after colony
602 formation (Fig. 6E, Supplementary Table 5).

603 Overall, these results indicate that inoculation with individual gut microbes is sufficient to
604 cause increases or decreases in foraging intensity between worker bees at both the group- and
605 individual- level.
606

607 **Discussion**

608 Here, we show that the gut microbiome plays an important role in modulating foraging
609 behavior in the honey bee. Specifically, our results indicate that differences in honey bee gut
610 microbial communities influence honey bee division of labor by affecting foraging intensity, but
611 not rate of behavioral maturation. This conclusion is supported by both microbe abundance and
612 behavioral results: *B. asteroides* is found in higher relative abundance in foragers compared to
613 nurses, and inoculation with this species caused bees to have increased foraging intensity
614 compared to microbiota-depleted controls. Similarly, *B. mellis* and *L. melliventris* are found lower
615 in abundance in foragers compared to nurses, and inoculation with either of these species caused
616 bees to have decreased foraging intensity compared to microbiota-depleted controls.

617 From a technical perspective, the observation that single microbe inoculations can cause
618 either an increase or a decrease in foraging intensity indicates that it is unlikely that the results
619 reported here are due to an artifact of the microbiological or behavioral methods we used. In
620 addition, the congruence of our microbe abundance and behavioral results indicate that single
621 microbe inoculations, although they do not represent natural microbiome variations, provide a
622 useful method for functional analysis.

623 Overall, our behavioral results show the strongest effects on the first and second days
624 after experimental colony formation, indicating that the most robust effects of microbiome on
625 behavior occur within the first few days after inoculation. We suspect that this is due to changing
626 social dynamics in the experimental honey bee colonies over the six day behavioral tracking
627 period. Although we did not measure gut microbiomes post colony introduction, it is likely that

628 changes in the gut microbiomes of the experimental bees occurred after colony formation due to
629 "homogenization" between individuals due to "trophallaxis," the sharing of gut fluids that contain
630 nutritive and signaling molecules, and/or colonization by microbes acquired from the colony
631 environment or non-experimental bees. Therefore, experimental treatment effects on behavior
632 may be weak or non-existent on later experimental days. Changes in colony dynamics, such as
633 the pool of available foragers, may also account for weak behavioral effects observed later in the
634 experiment. Foraging is an energetically costly task and is associated with high mortality
635 compared to in-hive behaviors [110–112]. Indeed, across all types of experimental colonies in our
636 studies, foragers that began foraging early after colony formation had an earlier last day of
637 foraging (which likely corresponds with day of death [44, 74]) (Supplementary Fig. 5D-F). This
638 indicates that our experimental colonies had a typical high forager turnover.

639 We observed additional interesting changes in colony foraging force due to foraging
640 activity. In the case of *B. mellis* experimental colonies, only microbiota-depleted foragers that
641 began foraging on the first day had a significantly earlier last day of foraging compared to later
642 foragers (Supplementary Fig. 5E). In addition, microbiota-depleted elite foragers had a
643 significantly earlier first day of foraging compared to *B. mellis* inoculated elite foragers
644 (Supplementary Fig. 5G). These results indicate that in *B. mellis* experimental colonies, forager
645 turnover was associated with treatment such that microbiota-depleted foragers had their foraging
646 career at younger ages compared to *B. mellis* inoculated foragers. A similar but non-significant
647 trend in elite forager onset was also observed in *L. melliventris* colonies (Supplementary Fig. 5G).
648 We suggest that this likely accounts for the (non-significant) switch in group-level and elite
649 foraging intensity seen on the later experimental days in *B. mellis* (Fig. 5B, Fig. 5E) and *L.*
650 *melliventris* (Fig. 6B, Fig. 6E), but not *B. asteroides* (Fig. 4) experimental colonies.

651 Previous studies reported correlational differences in gut microbiome associated with
652 honey bee behavioral task [30–32] and behavioral maturation [50]. These studies also reported
653 associations of *Bifidobacterium*, *B. mellis* and *L. melliventris* with behavioral task [30, 32] and

654 ontogeny [31], supporting a link between these microbes and division of labor in honey bees.
655 Here we found that *B. mellis* and *L. melliventris* were higher in abundance in nurses compared to
656 foragers, matching results from [30, 32]. However, while we found that *B. asteroides* was higher
657 in relative abundance in typical-age foragers relative to over-age nurses, results in [30, 32] found
658 that *Bifidobacterium* was higher in relative [32] and absolute abundance [30] in nurses compared
659 to foragers. This difference may be due to the difference in taxonomic level used in analysis
660 between these studies and ours, as we found that other *Bifidobacterium* species were higher in
661 abundance in nurses compared to foragers (trend in Fig. 1C-D and Fig. 2D, significant in Fig. 2F).
662 In addition, these studies found an increase in *S. alvi* with age [31], and a higher abundance of
663 *Gilliamella* and *Klebsiella* associated with nurses [30], which match our abundance results (Table
664 1). Here, we provide evidence that at least some of these gut microbes play a causal role in
665 defining behavioral differences between honey bees. Therefore, the congruence of our
666 abundance results with other studies indicate that our behavioral results are likely generalizable
667 across populations of honey bees.

668 Our findings are consistent with a previously proposed model in which positive feedback
669 interactions cause adaptive changes in both gut microbiome composition and host behavior [32].
670 Under this model, we speculate that gut microbes could change in abundance due to time spent
671 foraging, likely in association with environmental exposure and/or changes in diet and metabolic
672 needs. According to this speculation, the change in microbiome would then feed back to influence
673 host foraging behaviors. We suggest that changes in gut microbe abundance (e.g. increases in
674 *B. asteroides* with decreases in *B. mellis* and *L. melliventris*) and host behavior interact to increase
675 foraging intensity. Modeling interactions in this way can provide the basis for future studies that
676 probe the neural and metabolic mechanisms by which *B. asteroides*, *B. mellis* and *L. melliventris*
677 affect host behavior.

678 Previous studies have shown that gut microbes may influence host behavior through a
679 variety of mechanisms, including production of metabolites that cause changes in host brain gene

680 expression or host production of neurotransmitters and hormones [7]. Within honey bees,
681 *Bifidobacterium*, *Bombilactobacillus* Firm-4 and *Lactobacillus* Firm-5 are the major fermenters of
682 pollen-derived compounds and have large effects on the abundance of metabolites in the hindgut
683 [46]. In addition, *B. asteroides* inoculation has been associated with an increased production of
684 two juvenile hormone derivatives [46]. Changes in gene expression and the production of
685 neurotransmitters and hormones, most notably juvenile hormone, are associated with the
686 regulation of honey bee division of labor [42, 44, 74, 95–98, 101, 107, 113, 114], and therefore it
687 is possible that gut microbes influence honey bee division of labor through these pathways.
688 Likewise, previous studies indicate that some host factors associated with honey bee division of
689 labor, such as social interactions and diet, may also modulate gut microbiome composition,
690 including the abundance of these three microbes [7, 30, 48, 115, 116]. Future research will work
691 to identify specific mechanisms by which honey bees and their gut microbiomes interact to
692 influence worker behavior. Overall, studies using the honey bee as a model have the potential to
693 further elucidate the microbiota-gut-brain axis, and achieve a more comprehensive understanding
694 of interkingdom interactions.

695 Together, our results suggest that in naturally occurring bee populations, increases in *B.*
696 *asteroides* with simultaneous decreases in *B. mellis* and *L. melliventris* may act synergistically to
697 increase the intensity of foraging behavior in individual bees. Honey bees, with a complex eusocial
698 life history, live in large perennial colonies and therefore collect large amounts of nectar and pollen
699 to ensure colony survival during periods when floral resources are not available. Thus, the
700 influence of these three microbes on honey bee foraging intensity indicates that host-microbe
701 interactions likely help sustain the perennial lifestyle of this eusocial insect. Future studies may
702 address the interaction between these three microbes, other members of the honey bee
703 microbiome, and between the microbiome and the host in order to gain a more naturalistic and
704 comprehensive understanding of the role of the gut microbiome in social insect division of labor.
705

Acknowledgements

We thank Nathan Beach, Sarah Magdalena Murphree, Sarai Stuart, Laura Kilikevicius, Rashmi Bajaj, Maha Syed and Anthony Cantu for field assistance, Sue Cobey and Dr. Osman Kaftanoglu for SDI queen rearing and inseminations, Ahmed Burki for assistance making qPCR standard plasmids, Alvaro Hernandez, Chris Wright and staff at the Carver Biotechnology Center for sequencing services, members of the Computer Network Resource Group of the Carl R. Woese Institute for Genomic Biology (UIUC) for computational support, Konrad Wilk for annotating missed barcodes, Richard B. Devine for contributions to video annotation software development, and the workers of Turker Nation for annotating honey bee trajectories. Funding: US National Science Foundation Division of Integrative Organismal Systems award number 2120378, PI: GER.

Competing Interests

The authors declare no competing interests.

Data Availability Statement: 16S rRNA sequencing data are available from the NCBI Sequence Read Archive (PRJNA958253) and all other data are available from Mendeley Data (DOI: 10.17632/f2s47y3nhn). Computer code for the barcode detector is available at https://github.com/gernat/btools.

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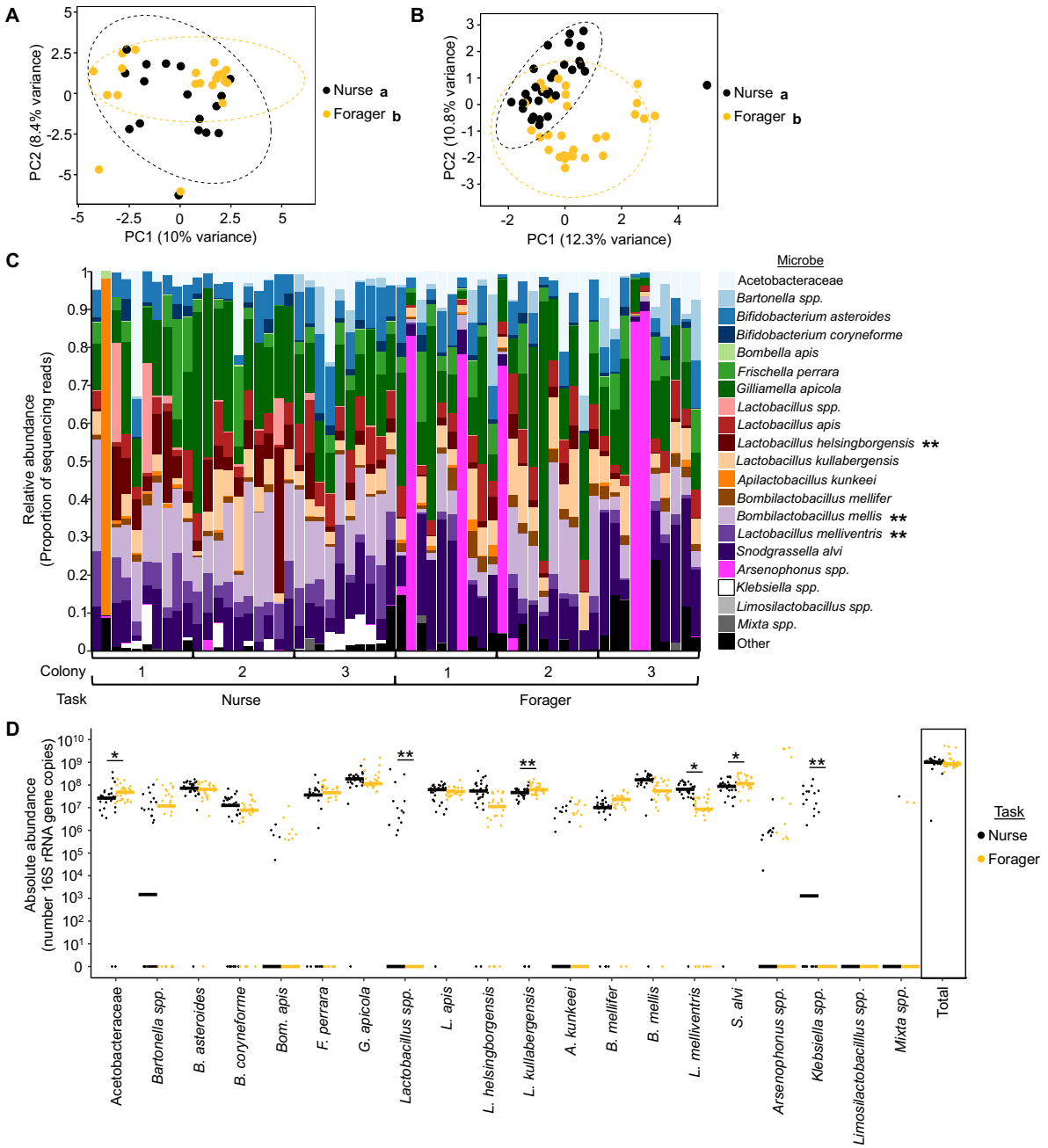
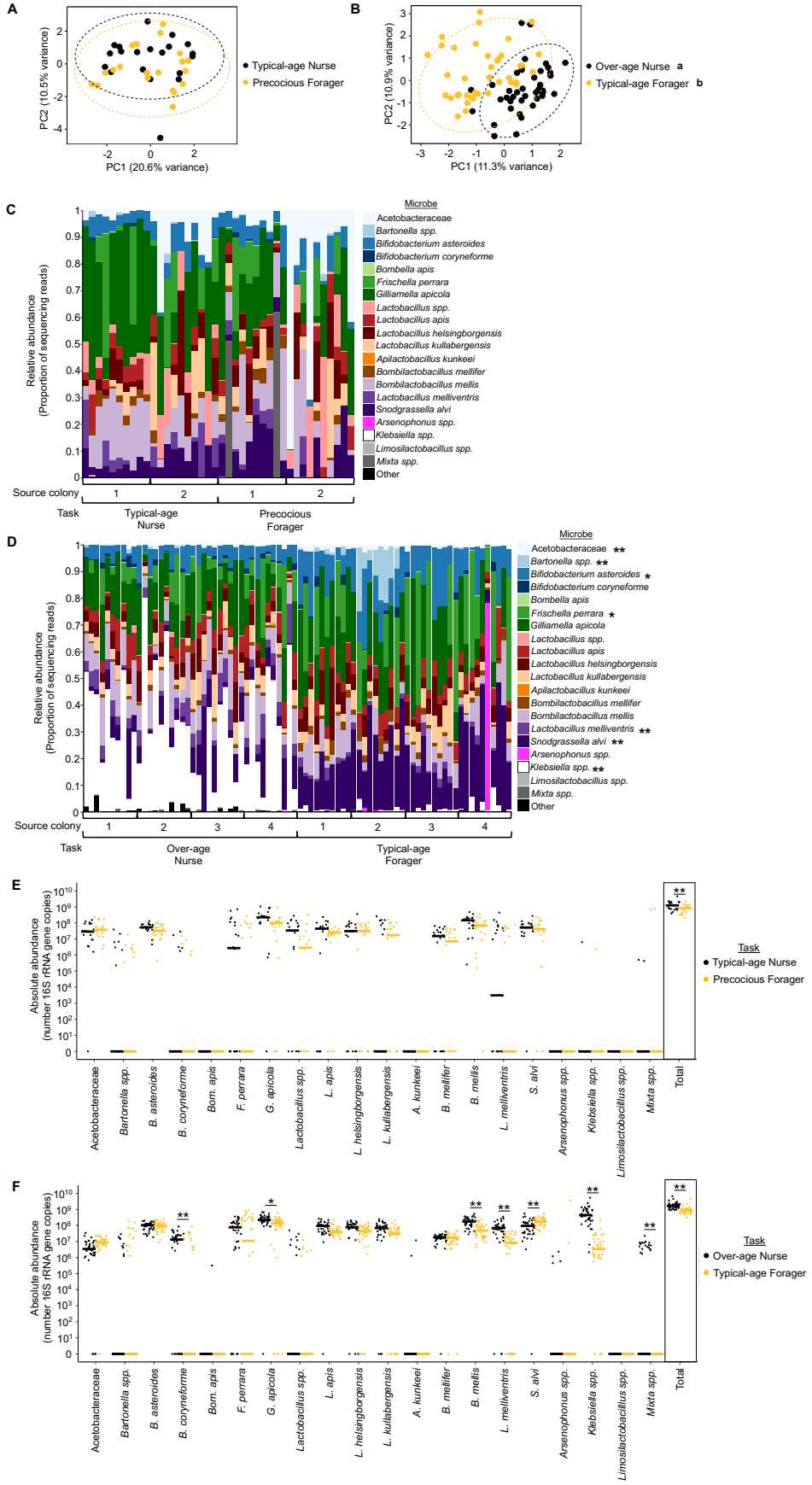
1008 # Figures



1009

1010 **Figure 1. Nurses and foragers from typical honey bee colonies differ in gut microbial**
1011 **community. (A-B)** Nurses and foragers differed in overall gut microbial community structure. (A)
1012 Reanalyzed data from Kapheim et al 2015. Two-way Permutation MANOVA using Aitchison
1013 Distance, Task: $F_{1,38}$ = 1.51, R^2 = 0.036, p = 0.016; Colony: $F_{4,38}$ = 1.72, R^2 = 0.165, p = 0.001,

1014 Task*Colony: $F_{2,38}$ = 1.17, R^2 = 0.056, p = 0.100. n = 1-12 bees/colony, 5 colonies. (B) New data.
1015 Two-way Permutation MANOVA using Aitchison Distance, Task: $F_{1,59}$ = 4.28, R^2 = 0.066, p =
1016 0.001; Colony: $F_{2,59}$ = 2.31, R^2 = 0.071, p = 0.001, Task*Colony: $F_{2,59}$ = 1.20, R^2 = 0.037, p =
1017 0.144. n = 10 bees/colony, 3 colonies. Depicted as Principal Components Analysis (PCA) plots.
1018 Lowercase letters in legends denote statistically significant groups. **(C)** Nurses and foragers
1019 differed in relative abundance of four individual microbial species (new data only). Depicted as
1020 stacked bar plots, with each bar representing a single bee's microbiome. Asterisks in legend: *,
1021 p≤0.05, **, p≤0.01, ANCOM-BC between nurses and foragers. See Supplementary Table 1 for all
1022 p-values. **(D)** Nurses and foragers differed in absolute abundance of four individual microbial
1023 species but not in the total normalized number of 16S rRNA gene copies (new data only). 10^x
1024 number of 16S rRNA gene copies, calculated by multiplying the relative abundance each microbe
1025 in each sample (determined through 16S rRNA sequencing) by the normalized number of 16S
1026 rRNA gene copies in the sample (determined through qPCR). Depicted as dot plots with all data
1027 points plotted, line represents median, n = 10 bees/colony, 3 colonies. *, p≤0.05, **, p≤0.01,
1028 Permutation ANOVA Test between nurses and foragers. See Supplementary Table 1 for all p-
1029 values.


A
PC2 (10.5% variance)
PC1 (20.6% variance)
Typical-age Nurse
Precocious Forager
B
PC2 (10.9% variance)
PC1 (11.3% variance)
Over-age Nurse a
Typical-age Forager b
C
Relative abundance
(Proportion of sequencing reads)
Microbe
Acetobacteraceae
Bartonella spp.
Bifidobacterium asteroides
Bifidobacterium coryneforme
Bombella apis
Frischella perrara
Gilliamella apicola
Lactobacillus spp.
Lactobacillus apis
Lactobacillus helsingborgensis
Lactobacillus kullabergensis
Apilactobacillus kunkeei
Bombilactobacillus mellifer
Bombilactobacillus mellis
Lactobacillus melliventris
Snodgrassella alvi
Arsenophonus spp.
Klebsiella spp.
Limosilactobacillus spp.
Mixta spp.
Other
Source colony
Task
Typical-age Nurse
Precocious Forager
D
Over-age Nurse
Typical-age Forager
E
Absolute abundance
(number 16S rRNA gene copies)
Task
Typical-age Nurse
Precocious Forager
Total
F
Over-age Nurse
Typical-age Forager

1030

1031 **Figure 2. Age-matched nurses and foragers differ in gut microbial community. (A-B)** Age-
1032 matched typical-age nurses and precocious foragers from a single cohort colony did not differ in
1033 gut microbial community structure at about one week of age (A), but age-matched over-age
1034 nurses and typical-age foragers significantly differed in gut microbial community structure at three
1035 weeks of age (B). 1 week: Two-way Permutation MANOVA using Aitchison Distance, Task: $F_{1,39}$
1036 = 0.92, R^2 = 0.023, p = 0.519; Source colony: $F_{1,39}$ = 2.60, R^2 = 0.064, p = 0.004, Task*Colony:
1037 $F_{1,39}$ = 1.15, R^2 = 0.028, p = 0.296. n = 10 bees/source colony, 2 source colonies. 3 weeks: Two-
1038 way Permutation MANOVA using Aitchison Distance, Task: $F_{1,79}$ = 6.49, R^2 = 0.074, p = 0.001;
1039 Source colony: $F_{3,79}$ = 1.82, R^2 = 0.062, p = 0.001, Task*Colony: $F_{3,79}$ = 1.23, R^2 = 0.042, p =
1040 0.084. n = 10 bees/source colony, 4 source colonies. Depicted as PCA plots. Lowercase letters
1041 in legends denote statistically significant groups. **(C-D)** Age-matched typical-age nurses and
1042 precocious foragers from a single cohort colony did not differ in relative abundance of individual
1043 microbial species (C), while age-matched over-age nurses and typical-age foragers differed in
1044 relative abundance of five individual microbial species at three weeks of age (D). Depicted as
1045 stacked bar plots, with each bar representing a single bee's microbiome. Asterisks in legend: *,
1046 $p \leq 0.05$, **, $p \leq 0.01$, ANCOM-BC between nurses and foragers. See Supplementary Table 2 for all
1047 p-values. **(E-F)** Age-matched typical-age nurses and precocious foragers from a single cohort
1048 colony did not differ in absolute abundance of individual microbial species but did differ in the total
1049 normalized number of 16S rRNA gene copies (E), while age-matched over-age nurses and
1050 typical-age foragers differed in absolute abundance of six microbial species and the total number
1051 of 16S rRNA gene copies at three weeks of age (F). 10^x number of 16S rRNA gene copies,
1052 calculated by multiplying the relative abundance each microbe in each sample (determined
1053 through 16S rRNA sequencing) by the normalized number of 16S rRNA gene copies in the sample
1054 (determined through qPCR). Depicted as dot plots with all data points plotted, line represents
1055 median, n = 10 bees/source colony, 2 source colonies (1 week) or 4 source colonies (3 weeks).

1056 *, $p \leq 0.05$, **, $p \leq 0.01$, Permutation ANOVA Test between nurses and foragers. See

1057 Supplementary Table 2 for all p-values.

1058

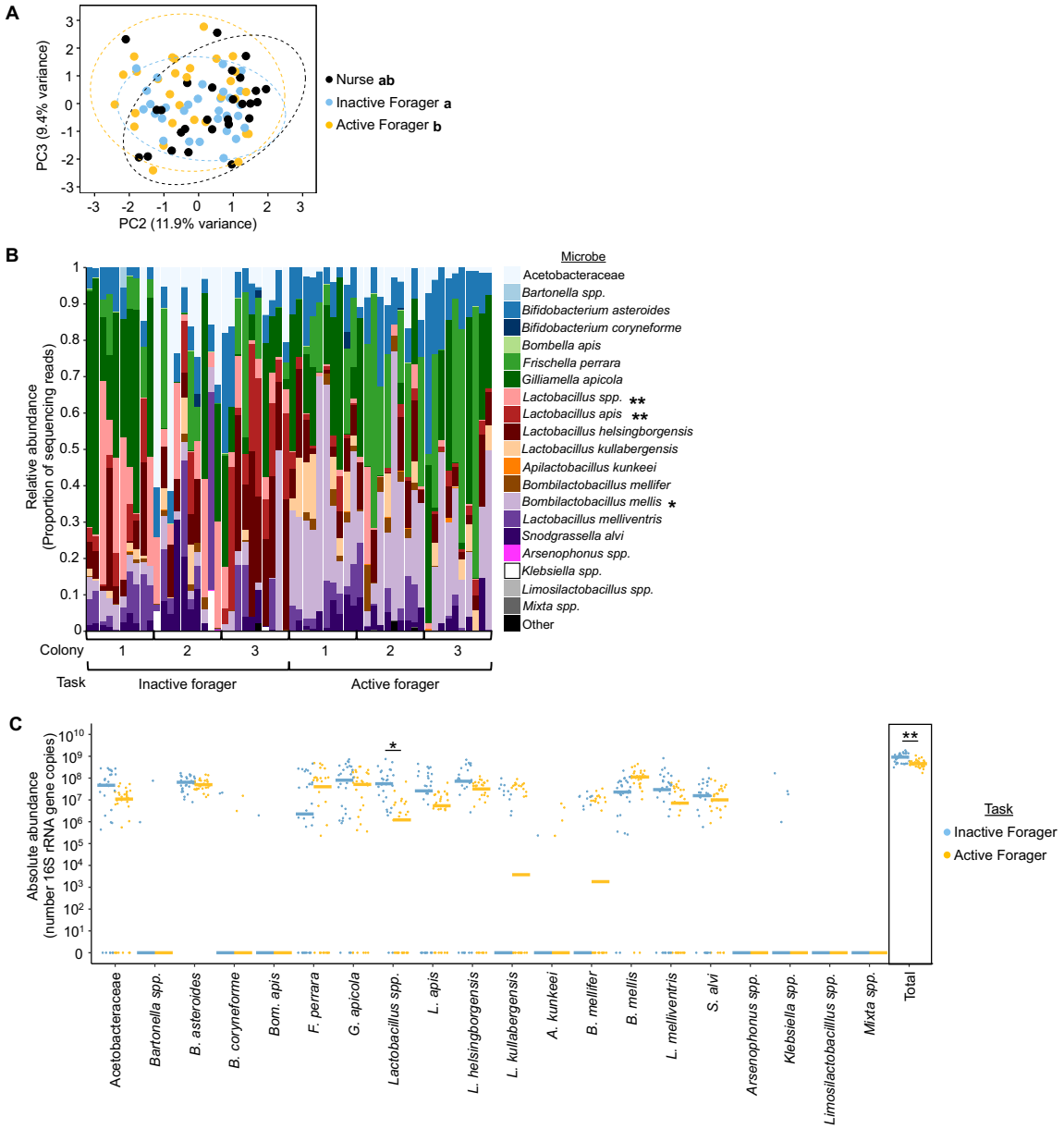


Figure 3. Age-matched inactive and active foragers differ in gut microbial community structure, but not the abundance of individual microbes. (A) Age-matched nurses and inactive foragers were similar in gut microbial community structure, while age-matched active and inactive foragers differed in gut microbial community structure. Two-way Permutation MANOVA using Aitchison Distance, Task: $F_{2,86}$ = 1.84, R^2 = 0.038, p = 0.011; Colony: $F_{2,86}$ = 6.02, R^2 =

1066 0.124, p = 0.001, Task*Colony: $F_{4,86}$ = 0.798, R^2 = 0.033, p = 0.883. n = 10 bees/colony, 3
1067 colonies. Depicted as PCA plot. Lowercase letters in legends denote statistically significant
1068 groups as determined by Pairwise Permutation MANOVA. **(B)** Age-matched inactive and active
1069 foragers did not differ in the relative abundance of individual microbial species (B). Depicted as
1070 stacked bar plots, with each bar representing a single bee's microbiome. Asterisks in legend: *,
1071 p≤0.05, **, p≤0.01, ANCOM-BC between inactive and active foragers. See Supplementary Table
1072 4 for all p-values. **(C)** Age-matched inactive and active foragers did not differ in absolute
1073 abundance of individual microbial species but did differ in the total normalized number of 16S
1074 rRNA gene copies. 10^x number of 16S rRNA gene copies, calculated by multiplying the relative
1075 abundance each microbe in each sample (determined through 16S rRNA sequencing) by the
1076 normalized number of 16S rRNA gene copies in the sample (determined through qPCR). Depicted
1077 as dot plots with all data points plotted, line represents median, n = 10 bees/colony, 3 colonies. *,
1078 p≤0.05, **, p≤0.01, Permutation ANOVA Test between inactive and active foragers. See
1079 Supplementary Table 4 for all p-values.
1080

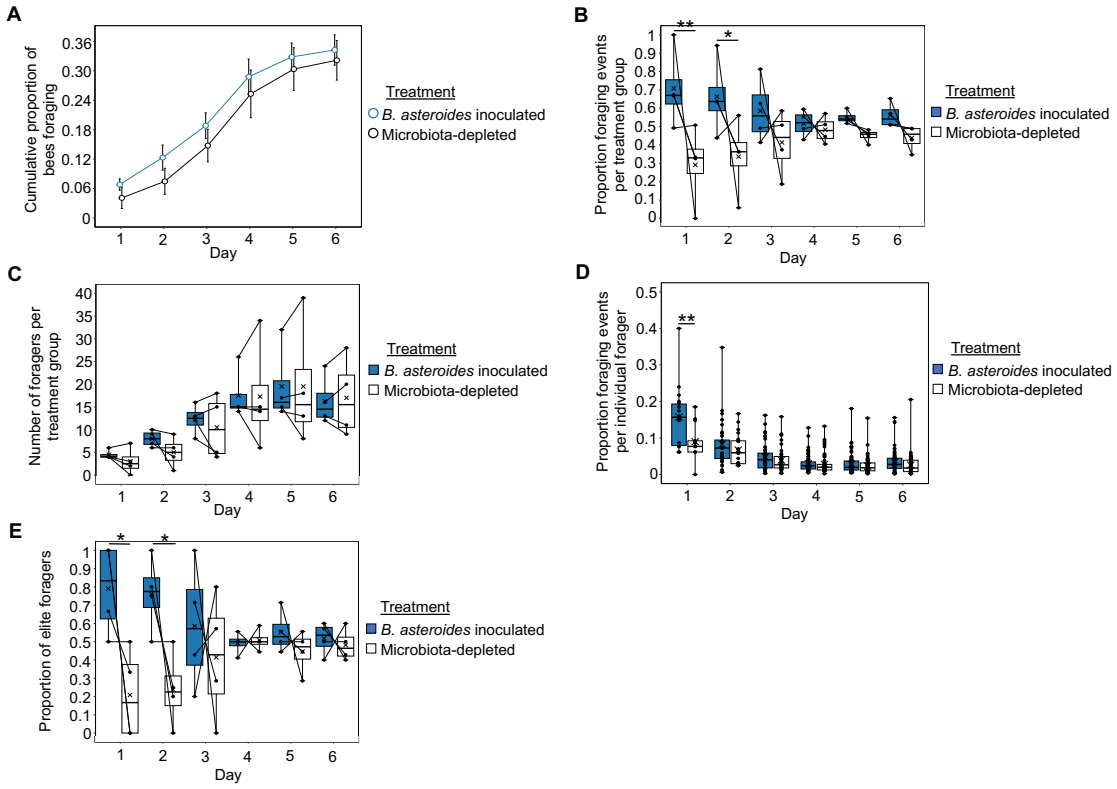


Figure 4. Bees inoculated with *B. asteroides* do not differ from microbiota-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *B. asteroides* inoculated bees did not differ from microbiota-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = -0.331, p = 0.741. **(B)** *B. asteroides* inoculated foragers, as a group, performed the majority of foraging trips for the colony on the first and second days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,33}$ = 21.16, p < 0.001, Day: $F_{5,33}$ = 0, p = 1, Treatment*Day: $F_{5,33}$ = 1.987, p = 0.107. See Supplementary Table 5 for Pairwise comparisons. **(C)** *B. asteroides* inoculated bees represented a similar number of foragers as microbiota-depleted bees on all days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,33}$ = 0.409, p = 0.527, Day: $F_{5,33}$ = 12.095, p < 0.001, Treatment*Day: $F_{5,33}$ = 0.140, p = 0.982. See Supplementary Table 5 for Pairwise comparisons. **(D)** Individual *B. asteroides* inoculated foragers performed a majority of foraging trips for the colony the first day after experimental colony formation. Generalized Linear Mixed Effects Model

with log-normal distribution, Treatment: $\chi^2 = 4.985$, $p = 0.026$, Day: $\chi^2 = 382.933$, $p < 0.001$, Treatment*Day: $\chi^2 = 26.315$, $p < 0.001$. See Supplementary Table 5 for Pairwise comparisons. **(E)** *B. asteroides* inoculated bees represented a higher proportion of elite foragers than microbiota-depleted bees on the first and second days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,33} = 15.799$, $p < 0.001$, Day: $F_{5,33} = 0$, $p = 1$, Treatment*Day: $F_{5,33} = 3.151$, $p = 0.020$. See Supplementary Table 5 for Pairwise comparisons. (A) depicted as survival plot. All other data depicted as box plots with data points plotted, thick horizonal line represents median, x represents mean, whiskers represent the minimum and maximum values, thin lines connect paired points for each colony, n = 4 colonies. Asterisks used to denote comparisons between treatment groups on each day only: *, $p \leq 0.05$, **, $p \leq 0.001$.

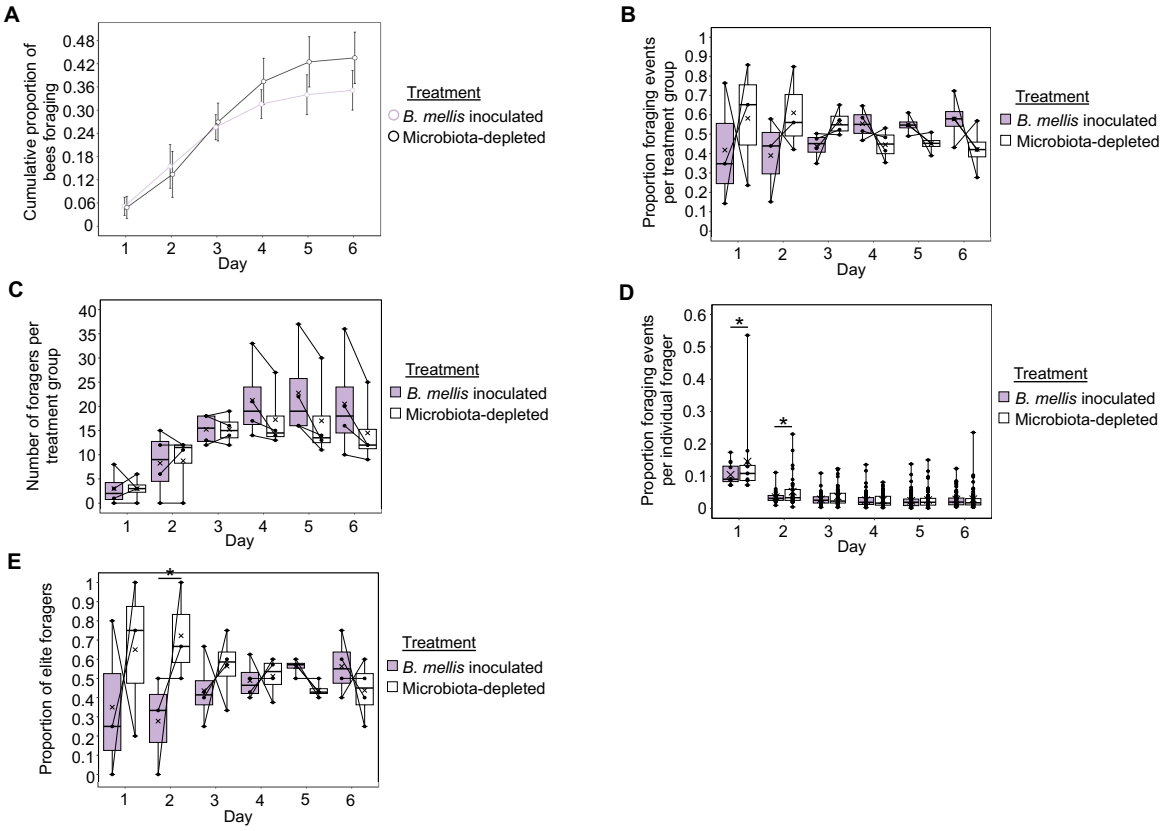


Figure 5. Bees inoculated with *B. mellis* do not differ from microbiota-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *B. mellis* inoculated bees did not differ from microbiota-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = 1.525, p = 0.127. (**B)** *B. mellis* inoculated foragers, as a group, performed a similar proportion of foraging trips for the colony as microbiota-depleted bees all days after experimental colony formation, with a marginal effect of performing a minority of foraging trips for the colony on the second day after colony formation. Linear Mixed Effects Model, Treatment: $F_{1,29.091} = 0.036$, p = 0.851, Day: $F_{5,29.719} = 0$, p = 1, Treatment*Day: $F_{5,29.091} = 1.981$, p = 0.111. See Supplementary Table 5 for Pairwise comparisons. **(C)** *B. mellis* inoculated bees represented a similar number of foragers as microbiota-depleted bees on all days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,33} = 3.183$, p = 0.084, Day: $F_{5,33} = 14.918$, p < 0.001, Treatment*Day: $F_{5,33} = 0.765$, p = 0.582. See Supplementary Table

1119 5 for Pairwise comparisons. **(D)** Individual *B. mellis* inoculated foragers performed a minority of
1120 foraging trips for the colony on the first and second days after experimental colony formation.
1121 Generalized Linear Mixed Effects Model with log-normal distribution, Treatment: $\chi^2 = 3.626$, $p =$
1122 0.057, Day: $\chi^2 = 255.497$, $p < 0.001$, Treatment*Day: $\chi^2 = 6.552$, $p = 0.256$, See Supplementary
1123 Table 5 for Pairwise comparisons. No statistical outliers were detected in these data. **(E)** *B. mellis*
1124 inoculated bees represented a lower proportion of elite foragers than microbiota-depleted bees
1125 on the second day after experimental colony formation. Linear Mixed Effects Model, Treatment:
1126 $F_{1,29.091} = 1.896$, $p = 0.179$, Day: $F_{5,29.719} = 0$, $p = 1$, Treatment*Day: $F_{5,29.091} = 2.183$, $p = 0.083$.
1127 See Supplementary Table 5 for Pairwise comparisons. (A) depicted as survival plot. All other data
1128 depicted as box plots with data points plotted, thick horizonal line represents median, x represents
1129 mean, whiskers represent the minimum and maximum values, thin lines connect paired points for
1130 each colony, n = 4 colonies. Asterisks used to denote comparisons between treatment groups on
1131 each day only: *, $p \leq 0.05$, **, $p \leq 0.001$.
1132

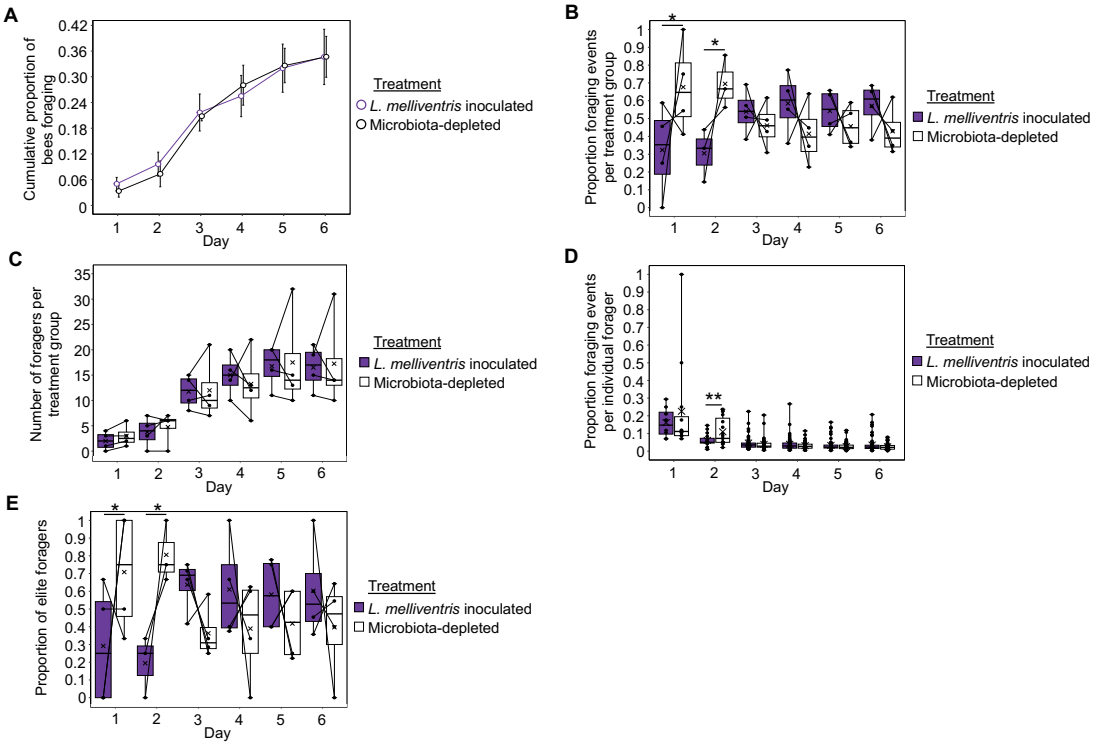


Figure 6. Bees inoculated with *L. melliventris* do not differ from microbiota-depleted bees in behavioral maturation (age at onset of foraging), but do differ in foraging intensity. (A) *L. melliventris* inoculated bees did not differ from microbiota-depleted bees in age at onset of foraging. Cox Proportional Hazards, z = -0.435, p = 0.664. (**B**) *L. melliventris* inoculated foragers, as a group, performed the minority of foraging trips for the colony on the first and second days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,31.021} = 0.351$, p = 0.558, Day: $F_{5,31.443} = 0$, p = 1, Treatment*Day: $F_{5,31.021} = 4.169$, p = 0.005. See Supplementary Table 5 for Pairwise comparisons. **(C)** *L. melliventris* inoculated bees represented a similar number of foragers as microbiota-depleted bees on all days after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,33} = 0.079$, p = 0.781, Day: $F_{5,33} = 18.923$, p < 0.001, Treatment*Day: $F_{5,33} = 0.132$, p = 0.984. See Supplementary Table 5 for Pairwise comparisons. **(D)** Individual *L. melliventris* inoculated foragers performed a minority of foraging trips for the colony the second day after experimental colony formation. Generalized Linear Mixed Effects

1147 Model with log-normal distribution, Treatment: $\chi^2 = 0.141$, $p = 0.707$, Day: $\chi^2 = 334.831$, $p < 0.001$,
1148 Treatment*Day: $\chi^2 = 18.336$, $p = 0.003$, See Supplementary Table 5 for Pairwise comparisons.
1149 No statistical outliers were detected in these data. **(E)** *L. melliventris* inoculated bees represented
1150 a lower proportion of elite foragers than microbiota-depleted bees on the first and second days
1151 after experimental colony formation. Linear Mixed Effects Model, Treatment: $F_{1,31.021} = 0.0006$, p
1152 = 0.981, Day: $F_{5,31.443} = 0$, $p = 1$, Treatment*Day: $F_{5,31.021} = 3.961$, $p = 0.007$. See Supplementary
1153 Table 5 for Pairwise comparisons. (A) depicted as survival plot. All other data depicted as box
1154 plots with data points plotted, thick horizonal line represents median, x represents mean, whiskers
1155 represent the minimum and maximum values, thin lines connect paired points for each colony, n
1156 = 4 colonies. Asterisks used to denote comparisons between treatment groups on each day only:
1157 *, $p \leq 0.05$, **, $p \leq 0.001$.
1158

	Relative abundance			Absolute abundance		
Microbe	**Typical colonies**	**SCC Replicate 1 (week 3)**	**SCC Replicate 2 (week 3)**	**Typical colonies**	**SCC Replicate 1 (week 3)**	**SCC Replicate 2 (week 3)**
Acetobacteraceae		lower abundance	lower abundance	lower abundance		lower abundance
Bartonella spp.		lower abundance				
B. asteroides		lower abundance	lower abundance			
B. coryneforme					higher abundance	
Bom. apis						
F. perrara		lower abundance				
G. apicola					higher abundance	higher abundance
Lactobacillus spp.				higher abundance		
L. apis						higher abundance
L. helsingborgensis	higher abundance					
L. kullabergensis				higher abundance		higher abundance
A. kunkeei						
B. mellifer						
B. mellis	higher abundance				higher abundance	higher abundance
L. melliventris	higher abundance	higher abundance	higher abundance	higher abundance	higher abundance	
S. alvi		lower abundance		lower abundance	lower abundance	
Arsenophonus spp.						
Klebsiella spp.		higher abundance	higher abundance	higher abundance	higher abundance	higher abundance
Limosilactobacillus spp.						
Mixta spp.					higher abundance	

Table 1: Summary table of individual microbe abundance results. Microbes that significantly differed in relative (left) and absolute (right) abundance measures in nurses relative to foragers in typical colonies and individual single cohort colony replicates. Higher abundance, higher in abundance in nurses relative to foragers (pink). Lower abundance, lower in abundance in nurses relative to foragers (blue).

Test Microbe	Colony Replicate	Overall Colony Gini Coefficient	Day 1 Gini Coefficient	Day 2 Gini Coefficient	Day 3 Gini Coefficient
B. asteroides	1	0.376	0.056	0.116	0.425
B. asteroides	2	0.362	0.352	0.32	0.278
B. asteroides	3	0.415	0.133	0.445	0.368
B. asteroides	4	0.305	0.199	0.303	0.242
B. mellis	1	0.339	0.13	0.32	0.304
B. mellis	2	0.459	0.304	0.453	0.355
B. mellis	3	0.31	NA	NA	0.31
B. mellis	4	0.302	0.126	0.23	0.363
L. melliventris	1	0.398	0.186	0.271	0.365
L. melliventris	2	0.283	NA	NA	0.281
L. melliventris	3	0.419	0.167	0.386	0.415
L. melliventris	4	0.391	0.174	0.453	0.267

Table 2: Gini coefficients for each experimental colony depicted in Figures 4-6. Gini coefficients, representing the degree of skew in foraging intensity between individual foraging bees, where 0 represents complete equality in foraging intensity between individuals and 1 represents complete inequality in foraging intensity between individuals, measured for each experimental colony across all days ("Overall Colony Gini Coefficient"), and for each experimental colony on each individual day ("Day X Gini Coefficient"). NAs represent days with bad weather in which foraging did not occur.