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CHARACTERIZATION OF THE HONEY BEE MICROBIOME THROUGHOUT THE QUEEN-REARING PROCESS

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Abstract:

The European honey bee (*Apis mellifera*) is used extensively to produce hive products and for crop pollination, but pervasive concerns about colony health and population decline have sparked an interest in the microbial communities that are associated with these important insects. Currently, only the microbiome of workers has been characterized, while little to nothing is known about the bacterial communities that are associated with queens, even though their health and proper function is central to colony productivity. Here, we provide a large-scale analysis of the gut microbiome of honey bee queens, during their developmental trajectory and through the multiple colonies that host them as part of modern queen-rearing practices. We found that queen microbiomes underwent a dramatic shift in size and composition as they aged and encountered different worker populations and colony environments. Queen microbiomes were dominated by enteric bacteria in early life but were comprised primarily of α-proteobacteria at maturity. Furthermore, queen gut microbiomes did not reflect those of the workers who tended them and, indeed, they lacked many of the bacteria that are considered to be "core" to workers. While worker gut microbiotas were consistent across the unrelated colony populations sampled, the microbiotas of the related queens were highly variable. Bacterial communities in mature queen guts were similar in size to those of mature workers and were characterized by dominant and specific α-proteobacterial strains known to be associated with worker hypopharyngeal glands. Our results suggest a model in which queen guts are colonized by bacteria from

53 workers' glands, in contrast with routes of maternal inoculation for other animal

54 microbiomes.

55

56 **Introduction:**

57

58 Honey bees (*Apis* spp.) are characterized by highly partitioned reproductive division of
59 labor, where a single queen lays the eggs that give rise to virtually all members of her
60 colony and her daughters—the workers—execute all other laborious jobs, including that
61 of caring for her offspring [1]. As the sole caregivers in the colony, workers share food
62 extensively with one another, consuming their colony's food reserves and then
63 distributing nutrients in various forms to their queen, other workers, and reproductive
64 males (drones) [2]. For all three of these castes, workers share food with adults through
65 trophallaxis (mouth-to-mouth food transfer of liquids from one worker's gut to
66 another's) or by feeding developing larvae 'brood food' (glandular secretions derived
67 from consumed nutrients). Because of these mechanisms of food distribution, a honey
68 bee colony is often considered to have a 'social stomach'. Studies with tracers show
69 rapid distribution (<24 hours) of food from small numbers of individuals to many, if not
70 the majority, of colony members across all ages and castes [3-7]. Nurse-age bees
71 (typically less than 10-days-old) are the primary consumers of pollen [8], thus they are
72 the main distributors of pollen-based nutrients to adults and the brood that they rear [3,
73 5]. Nectar is also distributed among colony members; foragers bring it back to the hive
74 in their crops (foregut) and it is then handled by bees of various ages, either to feed
75 adults and larvae or to store in wax combs and ripen into honey [7]. This continual
76 exchange of food among nestmates during all stages of their lives makes the
77 establishment and maintenance of gut microbiomes particularly complex to understand

across castes and for individuals as they age. However, the microbial communities that are associated with honey bees are considered to have an important influence on nutrient availability in colonies (although the specific role of microbes is uncertain [9]). As such, there is growing interest in elucidating the microbial communities within insect societies using culture-independent techniques.

Presently, gut microbiomes have been best explored in workers of the European honey bee (*Apis mellifera*) [10]. Workers have a characteristic microbial community that is composed predominantly of three major bacterial phyla (Firmicutes, Proteobacteria, and Actinobacteria) that are transcriptionally active within the honey bee gut [11]. Within these larger taxonomic groupings, several honey bee-specific families and genera have been identified [12-14]. The core microbiome of the adult worker has been characterized as being comprised of a small number of bacterial clades, some with new genus and species designations [12, 15, 16]. These core clades have been referred to as Firm-4, Firm-5 (within the Firmicutes), Bifido (within the Actinobacteria), and Alpha-2.1, Alpha-2.2, Alpha-1, Beta, Gamma-1, and Gamma-2 (within the Proteobacteria) [12]. Although several other bacterial species are found to be associated with honey bee workers, these specific clades are consistently found across different geographic regions and throughout different seasonal samplings [12, 13, 17]. A number of beneficial interactions among these microbes and the honey bee have been suggested, including increased metabolic functionality; these microbes may degrade complex polysaccharides that are otherwise inaccessible to the host organism [10]. Indeed, the

bacterial community encodes and expresses β-glucosidase genes specific to the breakdown of cellulose [10, 11]. Another potential benefit of the honey bee microbiome includes protection from invading pathogens through facilitation of the immune response [18] or exclusionary effects [19-21].

In contrast, virtually nothing is known about the microbial communities that are associated with queens, even though their health and proper function is central to the productivity of their colonies. Complicating such studies are the realities of queen production in modern apiculture, where colonies are managed intensively to yield hive products (e.g., honey) and particularly for crop pollination. In managed colonies, the natural process of queen replacement—where an aging queen is superseded by a daughter queen, raised by her worker sisters from within the larval ranks —is typically prevented by beekeepers. Instead, intentionally bred queens are artificially introduced to a colony by beekeepers only after taking a circuitous path through several other related and unrelated host colonies [22, 23].

Queens from different genetic sources (known as 'grafting' sources) are reared as larvae in 'cell builders', unrelated colonies whose queenless workers nurse larval queens until they are sealed into their cells (~1 week). Each sealed cell is then transferred to a small nucleus colony or 'mating nuc', where another set of unrelated workers support the queen through the first week or more of her adult life. Once she has mated and started laying eggs (typically 2–3 weeks), she is eventually transferred into a final host colony of

122 queenless, unrelated workers, who care for her as she beings to lay eggs for the new
123 colony. Eventually her worker progeny replace the resident, unrelated worker
124 population as the initial colony population dies. Queens do not feed themselves at any
125 point during this process; both larval and adult queens receive food products from many
126 different workers who care for them. Larval queens are reared by nurse-aged workers
127 and, as adults, they spend most of their time in the brood area of the nest, where they
128 continue to be fed by nurse-aged bees from the resident worker population [24, 25].
129 Thus, in addition to understanding the general characteristics of queen microbiomes
130 relative to the worker caste, we must also understand how their exposure in managed
131 colonies to different populations of workers shapes their microbiomes over the course
132 of their lives.
133
134 Here we present the first description of the bacterial communities that are associated
135 with honey bee queens that have navigated the complicated queen-rearing process
136 typically employed by beekeepers. We used 16S rRNA gene amplicon sequencing to
137 identify milestones in the development of their microbiome and to evaluate the relative
138 impact on their bacterial communities of the unrelated and related worker populations
139 that they encounter over their lifetimes. In addition, we use quantitative PCR to
140 elucidate the relative numbers of 16S rRNA gene copies in queen microbiomes as they
141 are hosted by different populations of workers during their larval and adult
142 development. Our data reveal many intriguing aspects of the development of the queen
143 gut microbiome. Most notably, queens experience a dramatic shift in the composition of

144 their bacterial communities as they age, especially between larvae and adults and,
145 interestingly, these communities do not reflect the microbial profiles of the workers
146 who cared for them. Moreover, while unrelated populations of host workers had
147 relatively similar gut microbiomes, the gut microbiomes of the related queens that they
148 tended were far more variable across individuals. Queen microbiota were dominated by
149 honey bee specific α-proteobacteria and lacked the canonical "core" gut microbiome
150 that is associated with honey bee workers. Because queens are fed from worker
151 hypopharyngeal glands, and because bee-specific α-proteobacteria are heavily
152 associated with this gland, our results suggest a model in which queen guts are
153 colonized by bacteria from a specific worker organ. This result contrasts with routes of
154 inoculation for mammalian microbiomes, where the maternal environment is the source
155 of bacteria for the next generation [26-28].
156
157
158 **Methods:**
159
160 *Queen rearing and sample collection*
161
162 The field research was conducted at the North Carolina State University Lake Wheeler
163 Honey Bee Research Facility (Raleigh, North Carolina). A single honey bee colony was
164 chosen as the sole source of all reared queens to minimize genetic variance among
165 individuals within the focal caste. Our general approach was to track these queens

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throughout their larval and adult development, sampling a subset of queens and host workers each time queens encountered a new colony in order to characterize and compare microbiomes across castes and host populations.

The experiment began by first marking with paint a single cohort of newly emerged workers from the single queen source colony (henceforth referred to as the 'grafting source'). These workers emerged from sealed cells over a 24-hour period in an incubator set at broodnest conditions (34°C and ~50% R.H.), then were paint marked as 1-day-old adults and reintroduced within hours to their natal hive (n > 200 marked workers). Five days later, 45 female larvae were transferred ("grafted") from their cells into plastic queen cells following standard queen-rearing techniques (e.g.,[23]). All worker larvae were <24-hours-old to ensure that the resultant queens were of high reproductive potential (see [29]). Larval transfer was accomplished without 'priming' each queen cell with royal jelly (known as "dry grafting") to avoid potential microbial cross-contamination, and the grafting needle was also dipped in 95% EtOH and flamed with a lighter in between larval transfers for the same reason. An additional 24 larvae of the same age were sampled from the grafting source directly into cryopreservation tubes, which were immediately submerged in liquid nitrogen. The tubes were later decanted from the liquid nitrogen and stored in a -80°C freezer until further processing. On the same day as grafting and continuing into the next, marked workers (now 5-days-old) were individually collected from the inner combs of the grafting source colony and placed into separate glass vials and cooled on ice until immobile. Once chilled, the mid-

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188 and hind-gut were removed from each worker by gently pulling on the stinger with
189 sterilized metal tweezers until the lower organs of the GI tract were extracted (n = 100
190 grafting-source workers). The gut from each worker bee was then placed into an
191 individually labeled cryotube, immediately flash frozen in liquid nitrogen, and later
192 stored in a -80°C freezer until further processing (see [21]). Thus, 5-day-old workers
193 were sampled from the grafting-source colony, which is an age when workers are likely
194 to be nurse bees that provision larvae with brood food, including the young focal queen
195 larvae [25]. After all larvae were grafted, the queen cells were placed into a queen-less
196 'cell builder', where its resident workers reared the unrelated queen larvae to pupation
197 over the next 7 days. Two days prior to the transfer of queen cells to the cell builder, a
198 cohort of same aged, newly emerged workers from the cell builder were paint marked
199 as described previously.
200
201 Five days later, mid- and hind-guts were collected from marked workers in the cell
202 builder, as described already (n = 100 cell-builder workers). These 5-day-old workers in
203 the common cell builder were therefore most likely to have been actively provisioning
204 the developing queen larvae with royal jelly over the majority of their larval period.
205 Three days later, after the queen cells were sealed by workers so that the queens could
206 pupate, the cells were moved into an incubator to complete their development.
207
208 The day prior to their emergence of queens from sealed cells, 30 queen cells were
209 transferred into 30 separate 'mating nucleus' colonies that had been established from

210 six unrelated colonies (5 mating nuclei per source colony). Each mating nucleus
211 contained 500-2,000 adult worker bees of unknown age and three mini-frames of brood
212 and stored food (honey and pollen); these workers cared for the queen during the early
213 period of her adult life, while she mated, and when began to lay eggs. The remaining
214 queen cells were placed into separate glass test tubes so that the queens could be
215 captured upon their emergence in the incubator. These queens therefore had no
216 contact with worker populations after leaving the cell builder. The following day, 13
217 newly emerged 'cell builder' queens were collected from the incubator and their mid-
218 and hind guts were removed. Unlike workers, the guts of which could be easily
219 extracted by gently pulling on the sting shaft, each queen was separately dissected with
220 sterilized microscissors and forceps in order to obtain their GI tracts, which were
221 immediately flash-frozen as above.
222
223 Between 10 to 20 days following queen emergence in their respective mating nucleus
224 colonies, adult worker bees were sampled from each mating nucleus for their gut
225 contents following the procedure outlined above. Unlike the workers sampled from the
226 grafting source and cell builder, these workers were of unknown age because sufficient
227 emerging workers from each small unit could not be adequately obtained. Of the 30
228 mating nuclei that were established, several were not successful because the queens did
229 not emerge from their cells properly, were not accepted by the workers upon
230 emergence, or failed to successfully mate on their mating flights and begin oviposition.
231 A total of 13 mating nuclei had samples for 100 'mating nucleus' workers collected per

232 colony (one unit yielded only 81 workers because of a limited worker population at the
233 time of sampling). At this time, five queens were also destructively sampled from their
234 mating colonies as outlined above (henceforth referred to as the 'mating nuc queens'),
235 each from a different source of the six source colonies originally used to create the
236 nuclei.
237
238 The remaining 8 mated and laying queens were then removed from their respective
239 mating nuclei, placed into separate queen cages, and introduced following standard
240 techniques into new field colonies [23]. All of the 'final' colonies were unrelated to each
241 other and all other colonies in the experiment. After two days of acclimation, the five
242 surviving queens were released from their cages into their final colonies and visually
243 verified as accepted by the resident unrelated workers (three of the queens died in their
244 cages and thus were not accepted by the workers). Two weeks after introduction,
245 frames of emerging workers were collected from each hive and emerged in the
246 incubator to paint-mark and reintroduce a cohort of age-matched workers in each, as
247 described above. The mid- and hindgut were removed from a sample of these workers
248 five days later, as described previously (n = 100 'final colony prior to offspring
249 emergence' workers per colony). Several of the remaining queens were also sampled at
250 this time, following the methods described above (n = 3 'final colony before offspring
251 emergence' queens). Two weeks later, a second cohort of newly emerged resident
252 worker bees was similarly marked in each of the remaining colonies, when emerging
253 brood were the genetic offspring of the 2 remaining focal queens. These marked

254 workers were sampled and their guts removed five days later, as described above (n = 2
255 'final colony after offspring emergence' workers per colony). At this time, the final
256 remaining two queens were also sampled for their gut contents (henceforth referred to
257 as 'final colony after offspring emergence' queens).
258
259 *DNA extraction and amplicon library generation*
260
261 Queen and larvae were processed individually and workers from each colony were
262 processed in pools of 10. In addition, we also processed and sequenced individual
263 workers (10 each) in order to account for sequencing depth when comparing to
264 individual queens. DNA was extracted from all samples using a modified liquid-nitrogen
265 protocol. Briefly, sterilized, ceramic mortar and pestles were pre-cooled in liquid
266 nitrogen and samples were ground into a fine powder. This powder was added to Tris-
267 EDTA with added proteinase-K (at 0.025 µg/mL final) and incubated for 1 hour at 50°C.
268 After this incubation, a phenol-chloroform extraction was performed twice before
269 ethanol precipitation. DNAs were resuspended in Tris-EDTA and cleaned using a column-
270 based genomic clean-up kit (Zymo) according to instructions. DNAs were then quantified
271 (using an Epoch Take3 plate) and stored at -80°C before use in PCR.
272
273 Polymerase chain reaction using barcoded Illumina primers followed the Earth
274 Microbiome protocols [30] with the following differences: HF Phusion polymerase mix
275 (New England Biolabs, Ipswich, Massachusetts, U.S.A.) was used and 3% DMSO was

276 added to the reactions before cycling at 98°C for 45 s, 50°C for 60 s, and 72°C for 90 s.
277 Amplifications were performed in triplicate and pooled before normalization based on
278 pico-green quantification.
279
280
281 *Sequencing and bioinformatics analysis*
282
283 Pooled amplicons were sequenced at the Indiana Center for Genomics and
284 Bioinformatics core facility (Bloomington, Indiana, U.S.A.) using an Illumina MiSeq and
285 250 paired end cycles. Adapter sequences were removed from all reads before raw
286 processing of data (using the program suite Mothur). We utilized the Schloss SOP [31],
287 accessed September, 2014, for MiSeq data utilizing the previously described honey bee
288 specific training set combined with the current Greengenes release for classification of
289 OTUs [14]. Briefly, contigs were generated using the make.contigs command and
290 sequences were screened for ambiguous basepairs and length using screen.seqs.
291 Unique seqs were pre-clustered based on 2 nucleotide differences. Chimeras were
292 detected and removed using the chimera.uchime command and lineages found in blank
293 water samples (*Halomonas* and *Shewanella*) were removed, as were sequences
294 classified as chloroplasts, mitochondria, Archaea, or eukaryotes. All samples were
295 rarified to 5,000 sequences (the size of the smallest library). All OTU-based analyses
296 were also performed in Mothur including rarefaction, heatmap creation, analyses of
297 molecular variance, UniFrac analyses, and principle component analyses. Statistical tests

298 beyond those performed in Mothur were implemented using SPSS (IBM, Armonk, New
299 York, U.S.A.). Raw sequencing reads have been deposited in the DDBJ (BioProject ID#
300 PRJDB3520).
301
302 *Quantitative PCR analysis*
303
304 Quantitative PCR was performed on the DNA extracted from each sample to detect the
305 bacterial titer (using standardized calibration curves) using an Applied Biosystems Step
306 One Real-time PCR system and SybrGreen chemistry (Thermo Fisher Scientific, Waltham,
307 Massachusetts, U.S.A.). Calibration curves were generated using cloned 16S rRNA gene
308 fragments (in pPCR-TOPO vectors) that were amplified, cleaned, and pico-green
309 quantified. We used 16S rRNA primers for the bacterial fraction (331F:
310 TCCTACGGGAGGCAGCAGT; 797R: GGACTACCAGGGTATCTAATCCTGTT) [32] with the
311 following cycling temperatures: 95°C for 10 min, then 40 cycles of 95°C for 15 seconds,
312 and 60°C for 1 minute. Reactions were performed in triplicate and any biological
313 replicates with Ct standard deviations above 0.5 were removed from the analysis.
314
315
316 **Results:**
317
318 *Sequencing statistics and overall diversity metrics*
319

320 Our sampling regime resulted in a total of 59 distinct barcoded amplicons across two
321 castes (queens and nurse-aged workers) and from each of five different hive
322 environments (grafting source, cell builder, mating nucleus, and final colonies before
323 and after queens began laying), corresponding to five different developmental stages
324 for queens (larvae, newly emerged queens, maturing queens in the mating nucleus, and
325 laying queens before and after their offspring emerged). A single MiSeq PE run of 250
326 cycles resulted in 11,167,225 reads, of which 7,053,677 (63%) passed stringent quality
327 thresholds (see Methods). The dataset was rarefied (i.e., subsampled to the size of the
328 smallest library) to 5,000 sequences and after alignment and clustering, we identified a
329 total of 264,865 unique sequences and 897 OTUs (at 97% identity) across the entire
330 dataset. Of these OTUs, the top 20 comprised >96% of the total dataset, so the rest of
331 our analyses focused on these top 20 OTUs. Below, we explore the bacterial
332 composition and diversity found in each of the castes and at each developmental time
333 point, focusing on differences between queens and workers.
334
335
336 *The honey bee queen gut microbiome shifted substantially during development*
337
338 We sought to determine microbial signatures of queen development and therefore
339 began by characterizing the communities that were found across our biological
340 replicates for each developmental stage. Honey bee workers are known to harbor
341 distinctive populations of bacteria [13], including the following core bacteria: Firm-5,

342 Firm-4, Beta, Gamma-1, Bifidobacterium, Alpha-2.1, Alpha-2.2, Alpha-1, and these same
343 phylotypes were found associated with queens throughout the rearing process.
344 However, we observed that their microbiome changed dramatically during development
345 and as they moved between host colonies. Specifically, larval queen microbiome
346 libraries were dominated (~78% abundance) by enteric bacteria such as *Escherichia* and
347 Gamma-1 (*Gilliamella*), with relatively small proportions (~19%) of other core bacteria
348 (such as Firm-5, Firm-4, Bifidobacterium, Beta, and the α-proteobacterial groups Alpha-
349 2.1, Alpha-2.2 and Alpha-1; Figure 1). Amplicons generated from newly emerged queens
350 also contained large proportions of enteric bacteria (such as *Escherichia*: 1837.7
351 reads/sample) and, although we were able to detect in all queens some members of the
352 characteristic microbiome of honey bee workers (such as Firm-5, Firm-4, Gamma-1,
353 Alpha-2.1, and Alpha-2.2), these sequences appeared at much lower frequencies than
354 *Escherichia* (minimum and maximum sequences seen in rareified libraries: Firm-5 = 30-
355 366; Firm-4 = 9-97; Gamma-1 = 9-122; Alpha-2.1 = 7-530; Alpha-2.2 = 4-237, Figure 1).
356 Amplicons generated from newly emerged queens had small proportions of α-
357 proteobacteria (means for Alpha-2.1 and 2.2: 120.5 and 49.4 sequences per sample).
358 Newly emerged queen gut microbiomes from the cell builder colony therefore greatly
359 resembled those generated for larval queens in the grafting source.
360
361 In contrast to microbiomes found associated with larval and newly emerged queens,
362 amplicon libraries from mature queens (both from the mating nucleus and the final
363 colonies, before and after the queens' own genetic offspring were present in colonies)

364 were characterized by a large proportion of α-proteobacterial sequences (~46% Alpha-
365 2.1 and ~25% Alpha-2.2, Figure 1). Specifically, two OTUs dominated the mature queen
366 amplicon libraries and were classified as well-known honey bee associated α-
367 proteobacteria, the Alpha-2.2 and Alpha-2.1 clades (mean and SE for each, respectively:
368 2,252 ± 604 reads; 1,219 ± 520 reads; Figure 1). Queens were much less likely to be
369 colonized by the related Alpha-1 clade (found in low quantities in only five of the 10
370 mature queen that were sampled: 1.4 ± 0.4 reads, or 0.03% of their bacterial
371 community). We saw no significant difference in microbiome composition between
372 queens from the mating nucleus or from the final colonies (t-test results $p > 0.05$;
373 Kruskal-Wallis tests not significant). Therefore, the subsequent analyses pooled queen
374 samples from the mating nucleus and the final colonies (referred to as "mature queens"
375 throughout).
376
377
378 *Gut microbiomes differed between queens and workers*
379
380 In order to contextualize gut microbiomes of the queens, we also analyzed gut
381 microbiomes of the workers who were of nursing age and may have participated in the
382 rearing of these queens. All of the workers sampled in this study harbored well-known
383 bacterial community members, in relative proportions expected based on previous work
384 [12, 13, 17]. Of the top-20 OTUs that were classified and identified from workers in our
385 study, 11 are known members of the honey-bee-associated community and are included

386 in the clades Firm-5, Firm-4, Beta, Gamma-1, Bifidobacterium, Alpha-2.1, Alpha-2.2, and
387 Alpha-1. These core members represented 84% of the classified reads in this
388 subsampled worker dataset and all of the libraries generated for workers sampled in
389 this study contained sequences that were classified as Firm-5, Bifidobacterium, Gamma-
390 1, Alpha-2.1 and Beta. Additionally, we also identified several unclassified
391 Enterobacteriales, Enterobacteriaceae, and Lactobacillales that were present at lower
392 frequencies and more sporadically than the core microbiome. One of these OTUs was
393 found consistently across all sampled colonies (Lactobacillaceae *incertae sedis*; average
394 number of sequences per queen = 103.6, max = 1,926).
395
396 Worker microbiomes were significantly different from those that were found in the
397 queens for which they cared; Kruskal-Wallis tests determined nearly all top-20 OTUs
398 differed between workers, larvae, newly emerged queens, and mature queens (df = 3; p
399 < 0.05; except for *Lactobacillales incertae sedis;*) (Figure 2). To visualize similarities
400 between microbial communities from each of these sampled environments, we
401 performed both weighted and unweighted UniFrac analyses, which compares bacterial
402 community composition between environments (based on phylogenetic relatedness of
403 these microbes, weighting relative abundance or not). Amplicon libraries sequenced
404 from worker bee digestive tracts clustered to the exclusion of those from larvae and
405 queens (Figure 3; Unifrac weighted analysis $p < 0.001$ for each pairwise comparison).
406 This means that workers microbiomes were more similar across unrelated colonies than
407 they were to the queens that they hosted (which were unrelated or related to them,

408 depending on the sampling point). Interestingly, within the queens, we saw a clear
409 developmental progression in microbiome composition; mature queens clustered with
410 some of the newly emerged adult queens, and some newly emerged queens clustered
411 with larvae (Figure 2). Therefore, development seemed to have a strong effect on the
412 microbial communities that were seen in queens.
413
414 To further visualize similarities and also to statistically determine which microbial
415 community members contributed to the differences observed across our samples, we
416 performed a principle components analysis (Figure 4). Again, we observed that
417 clustering of our amplicon libraries was dependent on developmental stage and was
418 strongly influenced by caste, such that worker samples clustered to the near exclusion
419 of mature queen samples (the first component accounted for 29.7% of the total
420 variance while the second was 11.2%). We further identified the microbiome members
421 that might be contributing to the specific clustering of these two communities, based on
422 caste and developmental stage, by identifying statistically significant differences in pair-
423 wise comparisons of microbiome composition. We identified the following classified
424 OTUs to be significantly different between workers and mature queens: Firm-4,
425 Enterobacteriaceae, Beta, Gamma-1, Bifidobacteriaceae, Alpha-2.1, Alpha-1,
426 Enterobacteriales, unclassified bacteria, unclassified proteo, Bacilli, Lactobacillales,
427 Bifidobacterium (based on Kruskal-Wallis and subsequent Mann Whitney U-tests
428 producing statistically significant differences between workers and mature queens, $p <$
429 0.05). To test the hypothesis that developmental stage and caste contributed to a

430 distinguishing microbiome profile, we performed a step-wise discriminant function
431 analysis utilizing abundance data from the top-20 OTUs in the dataset. This analysis
432 showed that microbiome composition could readily categorize each sample as a larva, a
433 newly emerged queen, a mature queen, or a worker (Wilks's lambda coefficient = 0.001;
434 $\chi^2 = 404.8$; df = 36; $p < 0.001$).
435
436
437 *Queen replacement may affect gut microbiome profiles of workers*
438
439 We expected to observe a "microbial signature" in the queens that reflected in some
440 way their interactions with the workers who attended to them throughout the rearing
441 process. Interestingly, we did not see a resemblance between the queen microbiomes
442 and the worker populations that reared them at any stage of their development. In
443 contrast, we saw a statistically significant change only in the worker microbiome
444 composition in the final colonies, when colony populations shifted from workers who
445 were unrelated to their queen to the queen's offspring (Figure 1B). Specifically, we saw
446 a statistically significant reduction in the proportion of Firm-5 found in these worker
447 bees (t-test, $p < 0.02$) and a qualitative increase in the proportion of Alpha-2.1 (with the
448 removal of one outlier, $p < 0.04$) for four colonies sampled after the emergence queen-
449 produced workers. Importantly, in one colony where we sampled workers before and
450 after the emergence of the offspring of the queen, we were also able to sample the
451 associated mature, laying queen. For this one colony, we did not see a significant shift in

452 worker microbiome composition between unrelated host workers and subsequent
453 offspring of the queen (Figure 1A). Because of the nature of destructive sampling, which
454 was necessary to complete this study, we were unable to increase the sample size for
455 this last time point (only 2 queens were sampled after they began laying and only 4
456 worker colonies were sampled after queens began laying). Therefore, although this
457 result is interesting and deserves future study, it should be cautiously interpreted.
458
459
460 *Queen gut microbiomes were variable in size and in diversity*
461
462 In order to more deeply characterize microbiome variability across queens, we
463 calculated pairwise Bray Curtis dissimilarity values within each of our sampled
464 developmental stages (Figure 1C). Based on this metric, we found that microbiome
465 composition was significantly more consistent across the unrelated populations of adult
466 workers than the related queens at each developmental stage they were sampled
467 (pairwise t-tests using Bonferroni correction, $p < 0.01$). Amplicons from queen larvae
468 were the most variable across individuals with regard to composition, showing the
469 largest Bray Curtis similarity distributions and highest means (Figure 1C). In contrast, as
470 the queens matured, their microbial compositions became more consistent across
471 individuals, with pair-wise differences between samples settling on a Bray Curtis
472 similarity of 0.67. Importantly, mature queens did not differ from workers with regard
473 to the average number of OTUs found within their digestive tracts (Table 1).

474

475 We wondered if the variability in microbiome composition between individual mature
476 queens might reflect stochastic sampling of transient and rare members; if queen
477 microbiomes have few bacteria, the amplicons resulting from these environments might
478 increase the variability observed across sampled queens. Sequencing libraries
479 generated from few bacteria would result in deeper sequencing of rare members
480 compared to libraries generated from environments dominated by a single species. In
481 order to investigate the number of microbes found in adult workers and queens during
482 their development, we performed a quantitative PCR analysis using the 16S rRNA gene
483 (Figure 5). The development of the worker bee microbiome, like that of many animals,
484 follows a well-known trajectory where the number of bacteria colonizing the animal
485 increases over time [33-38]. We found a similar increase in community size in queens as
486 they developed from larvae into mature adults (Figure 5). However, there was no
487 difference in the total number of 16S rRNA gene copies between these same mature
488 queens and the workers in the colonies that hosted them at the final sampling time
489 point. Instead, we saw a broader range of 16S rRNA gene copies associated with mature
490 queens than what was found in workers; queens were less consistent in both
491 composition and total number of microbiome members (Figure 5).

492

493

494 **Discussion:**

495

496 Although the developmental trajectory of honey bee queens in a highly managed setting
497 is complex, some straightforward insights can be gleaned about the establishment of
498 their gut microbiomes throughout this process. First, queen microbiomes changed
499 dramatically as queens aged and they encountered different colony environments, with
500 the greatest shifts in community composition occurring between young queens (larvae
501 and newly emerged adult queens who had not yet contacted more workers) and
502 mature, laying queens. While the microbiomes of the former were dominated by enteric
503 bacteria, the latter were comprised primarily of α-proteobacteria. Within the
504 boundaries of these broad generalizations, there was greater variability across the
505 related queens that we sampled than among the unrelated workers who hosted them,
506 which showed the core worker microbiota that has been documented previously [12-
507 14], even though queens at maturity had communities that were similar to workers in
508 size and diversity. Secondly, we found little evidence that the bacterial communities of
509 queens reflected those of the workers who were of the age to tend them; queen
510 microbiotas clustered reliably by developmental stage, but separately from adult
511 workers. Our study identifies a unique and numerically rich microbial signature for
512 queens that changed as queens aged in a way that was not linked to the gut microbiome
513 of any of the workers that they encountered over their lifetime.
514
515 Several studies have now documented the progressive development of the honey bee
516 worker microbiome from the larval to the adult stage. Initial attempts, based on PCR,
517 showed little amplification overall from larvae and relatively few of the bacterial groups

518 that have previously been associated with the core microbiome of adult workers [17].
519 However, other laboratories have been successful in culturing bacteria from surface-
520 sterilized larvae [19, 39, 40], although even these culture-based studies support the
521 hypothesis that larvae are not colonized consistently with the "core microbiome" of
522 adults. Similarly, newly eclosed workers are also believed to lack the characteristic
523 microbiome of adults. In a healthy hive, over the span of a few days, young workers are
524 colonized with bacterial phylotypes that are characteristic of adults [33] and interaction
525 with hive components, and with fecal material from adult bees, facilitates the
526 transmission of these bacterial phylotypes [39]. In some ways, the development of the
527 queen microbiome mirrors that of workers. Specifically, larval queens do not host the
528 canonical honey-bee-associated bacteria community, and gut microbiomes of newly
529 emerged queens (adults who have not yet had contact with more workers) resemble
530 the depauperate communities of larvae (Figures 1-3). However, by the time queens
531 mature (either in the mating nucleus or in their final colonies), they have developed a
532 queen-specific microbial signature, where libraries are dominated by alpha-
533 proteobacteria.
534
535 The route of transmission of the queen microbiome is likely quite distinct from that of
536 the worker microbiome. Compared to worker larvae, larvae destined to be queens are
537 fed higher quantities of royal jelly, a protein-rich secretion from worker hypopharyngeal
538 glands containing proteins that may alter DNA methylation (via Dnmt2) [41, 42]. This
539 difference in diet is directly responsible for the developmental differences found

540 between workers and queens, and this caste distinction is likely modulated by genome-
541 wide methylation patterns that differ during development [43]. After queens mature,
542 they do not feed on bee bread or nectar directly, unlike the nurse-age workers that
543 were sampled here and by others [44]. Instead, queens continue to be fed royal jelly by
544 the workers who attend them, who are typically of nurse age [25]. Additionally, workers
545 attending the queen dispose of her fecal material, as well as clean and groom the
546 queen, thus likely diminishing her contact with hive-associated bacteria, as workers
547 would experience. Presumptions about queen rearing and care within the colony fit well
548 with our observation that worker gut microbiomes do not resemble those of the queen,
549 based on caste differences in exposure and hive experiences.
550
551 There may be another route by which worker-associated microbes may influence the
552 queen. Interestingly, one component of worker hypopharyngeal gland secretions that is
553 not well investigated is a specific acetic acid bacterium, *Candidatus Parasaccharibacter*
554 *apis* (within the Alpha-2.2 clade [40]). This bacterial species is associated with young
555 worker larvae (that are primarily fed royal jelly) and is also found in high numbers in the
556 hypopharyngeal glands of nurse bees (~30% of the bacterial community) as well as in
557 royal jelly (~40% of the bacterial community) [40]. Interestingly, Alpha-2.2 is often
558 recovered from libraries constructed from worker guts, but usually in low proportions.
559 Indeed, in the libraries from worker digestive tracts that were sampled here, Alpha-2.2
560 was found to comprise ~2% of the bacterial community on average. Finally, although
561 royal jelly is known for its antiseptic properties, Alpha-2.2 strains can be cultivated in

562 the presence of royal jelly [10]. This result suggests that, while Alpha-2.2 can be found in
563 the gut environment, their primary niche within the honey bee is the hypopharyngeal
564 gland. So what might *Parasaccharibacter apis* be doing for queens? The genome of a
565 representative honey-bee-associated strain of Alpha-2.2 has been recently sequenced
566 and annotated [45], and suggests speculative roles for the bacterium within queen bees.
567 For example, based on genomic content, the bacterium is believed to prefer micro-oxic
568 environments and, under fermentative conditions, to produce lactate (via L-lactate
569 dehydrogenase), acetoin (via acetolactate synthase), and 2,3-butanediol (via acetoin
570 dehydrogenase). These fermentative products, also thought to be produced by other
571 honey-bee-associated microbes [11], could potentially impact queen physiology and
572 development, through as-of-yet-unknown mechanisms.
573
574 The implications of our findings on the commercial-production apiculture industry are in
575 many ways reassuring. Because the queen microbiome does not reflect the workers
576 within a specific colony or even that of the worker caste, the physical movement of
577 queens from one colony environment to another does not seem to have any major
578 effects on either queen-gut or worker-gut communities. Thus, we have no evidence that
579 beekeepers who regularly replace their queens from outside genetic sources
580 detrimentally affect their colonies by disrupting the gut microfauna of a particular
581 genetic line or colony unit. Moreover, our results seem to support several general
582 recommendations for queen rearing, namely that even the youngest grafted larvae are
583 colonized by the appropriate gut bacteria (i.e., those that are present at later stages of

584 queen development, although we cannot eliminate differences that might be induced
585 by late grafting) and that large populations of nurse-aged worker bees in 'cell builder'
586 colonies are important for adequately provisioning queen larvae with royal jelly, which
587 we speculate could be an important source of their early microbiota members.
588
589 Maternal (or vertical) transmission is a common, if not nearly universal, mode of
590 acquisition for the host-associated microbiome in mammals [46-50]. In humans, for
591 example, mode of delivery can dramatically impact an infant's microbiome composition;
592 infants born through natural delivery have a microbiome composition that resembles
593 that of the mother's vaginal microbiota, while infants born via Cesarean section harbor
594 communities resembling the skin [26]. However, this does not preclude the acquisition
595 of microbes from the environment (or horizontally) during development of the animal
596 host. Indeed, there is evidence that in many animal groups, including insects,
597 individuals acquire their microbiome through horizontal transmission, for example from
598 their surroundings [38, 51], via coprophagy [52-54], or through interaction with
599 congeners [55]. Our data suggest that, unlike the majority of animals, maternal
600 transmission of the microbiome (the passing of microbiota from the queen to her
601 offspring) does not occur in the honey bee; the queen honey bee is not a source of
602 inoculum for her workers' microbiome because she does not harbor the diversity of
603 bacterial groups that are core to the adult worker bee (Figures 1,2) nor does she
604 participate directly in the rearing of her offspring. In contrast, our data suggest a model
605 in which this single reproductive member of the colony is inoculated by worker bee

caregivers, possibly via a specific organ containing a microbial composition quite distinct from that found within worker digestive tracts. Therefore, honey bees, like social bumble bees, likely transmit their microbiome from one generation to the next through horizontal social contact and interaction with the nest (or hive components) [33, 55], although in a way that is heavily mediated by caste membership.

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620

621 **Table 1.** Diversity metrics for microbial communities found for queens across the

622 developmental stages of queen rearing and for the workers that were associated with

623 them at each stage. Average number of 97%-identity OTUs differs dramatically between

624 larvae and newly emerged queens versus workers (t-tests are the results of pairwise

625 comparisons between diversity, i.e., OTUs, of worker microbiomes and queen

626 microbiomes at each developmental stage).

627

	Larvae	**Newly Emerged Queens**	**Mature Queens**	**Workers**
OTUs (97%, avg)	48.5	110.7	26.1	26.9
InvSimpson	3.67	12.41	1.88	5.45
t-test (p-val)	0.007	< 0.0001	0.90	----

628

629

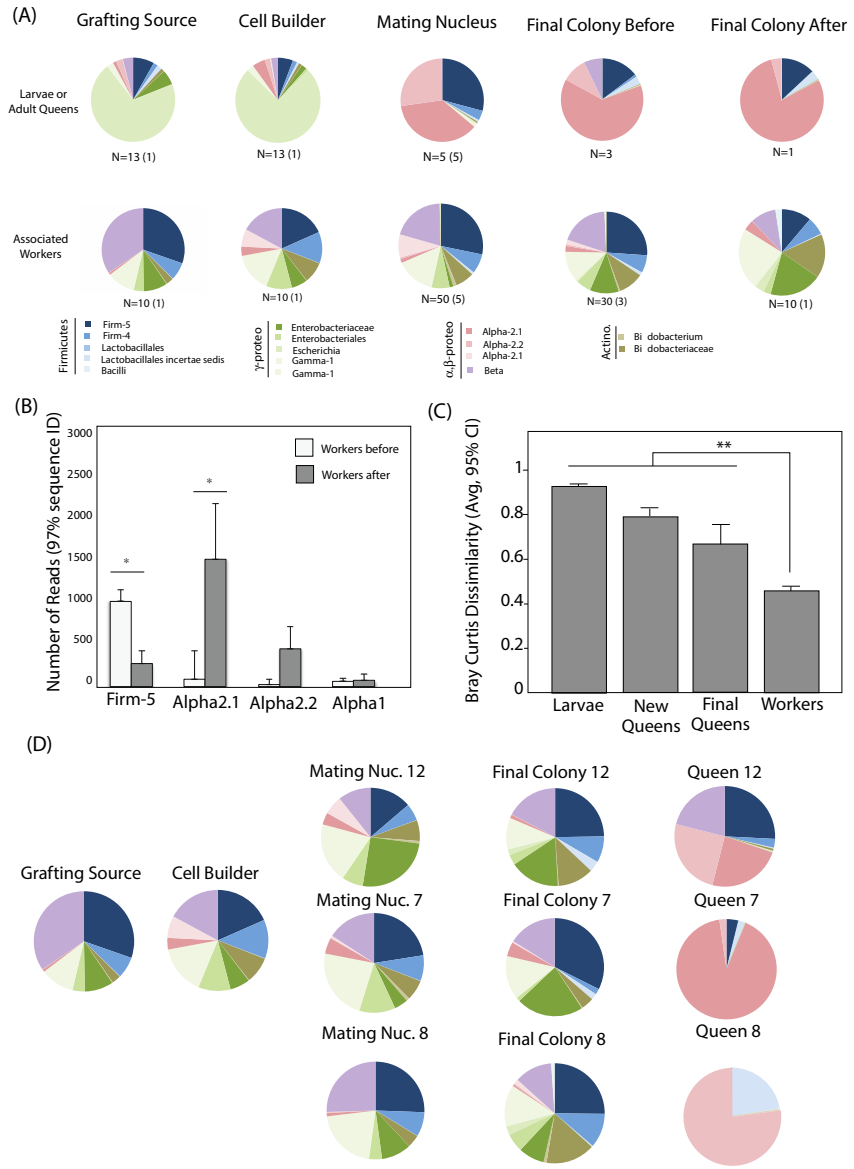


Figure 1. (A) Pie chart depiction of the microbial composition of young larvae (from the grafting source colony, destined to be reared into queens) and queens (newly emerged

634 and mature), and their associated workers, at each stage of queen development. N =
635 number of individuals; number of colonies in parentheses (B) Workers exhibited a
636 relatively consistent microbiome profile, although workers that were the offspring of
637 laying adult queens (workers after) exhibited larger proportions of Alpha-2.1 and fewer
638 Firm-5 compared to workers present in final host colonies before queen progeny
639 emerged (workers before). (C) Bray Curtis dissimilarity metrics for pairwise comparisons
640 between sampled communities were significantly different between workers and all
641 other sampled castes (pairwise t-tests; $p < 0.01$ with Bonferroni correction), supporting
642 the assertion that microbiomes of unrelated worker populations were more consistent
643 across the host colonies than were the microbiomes of the related queens over the
644 course of their development. (D) Visual depiction of consistency in microbiome
645 composition between workers across eight different colonies compared to three mature
646 queens. In this series, all individual queens interacted with the same populations of
647 grafting-source and cell-builder workers, but each were moved to their own mating
648 nucleus and final host colony (sampled before emergence of queens' genetic offspring,
649 which is depicted here).
650

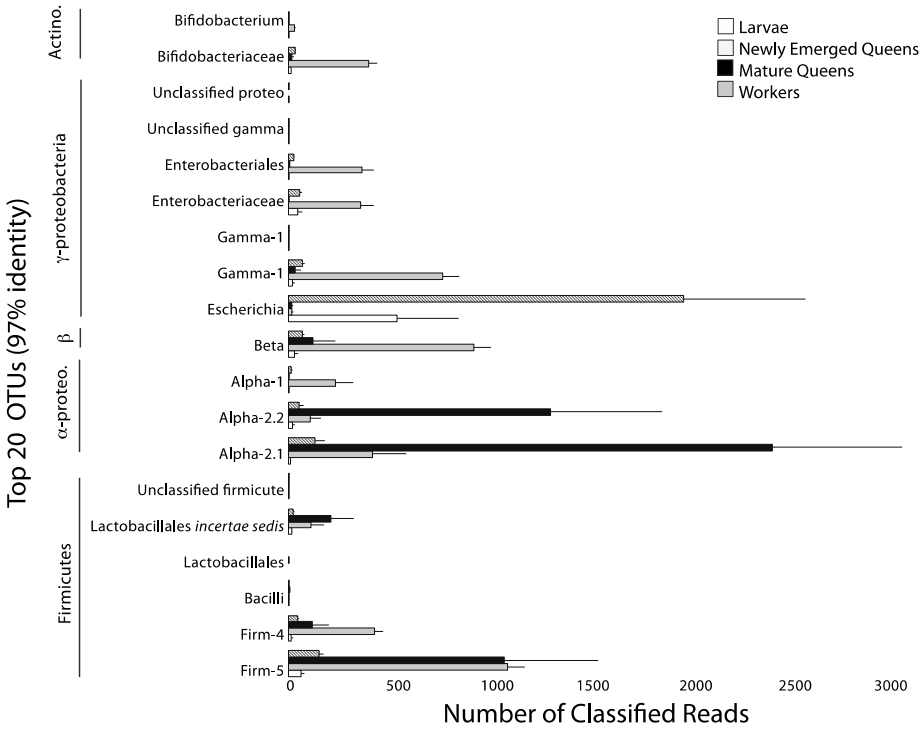


Figure 2. The distribution of the top-20 bacterial operational taxonomic units found across all samples and comprising >96% of the data are shown. The prevalence of 19 of these top-20 OTUs (excluding *Lactobacillales incertae sedis*) were found to distinguish worker and queen microbiomes (Kruskal-Wallis, df =3, $p < 0.05$). Libraries from mature queens are characterized by a bacterial community in which α-proteobacteria predominate. Error bars = SE of the mean across all sampled libraries.

Figure 3. UniFrac analysis of bacterial communities found across sampled environments including larvae transferred from the grafting source (GS), newly emerged queens from the cell builder (CB), and mature queens in their mating nucs (MN) or final colonies (FC). Communities from workers interacting with queens at each stage of development did not cluster with their respective queens but instead claded separately (Unifrac weighted analysis $p < 0.001$ in all pairwise comparisons between queens, workers, and larvae). Outliers are denoted with *.

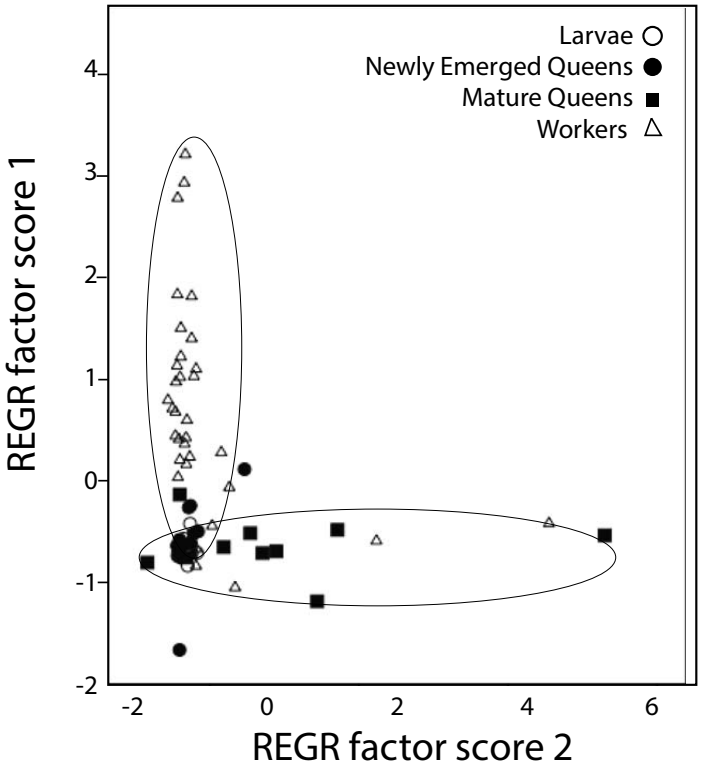
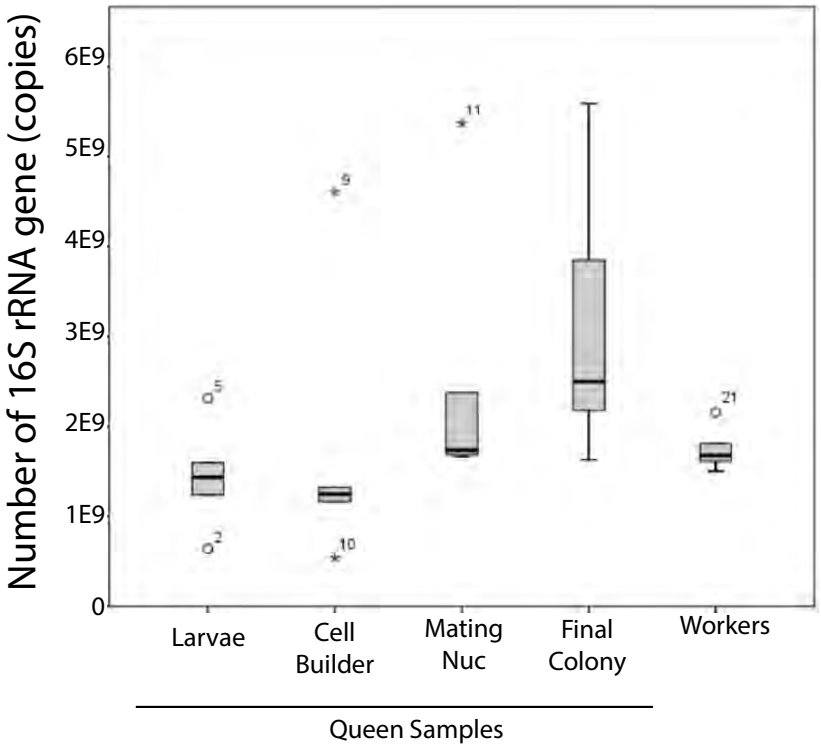


Figure 4. Principle components analysis clustered the microbial communities of workers separately from those of mature queens, while newly emerged queens and larvae clustered together. Ellipses presented to highlight visual pattern only.



673

674 **Figure 5.** Total number of 16S rRNA gene copies (as detected by quantitative PCR using
675 16S rRNA gene primers) from queens and workers (N = 5 for each caste and
676 developmental stage). Mean total number of bacteria colonizing queens was influenced
677 by age (Kruskal-Wallis: $\chi 2 = 8.0$; df = 3; p = 0.046); larval queens hosted fewer bacteria
678 than final queens (Mann-Whitney U-test: U = 2; Z= -2.2; p = 0.032). Mean total number
679 of bacteria in mature queen digestive tracts did not differ significantly from the mean
680 total number of bacteria colonizing workers (F = 1.5; df = 1,4, p = 0.24). Symbols and
681 numbers represent outliers.

682

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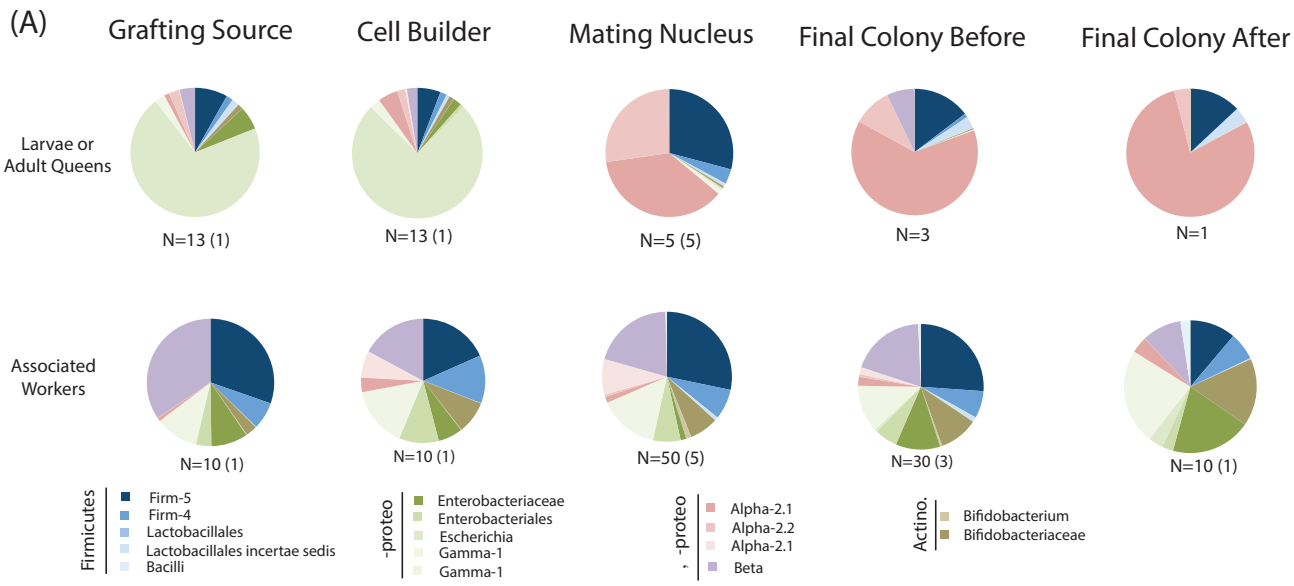
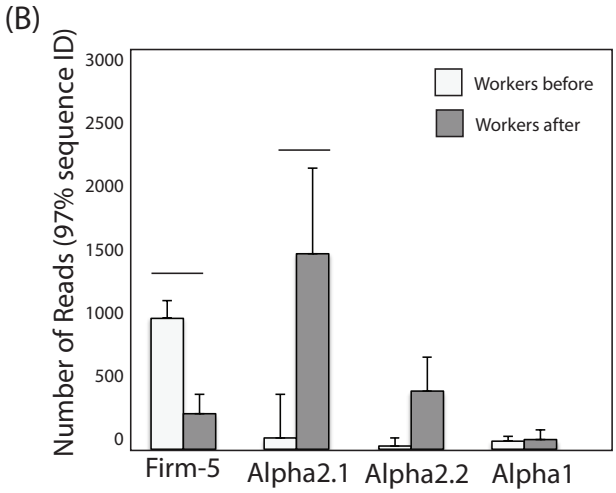
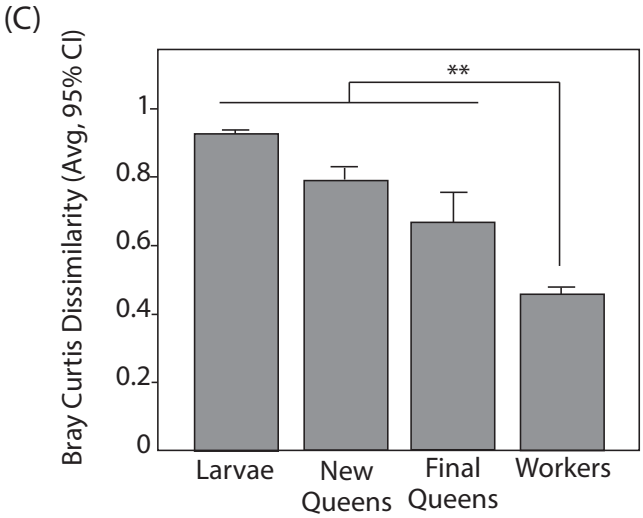
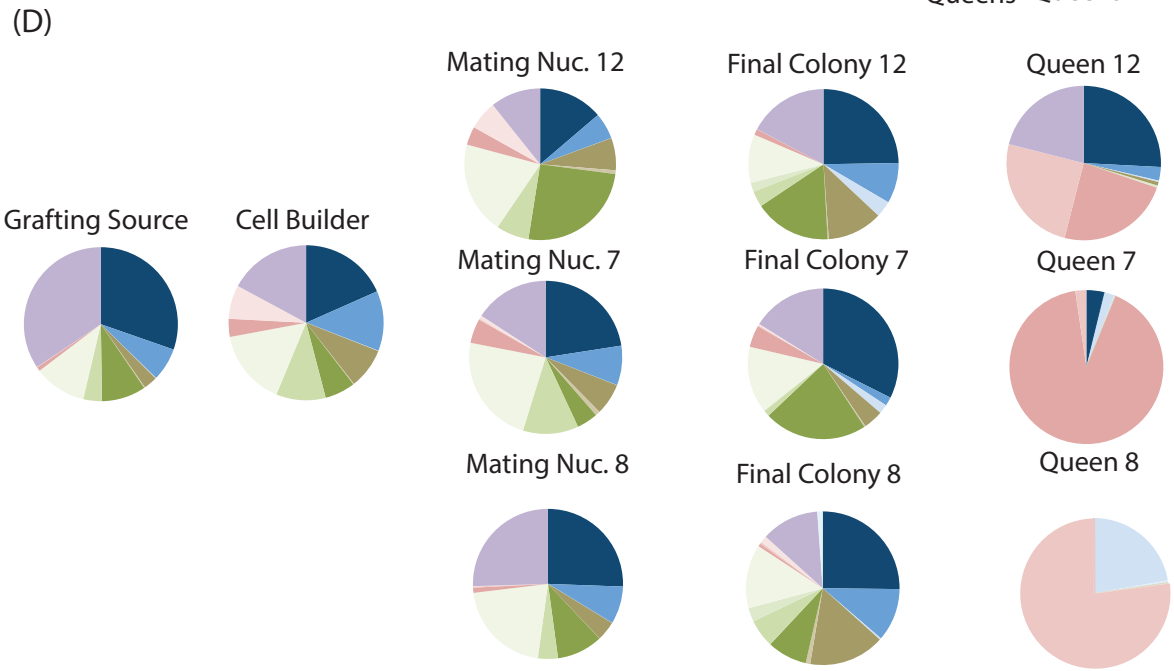
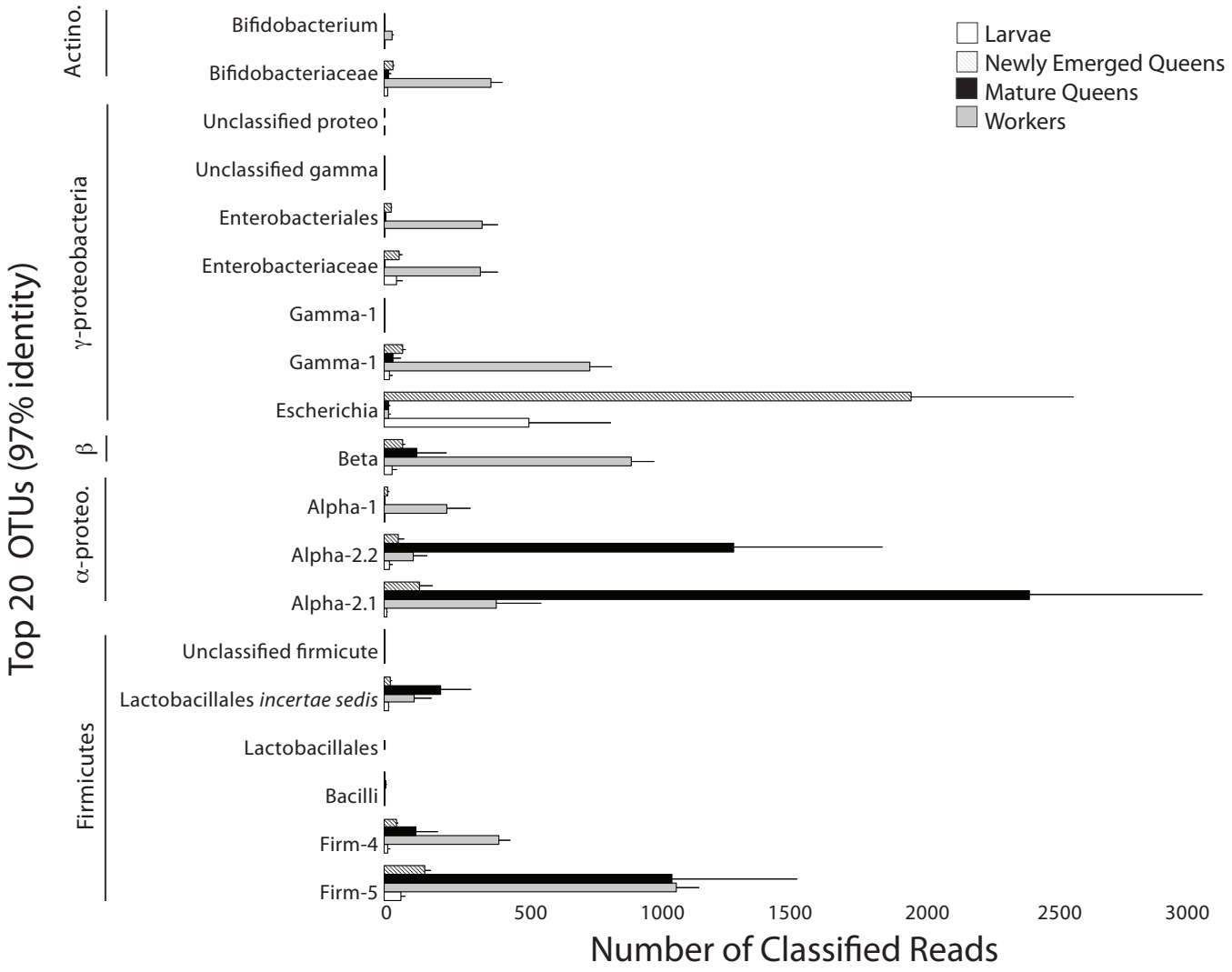
859
860


(A)
Grafting Source
Cell Builder
Mating Nucleus
Final Colony Before
Final Colony After
Larvae or Adult Queens
N=13 (1)
N=13 (1)
N=5 (5)
N=3
N=1
Associated Workers
N=10 (1)
N=10 (1)
N=50 (5)
N=30 (3)
N=10 (1)
Firmicutes
Firm-5
Firm-4
Lactobacillales
Lactobacillales incertae sedis
Bacilli
-proteo
Enterobacteriaceae
Enterobacteriales
Escherichia
Gamma-1
Gamma-1
-proteo
Alpha-2.1
Alpha-2.2
Alpha-2.1
Beta
Actino.
Bifidobacterium
Bifidobacteriaceae

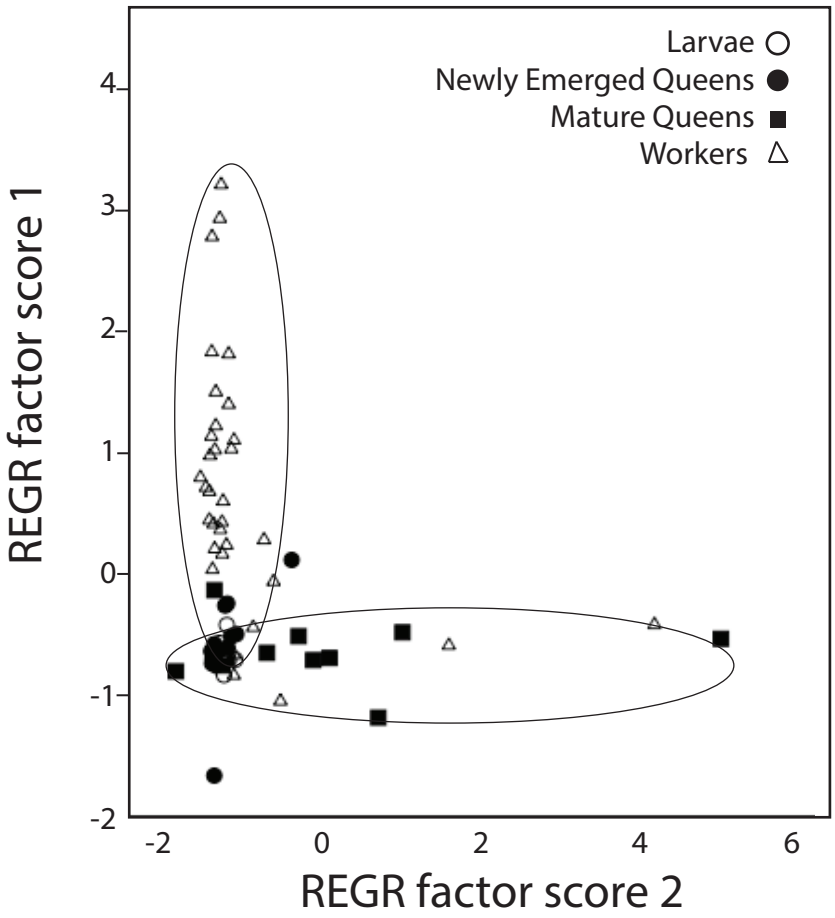

(B)
Number of Reads (97% sequence ID)
3000
2500
2000
1500
1000
500
0
Workers before
Workers after
Firm-5
Alpha2.1
Alpha2.2
Alpha1

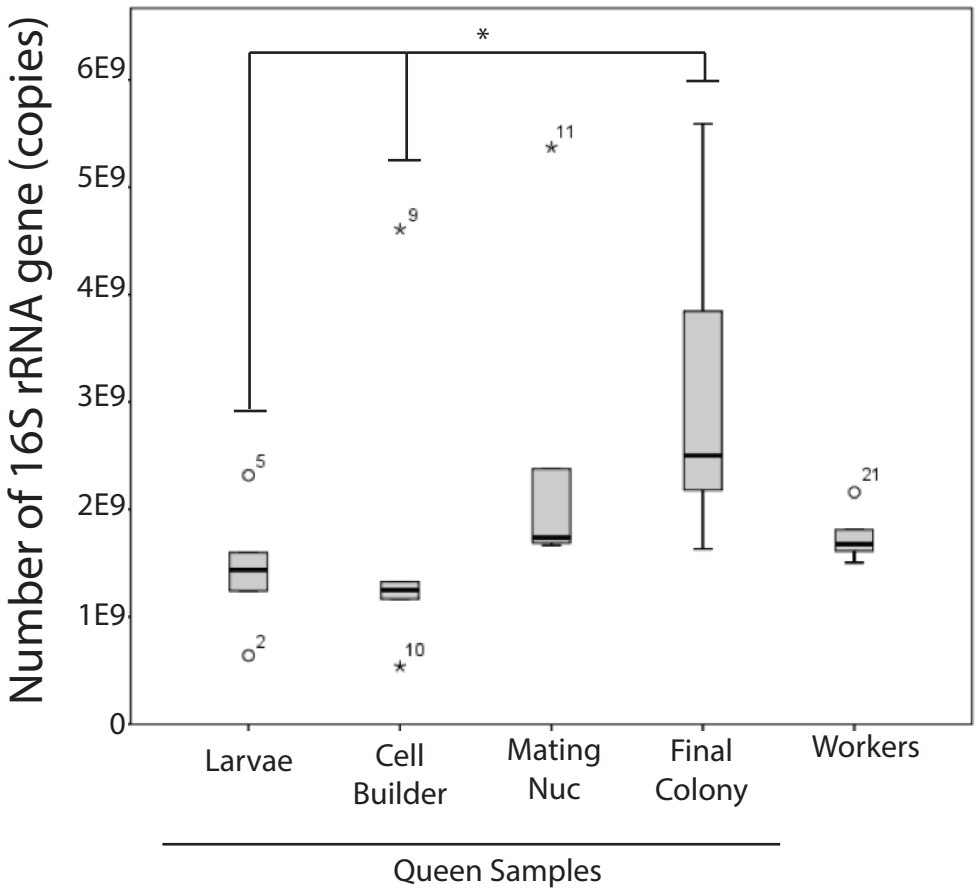

(C)
Bray Curtis Dissimilarity (Avg, 95% CI)
**
1
0.8
0.6
0.4
0.2
0
Larvae
New Queens
Final Queens
Workers


(D)
Grafting Source
Cell Builder
Mating Nuc. 12
Final Colony 12
Queen 12
Mating Nuc. 7
Final Colony 7
Queen 7
Mating Nuc. 8
Final Colony 8
Queen 8


Top 20 OTUs (97% identity)
Actino.
γ-proteobacteria
β
α-proteo.
Firmicutes
Bifidobacterium
Bifidobacteriaceae
Unclassified proteo
Unclassified gamma
Enterobacteriales
Enterobacteriaceae
Gamma-1
Gamma-1
Escherichia
Beta
Alpha-1
Alpha-2.2
Alpha-2.1
Unclassified firmicute
Lactobacillales *incertae sedis*
Lactobacillales
Bacilli
Firm-4
Firm-5
Larvae
Newly Emerged Queens
Mature Queens
Workers
0
500
1000
1500
2000
2500
3000
Number of Classified Reads

MN30Q
MN2Q
FC20QB
FC23QA
FC7QB
FC19WB *
FC17WA *
MN22Q
MN15Q
MN11Q
FC8QA
FC13WA *
FC12QB
Mature Queens
CB5Q
CB9Q
CB11Q
CB6Q
CB4Q
CB7Q
CB3Q
GS7Q
GS5Q
CB13Q
CB10Q
CB8Q
CB1Q
CB2Q
CB12Q
Newly Emerged Queens
GS3Q
GS17Q
GS16Q
GS22Q
GS12Q
GS9Q
GS15Q
GS13Q
GS10Q
GS6Q
GS11Q
Larvae
MN8W
CB77Q *
FC20WB
MN15W
GSW
CBW
MN2W
MN22W
MN11W
FC23WB
FC8WB
FC12WB
FC7WB
FC17WB
MN7W
MN15W
FC19WB
MN10W
CBW
MN30W
FC13WB
MN13W
CBW
FC13WA
MN12W
FC23WA
CBW
CBW
MN30W
CBW
Worker Bees
0.05


Larvae
Newly Emerged Queens
Mature Queens
Workers
REGR factor score 1
REGR factor score 2
4
3
2
1
0
-1
-2
-2
0
2
4
6


Number of 16S rRNA gene (copies)
6E9
5E9
4E9
3E9
2E9
1E9
0
*
11
9
5
21
2
10
Larvae
Cell Builder
Mating Nuc
Final Colony
Workers
Queen Samples