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Authors
Hammer, Tobin J
Easton-Calabria, August
Moran, Nancy A

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Microbiome assembly and maintenance across the lifespan of bumble bee workers

Tobin J. Hammer[1,2*], August Easton-Calabria[3], Nancy A. Moran[2]

[1]Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA 92697

[2]Department of Integrative Biology, University of Texas at Austin, Austin, TX 78703

[3]Department of Entomology, University of Wisconsin-Madison, Madison, WI 53706

*Corresponding author: hammert@uci.edu

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Abstract

How a host's microbiome changes over its lifespan can influence development and aging. As these temporal patterns have only been described in detail for a handful of hosts, an important next step is to compare microbiome succession more broadly and investigate why it varies. Here we characterize the temporal dynamics and stability of the bumble bee worker gut microbiome. Bumble bees have simple and host-specific gut microbiomes, and their microbial dynamics may influence health and pollination services. We used 16S rRNA gene sequencing, qPCR, and metagenomics to characterize gut microbiomes over the lifespan of *Bombus impatiens* workers. We also sequenced gut transcriptomes to examine host factors that may control the microbiome. At the community level, microbiome assembly is highly predictable and similar to patterns of primary succession observed in the human gut. But at the strain level, partitioning of bacterial variants among colonies suggests stochastic colonization events similar to those observed in flies and nematodes. We also find strong differences in temporal dynamics among symbiont species, suggesting ecological differences among microbiome members in colonization and persistence. Finally, we show that both the gut microbiome and host transcriptome—including expression of key immunity genes—stabilize, as opposed to senesce, with age. We suggest that in highly social groups such as bumble bees, maintenance of both microbiomes and immunity contribute to the inclusive fitness of workers, and thus remain under selection even in old age. Our findings provide a foundation for exploring the mechanisms and functional outcomes of bee microbiome succession.

Introduction

Understanding how and why microbial communities change over time is a fundamental goal of microbial ecology (1–3). For host-associated microbiomes, the local environment can change dramatically across the host lifespan, influencing their temporal dynamics (4–8). These dynamics may also have functional consequences, possibly influencing or regulating host development and life history processes (5, 9–11). We have an increasingly clear picture of microbiome succession in humans and in certain models for biomedical and symbiosis research [e.g., (12–18)], for which a range of methods have been used to describe dynamics of both microbes and host processes in great detail. Patterns can vary substantially across hosts. For example, in primary succession, stochastic colonization dynamics observed in *D. melanogaster* and *C. elegans (13, 14, 19)* contrast with predictable gut microbiome assembly in human infants and honey bees (20–22). On the other hand, convergent patterns are also observed, especially with respect to microbiome maintenance in old age. In humans, gut microbiome composition becomes more variable in the elderly, with losses of core symbiont species (23–26). In lab models, gut microbiomes also shift (though in various ways) in old age (17, 27–29); these shifts may constitute a form of senescence, both responding and contributing to deterioration of gut physiology and immunity (28, 30, 31). However, given major biological differences, it is difficult to explain why we see divergent or convergent successional trajectories among these groups.

Marker gene-based studies of microbiome succession from a greater diversity of hosts suggest a much broader array of temporal patterns in nature. For example, microbiome assembly can differ even between closely related hosts [e.g., humans and chimps (32)], and in some hosts, microbiome senescence does not seem to occur (33). However, typical marker gene (e.g., 16S rRNA) amplicon sequence datasets lack information on taxa not amplified by the chosen primer set, on absolute abundance, and on activity (e.g. live versus dead, replicating versus dormant).

Short amplicon datasets of conserved genes also lack phylogenetic resolution below the species (i.e. ASV) level, masking subspecies or strain-level dynamics. Furthermore, many of these studies lack data on host processes that might impact microbes, such as immune responses, especially at spatial and temporal scales relevant to microbial dynamics. For a general understanding of how and why host-associated microbiomes change over time, it is crucial to develop a broader range of host-microbiome systems, studied with a comprehensive set of host- and microbe-level analyses.

Eusocial corbiculate bees (honey bees, bumble bees, and stingless bees) are a promising group for comparative, in-depth studies of microbiome dynamics. First, these bees are key pollinators in natural and agricultural ecosystems, and bacterial symbionts have functional roles in host health (34–36). Therefore, the dynamics of these microbes could have important consequences for bees as well as plants. Bees are threatened by a variety of anthropogenic stressors (37), and baseline temporal variability in the microbiome needs to be measured in order to observe perturbations and study resilience (38, 39). Second, these three bee clades are related, are ecologically similar in many respects, and share some conserved symbiont taxa, but they also differ in key life history and ecological traits, as well as in the composition and functional potential of their microbiomes (34, 35, 40). This contrast among close relatives provides an opportunity to study how host traits shape the evolution of microbiome dynamics. Third, social bees have host-specific and very simple gut microbiomes, dominated by just a few core bacterial lineages (41). This simplicity makes it easier to delve below community-level patterns to study the temporal dynamics of individual species and strains within the microbiome. Distinct microbial taxa may exhibit different life history strategies that shape colonization and persistence

69 [e.g., (42–45)]. These strategies are poorly understood for microbes within host-associated
70 communities, but are likely important for shaping community-level succession (45).
71 Gut microbiome temporal dynamics have been relatively well-studied in the Western
72 honey bee (*Apis mellifera*). The gut microbiome is completely different between larvae and adult
73 workers (46), and it continues to change in composition and abundance as workers age (15, 22,
74 47–49). However, as *A. mellifera* workers go through a highly stereotyped sequence of tasks
75 over time (age polyethism) (50), age and task effects are difficult to disentangle. For example,
76 honey bees do not defecate until they become foragers and leave the hive for the first time (51);
77 this could contribute to a decrease in microbial abundance from young nurses to older workers
78 (47). Indeed, differences in task performance alone (in-hive tasks versus foraging) are associated
79 with microbiome differences in age-matched workers (52). Furthermore, the oldest workers are
80 those who overwinter and enter a phase with distinct metabolic, immunological, thermal, and
81 behavioral (e.g., lack of defecation) characteristics (47, 53, 54). The gut microbiome can be quite
82 stable into old age in these long-lived workers (55). It is unclear to what extent gut microbial
83 succession in *A. mellifera* will extend to other bees that lack strong age polyethism and unique
84 overwintering phenotypes.
85 Bumble bees (*Bombus* spp.) differ from honey bees in many ways that likely relate to
86 microbiome dynamics (35). Workers exhibit comparatively weak age polyethism (temporal
87 division of labor); tasks are generally carried out by workers of all ages, though some tasks are
88 more likely to be performed at certain ages (56). Symbionts are transmitted between generations
89 by a single queen, instead of by a large group of workers as in honey bees; this changes the
90 bottleneck size, and potentially, selection on caste-specific maintenance processes (35). They
91 also lack certain bacteria characteristic of honey bees and have gained *Candidatus*

92 Schmidhempelia bombi (hereafter, *Schmidhempelia*) (41, 57). A unique practical advantage of
93 bumble bees is that full colonies can be reared indoors. This provides an opportunity to study
94 intrinsic aging processes under optimal conditions, in the absence of environmental variation,
95 and to sample microbiomes of very old bees that would normally be rare due to extrinsic
96 mortality. Moreover, the bumble bee gut microbiome seems uniquely prone to disturbance: field-
97 collected workers are often found to lack the core symbionts and instead harbor opportunistic
98 environmental bacteria (35, 58, 59). This phenomenon has been linked to colony age, but old
99 colonies will also tend to have older workers on average (59). Whether microbiome disturbance
100 is due to individual senescence has not been fully resolved.

101 Previous work has outlined the early stages of gut microbiome succession in bumble bees
102 (60–62), but there is no information on what happens to the microbiome in old age. We also lack
103 information on the temporal dynamics of endogenous processes (e.g., immunity) in the bee gut
104 that control gut microbes, and may be controlled by them (63, 64). Gut physiology and immunity
105 senesce in many animals [e.g., (8, 28, 30, 65)], but these processes—and senescence generally—
106 may operate quite differently between different castes of eusocial insects (66, 67). In bumble
107 bees, a solitary queen founds the colony and produces cohorts of (mostly) nonreproductive
108 workers; reproductive offspring (queens and males) are produced toward the end of the colony
109 cycle (68). Given that: *i*) age-specific survival probabilities are similar over much of the bumble
110 bee worker lifespan (69, 70), *ii*) even old workers contribute to colony reproduction (56), and *iii*)
111 there is a need to transmit the core gut symbionts—and not pathogens or parasites—to new
112 queens (35), one may expect only minimal senescence of worker microbiomes, gut physiology,
113 and immunity. Indeed, some aspects of systemic immunity in bumble bees remain stable or
114 increase with age (71).

Our main research questions are: how is the bumble bee gut microbiome assembled and maintained through the lifespan—are patterns predictable, and are they convergent with other host systems? Do species within the gut microbiome vary in their temporal dynamics, and can this give us clues into ecological differences? How does the host's gut transcriptional landscape change in concert with the microbiome? And, is the microbiome disturbance that is widely observed in wild bumble bee populations due to individual senescence? To address these questions, we conducted a cross-sectional microbiome and transcriptomic survey of *Bombus impatiens*, focusing on dynamics during the adult stage of workers. We used high-temporal-resolution sampling and a variety of molecular methods (16S rRNA amplicon sequencing, metagenomics, qPCR, RNAseq) to provide a detailed characterization of microbiome succession and gut processes over the lifespan. Our findings develop bumble bees as a case study with which to compare dynamics with other social insects and hosts generally, and have implications for microbiome disturbance and bumble bee health.

Materials and Methods

1. Bumble bee rearing

For the main study, three commercially reared bumble bee (*Bombus impatiens*) colonies were obtained from Koppert Biological Systems and reared in the laboratory. Upon arrival, all of the cocoons (containing worker pupae) present in each colony were moved to separate containers in a 35 °C incubator. We monitored the cocoons daily, and marked all newly emerged adult worker bees with numbered tags, affixed with wood glue to the thorax. Tagged bees were then

returned to their colony of origin. Three newly emerged bees per colony were sampled (see below) instead of returned to the colony. To maintain colonies, we provided non-sterile pollen dough (ground pollen mixed with syrup) every 3-4 days. Non-sterile sucrose syrup (50% w/v) was provided ad libitum through an enclosed foraging area connected to the main nest.

We used cross-sectional sampling to measure changes in gut microbiomes and transcriptomes over the worker lifespan (Fig. S1). For the first week of adult life, we sampled one bee per colony per day, in order to have higher temporal resolution for the colonization phase, which we expected to be dynamic. Thereafter, for colonies B and Y—which had more tagged bees available, because more pupae were present—we sampled one bee every other day in age (e.g. 9, 11, 13 days old). For colony W, sampling occurred every fourth day in age. Sampling entailed anesthetizing bees on ice and removing the gut with 70% ethanol-sterilized forceps. The midgut and hindgut were separated at the pylorus and each stored in 0.1 ml DNA/RNA Shield (Zymo) at -80 °C until nucleic acid extractions.

Sampling continued until all of the originally tagged bees had either died or been collected—up to 59 days old (colonies Y and W) or 75 days old (colony B) (Fig. S1). These maximum ages are similar to, or greater than, the average lifespan for indoor-reared workers of *Bombus impatiens* (72, 73) and other *Bombus* species (74, 75). They greatly exceed the average lifespan of free-foraging bumble bee workers (76–78).

A smaller set of samples were collected from colonies reared from field-collected queens of *B. impatiens* (3 colonies) and *B. ternarius* (1 colony). Queens were collected from New Hampshire, USA (*B. impatiens*: 44.221788, -71.735138; *B. ternarius*: 44.221034, -71.774747). They were then reared in small Ziploc containers in the closet of a private residence at ~60% relative humidity and at 28 °C. The colonies were fed pollen and nectar as described above.

Newly eclosed (emerged) bees were tagged and returned to the colony, and combined midgut and hindgut samples were collected from younger (4-14 days old) and older (37-47 days old) workers and males. Samples were stored in 95% ethanol at -20 °C. Finally, we also sampled 11 larvae from two additional commercial *B. impatiens* colonies. Whole larvae were stored in 95% ethanol at -20 °C.

2. Nucleic acid extractions and qPCR

All samples were homogenized with a sterile pestle prior to extractions. For hindguts sampled from the commercial colonies, we extracted both DNA and RNA using the Zymo Quick-DNA/RNA kit, following the manufacturer's protocol. For all other samples we extracted only DNA using the ZymoBIOMICS DNA kit. Six extraction blanks and three cross-contamination controls (0.l ml of a OD_{600} 10.0 suspension of *Sodalis praecaptivus* cells in PBS) were included alongside the gut samples.

For hindguts and midguts of the commercial bees, bacterial titers were measured by SYBR Green-based quantitative PCR targeting the 16S rRNA gene (with universal 27F/355R primers), as described in ref. (22). Absolute copy numbers were calculated using standard curves generated from serially diluted plasmid DNA carrying the target gene. Estimates of copy numbers per gut sample were calculated by multiplying values from qPCR reactions (containing 1 µl template) by the volume of gDNA eluted from each extracted sample.

3. Library prep and sequencing

For 16S rRNA gene sequencing, gDNA (excepting larval samples) were first PCR-amplified using universal primers targeting the V4 region (515F/806R) and conditions as detailed in ref. (79). Addition of dual-indexed barcodes, magnetic bead purifications, and additional library preparation steps also followed the protocols in ref. (79). Libraries (including the extraction blanks and three PCR no-template controls) were pooled and sequenced on an Illumina iSeq with 2 x 150 chemistry. Samples were split among three separate sequencing runs, as listed in the supplementary metadata file. For larvae, library prep and 16S rRNA gene (V4 region) sequencing (Illumina NovaSeq 2 x 250) were conducted separately by Novogene.

A total of 57 hindguts from the commercial colonies, spanning the range of ages in our sample set (including newly emerged bees), were selected for RNAseq. Library prep for host mRNA sequencing was conducted by Novogene using the NEB Next Ultra II RNA library prep kit. Libraries were sequenced on an Illumina NovaSeq with 2 x 150 chemistry, resulting in an average of 23.7 M raw paired-end reads per sample. The same set of hindguts used for RNAseq were initially selected for shotgun metagenomics, excepting the newly emerged bees, which had very low amounts of bacterial DNA. Five gDNA samples did not pass QC, and three of these were replaced by other hindgut samples from bees "adjacent" in age, for a total of 46 samples. Library prep was conducted by Novogene, using the NEB Next Ultra II DNA Library prep kit. Libraries were sequenced on an Illumina NextSeq with 2 x 150 chemistry, with an average of 22.4 M raw paired-end reads per sample.

4. Bioinformatic analyses

16S rRNA gene amplicons from the three iSeq runs were combined for data processing. Adapters and primers were removed using cutadapt (80). Sequences were then quality-filtered, trimmed, and denoised to generate amplicon sequence variants (ASVs) by DADA2 (81). Only the forward reads were used, as the reverse reads were poor quality. Taxonomy was assigned to ASVs using the SILVA v. 138.1 database (82). Data processing and analysis was conducted in R v. 4.1.1, following the general approach described in ref. (83). Additional detail is provided in the Supplemental Methods.

The workflow for processing the RNAseq data, from raw reads to gene-level counts, is described in the Supplemental Methods. Analysis of count data in R followed the general approach of ref. (84), using limma (85) and edgeR (86) packages. Genes were filtered using the filterByExpr function, with normalization factors calculated by the TMM method. To use pairwise differential expression analyses, we grouped bees into four age classes: new: 0-1 days, N = 12; young: 3-19 days, N = 16; middle: 23-43 days, N = 15; old: 47-75 days, N = 14. Age classes were delineated such that they would have roughly similar sample sizes, and were chosen before statistical analysis of the data. We calculated the number of differentially expressed genes (DEGs) between age classes using linear models of log counts-per-million (log-CPM) values in limma. The design matrix (~ 0 + age + colony) and contrasts were designed for pairwise comparisons of sequential age classes (e.g., young versus middle-aged). DEGs were defined as genes with a p value <0.05 after false discovery rate (FDR) adjustment for multiple comparisons. To analyze expression patterns of genes that might be linked to microbiome dynamics, we focused first on antimicrobial peptides (AMPs) and dual oxidase, which generates reactive oxygen species (ROS). AMPs and ROS are major effectors in the insect gut epithelial immune response, and are known to regulate gut microbes in bumble bees and other insects (87–89). To

229 investigate how host immune regulation may change with age, we also analyze key genes in the
230 Imd and Toll pathways, which control expression of these effectors (88, 90). The specific genes
231 we included are listed in Table S1.
232 The workflow for processing the shotgun metagenomic data is described in the
233 Supplemental Methods. phyloFlash (91) was used to analyze the non-bacterial taxonomic
234 composition of SSU rRNA genes. To assemble the data, we used megahit (92) (default
235 parameters) for single-sample assemblies (93). After binning (see Supplemental Methods), we
236 used dRep (94) to obtain a dereplicated set of 15 high-quality, approximately subspecies-level
237 (95) bacterial metagenome-assembled genomes (MAGs) with an average nucleotide identity
238 (ANI) threshold of 98% (Table S2). MAGs were classified using GTDB-Tk (96). For further
239 analyses, we mapped each sample's reads against the concatenated set of MAGs using bowtie2.
240 The relative abundance of each MAG in each sample, normalized by sequencing depth, was
241 measured as the number of reads per kilobase per million mapped reads (RPKM). inStrain (95)
242 was used to resolve strain-level diversity. Specifically, we characterized strain-level clusters
243 (generated by the inStrain compare function) belonging to each of the MAGs, and visualized
244 their distribution across bee gut samples using cytoscape (97). The default Prefuse Force
245 Directed Layout was used to visualize the bee-strain network shown in Fig. 4. We also
246 conducted a non-clustering-based and MAG-specific analysis of strain sharing with 99.99%
247 population ANI as a cutoff for differentiating strains. Finally, we used iRep (98) to estimate the
248 instantaneous population-average replication rates for MAGs of *Schmidhempelia* and
249 *Gilliamella*, the two taxa that varied in abundance with age. These data can provide insight into
250 the relative contributions of cell replication and mortality to bacterial population dynamics (98).
251 Additional detail for these analyses is included in the Supplemental Methods.

252

253 5. Statistical analyses

254

255 To model changes in bacterial titer with age, we fit logistic curves to the data using the
256 SSlogis and nls functions in R. To test whether changes in community composition were
257 associated with age and colony, we used distance-based redundancy analysis (db-RDA) with the
258 Bray-Curtis dissimilarity metric, as implemented in the vegan package (99). To identify bacteria
259 whose relative abundance changed with age after the colonization phase, we focused on only the
260 eight dominant genera shown in Fig. 2, leaving aside very low-abundance taxa (< 1% mean
261 relative abundance across gut samples) that are less likely to influence host function or overall
262 microbiome dynamics. Then we conducted Spearman's correlations and adjusted p values using
263 FDR. For the three taxa with an FDR-corrected p value < 0.05, we used linear mixed effects
264 models to further test whether age predicted changes in relative abundance, including colony as a
265 random effect. The latter approach was also used to test whether the relative abundance of
266 *Schmidhempelia* and *Gilliamella* MAGs varied with age. Strain partitioning by colony versus age
267 (the four discrete age classes described above) was analyzed by the following method: for all
268 MAGs, chi-squared tests were conducted to test whether bees belonging to the same colony or
269 age class tended to have a higher number of shared strains; p values from these tests were
270 corrected for multiple comparisons by FDR. To model replication indices of *Schmidhempelia*
271 and *Gilliamella* as a function of age and colony, we first conducted linear regressions including
272 the interaction term; these did not provide a significantly better fit to the data than models lacking
273 an interaction, so the results we report are from the latter. We used the glht function in the

multcomp package for posthoc tests of *Schmidhempelia* replication indices among the three colonies.

Results

We focused our study on changes with aging during the adult stage, when the characteristic gut microbiome is known to be present (35). As microbiome colonization in adult bees could be influenced by larval symbionts that persist through metamorphosis (5), we also characterized microbiomes in larvae. Larval microbiomes are dominated by *Lactobacillus* and *Apilactobacillus* (Fig. S2), which are also present in the gut of adult worker bees (Fig. 2A). Despite this overlap, microbiomes are largely restructured across metamorphosis, with other adult-associated bacteria very rare in larvae (mean relative abundances in 16S rRNA amplicon libraries: *Schmidhempelia*, 9.92×10^{-4}; *Gilliamella*, 4.12×10^{-5}, *Snodgrassella*, 3.97×10^{-3}). Newly emerged adults (< 24 hours post-emergence) have very few bacteria in either the midgut or hindgut (Fig. 1A). 16S amplicon profiles (Fig. S3) show large proportions of reagent contaminants, such as *Burkholderia*, the most abundant taxon in our extraction blanks (see Supplemental Methods), further indicating a scarcity of bacteria in these bees' guts (100). These < 24-hour-old bees are not included in further 16S-based analyses. As bees mature, the gut bacterial community exhibits logistic growth, stabilizing after approx. 4 days, with much higher abundances in the hindgut than in the midgut (Fig. 1A). Therefore, we focus on the adult worker hindgut in the following analyses, which involve commercially reared colonies unless otherwise noted. Alpha diversity also increases quickly in young bees, from a monodominance of *Schmidhempelia* to a stable community of ~8 bacterial groups (Fig. 2A). There was no evidence of a change in absolute abundance or alpha diversity in old bees (Figs. 1A, 1B). These patterns

297 are highly consistent among the three replicate colonies (Figs. 1, 2). Community composition
298 also changes with age (db-RDA, F= 17.9, $p < 0.001$) (Fig. 1C), and only weakly differs between
299 the three replicate colonies (db-RDA, F = 2.15, p = 0.036).
300 Despite exposure to microbes present in the diet and rearing environment, gut
301 microbiomes of workers from both commercial *B. impatiens* and wild-queen-derived *B.*
302 *impatiens* and *B. ternarius* colonies are almost entirely dominated by the core, host-specialized
303 bacterial taxa known to be prevalent in bumble bees (35) (Figs. 2A, S3). Bacteria previously
304 observed in microbiome-disrupted bumble bees, such as Enterobacteriaceae (58, 101, 102) and
305 *Fructobacillus (59, 103)*, are virtually absent from the 16S rRNA gene amplicon datasets,
306 including commercial bee midguts (Fig. S4) and hindguts (Fig. 2A) and wild-queen-derived
307 colonies (Fig. S5). The single exception is a male bee from one of the latter colonies, which has a
308 large proportion of *Klebsiella* (Enterobacteriaceae) and fungal sequences (Fig. S5).
309 After the colonization phase, hindgut microbiome composition is generally stable
310 throughout the adult stage (Fig. 2A), with only three taxa changing in relative abundance:
311 *Schmidhempelia* steadily decreases (t = -4.47, $p < 0.001$) (Fig. 2B) while *Gilliamella* (t = 7.02, p
312 < 0.001) (Fig. 2B) and *Bombiscardovia* (t = 3.05, p = 0.003) increase. These are relative
313 abundances, derived from compositional 16S rRNA amplicon profiles. Changes in relative
314 abundances of taxa can be misleading when the absolute abundance of the entire community
315 changes [e.g., (104)]. Indeed, using taxon-specific population sizes estimated by correcting
316 relative abundances with qPCR data, *Bombiscardovia* does not significantly increase with age
317 after the colonization phase (t = 1.31, p = 0.19) (Fig. S6). Otherwise, similar patterns are found:
318 *Schmidhempelia* decreases, *Gilliamella* increases, and other dominant bacterial taxa generally

remain stable (Fig. S6). *Schmidhempelia* and *Gillamella* show the same pattern in *B. impatiens* colonies reared from wild queens (Fig. 2C).

Metagenomic data provide further support for a *Schmidhempelia*/*Gilliamella* transition with age. Using read mapping to metagenome-assembled-genomes (MAGs) as another compositional measure of bacterial abundance, we find the same switch (*Schmidhempelia*: $t = -3.27$, $p = 0.002$; *Gilliamella*: $t = 4.62$, $p < 0.001$) (Fig. 3). Metagenomes also show that gut microbiomes are dominated by core bacteria. All of the MAGs belong to bee-specific bacterial taxa (Table S2); SSU rRNA genes from fungi (homologous to bacterial 16S rRNA genes) are generally rare relative to those from bacteria, though with elevated proportions in a few of the youngest and oldest bees in our sample set (Fig. S7). SSU rRNA genes from other non-bacterial microbes are practically non-existent. We also detect diet-derived plant sequences. Excepting the youngest bees, proportions of plant sequences are generally low and do not show any clear trends with age (Fig. S7).

Analysis of amplicon sequence variants (ASVs), the finest level of resolution available with our 16S sequencing approach, shows that the major core taxa comprise only a single ASV generally ubiquitous across samples (Fig. S8). We used metagenomic data to reveal further layers of diversity beyond ASVs. Some (but not all) of the major bacterial groups comprise multiple MAGs with < 98% ANI ["subspecies", following (95)] (Fig. 3, Table S2). Using inStrain, which compares single-nucleotide variants between samples' reads aligned to a common reference (95), we find that MAGs contain additional strain-level diversity. For most MAGs, this diversity is clearly partitioned by colony, but not by age (Fig. 4, Fig. S9). All of the MAGs from Gram-negative bacterial taxa (*Snodgrassella*, *Schmidhempelia*, *Gilliamella*), but only some of those from Gram-positive taxa, are more likely to be shared within than between colonies (FDR-

adjusted $p < 0.05$) (Fig. S9). We also used metagenomic data to examine *in situ* population-average replication rates, focusing on the two taxa that shift with age. *Schmidhempelia* has much lower replication indices in colony B (posthoc pairwise contrasts: W vs. B, $t = 10.69$, $p < 0.001$; Y vs. B, $t = 14.29$, $p < 0.001$; W vs. Y, $t = 1.35$, $p = 0.38$) (Fig. 5). There is also a weak negative effect of age on *Schmidhempelia* replication ($t = -2.65$, $p = 0.013$). *Gilliamella* replication indices do not significantly differ between colonies ($t = -1.32$, $p = 0.22$) or due to age ($t = 0.197$, $p = 0.89$) (Fig. 5), although sample sizes are also smaller due to lower coverage.

Host gene expression profiles in the hindgut change as bees mature and reach "middle age" (~3-6 weeks old) (Fig. 6A). Between newly emerged and young bees, and young and middle-aged bees, there are 2696 and 6136 differentially expressed genes (DEGs), respectively. Thereafter, gut gene expression profiles do not change with age in a consistent way (Fig. 6A): there are zero DEGs comparing middle-aged and old bees. Of the immunity effectors we analyzed, most show low levels of gene expression in newly emerged bees, with upregulation in older age cohorts (Fig. 6B). Dual oxidase [which generates reactive oxygen species (ROS) (87, 88)], and three of the four antimicrobial peptides, increase in expression as bees mature. Catalase, which degrades ROS to maintain redox balance (105), is highly expressed in newly emerged bees—possibly to prevent self-harm in the absence of abundant microbial cells (64)—and is subsequently downregulated (Fig. 6B). Signaling genes in the Imd and Toll pathways show variable patterns. *Imd* and *relish* decrease in expression with age, while *cactus* and *dorsal* expression do not significantly differ between any age classes (Fig. S10).

Discussion

Our study provides an initial picture of how the bumble bee worker microbiome changes throughout adult life, and how these changes correlate with the expression of key host genes. Overall, the gut microbiome and host transcriptome are highly dynamic during the initial assembly phase. These changes continue over the longer-term maintenance phase, but their magnitude and direction vary among symbiont species and host genes. Both the microbiome and transcriptome appear to stabilize, as opposed to senesce, in old age. We discuss each of these phases in turn.

Assembly

In adult bumble bees, microbiome assembly appears to be an example of primary succession. Amounts of bacterial DNA in newly emerged adult guts are very low (Fig. 1A), and previous work finding these guts generally devoid of culturable bacteria (106, 107) suggests that at least some of this DNA derives from nonviable cells. Larvae harbor *Lactobacillus* and *Apilactobacillus* (Fig. S2), taxa also present in adult guts (Fig. 2A). Although transmission through metamorphosis is theoretically possible (5), these bacteria may instead be cleared during pupation and reacquired from the nest environment by newly emerged adults. Other dominant bacteria in adult guts such as *Schmidhempelia, Snodgrassella*, and *Gilliamella* were either very rare or absent from larvae, indicating *de novo* colonization of adults. Developmental restructuring of the microbiome has been found in other *Bombus* species (108) and in *Apis mellifera (46)*, but why it occurs is not fully clear. Larvae and adults interact through trophallaxis, and both consume pollen and honey from communal stores in the nest (68, 75, 109). Thus, nutritional and microbial inputs into the gut are likely similar. Potentially, aspects of larval

388 gut morphology, physiology, or immunity create less hospitable conditions for colonization by
389 the core symbionts of adults. The trypanosomatid *Crithidia bombi*, a common gut parasite in
390 adult bumble bees, is also unable to infect larvae (110). High osmotic potential in the larval gut
391 has been suggested to inhibit *Crithidia* infection (110), and may be a factor inhibiting bacterial
392 colonization as well.

393 Coupled with a low expression of immunity effectors (Fig. 6B), the low abundance of
394 pathogen-protective (111, 112) core gut bacteria suggests that newly emerged adults are
395 particularly vulnerable to microbiome disruption. Similarly, human infants are prone to
396 infections while their immune system, microbiome, and gut microenvironment mature (113). The
397 microbiome disruption phenomenon widely observed in field-collected bumble bee workers as
398 well as queens (35) may begin during the assembly phase. In *Bombus griseocollis*, workers do
399 not leave the nest for the first couple of days after emergence; most activities, including foraging,
400 begin by the fourth or fifth day (56). By this point, the core gut microbiome is established and
401 expression of immune effectors has increased (Fig. 1, Fig. 2, Fig. 6B). The timing of the onset of
402 foraging therefore limits direct exposure to stressors during this vulnerable period. However,
403 environmental microbes and chemicals are present in food stores and other substrates, presenting
404 an opportunity for microbiome perturbations even in bees restricted to the nest.

405 Microbiome assembly dynamics in bumble bees are both predictable and convergent with
406 other hosts. Temporal patterns of microbiome abundance, diversity, and composition (Fig. 1) are
407 highly similar among replicate colonies. Moreover, these patterns are evident despite our cross-
408 sectional study design, suggesting that temporal variation in microbiomes outweighs
409 interindividual variation. Early successional patterns showed similarities to those observed in
410 honey bees (22) and human infants (4, 20, 104, 114) and, more generally, to heterotrophic

411 microbial communities supplied with external carbon sources (3). However, there are also
412 marked differences with gut microbiome assembly in other invertebrates, such as flies (*D.*
413 *melanogaster*) and nematodes (*C. elegans*). In these hosts, bacterial colonization is highly
414 stochastic and can lead to microbiome compositions that are stably distinct among individuals
415 (13, 14, 19). These hosts also generally harbor non-host-restricted, flexible, environmentally
416 acquired gut microbiomes (115). In contrast, the symbiosis between social bees and their gut
417 microbes is ancient and specific (34, 41). By living in dense colonies, social bees enrich their
418 local environment with core symbionts, favoring predictable assembly. Functional redundancy
419 among bacterial species may also be lower in social bees than flies and nematodes, possibly
420 selecting for stronger host control over microbiome establishment.

421 Despite predictable assembly at the community level (Figs. 1, 2), we also observe
422 evidence for stochastic colonization at the strain level. Strain-level diversity is clearly partitioned
423 between the three replicate colonies (Fig. 4), a pattern not evident in the ASV (Fig. S8) or
424 subspecies (Fig. 3) data. Notably, all Gram-negative bacterial genomes exhibited significant
425 colony partitioning, while only some of the Gram-positive genomes did so (Fig. S9). Gram-
426 positive bacteria may be more likely to survive outside the host, facilitating dispersal among
427 colonies. Similarly, Gram-positive gut bacteria of honey bees can be transmitted via hive
428 surfaces, with less reliance on social contact than Gram-negative species (22). Differences in
429 social structuring among mammalian microbiome members have also been linked to bacterial
430 physiology (116, 117).

431 There are multiple potential explanations for the origin of the colony-partitioning pattern.
432 One is an interaction between host and symbiont genotypes (106). There may also be genotype-
433 by-environment effects; to give one example, bee colonies of different sizes may have different

thermoregulatory capacities and temperatures (118); this could act as an ecological filter for strains with different thermal tolerances (107). In addition to intrinsic physiological differences between strains, differences in temperature or other environmental factors may explain why the inferred replication rates of *Schmidhempelia* differed substantially between colonies (Fig. 5). A final explanation is founder (or foundress) effects. Bumble bee colonies are initiated by a single foundress queen, who is the source of gut symbionts for her offspring (35, 60). A diverse pool of strains may be stochastically sorted into a single foundress queen's gut, with the established population resistant to subsequent invasion [i.e., priority effects (2)]. This process may be analogous to the neutral bottlenecking described for bacterial strain partitioning among skin pores (119) or the stochastic colonization of individual guts of flies and nematodes (13, 14, 19).

Maintenance

Gut microbiome abundance and composition generally stabilize after the colonization phase in newly emerged adults. However, the ratio of two of the core symbiont species, both members of the family Orbaceae, continues to shift with age. The worker gut starts as a near monoculture of *Schmidhempelia* (Fig. 2A). This finding matches previous work: in the *B. impatiens* genome project, which used DNA from a one-day-old male, the only bacterial genome with substantial representation belonged to *Schmidhempelia (57)*. Over time, *Schmidhempelia* progressively declines in relative abundance, while *Gilliamella* increases. This shift is evident in both the amplicon (Fig. 2B) and metagenome datasets (Fig. 3) and across the three replicate colonies. We observe the same pattern in wild-derived colonies (Fig. 2C), suggesting that it is a common feature of microbiome succession in *B. impatiens*.

The functional consequences of the switch from *Schmidhempelia* to *Gilliamella* are unknown. In honey bees, *Gilliamella* can ferment pollen cell wall components (120), with products (short-chain fatty acids) potentially providing bees with a supplemental energy source and lowering gut pH (121). *Gilliamella* likely perform similar functions in bumble bees, although bumble bee-derived strains have fewer capabilities for degrading and fermenting pollen components (35, 122). Acidification is thought to limit infection by *Crithidia bombi*, a trypanosomatid parasite of bumble bees (123). An increase in *Gilliamella* may thus contribute to the metabolism and defense of older bees.

Presumably, changes in *Schmidhempelia* abundance over time also affect hosts, as well as other gut microbial species. Differences between *Schmidhempelia* and *Gilliamella* metabolism are evident from genome analyses (57, 120). Whereas *Gilliamella* is a facultative anaerobe with an intact TCA cycle, *Schmidhempelia* is inferred to be an obligately anaerobic fermenter, producing acetate and other short-chain fatty acids (57). These products would acidify the gut, potentially inhibiting parasites and facilitating subsequent colonization by core symbionts. However, as *Schmidhempelia* has not been cultured (35), we lack experimental evidence for its effects on hosts or other microbes.

The temporal dynamics of *Schmidhempelia* and *Gilliamella* point to distinct life history strategies, perhaps exemplifying the competition-colonization trade-off shown in various microbial communities [e.g., (42–44)]. For example, *Schmidhempelia* may be a pioneer colonizer or ruderal (124), one that is good at dispersing to and exploiting unoccupied gut habitat. *Gilliamella* may be a better competitor, successfully excluding *Schmidhempelia* with time. The nature of this competition remains to be determined. *Schmidhempelia* replication rates appear to be generally stable with age after the colonization phase (Fig. 5), suggesting that declines in

480 population size are driven by increased mortality over time, rather than by a dwindling resource
481 supply slowing replication. Increased mortality could be due to interference competition, where
482 *Gilliamella* directly antagonizes *Schmidhempelia*, possibly by using type VI secretion systems
483 [possessed by both species (57, 125)] or other means. It could also be due to apparent
484 competition, where *Gilliamella* growth induces increased expression of host immune responses
485 (Fig. 6B) that are more harmful to *Schmidhempelia* than to *Gilliamella* (64). Although the
486 mechanisms are unknown, our data support the existence of variation in life history strategies
487 within the gut microbiome. Such differences are likely to be important drivers of coexistence and
488 community function.

489 As with the microbiome, gene expression profiles in the hindgut are dynamic up to ~3-6
490 weeks of age ("middle age"), with many differentially expressed genes between newly emerged,
491 young, and middle age (Fig. 6A). Multiple genes involved in production of antimicrobial
492 peptides (AMPs) and reactive oxygen species (ROS), key components of gut epithelial immunity
493 (87, 88), increase in expression over this time frame (Fig. 6B). In contrast, components of Imd
494 and Toll signaling pathways either decrease in expression or remain stable with age (Fig. S10).
495 Pathogen infection induces these pathways, which then activate immune effectors (88, 90). In
496 this experiment, non-core microbes are almost entirely absent from the hindgut (Fig. 2A), so
497 induction by pathogens is expected to be minimal. Potentially, the temporal patterns we observe
498 could be due to a shift from low (but more inducible) effector expression to high (and more
499 constitutive) expression with age. These patterns contrast with systemic (hemolymph) immune
500 defenses, which decrease with age in bumble bees (71, 126). Differing selective pressures on
501 defense could underlie this discrepancy; for example, gut infection may be more likely to occur
502 or more likely to spread to nestmates (via feces) than hemolymph infection. Currently however,

503 comparisons between datasets are complicated by the fact that colony age may influence
504 immunity independently of individual age (71).
505 Changes in gut immunity (Fig. 6B, Fig. S10) appear to be an intrinsic property of aging
506 in *B. impatiens* workers, as they occur despite continuous food availability, static environmental
507 conditions in the laboratory, and an apparent lack of pathogen infection. As hypothesized for
508 systemic immunity (71), they may represent a plastic adjustment of host defense. For example,
509 increases in constitutive expression of AMPs and ROS may have evolved in response to
510 heightened infection risk with age. An alternative hypothesis is that increases in immune
511 effectors represent unregulated inflammation, a common feature of animal immunosenescence
512 (65). In *D. melanogaster*, increased AMP expression with age is linked to increased gut bacterial
513 load and to deteriorating gut integrity (8, 29). However, total gut bacterial load in bumble bees is
514 stable (Fig. 1A), and the only taxon that increases in abundance is *Gilliamella* (Fig. 2B, Fig. 3,
515 Fig. S6), one of the core bumble bee-specialized symbionts (35). While *Gilliamella* may induce
516 bee AMP expression (64), such a response with age could be interpreted as a sign of
517 strengthening, as opposed to deteriorating, immunity.
518 Unusually, these changes in immunity (and other endogenous processes) decelerate with
519 age. No genes are differentially expressed in the hindgut between middle-aged and old bumble
520 bees (Fig. 6A). In contrast, transcriptomic changes in old age have long been observed in
521 *Drosophila*, *C. elegans*, mice, and humans [e.g., (127–130)]. In a fish model, the gut
522 transcriptome is also markedly different toward the end of the lifespan, and is associated with
523 upregulated immunity and an enrichment of potentially pathogenic bacteria (27).
524 Gut immunity and microbiomes are likely to covary, and we find that microbiome
525 dynamics also slow as bees enter old age. This stability contrasts with the major microbiome

changes observed between life stages (Fig. S2) and earlier in the adult stage (Fig. 1, Fig. 2). Total microbial abundance is stable in old bees (Fig. 1A), and there is no evidence of microbiome disruption—with the exception of a single male from a wild-derived colony (Fig. S5)—or loss of any symbionts besides *Schmidhempelia* (Fig. 2). All bees were reared indoors, and indoor-reared bumble bees have been shown to have lower gut microbiome diversity (35, 101, 102). However, the bees studied here were exposed to non-core microbes in their food and rearing environment, and previous work has documented occasionally large numbers of Enterobacteriaceae and other non-core bacteria in indoor-reared *B. impatiens* when exposed to stressors (131–133). The microbiome stability we observe in old bees indicates a lack of intrinsic senescence processes that would disrupt core symbionts and allow invasion, rather than simply a lack of exposure to non-core microbes. Bumble bees therefore contrast with humans (23–26), as well as other animals such as flies, mice, and fish (17, 27–29), which exhibit microbiome senescence (or at least community-wide shifts during aging) even when reared in the laboratory. Our data also weigh against the hypothesis that individual senescence underlies the microbiome disturbance observed in wild bumble bee populations. As mentioned above, it is the youngest bees that appear to be the most vulnerable. These results support previous work finding microbiome disruption to be concentrated in young bumble bees (102).

There are many potential proximate causes of microbiome stability in old age. Communal living may buffer microbiome disturbances by providing a continuous source of microbes that can be transmitted between individuals or via a shared social environment, such as a nest (116, 117, 134, 135). In our experiment, diet was kept constant, and bees appear to consume pollen even in old age based on the presence of plant DNA in metagenomes (Fig. S7) and observations of gut color. Our transcriptomic data also suggest that the gut microenvironment stabilizes after

549 bees reach middle age (Fig. 6A). In addition to inoculation from nestmates, a steady resource
550 supply, structural integrity, and maintenance of immune responses in the gut (Fig. 6B) likely
551 help maintain stable core microbiomes. One caveat is that these bees were not able to fly, a factor
552 that should be addressed in future work. Bee flight is metabolically costly, reduces lifespan, and
553 affects systemic immune responses (136–138). Free-foraging honey bee workers do exhibit
554 changes in gut microbial abundance, composition, and replication rates with age (15, 22, 47, 48,
555 55). On the other hand, as noted earlier, temporal changes in the honey bee gut microbiome may
556 be primarily driven by a shift from performing in-nest tasks to foraging (52). In overwintering
557 honey bee workers, which do not forage much, if at all, the gut microbiome is largely stable into
558 old age (55).
559 In the bumble bee gut, senescence of the microbiome and of endogenous processes (such
560 as immunity) appears to be either absent, or compressed into such a short window that we did not
561 observe it. We hypothesize that this is explained by the unique selection pressures that
562 accompany eusociality. Evolutionary theories of aging suggest that in a non-social host
563 organism, i) selection against late-acting, deleterious variants—either host alleles or microbes—
564 should be weak, and ii) such variants may trade off with early-life, pre-reproductive benefits
565 (139–141). The situation is different in bumble bee workers, which often complete their entire
566 life cycle before colony reproduction occurs at the end of the season (68). According to theory,
567 the strength of selection should be maximal up until the onset of reproduction (141). In eusocial
568 insects, what counts is the colony's production of sexual offspring (142), as workers are usually
569 sterile. Hence, for most of the colony lifespan, maintenance of microbiomes and immunity in
570 workers should be under strong selection even in old age, given their expected effects on
571 inclusive fitness (i.e., overall colony reproductive success). Core gut symbionts may contribute

indirectly (e.g., via nutrition) to worker performance—brood care, foraging, defense, etc.—which in turn will affect production of new queens and males at the end of the colony cycle. Workers may also benefit their reproductive siblings (the new queens and males) by acting as a vector for core symbionts, and not for pathogens or parasites. Microbiomes of at least some other highly social animals do not appear to become destabilized in old age (32, 33, 55), raising the question of whether group living contributes to differences in microbiome senescence. In general, organisms display diverse patterns of mortality and reproduction with age (143), and such diversity appears to extend to microbiome dynamics.

Variation in microbiome dynamics may also be expected within species, especially in eusocial insects, which contain castes subject to unique selection pressures (144). Our study focused exclusively on the nonreproductive worker caste, but future work should examine how microbiomes change with age in reproductives. In honey bees, these dynamics differ between queens and workers [e.g., (145–147)]. Queen-worker differences may also apply to bumble bees, even though—unlike honey bees (34)—bumble bee queens acquire gut bacterial communities compositionally similar to those of workers (60, 148). In *Bombus lantschouensis*, pre-diapause queens show large decreases in core gut symbionts with age (61), strongly contrasting with the stability we observe in *B. impatiens* workers. Potentially, only a small number of core symbionts are needed for successful transmission, favoring a reduction in titer before diapause (e.g., (149)). Queen-worker differences in microbiome dynamics may also be related to immunity. For example, queens have been reported to exhibit stronger resistance to gut parasite infection, and distinct immune activity in hemolymph, relative to age-matched workers (150).

Conclusions

Even in the relatively simple gut microbial communities of laboratory-reared worker bees, we see a complex assortment of temporal patterns that differ between symbiont taxa, vary with phylogenetic scale, and decelerate as hosts age. Some of these patterns are convergent with those in other hosts. At the level of symbiont species and genera, assembly is predictable, with dynamics similar to those of human infant gut microbiomes. At the strain level, assembly resembles the stochastic colonization dynamics observed in flies and nematodes. We also find unique temporal patterns that contrast with those in other hosts: in bumble bee workers, neither gut microbiomes nor gut immunity appear to senesce. This stability may be due to the important contributions of each to inclusive fitness, even in old age. Temporal dynamics differ markedly among bacterial symbiont species, suggesting distinct ecological strategies within the microbiome for colonization and persistence. Many of the patterns we observe would be undetectable by 16S rRNA gene sequencing, emphasizing the need to use quantitative and higher-resolution methods to study microbiome dynamics. We also characterize the transcriptomic landscape of the bumble bee gut, finding that expression of genes involved in immunity (and other processes) changes in similar ways to the microbiome over host age—likely due to bidirectional feedbacks or to common selection pressures acting on both. A priority for future work is to determine the mechanisms underlying these microbial and immunological dynamics, and to assess functional consequences for bumble bee health and pollination services.

Author Contributions

TJH and NAM designed the research. TJH conducted the commercial bee rearing and sampling, molecular methods, bioinformatics, and statistical analyses. AEC reared and sampled bees from the wild-queen-derived colonies. The manuscript was drafted by TJH and subsequently revised and approved for submission by all authors.

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Data Accessibility Statement

Raw reads from 16S rRNA gene sequencing, metagenomics, and RNAseq are deposited in the NCBI SRA (BioProject PRJNA849590). Sample metadata, qPCR data, processed 16S data (ASV tables and sequences), gene-level counts of mapped reads, other raw data files, and R code are available from the Dryad repository (https://doi.org/10.5061/dryad.gb5mkkws9).

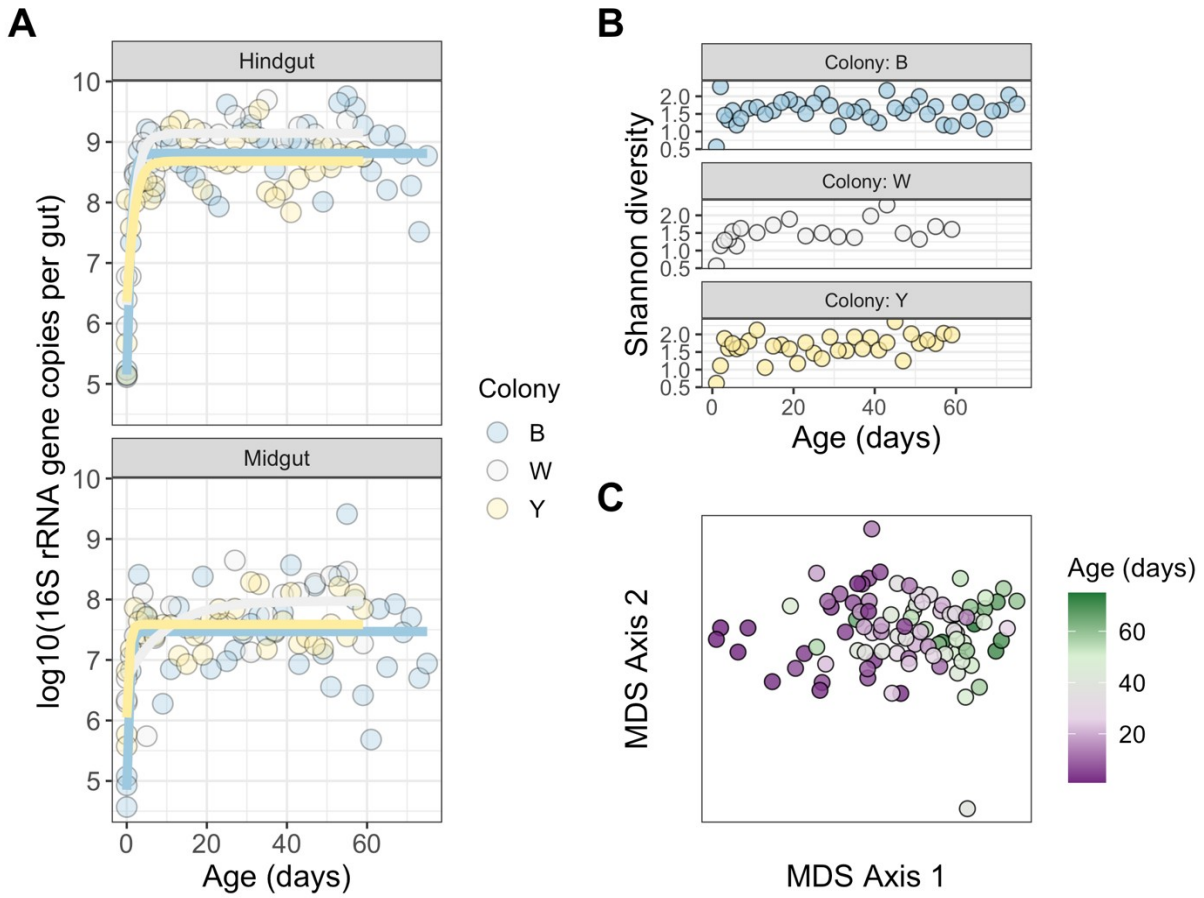

Figure 1. Changes in gut microbiome abundance, diversity, and composition over the worker lifespan. A total of 103 bees were sampled, consisting of 44, 23, and 36 from colonies B, W, and Y, respectively. A) qPCR-based measurements of bacterial titer as a function of age, showing patterns for each replicate colony and gut region. Solid lines are logistic curves fitted to the data. B) Alpha diversity of bacterial communities in hindguts of ≥ 1-day-old bees only, characterized by 16S rRNA gene sequencing. C) Beta diversity of the same hindgut samples visualized as an ordination (non-metric multidimensional scaling) of Bray-Curtis dissimilarities.

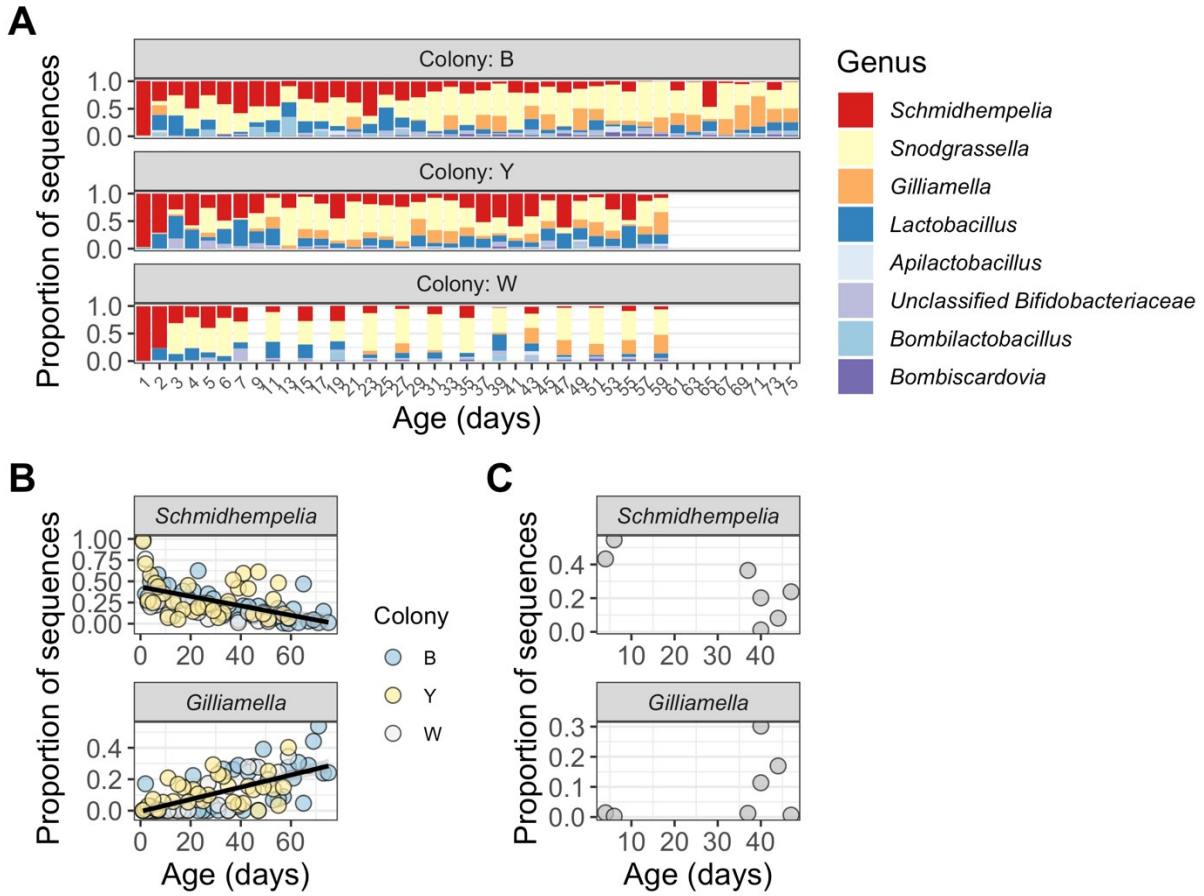

Figure 2. Dynamics of dominant hindgut microbiome taxa (≥ 1% mean relative abundance across samples) over the lifespan. A) 16S-based relative abundances of the top genera. A total of 94 bees are shown, consisting of 41, 20, and 33 from colonies B, W, and Y, respectively. One taxon belonging to the Bifidobacteriaceae was not classified to the genus level using the SILVA database. Also note that the sampling interval varied among the three colonies (see Methods and Fig. S1). B) 16S-based relative abundances (in the same hindgut samples) for *Schmidhempelia* and *Gilliamella*, the only two taxa that varied significantly with age. Lines are linear models fitted to the data, with 95% confidence intervals in gray. C) Relative abundances of *Schmidhempelia* and *Gilliamella* in whole guts of seven workers from three *Bombus impatiens* colonies reared from wild queens.

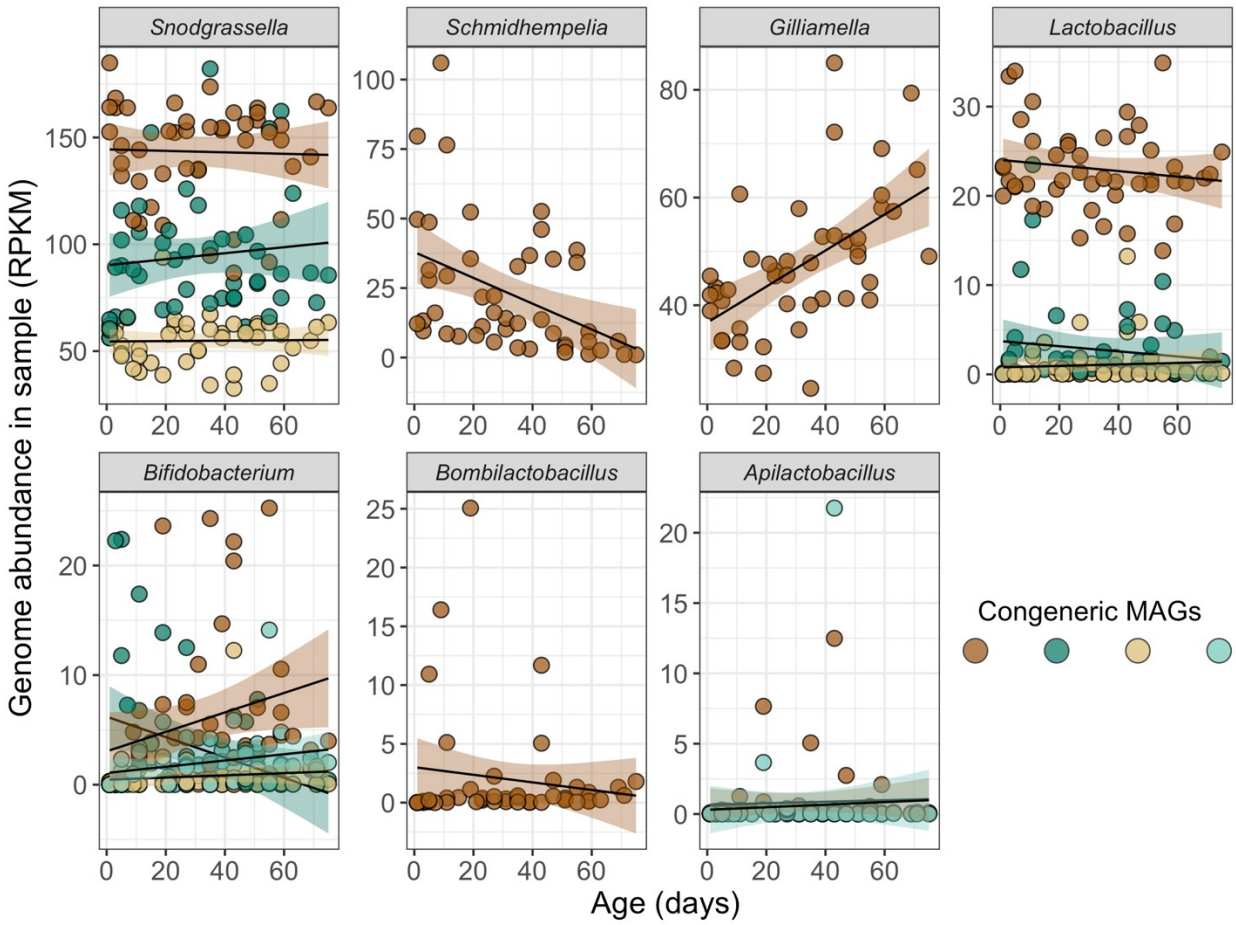

Figure 3. Coverage-based abundance estimates of all metagenome-assembled genomes (MAGs) in 46 worker hindgut samples from the three commercial colonies. Abundance for a given sample is normalized to sequencing depth and MAG size, by measuring reads per kilobase per million mapped reads (RPKM). Lines are linear models fitted to the data, with 95% confidence intervals. Some genera contain multiple MAGs with < 98% average nucleotide identity; in these cases, congeneric MAGs are shown in different colors. MAGs are listed and described in Table S2.

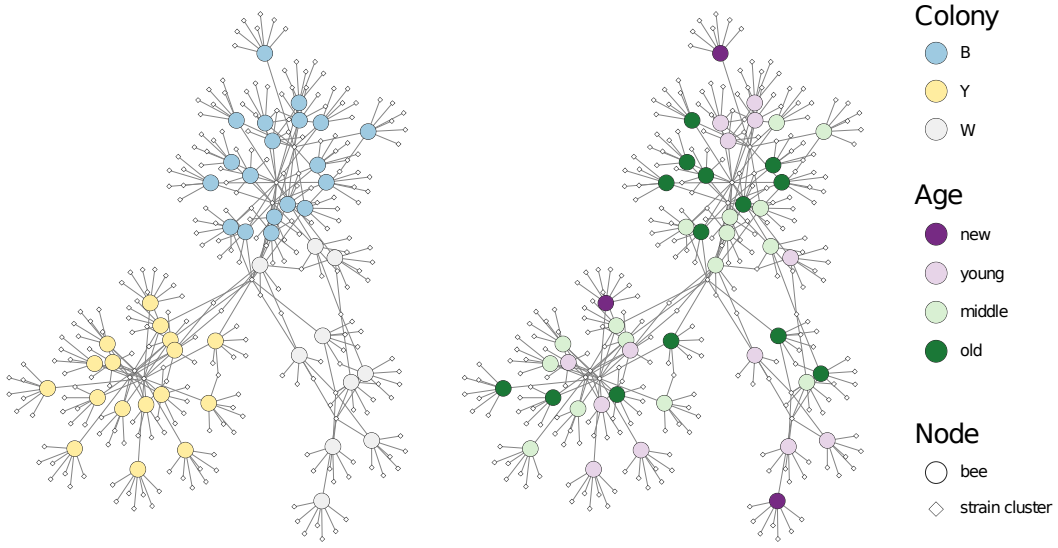

Figure 4. Networks of bacterial strain composition in the 46 worker hindgut metagenomes, showing bee gut sample (large circle) grouping by colony versus age class. Strain clusters (small diamonds) from all MAGs are shown; strain sharing within and between colonies is shown for each MAG individually in Fig. S9. Clusters are derived from hierarchical clustering of pairwise comparisons of population ANI, a metric calculated by inStrain (see Methods).

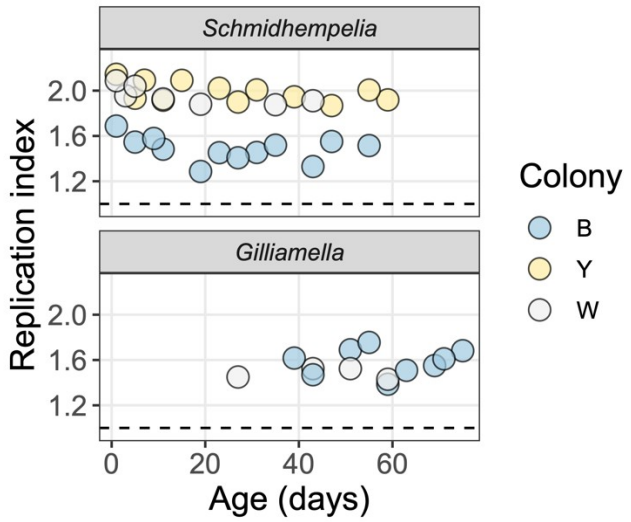

Figure 5. Instantaneous population-average replication rates, estimated for *Schmidhempelia* and *Gilliamella*, the two taxa that vary in abundance with age. A replication index value of 1.5 corresponds to half of the cells making one copy of their genome; with a value of 2, all cells are making one copy [see ref. (98)]. However, note that these are population averages, and bacteria can make multiple copies of their genome simultaneously. Some data points are missing due to low coverage of the MAG in a given sample.

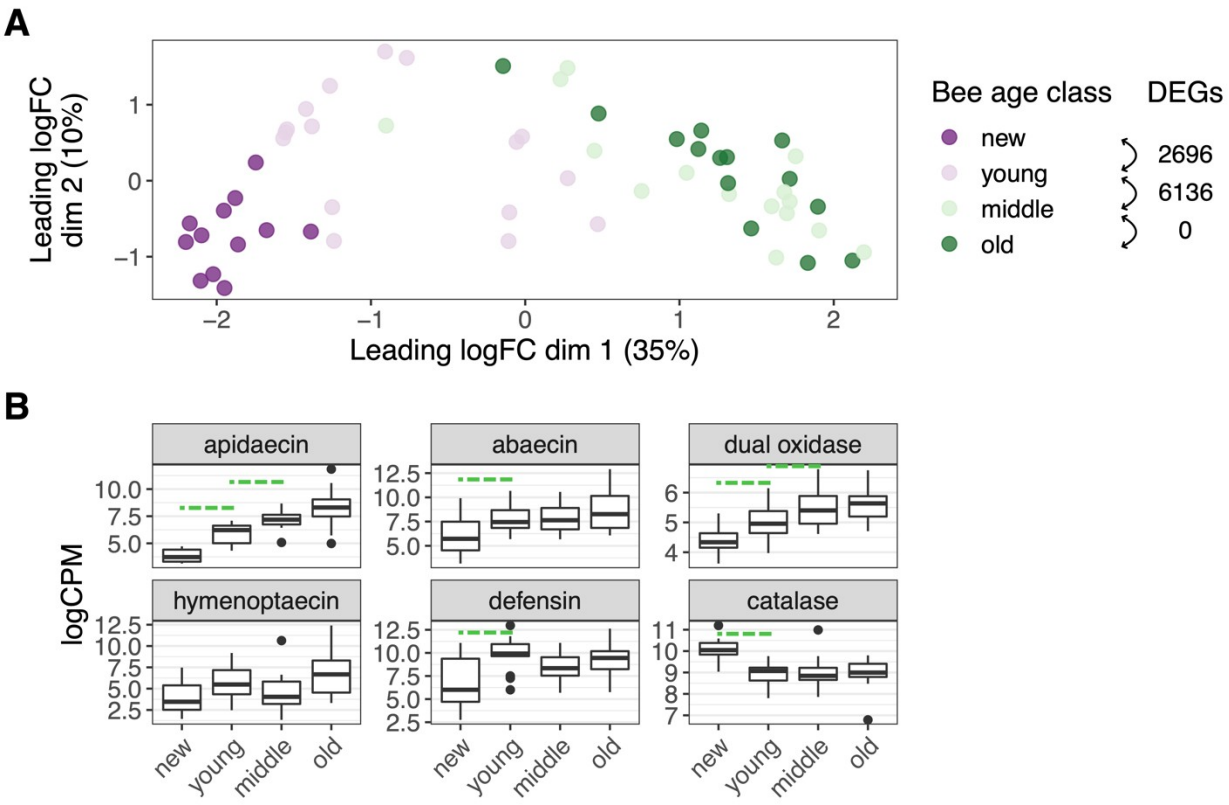

Figure 6. Dynamics and stability of host hindgut gene expression over the lifespan. Ages and sample sizes of age classes: new: 0-1 days, N = 12; young: 3-19 days, N = 16; middle: 23-43 days, N = 15; old: 47-75 days, N = 14. A) Principal coordinates analysis showing similarity in gene expression profiles between bees of different age classes. Similarity is quantified as leading log2-fold changes, which are defined as the quadratic mean of the largest log2-fold changes between a pair of samples. The number of differentially expressed genes (DEGs) is shown for each pair of sequential age classes. B) Expression levels of key immunity genes (Table S1) normalized to library size (log2 counts per million) over bee age. Dashed lines show significant differences in expression between sequential age classes (FDR-adjusted $p < 0.05$).