

Gut and Colony Microbiota of Honey Bees *Apis mellifera*: Social Immunity, Opportunistic Disease and Survival Overwinter.

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Research

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Abstract

Background

Overwintering is a major contributor to honey bee colony loss and involves changes in environmental conditions, host physiology and group behavior that influence disease susceptibility. Honey bees possess a secretory head gland that interfaces with the extended colony environment on many levels, producing pro-oxidants, antioxidants and antimicrobial peptides. With the coming of winter, colonies produce a long-lived (diutinus) worker phenotype that survives until environmental conditions improve. We used a known-age worker cohort to investigate microbiome integrity and social gene expression of diutinus workers overwinter. We provide additional context by contrasting host-microbial interactions from warm outdoor and cold indoor overwintering environments.

Results

We produce the first evidence that social immune gene expression is associated with the core hindgut and colony microbiota in honey bees, and highlight the midgut as a target of opportunistic disease overwinter. We discovered a distinct physiological and microbiological trajectory for diutinus workers that differs drastically from younger, short-lived workers in the colony. Diutinus bees were associated with decreased fungal load and decreased bacterial diversity, and increased core microbiota and longevity. Colonies overwintered indoors maintained a stable or improved microbiota structure and complimentary gene expression overwinter. In contrast, workers from colonies overwintered outdoors in warm southern conditions possessed changes co-occurring throughout the alimentary tract microbiota that suggest opportunistic disease progression and resistance in diutinus workers, but susceptibility to opportunistic disease in younger workers that emerged during the winter, including increases in Enterobacteriaceae, fungal load and bacterial diversity abundance.

Conclusions

Our results highlight social selection pressures that shaped the colony and hindgut microbiome with evolution to a perennial life history. The results are consistent with a “group level” explanation of social immunity, including host associations with the colony microbiota, and a social immune response by long-lived diutinus workers to accompany microbial opportunism. The cost/benefit ratio associated with limited expression of the diutinus phenotype may be a strong determinant of colony survival overwinter. The relationship of colony and gut microbiota with social immune function highlights the range of host-microbial interaction associated with the honey bee superorganism, and its potential influence on colony health, disease resistance and gut integrity.

Background

Social insects are under strong selection to evolve antibiotic mechanisms that result in group hygiene, or social immunity [1,2]. There are fascinating social insect symbioses evolved to control the abundance of

particular microbial species throughout the nest, hive or social environment [3–6]. The social context of disease susceptibility involves multiple factors that shape the evolution of life history, including resident microbial symbionts and resistance to opportunistic disease [7–10]. Many opportunistic disease states are associated with changes in core hindgut structure or extended “colony” microbiota overgrowth, highlighting the importance of microbiome integrity or taxonomic membership in disease susceptibility. How, when and where opportunistic pathogens can invade the host depends in part on their ability to occupy sub-optimal or fringe niches with consistent host exposure, to form relationships with, or outcompete other resident microbes, and to evade host defenses [8,11,12]. The primary function of the eukaryotic microbiome is protection from pathogens, and many factors may weaken the core microbiota rendering the host organism susceptible to disease [12–16]. In humans, the skin and nasal pharyngeal microbiome are considered the first line of defense against pathogen colonization and infection [17]. Analogous to these protective human niches, the honey bee is host to a variety of microbiotas associated with a type of “social skin” that occurs throughout the colony and hive [4,13,14,18,19]. This social or colony microbiota is associated with nutrient processing and information sharing including the structurally complex worker mouthparts that perform these tasks and a secretory hypopharyngeal gland (HPG) in the worker head. The HPG produces a highly nutritious and bioactive jelly substance containing pro-oxidants, antioxidants and antimicrobial peptides that interface constantly with the microbiota and social network [4,14,18]

A recently emerged model for gut microbiome studies, the honey bee worker hindgut microbiota is highly predictable by both taxonomy and structure, comprised almost exclusively of five core phylotypes represented in every study [7,12,20–24]. Occurring downstream of excreted host waste, the ileum and rectum each support a distinct microbiota composed of three core phylotypes, and referred to as the “core hindgut microbiota” throughout this manuscript; In the rectum, *Lactobacillus* firm5, *Lactobacillus* Firm4, *Bifidobacterium asteroides* dominate, while *Snodgrassella alvi*, *Gilliamella apicola* and *Lactobacillus* Firm5 dominate the ileum. These five hindgut phylotypes are essential to the health of the individual worker and colony, and occur throughout the colony and hive environment, facilitating generational transmission [25–27]. Similar to the hindgut, a variety of niches throughout the colony and hive also contain a unique and predictable set of phylotypes [4,19,22,28–30]. The honey bee maintains a strict relationship with the microbes in its hindgut, and we predict similar functional pressure exists to control the microbiota of the colony environment.

Honey bees are a “colony in a hive”[31]. The colony is the animated genome of *Apis mellifera*, the hive is a built structure that contains developing young larvae, pupae and stored food. Due to physical continuity, the colony (and gut) microbiota persists in and on the bodies of adult worker bees; in the secretory glands, on the mouthparts, on the exoskeleton, and in the guts and hemolymph. Colonies reproduce by budding when thousands of adult worker bees depart the overgrown parent hive and build a new hive to perpetuate the lineage. Larvae are left behind in this process, perhaps one reason that larval microbiomes are unpredictable relative to that of workers [19,32]. From a perspective of microbial evolution, worker bees are the continuous microbial niche. With the establishment of a new hive, the colony microbiome carried by the workers populates the hive environment. Referred to as the colony

microbiota throughout this manuscript, we argue that a variety of low abundance niches throughout the colony and hive possess a host-adapted microbiome with a large impact on colony health. The colony microbiome populates beebread, honey, jelly, queens and larvae [13,14,18,19,22,28,32,33]. It is comprised of hundreds of microbial species that only attain high numbers at a particular space and time, or in the context of opportunism and disease [34–40]. Here we hypothesize that the microbiotas that populate the colony and hive are co-adapted to the honey bee environment including host-generated enzymes and peptides that function in social immunity [41].

A combination of selective culturing and next generation sequencing provides insight into the breadth and diversity of the colony microbiota. A typical 16S rRNA gene amplicon library of the whole worker gut reveals a taxonomic handful (5-9) of phylotypes (OTUs) that account for 95-97% of the whole gut microbiota [7]. The remaining 3-5% tail of the distribution, also referred to as the rare biosphere, can contain thousands of bacterial species, many of which are easily cultured [18,19,22,38,40,42,43]. This “rare biosphere” [44] is comprised of both microbes from the pollination environment and a more predictable set of phylotypes adapted to the colony and hive environment. A recently discovered exemplar, *Serratia marcescens* (Enterobacteriaceae), was shown to possess a suite of honey bee colony-associated survival genes not found in any other strain of *S. marcescens* [45]. Although rarely apparent with next generation sequencing of the whole gut, this bacterial strain is a very successful and widespread opportunist of honey bees, becoming highly pathogenic when the integrity of the gut microbiota or host health is compromised [12]. Consistent with this finding, the hive is host to a variety of Enterobacteriaceae and other groups of well-known opportunistic or pathogenic Gammaproteobacteria [43,46,47]. Many of these OTUs occur frequently across studies suggesting they are part of the taxonomically consistent colony microbiota.

Core to the colony microbiota, *Lactobacillus kunkeei* and *Parasaccharibacter apium* (synonym for *Bombella apis*) reflect a deep ecological and evolutionary history with bee species and the pollination environment [18,32,48–51]. These two species are part of a healthy larval and queen microbiota [33]. Their growth is either enhanced or unaffected by honey and jelly, while taxonomically similar non-colony microbes are inhibited [32]. *P. apium* dominates the mouthparts of workers and queens, and can inhibit specialized fungal pathogens [15,33,52] suggesting a co-evolved protective role. Our research suggests that these two bacteria, among many others, are in somewhat continuous contact with hygienic structures of workers, larvae and queens like the mouthparts, midguts, social stomach, HPG, and leg combs. Well adapted to high solute concentrations and bioactive properties of the hive, the colony microbiota may survive or even proliferate in antimicrobial hive materials like plant resins (propolis), jelly, and honey [4,32,53,54].

Honey bee life-history and aging is extremely plastic; queens can live for years, and workers also exhibit considerable longevity differences associated with age, task, overwintering and forage dearth [55–58]. Similar to other model systems, the hindgut microbiota tracks the physiology of honey bee behavioral and reproductive phenotypes associated with age and nutritional state [30,33]. Worker bees acquire their highly structured hindgut microbiota in the first 1-4 days of adult life concurrent with the consumption of

beebread; pollen, honey and worker secretions [25,26]. Host digested beebread is converted to an internal storage molecule, vitellogenin (Vg), a large lipo-glyco-phosphoprotein. The role of Vg varies by tissue and caste and is associated with antioxidant, antimicrobial, and anti-inflammatory properties [59]. Workers raised in sterile conditions and fed sterile beebread do not establish a typical gut microbiota, and show deficient central metabolism including decreased insulin signaling and Vg production [27], and as a social consequence, deficient HPG secretions.

The HPG can express a broad variety of nutritional and antimicrobial combinations in response to colony needs. Jelly contains antimicrobial peptides and social hygienic enzymes glucose oxidase (GOX), and superoxide dismutase (SOD). Mixed with glucose in honey, GOX produces gluconic acid and hydrogen peroxide. The honey bee social environment is coated with GOX and H₂O₂ providing a generalized social immune barrier [1,60–62]. Phenotypic plasticity within the worker caste is reflected in HPG gene expression; young nurses produce copious amounts of nutrient rich jelly, but the HPG shrinks in older foragers and secretes enzymes associated with honey processing [63,64]. The HPG of workers can resume past physiological states associated with youth, or shift forward to assume the task of an older bee [65]. The gland can also react more proximally to colony-level challenge associated with a variety of social processes like emergency queen rearing [66,67]. In addition to providing shared nutrition, jelly may transmit immune training molecular patterns across generations, extend life expectancy, modulate microbiota structure and prevent opportunistic disease [67–69]. Gene expression associated with individual and social immunity is costly, and only generated by well-nourished individuals [61,70]. Because internal vitellogenin stores overwinter rely on nutrition in the fall, the availability of pollen on the landscape is the major factor in brood production, colony growth and disease resistance [71].

Honey bee colonies respond rapidly to environmental conditions. The proximal cue of artificial rainfall causes a shift to conservation physiology in a matter of hours [72]. Similarly, persistent low temperatures shift physiology towards colony thermoregulation and resource conservation including the production of the long-lived diutinus worker phenotype. Diutinus workers consume and digest pollen in the fall, or at the beginning of a pollen dearth, then store the nutrition internally throughout the winter months to provide for the growing colony when environmental conditions improve. With the coming of winter, pollen diversity and nutrition disappear completely across the northern landscape, and decrease drastically on the southern landscape. While colony function in cold winter environments is well defined, mild overwintering conditions found in the southern US involve a variety of indistinct or conflicting environmental cues, including warm daily temps, and the availability of pollen and nectar on the landscape. Subsequently, brood rearing over winter commonly discontinues in northern climates, but continues at significantly reduced levels in southern climates, stimulating foraging behavior throughout the winter [73,74]. The presence of brood in the hive environment accelerates both behavioral and cellular senescence among worker bees [55]. After approximately 10 days of foraging, workers experience a sharp increase in mortality [55]. The forager gut microbiota differs from that of a nurse, likely due to task-associated metabolism and senescence [30,33]. Incomplete nutrition and fluctuations in temperature or water availability associated with foraging and brood rearing overwinter may also affect the colony and

gut microbiota producing an environment conducive to the growth of opportunists. Colonies weakened by less than adequate nutrition and cold temperatures are more susceptible to disease and dysentery, and if they survive the winter, may build back slowly in the spring.

In northern climates, the cohort of workers emerging in early winter express the diutinus phenotype, and can live 8X longer (240 days) than a worker during spring/summer colony growth [75,76]. Although a critical point in the life cycle, variation in gut and colony microbiota and physiology that accompanies this process is relatively unknown, as is the HPG gene expression of diutinus workers overwinter [77,78]. A recent investigation of the diutinus hindgut microbiota implied healthy overwintering physiology in cold (7°C) indoor climates, but the proliferation of opportunistic bacteria associated with southern outdoor overwintering [8]. However, the opportunistic microbes were at extremely low abundance in the hindgut relative to the core microbiota. In this contribution, we investigate these same samples more deeply by examining the change in worker mouth and midgut microbiotas, and detailing the associated change in gene expression from the hypopharyngeal gland, a secretory gland that interfaces with the extended social environment on many levels. Like other examples from social insects [5,6], we hypothesize that HPG social secretions nurture symbionts native to the alimentary tract, but are negatively associated with opportunists in the colony environment.

Methods

To gain an understanding of host-microbial interaction overwinter, we performed two separate but related experiments (Fig. 1). We designed the first experiment to provide perspective on the relationships of mouth and midgut microbiota with social gene expression. We quantified HPG gene expression and the microbiotas associated with the long-lived diutinus phenotype in warm winter conditions; The second experiment compared HPG expression and various microbiota characteristics of the midgut associated with full-sized colonies placed in cold indoor climate-controlled wintering, and warm outdoor wintering. These results are associated with a published companion paper that sequenced the ileums and rectums from the same experimental samples [8]. Briefly, those results revealed little change in the hindgut microbiota of worker bees overwinter regardless of climate or age. However, the authors suggested the potential for compromised host physiology in warm southern climates, revealed by poor longevity overwinter and significantly increased fungal load correlated with an increase in putatively opportunistic Enterobacteriaceae. This manuscript more fully explores the microbiomes and host response associated with microbial opportunism and fungal growth.

Experiment 1: Known age cohort

We used four observation hives to follow a known age (KA) cohort and validate chronological age. Colonies comprised of three or four frames containing brood, plentiful honey and beebread, were kept in greenhouses at the Carl Hayden Bee Research Center, Tucson AZ with exposure to diurnal cycles, access to the foraging environment and a small space heater for rare nights below freezing. To control for age in the observation hive, newly emerged winged adults were sourced from brood frames of 20 healthy

colonies. Late stage pupae emerged naturally from their natal frame over a period of ≤ 24 hours while housed in a humidity (50%) and temperature-controlled (35°C) room. All newly emerged bees were collected into a single container and randomized prior to colony assignment. We painted the thoraces of 2,000 newly emerged bees and divided them equally among four observation colonies. Adult worker bees were introduced into observation hives in early December, then sampled from Dec 22nd 2015 - Feb 10th 2016. Samples of marked bees were collected at 19, 33, 50 and 70 days of age. Our 19 Day-old sample represents the age when workers have expelled the pollen from their guts and transitioned to foraging, while 33 days is the expected longevity of foragers. In the previous study, we estimated that only 10% of introduced bees survived until 50 days of age [8]. Thus our 50 and 70 day old samples were comprised of the longest-lived bees relative to average life expectancy in southern climates. From this sample set, we quantified the change in gene expression overwinter of host genes related to reactive oxygen species, and antimicrobial peptide (AMP) expression. The ileum and rectum microbiota from these same worker samples (KA) was remarkably stable from 19–70 days of age while fungal load and bacterial diversity decreased significantly overwinter in the hindgut.

Experiment 2: Overwintering climate comparison

In a second experiment, we hypothesized that overwintering climate (environment) affects the colony microbiota. More specifically, that the midgut microbiomes associated with warm vs. cold overwintering would differ as a result of temperature and task differences experienced by the hive. We compared the midgut microbiota of colonies from southern outdoor climates to colonies kept indoors overwinter in cold climate controlled conditions. Because honey bees are homeotherms, they regulate hive temperature by clustering together and shivering their flight muscles to generate heat [79]. Climate controlled overwintering of honey bee colonies is a highly successful strategy for commercial beekeepers, and much of the knowledge about wintering honey bees is focused on cold tolerance in northern climates. Colonies overwintered in warm southern climates are exposed to diurnal cycles of light and temperature, and can leave the hive to defecate and forage for resources. They typically maintain a weak foraging effort overwinter, but still consume simple sugars and cluster for warmth mostly at night. All colonies in this experiment had access to honey and beebread overwinter, but the cold winter colonies became broodless, while the warm winter colonies continued to forage and rear small numbers of brood throughout the winter [73, 74]. All full size colonies in both environments were 6 + frames of bees at the beginning of the experiment with no signs of disease.

The warm overwintering (WW) environment was the Santa Rita Experimental Range in southern Arizona 31°46'38"N, 110°51'47" W. We collected worker samples in mid-December 2015 and mid-February 2016. We placed cold winter (CW) colonies in a climate controlled storage facility at a constant 7°C and 25% relative humidity with no access to environmental cues. Worker bees were sampled just prior to entering the cold storage warehouse in Firth, Idaho, USA, 43°18'45" N, 112°09'20"W in early winter, mid-October 2015, and directly after they were removed from cold storage and transported to the almond orchards in late winter, mid-February 2016, near Snelling, California, USA, 37°31'33"N, 120°31'52"W. Thus, we assume that the CW worker samples represent the diutinus phenotype in early and late winter. From the edge of

the brood nest, we sampled worker bees from 16 colonies per time point, per climate for 64 total colony samples. The hindgut microbiotas of early winter bees did not differ by climate/locale but fungi increased significantly overwinter in the hindguts of WW samples, concurrent with a slight but significant increase in an unknown *Gilliamella* spp. and Enterobacteriaceae [8].

DNA analysis

Two honey bee worker tissues were used for microbial DNA analysis, the mouthparts and midguts. Immediately after being removed from -80 C individual bees were surface sterilized and dissected in 95% ethanol using sterile forceps and dissection scissors. We immediately placed all tissues into a 2ml bead beating tube containing ~ 100ul of 0.1mm silica beads and 300ul of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) and immediately frozen on dry ice. All dissected tissues were stored at -80 C. Prior to DNA extraction, each sample was bead beaten for a total of 2 min in 30 sec intervals. To each sample, 100 µl lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5 % Triton X-100, 80 mg/ml lysozyme, pH 8.0) was added and the samples were incubated at 37°C for 30 min. Total genomic DNA was extracted using a Fermentas GeneJet Genomic DNA Purification Kit (#K0722) following the protocol for gram-positive bacteria. Miseq amplification and analysis was performed as in [8]. We estimated the size of the bacterial/fungal communities in the ileum and rectum using degenerate primers [80, 81]. We amplified the 16s gene template using forward primer 27F (5'-AGAGTTTGATCCCTCAG-3') and reverse primer 1522R (5'-AAGGAGGTGATCCAGCCGCA - 3'). For fungal quantification, we extracted total genomic DNA from *S. cerevisiae* cells. The 18s gene template was amplified using forward primer PanFungal_18S_F (5'-GGRAAACTCACCAGGTCCAG - 3') and reverse primer PanFungal_18S_R (5'-GSWCTATCCCCAKCACGA-3'). We created plasmid vectors using Invitrogen's pCR™2.1 TOPO™ cloning vectors per the manufacture's specifications.

Microbiota sequencing

The V6-V8 region of the 16S rRNA gene was amplified using universal (degenerate) PCR primers 799F (acCMGGATTAGATACCCKG) and bac1193R (CRTCCMCACCTTCCTC). We amplified DNA using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD, USA) under the following conditions: 94C for 3 min, followed by 28 cycles of 94C for 30 s, 53C for 40 s and 72C for 1 min, with a final elongation step at 72C for 5 min. We used the PCR products to prepare DNA libraries following Illumina MiSeq DNA library preparation protocol. Sequencing was performed at the University of Arizona Genetics Core (UAGC) on a MiSeq following the manufacturer's guidelines. Resulting sequences were processed using MOTHUR v1.43. [82]. Forward and reverse reads were joined using the make.contigs command. Then the first and last five bases pairs were removed using the SED command in unix. Sequences were screened (screen.seqs) to remove any sequences containing ambiguous bases. Unique sequences were generated using the unique.seqs command. Sequences were aligned to Silva SSUREF database v102 [83] using the align.seqs command. Sequences not overlapping in the same region and columns not containing data were removed using the filter.seqs command. Sequences were pre-clustered using the pre.culster command. Chimeras were removed using UCHIME [84] and any sequences of non-bacterial origin were removed using the remove.seqs command. All remaining sequences were classified using the

classify.seqs command. All sequences with only one or two (single/doubletons) associated reads were removed using the AWK command in unix. A distance matrix was constructed for the aligned sequences using the dist.seqs command. Sequences were classified with the RDP Naive Bayesian Classifier [85] using a manually constructed training set containing sequences sourced from the greengenes 16S rRNA database [86], the RDP version 9 training set, and all full length honeybee-associated gut microbiota listed in NCBI. Operational taxonomic units (OTUs) were generated using the cluster command. Representative sequences for each OTU were generated using the get.oturep command.

Expression analysis

RNA was extracted from the hypopharyngeal glands of all individuals, and the midguts were used for microbiota sequencing. We also sequenced the microbiotas of mouthpart tissues from KA samples. Heads were surface sterilized and dissected in RNAlater™ using sterile forceps and dissection scissors. All samples were immediately placed into a 1.5ml centrifuge tube containing 200µL of Fermentas Lysis Buffer supplemented with β-mercaptoethanol and immediately frozen on dry ice. All samples were stored at -80°C. Total RNA was extracted using a Fermentas GeneJet RNA Purification Kit (#K0732) following the protocol for Total Insect RNA Purification. Complementary DNA (cDNA) was generated using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622) following the First Strand cDNA Synthesis protocol. We quantified the expression of eight genes relative to the typically used housekeeping gene β-actin: three antimicrobial peptides; Hymenoptaecin, Defensin-1 and Abaecin; three antioxidants; Catalase, Zn/Cu Superoxide Dismutase (Zn/CuSOD) and Mn Superoxide Dismutase (MnSOD); the redox “social immunity” enzyme Glucose Oxidase (GOX) and finally Vitellogenin (Vg) as a marker of nutritional state. Briefly, all reactions were performed in a 12ul volume (6ul of Bio Rad iTaq Universal SYBR Green Supermix, 0.5ul of 10uM forward and reverse primer [see [87] for primer details], 3ul of molecular grade water and 2ul of template). Cycle conditions were run as described in [87]; 95°C for 30 s and then 40x of 95°C for 20 s and 60°C for 30 s. Amplification was normalized to β-actin expression.

Nosema quantification

The midgut is the site of *Nosema* infection, a highly specialized fungal microsporidian that is a ubiquitous opportunist of honey bee midguts. To determine *Nosema* infection status in the midgut we ran a modified version of the qPCR protocol reported in [88]. For each 10µl reaction we used 5µl of Luna qPCR master mix (New England Biolabs), 0.25µM of each primer, 1µl of template and 3.5µl molecular grade water. We used *N. ceranae* specific primers NcF (AAGAGTGAGACCTATCAGCTAGTTG) and NcR (CCGTCTCTCAGGCTCCTTCTC) and *N. apis* specific primers NaF (GCCCTCCATAATAAGAGTGTCCAC) and NaR (ATCTCTCATCCCAAGA). To confirm efficiencies reported in [88] we ran a temperature and concentration gradient qPCR with a known *Nosema* rich DNA sample. Amplification efficiency was calculated by the CFX manager software and were close to 100% (97.1% and 98.3% respectively). Using a CFX96 real time system (BioRad, Hercules, CA) we ran the following thermocycler program: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds and 63°C for 30 seconds. Each sample and negative controls were run in triplicate. Using the same reagent mixture (with a 57°C annealing/extension step), we used actin specific primers ActinF (TGCCAACACTGTCCTTTCTG) and ActinR

(AGAATTGACCCACCAATCCA). We used the average of the three reactions to determine *N. ceranae* load relative to β -actin expression. We compared the relative qPCR measures to a previous microscopic assessment of Nosema abundance based spore counts from midgut samples of (CW) workers overwintered indoors at 7°C and 25% RH.

Statistical Analysis

We analyzed microbial community structure and abundance with two different approaches: MANOVA performed on centered log ratios and non-parametric Wilcoxon tests. The MANOVA accounts for microbiota structure while the Wilcoxon test examines absolute abundance without regard to microbiota structure. We compared differences in the microbial community structure by known chronological age (KA samples) as well as within, and between WW and CW environments. To allow the use of parametric multivariate analyses [89], we converted bacterial abundances to ratio abundances among all OTUs [90] using the software CoDaPack's centered log-ratio (CLR) transformation [91]. We compared microbiota structure by chronological age (known age cohort) and season (pre/post winter) within and between climates using one-way MANOVA. Pillai's Trace test statistic was used for all MANOVA's to account for deviations in normality and homogeneity of covariance. Subsequent univariate tests followed by FDR correction for multiple comparisons were used to explore differences between dependent variables.

We compared total microbial load for bacteria, fungi and Nosema using one-way ANOVA (FDR corrected for multiple comparisons). All analyses were conducted in either JMP_v13 (JMP_ 1989–2007) and/or SAS_v9.4 (2013). Pillai's Trace test statistic was used for all MANOVAs to account for deviations in normality and homogeneity of covariance. Statistically significant MANOVA results were further analyzed with pairwise ANOVA tests followed by FDR correction for multiple comparisons. We analyzed total cell number using pairwise Wilcoxon tests (Steel– Dwass correction for multiple comparisons). To normalize to absolute abundance, the proportional abundance of OTUs returned by amplicon sequencing was multiplied by the total bacterial 16S rRNA gene copies determined with qPCR for each individual tissue type. To account for variability in 16S copy numbers across taxa the 16S total was divided by the number of 16S rRNA gene copy numbers present within each bacterial genome. All core phylotypes contain four 16S rRNA gene copies except *B. asteroides* (2). Copy number of the remaining taxa were determined using the average copy number according to taxonomic level on NCBI: *L. kunkeei* = 5, *Parasaccharibacter apium*, a synonym of *Bombella apis* = 1, Actinobacteria = 2.9, Enterobacteriales = 2 and Xanthomonadaceae = 2.2. The remaining OTUs (14-6294) were summed, and assigned a gene copy number of 4.2, the average for all bacteria [92]. This value represents a general measure of diversity abundance as it contains > 99% of the OTUs. To examine hypotheses of host microbial interaction, we generated Spearman's rank correlation coefficient among the dependent variables independently for each treatment condition, and for the entire data set.

Results

Experiment 1: Known age cohort

We deep-sequenced the mouthpart and midgut microbiota of aging worker bees overwintered in southern Arizona. We sampled known age (KA) diutinus workers as the winter progressed at 19, 33, 50 and 70 days of age (Fig. 1, Experiment 1). Bacterial diversity on the mouthparts was greatest in 33-day-old bees and decreased in older bees (Fig. 2). Based on the total number of unique 16S rRNA gene sequences found in each niche, bacterial diversity was positively associated between the mouthparts and midguts of known age bees ($R_{sq} = 0.25$, $F_{2,36} = 12.0$, $p = 0.001$). The mean number of unique OTUs detected on the mouth not differ from that detected in the midgut and did not change with age ($n = 38$, mouth = 57, midgut = 62). Both the mouth and midgut environments were comprised of a taxonomic variety of bacteria, including all five of the core hindgut phylotypes (Fig. 3). The five core hindgut bacteria were strongly inter-correlated on both the mouth and the midgut, as were *P. apium* and *L. kunkeei* (Table S1). Based on a Wilcoxon rank sum test comparing 19/33 day old bees to 50/70 day old bees, bacterial load remained steady on the mouth with age but increased in the midgut ($W_{46} = 166$, $p = 0.03$). In contrast, total fungal load decreased slowly and significantly on the mouthparts of aging bees ($W_{46} = 114$, $p = 0.0004$) but remained steady in the midgut (Fig. 4). In both the mouth and midgut of aging bees, fungal load was positively associated with bacterial diversity abundance (Table S1).

The mouthpart microbiota was comprised of *P. apium*, Actinomycetales, Xanthomonadaceae, *Delftia* (Comamonadaceae), an unknown *Gilliamella spp.*, and *L. kunkeei*. Enterobacteriaceae was at low abundance on 95% of the mouthparts, and Actinomycetales, Xanthomonadaceae and *Delftia* had 100% prevalence and relatively even abundance. *P. apium* and *L. kunkeei* were both prevalent on the mouth but varied considerably in absolute abundance (Fig. 3, Table S2). The mouth microbiota of the aging bee (KA) differed from early to late winter. MANOVA results reflect the change in particular OTUs relative to other members of the microbiota, while Wilcoxon tests do not account for the group effect (Table S2). On the mouthparts of aging bees, Actinomycetales and the unknown *Gilliamella spp.* increased while Xanthomonadaceae decreased from twenty-six to nine percent of the total mouthpart community. Relative abundance of Enterobacteriaceae on the mouth also decreased with age (Fig. 3, Table S2). We examined the aging bees by predicted task, assuming 19–33 days old bees were potential foragers or young diutinus workers and 50–70 day old bees were long-lived diutinus workers. According to this grouping, diversity abundance on the mouth decreased with age, while the relative abundance of *L. firm5*, *L. firm4* and *B. asteroides* on the mouth also decreased with age (Table S2).

The midgut of aging diutinus workers supported Enterobacteriaceae, *P. apium*, an unknown *Gilliamella spp.* and *L. kunkeei*. The triad signature of Actinomycetales, Xanthomonadaceae, and *Delftia* was present in nearly every midgut at low relative abundance (Table S2). All five of the core hindgut bacteria increased with age (Fig. 3). *Snodgrassella alvi* and *G. apicola* increased significantly, while *L. firm5* and *B. asteroides* also increased, trending towards significance (Table S2). A putative midgut opportunist; Enterobacteriaceae also increased markedly with age. Although at low absolute abundance in KA midguts, the relative abundance of both *Delftia* (Comamonadaceae) and Xanthomonadaceae decreased with age overwinter. Both Lachnospiraceae and *Serratia* were at low abundance in the KA mouthparts and midguts, present in < 50% of samples (Table S2).

Nosema ceranae, a ubiquitous midgut pathogen, was at generally low absolute abundance in the midgut of aging bees. Many samples from all KA age groups did not rise above the limit of detection (Table S3). Nonetheless, *N. ceranae* levels differed significantly among age classes, peaking at 33 days of age and declining thereafter. Nineteen and 70-day-old bees contained significantly less *N. ceranae* than 33-day-old bees representing diutinus “mid-life” or forager-aged workers (Table S3).

Experiment 1: Known age cohort gene expression

To examine the hypothesis that gene expression of long-lived honey bees affects the colony and/or gut microbiome, we examined associations of HPG gene expression with the aging diutinus microbiota (KA samples) of the mouth and midgut overwinter. We determined the relative expression of HPG genes with a hypothesized role in structuring the colony microbiome. The relative expression of five genes involved in ROS generation and AMP production increased significantly with age (Table 1, Table S4). ROS associated gene expression had a strong and significant negative association with both bacterial diversity abundance and fungal load on the mouth (Table 2, Table S1). Actinomycetales increased significantly with age on the mouth and was positively associated with ROS gene expression. Xanthomonadaceae Decreased significantly with age on the mouthparts, and was negatively correlated with ROS associated gene expression (Table 2). Enterobacteriaceae abundance was also negatively associated with ROS gene expression on the mouth trending toward significance (Table 2, Table S1).

Table 1
Change (Δ) in hypopharygeal gland gene expression overwinter.

Change (Δ) overwinter	Warm Winter			Warm winter			Cold winter		
	Known age*			Random sample			Random sample		
Gene	Z	p	Δ	Z	p	Δ	Z	p	Δ
Nutritional State									
Vitellogenin	0.69	0.49	NC	-2.00	<i>0.05</i>	↓	0.42	0.68	NC
Antimicrobial Peptides									
Hymenoptaecin	2.69	<i>0.007</i>	↑	-1.52	0.13	↓	1.34	0.18	↓
Defensin-1	-0.67	0.53	NC	1.62	0.11	↓	2.18	<i>0.03</i>	↓
Abaecin	2.87	<i>0.004</i>	↑	-2.21	<i>0.03</i>	↑	1.42	0.16	↓
Oxidative State									
Glucose Oxidase	2.80	<i>0.005</i>	↑	0.02	0.98	NC	2.05	<i>0.04</i>	↓
Cu/ZnSOD	5.14	<i>0.0001</i>	↑	-0.58	0.56	NC	-0.18	0.86	NC
MnSOD	5.57	<i>0.0001</i>	↑	1.10	0.27	NC	-0.66	0.51	NC
* The change (Δ) in expression overwinter compares 19/33 days to 50/70 days of age. In the warm winter and cold winter environments, we compared randomly sampled in-hive workers in early vs. late winter. See Table S2 for details.									

Table 2

Correlations[†] of diutinus HPG immune gene expression with microbial abundance and metrics.

Gene	Known Age Mouthparts			Known Age Midguts		
	Dependent variable	Rs	p	Dependent variable	Rs	p
Nutritional State						
Vitellogenin	No strong associations			No strong associations		
Antimicrobial Peptides						
Abaecin	Actinomycetales	0.29	0.05	<i>Nosema ceranae</i>	-0.36	0.01
	<i>P. apium</i>	-0.24	0.11	<i>P. apium</i>	-0.33	0.04
				Bacterial abundance	0.30	0.05
Hymenoptacin	Actinomycetales	0.38	0.01	<i>Nosema ceranae</i>	-0.38	0.01
Defensin-1	<i>Lactobacillus kunkeei</i>	-0.31	0.04	<i>Lactobacillus kunkeei</i>	-0.30	0.07
Oxidative State						
GOX	Fungal abundance	-0.23	0.13	<i>Snodgrassella alvi</i> *	0.54	0.0004
	Diversity abundance	-0.32	0.03	<i>B. asteroides</i> *	0.45	0.004
	Xanthomonadaceae	-0.37	0.01	<i>Gilliamella apicola</i> *	0.44	0.006
	<i>P. apium</i>	-0.14	0.33	<i>Lactobacillus firm4</i> *	0.33	0.04
	<i>L. kunkeei</i>	-0.11	0.44	<i>Lactobacillus firm5</i> *	0.30	0.07
				<i>Nosema ceranae</i>	-0.40	0.007
Cu/Zn	Fungal abundance	-0.48	0.0008	Core hindgut bacteria*	0.36	0.05
SOD	Diversity abundance	-0.38	0.01	<i>Snodgrassella alvi</i>	0.53	0.0006
	Xanthomonadaceae	-0.50	0.0004	Bacterial abundance	0.33	0.02
	Enterobacteriaceae	-0.26	0.08	Enterobacteriaceae	0.32	0.05
	Actinomycetales	0.43	0.003	<i>Nosema ceranae</i>	-0.32	0.03

[†] Spearman's correlations based on diutinus worker (KA) samples. See Table S1 for a complete list of values.

* Values for the core hindgut bacteria are averages based on five OTUs (phylotypes); *S. alvi* is one of the five.

[^] Strongest detected relationship of mouth microbiota with Catalase expression

Gene	Known Age Mouthparts			Known Age Midguts		
Mn SOD	Fungal abundance	-0.48	0.0008	Core hindgut bacteria*	0.38	0.05
	Diversity abundance	-0.41	0.004	<i>Snodgrassella alvi</i>	0.54	0.0004
	Xanthomonadaceae	-0.55	0.0001	Bacterial abundance	0.31	0.04
	Enterobacteriaceae	-0.26	0.08	Enterobacteriaceae	0.36	0.03
	Actinomycetales	0.52	0.0002	<i>Nosema ceranae</i>	-0.30	0.04
CAT	Diversity abundance [^]	0.23	0.11	<i>Lactobacillus kunkeei</i>	0.43	0.007
				Bacterial Diversity	0.34	0.04
				<i>Lactobacillus Firm5</i>	-0.38	0.02
† Spearman's correlations based on diutinus worker (KA) samples. See Table S1 for a complete list of values.						
* Values for the core hindgut bacteria are averages based on five OTUs (phylotypes); <i>S. alvi</i> is one of the five.						
[^] Strongest detected relationship of mouth microbiota with Catalase expression						

The midgut showed very different host-microbial relationships than the mouthparts. Both GOX and SOD expression (CuZnSOD and MnSOD) were strongly and positively associated with abundance of the core five hindgut phylotypes in the midgut (Table 2, Table S1). The strongest positive relationship within this core group was *Snodgrassella alvi*, an obligate aerobe (Table 2). In contrast to the mouth, Enterobacteriaceae and ROS gene expression were positively correlated in the midgut. Because both the core hindgut bacteria and Enterobacteriaceae increased in the midguts of aging bees overwinter, total bacterial load in the midgut was also positively associated with ROS gene expression. The expression of both SOD genes was strongly intercorrelated in both the mouth and midgut. Both were also strongly and positively correlated with the group of five core hindgut bacteria, as was glucose oxidase, widely considered the standard enzyme for social hygiene (Table 2, Table S1).

Relative to actin, the expression of two of three measured AMPs increased significantly with age, Hymenoptaecin and Abaecin (Table 1). None of the tested AMPs were significantly correlated with total bacterial or fungal load on the mouth or midgut with the single exception of abaecin, positively correlated with midgut bacterial load (Table 2, Table S1). Most notably, not one of the five core hindgut bacteria was significantly associated with AMP expression on the mouth or in the midgut consistent with their long-term co-evolution with honey bee defensive peptides. However, defensin-1 was negatively correlated with *L. kunkeei* abundance on the mouth and in the midguts, and to a lesser degree, *P. apium*. Abaecin expression was negatively correlated with *P. apium* abundance in the midgut. Both Hymenoptaecin and Abaecin were positively associated with the abundance of Actinomycetales on the mouthparts (Table 2).

Experiment 2: Overwintering climate comparison

We sequenced the midgut microbiota of worker bees overwintered outdoors in southern Arizona or indoors at 7°C and 25% RH. These samples are referred to throughout the manuscript as the WW and CW samples (Fig. 1, Experiment 2), and are the same individuals sequenced for ileum and rectum microbiota in a companion manuscript[8]. Based on the total number of unique 16S rRNA gene sequences, or amplicon sequence variants (ASVs) found in each midgut, bacterial diversity increased significantly overwinter in the WW environment (Fig. 2, $t_{30} = 2.89$, $p = 0.008$, $\mu = 59$ early, 154 late), and decreased significantly in the CW environment; (Fig. 2, $t_{32} = 2.89$, $p = 0.007$, $\mu = 253$ early, 94 late). Both bacterial and fungal load overwinter remained unchanged in CW midguts, but increased significantly in WW midguts (Fig. 6, Bacteria: $W_{30} = 43$, $p = 0.003$, Fungi: $W_{30} = 42$, $p = 0.0008$). Fungal load was positively associated with diversity abundance, and both factors increased over winter in WW bees and decreased in CW bees.

Both WW and CW midgut environments were comprised of the same OTUs, including all five core hindgut phylotypes, *P. apium* = (*Bombella apis*), Actinomycetales, Xanthomonadaceae, *Delftia* (Comamonadaceae), and *L. kunkeei*. Actinomycetales, Xanthomonadaceae and *Delftia* had 100% prevalence and relatively even abundance across both groups (Fig. 5, Table S2). Core hindgut bacteria in the midgut increased in CW bees overwinter, but decreased in WW bees (Table S2).

The midgut microbiota from early to late winter differed between WW and CW climates. MANOVA results reflect the change in particular OTUs relative to other members of the microbiota, while Wilcoxon tests compare absolute abundance and do not account for the group effect (Table S2). According to Wilcoxon analysis, the CW midguts did not differ statistically from early to late winter when comparing OTU absolute abundance and correcting for multiple comparisons (Table S2). Near significance, *L. Firm4* increased and *L. kunkeei* decreased overwinter in WW samples. Considering OTU ratio abundance in CW samples, *L. firm5* and *L. firm4* increased significantly overwinter, and the other core bacteria trended in the same direction (Fig. 5). *L. kunkeei* decreased significantly in relative (ratio) abundance as did our measure of diversity abundance, indicating both a loss of diversity and a loss of diversity abundance relative to the midgut microbiota as a whole (Figs. 2 and 5). While the CW microbiota changed very little in terms of abundance and composition, the colonies overwintered in a warm outdoor environment changed drastically for both measures (Fig. 5).

Rare or absent in the CW samples Enterobacteriaceae and an unknown *Gilliamella* spp. were abundant in the midguts of late WW samples (Fig. 5). Increasing in absolute abundance from early to late winter (WW) were Enterobacteriaceae, an unnamed *Gilliamella* spp (3% different from either *G. apis* or *G. apicola*), *Gilliamella apicola*, *P. apium*, Actinomycetales and diversity abundance (Table S2).

Enterobacteriaceae and the *Gilliamella* spp. accounted for the vast majority of the bacterial increase in midgut microbiota size overwinter (Fig. 6, Table S2). Although at low abundance in WW midguts, Xanthomonadaceae decreased significantly overwinter. MANOVA comparisons indicate *L. Firm5* and *L. Firm4* decreased relative to the total microbiota. However, the WW microbiota increased 10X in size overwinter (Fig. 6), such that the absolute cell numbers of *L. Firm5* did not differ between treatments, but

the ratio abundance of L. firm5 relative to the whole community decreased significantly (Fig. 5). L. Firm5 and L. Firm4 decreased significantly in WW midguts overwinter when considered as a proportion of the community whole, as did the less abundant OTUs Xanthomonadaceae and *Delftia* (Comamonadaceae). Enterobacteriaceae, *Gilliamella* spp. and Actinomycetales increased in both absolute abundance, and relative to the total community (Table S2).

Associated with small mouthpart microbiomes, Actinomycetales was present in every midgut of the CW samples. Moreover, the CW samples had a signature of three inter-correlated OTUs associated with small, presumably healthy midgut microbiotas; Xanthomonadaceae, Actinomycetales, and *Delftia*, a Comamonadaceae. These bacteria appear in virtually every next generation sequencing dataset, and are typically more abundant in the HPG, royal jelly, and now seemingly the midguts of bees overwintered indoors at 7°C.

Levels of *Nosema ceranae* differed by overwintering climate (Table S3). Similar to many other microbial metrics, levels of *N. ceranae* remained unchanged from early to late winter in the CW samples. In the WW samples, *N. ceranae* increased significantly from early to late winter. Biologically, *Nosema ceranae* was at low abundance overwinter in both environments based on high Ct values and non-amplification of many samples (Table S3).

Experiment 2: Overwintering climate gene expression

In the CW indoor environment, HPG expression differed from early to late winter for two of seven genes (Table 1, Table S4). Glucose oxidase, and defensin-1 expression decreased significantly from early to late winter. The remaining genes either decreased slightly in expression or remained unchanged. Gene expression was associated with host microbial metrics. HPG expression of Defensin-1 decreased concurrently overwinter with bacterial diversity in CW midguts (Table 3). GOX expression decreased as *S. alvi* midgut abundance increased. For consideration independent of corrected p-value in the CW samples, *S. alvi* was positively associated with total bacterial cell abundance, and GOX expression was negatively associated with the abundance of fungi, *Bifidobacterium asteroides*, and *G. apicola* in the midgut. Catalase expression was negatively associated with bacterial diversity in the midgut (Table 3, Table S1).

Table 3

Correlations[†] of HPG immune gene expression with microbial abundance and metrics.

Category WW Midgut				CW Midgut		
Gene	Dependent variable	Rs	p	Dependent variable	Rs	p
Nutritional State						
Vg	Bacterial Diversity	-0.59	0.005	Bacterial Diversity	0.05	0.81
	Diversity Abundance	-0.61	0.003	Diversity abundance	-0.17	0.35
	Fungal Abundance	-0.56	0.006	Fungal Abundance	0.03	0.87
	Actinomycetales	-0.54	0.01	Actinomycetales	-0.35	0.05
Antimicrobial Peptides						
Abaecin	Xanthomonadaceae	-0.47	0.01	Xanthomonadaceae	-0.05	0.79
	Enterobacteriaceae	0.45	0.01	Enterobacteriaceae*	N/A	N/A
Hymen	Bacterial Diversity	-0.52	0.004	Bacterial Diversity	0.26	0.16
	Fungal Abundance	-0.48	0.007	Fungal Abundance	-0.08	0.67
	<i>Nosema ceranae</i>	-0.48	0.007	<i>Nosema ceranae</i>	0.25	0.18
	Actinomycetales	-0.39	0.04	Actinomycetales	-0.06	0.76
Defensin	Bacterial Diversity	-0.23	0.22	Bacterial Diversity	0.51	0.005
	<i>Nosema ceranae</i>	-0.40	0.02	<i>Nosema ceranae</i>	0.21	0.25
Oxidative State						
GOX	<i>Nosema ceranae</i>	-0.43	0.02	<i>Nosema ceranae</i>	0.01	0.99
	Bacterial Diversity	-0.37	0.05	Bacterial Diversity	0.28	0.15
	<i>G. apicola</i>	0.01	0.97	<i>G. apicola</i>	-0.37	0.04
	<i>B. asteroides</i>	0.34	0.07	<i>B. asteroides</i>	-0.39	0.03
	Fungal Abundance	-0.26	0.15	Fungal Abundance	-0.40	0.03
	<i>S. alvi</i>	-0.03	0.87	<i>S. alvi</i>	-0.45	0.01
MnSOD	<i>P. apium</i>	0.38	0.05	<i>P. apium</i>	0.09	0.63
CuZnSOD	<i>Nosema ceranae</i>	-0.43	0.01	<i>Nosema ceranae</i>	-0.10	0.58

† Values depict change overwinter in midguts of WW and CW samples. See Table S1 for a complete list of values.

* Enterobacteriaceae occurred with low frequency and abundance in cold winter samples.

Category WW Midgut		CW Midgut				
CAT	Bacterial Diversity	0.09	0.66	Bacterial Diversity	-0.39	0.04
† Values depict change overwinter in midguts of WW and CW samples. See Table S1 for a complete list of values.						
* Enterobacteriaceae occurred with low frequency and abundance in cold winter samples.						

In the WW outdoor environment, HPG expression differed from early to late winter for two of seven genes (Table 1, Table S4). As a marker of nutritional state, vitellogenin expression decreased significantly overwinter, while Abaecin expression increased. Vitellogenin expression was negatively correlated with late winter increases of bacterial diversity, bacterial diversity abundance, and fungal abundance (Table 3). Abaecin expression was negatively associated with Xanthomonadaceae abundance in the midgut overwinter, but positively associated with Enterobacteriaceae (Table 3). Hymenoptacin and Defensin-1 expression decreased markedly, but not significantly from early to late winter (Table S4). Both were negatively correlated with the midgut increase in *Nosema ceranae* abundance (Table 3). Hymenoptacin expression was negatively correlated with bacterial diversity, fungal abundance and Actinomycetales (Table 3). Oxidative state interactions associated with the WW microbiota were limited to *Nosema ceranae* and bacterial diversity, which became more abundant in late winter midguts concurrent with a significant decrease in GOX expression (Tables S1, S4).

Discussion

We describe a novel relationship in *Apis mellifera* involving the colony microbiota, the hindgut microbiota, and social gene expression. We suggest that the hypopharyngeal gland (HPG) performs a vital defense role in overwintering honey bees, on par with its established role in providing shared nutrition for colony members during periods of growth. More specifically, in diutinus workers, the abundance of the five core-hindgut bacteria were positively inter-correlated on the mouth and in the midgut throughout the aging process, showing strong age-related associations with the expression of HPG secreted antimicrobial peptides (AMPs), the redox enzyme glucose oxidase (GOX), and superoxide dismutase; Cu/ZnSOD and MnSOD (Table 1). We hypothesize that this gene expression may occur across many tissues, and represents a more generalized metabolic state of diutinus bees associated with aging and resistance to opportunism. Our results are similar to host microbial control seen in other social insect species including host secretions that nurture defensive symbionts [2, 6, 70, 93]. The results suggest one mechanistic hypothesis for the lack of antimicrobial peptides in the honey bee genome [94]. Much of the microbial variation and abundance in the gut and colony environment may be mitigated by the redox potential of the individual and the colony [1, 27].

In honey bees and other organisms, microbiota and associated disease states have evolved to exploit social context and distinct developmental and reproductive phenotypes [13, 32, 33, 66]. The microbiome of the worker mouth, social stomach, and midgut highlight the intimate connectedness of colony members via hygienic behavior and trophallaxis within the colony (food and information sharing). These

resource-laden niches provide a window into disease transmission, as compositional (dysbiotic) changes in these microbiotas are strongly correlated with host health and the colony microbiota [14]. The foregut (crop or social stomach) is a non-digestive expandable bag located between the mouth and midgut, used to share honey and jelly provisions with other colony members. Due to rapid shifts in osmolarity, redox potential, pH, and antimicrobial character, it is considered a harsh microbial environment [22]. In the foregut, we typically detect gram-positive core hindgut bacteria, Enterobacteriaceae, *Lactobacillus kunkeei* and *Parasaccharibacter apium*, but few others (Fig. 7). The microbial load of the foregut is low relative to the midgut and hindgut, but varies in size and diversity by worker task consistent with an antibiotic effect during nectar dehydration, and significantly greater microbial diversity and abundance in foragers[30]. In this study, the colony microbiota was evident in the midguts of CW samples, and on the mouthparts of aging diutinus (KA) workers typified by the occurrence of *P. apium*, *L. kunkeei*, Xanthomonadaceae, Actinomycetales, Comamonadaceae, and Enterobacteriaceae (Figs. 3, 5 and 7). These microbiotas are overtly similar to those described from the worker HPG and fresh royal jelly [14, 18], suggesting that the HPG is a continuous microbial niche that secretes the hindgut and colony microbiota in addition to nutrition, enzymes, and antimicrobial peptides. Regardless, these patterns indicate that HPG secretions played a prominent role in shaping the microbiotas of the mouth and midgut overwinter.

Our findings for the mouth and midgut are consistent with those presented in a companion paper that deep sequenced the ileum and rectum microbiotas from the same three (KA, CW, WW) sample sets presented here [8]. Considered collectively, the midguts of late winter bees in southern AZ were dysbiotic independent of age while the ileum and rectum (hindgut) microbiota remained largely undisturbed. Similarly, disruption of the midgut by *Nosema ceranae* infection does not necessarily result in large compositional changes to the hindgut microbiota [95]. However, it is clear from these and other results that distinct and disparate gut niches are altered synchronously by opportunism or perturbation [14]. Fungal abundance and bacterial diversity decreased significantly and collectively on the mouthparts, ileums and rectums of aging diutinus bees [8], (Fig. 4), and showed a strong negative association with the expression of GOX and both SOD genes from the HPG (Table 2). Resulting gene expression relative to the general character and taxonomy of all four alimentary tract niches suggests a systemic host-microbial interaction that functions throughout the entire alimentary tract. In support of this hypothesis, the hindgut microbiota in conventionalized bees is associated with significantly lower pH and redox potential throughout the entire gut [27].

The Redox Environment / ROS Gene Expression

Our results show that host responses to microbial opportunism involve antimicrobial peptides, and ROS associated gene expression (Table 1). Produced in response to social role, glucose oxidase (GOX) is omnipresent throughout social resource space, and is considered an immediate form of social immune response [61]. GOX functions as a pro-oxidant (generates reactive oxygen species; ROS) that thwarts microbial growth by converting omnipresent glucose into hydrogen peroxide and gluconic acid throughout the colony niche. ROS interfere with gram-negative iron metabolism, but gram-positive

species are more resilient [96]. This function is consistent with the occurrence and abundance of core hindgut species in the hive and colony environment. Of the core hindgut species, *Snodgrassella alvi* is least abundant throughout the social/hive environment [13], perhaps a result of ROS social dynamics. In general however, bacteria that have evolved with the gut environment appear resilient to GOX expression. The abundance of all five core-hindgut bacteria in the midgut were positively associated with the expression of GOX in the HPG highlighting a fundamental host-microbial relationship[27] that likely facilitates hindgut microbiome survival/establishment and limits opportunism (Table 2, Table S4). The concurrent upregulation of Vitellogenin (Vg), GOX, AMPs and SODs in the HPG is consistent with the aging diutinus host response to a microbially challenging overwintering environment (Table 1). Vitellogenin is an antioxidant [97], and superoxide dismutase (MnSOD, and CuZnSOD) are able to scavenge excess oxidants associated with metabolic demand or microbial challenge and convert them into less harmful molecules [98]. Of these two, only CuZnSOD is secreted by the HPG [99], while increased expression of MnSOD is an indication of increased mitochondrial function (Table 1).

In the southern overwintering environment, immune response in late winter differed between old and young bees, and the pattern of gene expression indicates that GOX and SOD secretion is metabolically expensive (Table 1, Table S4). Older diutinus bees had a strong immune response and low levels of fungal infection and bacterial diversity, while younger bees appeared less resistant to infection. Similar to the occurrence patterns of Enterobacteriaceae in WW and KA samples, older bees challenged with the midgut parasite *Nosema* developed more severe infections than younger bees, but survived infection better [100]. This is consistent with our findings wherein the longer-lived diutinus bees possessed the molecular resources (e.g. Vitellogenin) to combat infection. The hypothesis of poor larval nutrition overwinter is consistent with the expression patterns of Vg and other secreted gene products from the HPG across treatments [74]. While Vg expression in the HPG is unstudied, the overall expression patterns suggest that Vg expressed in the HPG may reflect a general nutritional state throughout the host organism. Vitellogenin is expressed in the midgut perhaps in response to opportunistic bacteria and fungi [101]. In older diutinus bees, Vg expression was positively associated with SOD expression suggesting the resources were available to mount an immune response (Table 1). In turn, the expression of both SOD genes was strongly associated with GOX production. In contrast, Vg expression in younger (WW) bees decreased significantly overwinter (Table 1, Table S4) and was negatively associated with increases in bacterial diversity and diversity abundance (Table 3, Table S1), suggesting that scarce resources were available to combat opportunism in this cohort. The indoor CW colonies showed no strong associations with Vg expression overwinter and no immune response, indicative of energy conservation and no microbial challenge. Although queen and queen-like (diutinus) phenotypes often possess higher mean ROS than workers in the form of H₂O₂, similar levels of fat body protein carboxylation between castes are maintained because queens or queen-like phenotypes can afford to mount an immune response, expressing antioxidant genes that include MnSOD and CuZnSOD [102]. There may be a similar explanation for the different response to opportunism between 50–70 day old (KA) workers and workers >30 days-old (WW) sampled in late winter. The younger (WW) bees that emerged during winter lacked the resources to mount an immune response [103], producing very different host-microbial interactions.

GOX expression overwinter appears critical to colony health; it increased significantly with age in the diutinus (KA) samples, remained unchanged overwinter in the WW samples, and decreased significantly overwinter in the CW samples (Table 1). The resulting host-microbial correlates by sample set suggest a fundamental ecological interpretation associated with the control of ROS in the colony and gut overwinter. To review, an obligate aerobe, *S. alvi*, is partnered with the metabolism of *G. apicola* in the ileum of a healthy worker host, contributing to the production of an anoxic hindgut environment [27]. We hypothesize that oxidative control of the midgut is associated with the increased abundance and structure of the core hindgut phylotypes in the midgut, as modeled for the ileum microbiota [27]. In the cold indoor environment, GOX expression decreased, while the abundance of core hindgut bacteria *S. alvi*, *G. apicola* and *B. asteroides* increased significantly. In fact, all five of the core hindgut bacteria increased as a correlated group in the CW midgut, while less abundant OTUs, and bacterial diversity decreased (Table S1, Figs. 2 and 5). The core five hindgut bacteria are well equipped to exploit low oxygen gut environments and many strains are capable of producing their own catalase and SOD [104, 105]. Several commensal bacteria, like *Lactobacillus* species secrete ROS into their surroundings, and this activity influences the growth of other commensal organisms including fungi. In the warm southern outdoors, GOX and SOD expression in the HPG of older diutinus workers (KA samples) increased significantly overwinter, associated with a reduction of fungi and bacterial diversity on the mouthparts (Table 2). In the late WW samples, comprised mostly of middle-aged bees born during the winter, GOX production was unchanged, concurrent with significant increases in *Nosema ceranae*, bacterial diversity and fungal abundance (Table 3). Again, the collective results indicate that CW colonies possessed low fungal abundance and a healthy microbiota and did not require an immune response. In contrast, WW colonies were dysbiotic, but could not afford to mount a strong immune response, while long-lived diutinus (KA) bees had the nutritional resources to mount an immune response to bacterial and fungal opportunism (Table 1).

AMP Expression in the HPG

Antimicrobial peptides were expressed differentially in the HPG by age and overwintering environment (Table 1). Although hymenoptacin and abecin are active against a variety of gram negative and enteric pathogens including Enterobacteriaceae [106], their expression in the HPG was unassociated with the majority of bacteria in the midgut or on the mouth of diutinus bees (Table S1). Most notably, none of the core-five hindgut bacteria were significantly associated with AMP expression, consistent with their long-term co-evolution with secreted honey bee defensive peptides (Table S1). However, social immune gene expression is costly and likely associated with careful regulation [61]. Both abecin and hymenoptacin were upregulated with age in diutinus bees (Table 1). We recorded a significant reduction in Xanthomonadaceae bacteria on the mouthparts of diutinus bees expressing significantly more antimicrobial peptides from their hypopharyngeal glands (Table 1, Fig. 3). Abaecin is highly effective against *Xanthomonas campestris*, a well-known plant pathogen [107]. Aging diutinus bees showed a significant reduction in the absolute and relative abundance of midgut Xanthomonadaceae, and a relative decrease in Xanthomonadaceae in the ileum and rectum [8]. The same Xanthomonadaceae OTU is prevalent and abundant in the microbiota of the HPG and jelly according to two studies [14, 18]. This

indicates a targeted host response to a colony opportunist but requires further study. Concurrent with increased upregulation of GOX, SOD, abaecin and hymenoptacin in older diutinus bees, the abundance of Xanthomonadaceae (an aerobe), fungal load, and bacterial diversity all decreased significantly on the mouthparts (Fig. 3, Table S2). Under the same conditions, Actinomycetales (an anaerobe) became significantly more abundant on the mouthparts. Actinomycetes are well known for antibiotic richness, and the suppression of fungal growth [108]. There is a wide variety of native and relatively unknown Actinobacteria in the social environment of honey bee colonies [22]. The associations of this particular Actinobacterial OTU with host-microbial metrics differed by sample set and niche, suggesting it is an influential member of the colony microbiota with a close association to colony fungi (Tables 2 and 3).

Cold indoor colonies overwinter

Cold indoor overwintering is a highly successful technique proven over many generations of beekeeping experience [74, 79]. It keeps the workers of the colony from flying or performing activities associated with brood rearing like larval nursing and pollen collection, behaviors that lead to metabolically expensive oxidative states that vary in severity by tissue, and contribute to early senescence [109, 110]. Cold indoor overwintering also disrupts the cycle of the ectoparasitic Varroa mite, associated with viral transmission, host immune suppression and oxidative damage [111, 112]. That the gut microbiome of CW bees did not shift overwinter suggests that the diutinus physiology of energy conservation is associated with microbiome stasis.

To effectively compare southern (KA and WW samples) to northern overwintering (CW samples), we briefly review the microbiota and general health of CW colonies overwintered indoors at 7°C. As a comparative example, all CW colonies overwintered successfully, and showed a broad collection of factors that distinguish them from the warm winter apiary. Our results for CW conditions indicate that we effectively sampled the early and late diutinus phenotype. The change overwinter was associated with gut microbial stasis, energy conservation, and a lack of microbial opportunism. The hindgut microbiota, intimately tied to host metabolism [27], was either improved overwinter or not diminished (Fig. 5), [8]. The whole gut microbiota (midgut, ileum, rectum) did not change in size, and retained its structure. Fungi remained low and constant overwinter throughout the entire alimentary tract, in the midgut, ileum and rectum (Fig. 6), [8]. Bacterial diversity throughout the gut decreased drastically (Fig. 2). Immune related HPG gene expression either did not differ, or decreased from early to late winter. The expression of genes associated with oxidative stress and microbial control remained constant in the HPG; both defensin-1 and GOX production decreased significantly overwinter consistent with a stable hive environment (Table 1); similar to other findings from healthy hives kept in cold winter conditions [113]. The abundance of *Nosema ceranae*, a destructive midgut parasite, also remained low and constant in the midgut overwinter (Table S3). The same Enterobacteriaceae and *Gilliamella* spp. found in dysbiotic WW samples were detected in CW samples, but at negligible prevalence and abundance, decreasing overwinter in the worker hindgut [8].

Comparing warm to cold winter environments

To provide context for the comparison of CW to WW overwintering environments, we reference the estimated age structure associated with the diutinus (KA) marked cohort. The outdoor overwintering environments experienced by KA and WW bees were highly similar, in southern AZ, subject to diurnal cycles and access to the pollination environment. However, the sampling associated with WW colonies was random with respect to age, while KA sampling was both age and time-specific. The collection of 19/33 day-old bees in early/mid-winter likely sampled many foragers, and some destined to live a longer “diutinus” life. Consistent with foraging behavior [13] the 19/33 day-old bees contained significantly more *Gilliamella apicola* and *Gilliamella* spp. compared to randomly collected in-hive bees from the same environment. The 50/70 day old bees sampled in mid-late winter were clearly diutinus bees expressing HPG enzymes associated with plentiful storage proteins [74], and microbial challenge. Independent of age, the midguts of both KA and WW treatment groups in late winter were overgrown with Enterobacteriaceae and *Gilliamella* spp. (Figs. 3 and 5). However, host immune gene expression and fungal abundance differed significantly between KA and WW samples. The mouthparts of diutinus (KA) bees showed a significant reduction in fungal load with age (Fig. 3), and this pattern was repeated in the hindgut (ileums and rectums) of these same bees [8]. In contrast, fungal load increased significantly overwinter in the midguts of randomly sampled WW bees (Fig. 6), concurrent with significant fungal increases recorded for the ileum and rectum [8]. Based on these results we suggest that longevity-associated (diutinus) physiology and associated HPG gene expression suppresses fungal growth and bacterial diversity throughout the entirety of the diutinus worker alimentary tract.

According to rough estimates from Maes et al.[8] the average age of workers in the WW colonies was < 30 days of age, and less than 10% of introduced workers in the KA cohort lived > 50 days. The younger bees that emerged during the winter in southern climates may have lacked the nutritional resources needed to produce the diutinus phenotype, although we did not control for nutrition. A question that emerges from our results would explore how conflicting proximal cues may affect colony division of labor under warm winter conditions. In the same colony, do particular genotypes nurse and forage while others enter the diutinus state? Our experiment would have benefitted by classifying the physiology and social role of diutinus bees and randomly collected cohorts, including what they had consumed and when, whether they were primarily nursing or foraging, and levels of storage proteins. It is also possible that variation in colony size, demography, and hive structure between KA and WW samples affected these results. In general, however, our results indicate greater microbial exposure and less microbial control in the warm outdoor environment (Fig. 2). The stark difference between CW and WW samples in gut bacterial diversity, microbiota structure and fungal load overwinter may be associated with early winter nutrition and colony function [73, 74].

Aging and opportunism in the midgut

The collective information suggests that the midgut is a likely target for opportunistic fungi and bacteria overwinter in southern climates. Based on absolute abundance, Enterobacteriaceae was the dominant bacterium in the midgut of both outdoor southern experiments sampled in late winter regardless of age or host gene expression. Enterobacteriaceae are part of the colony microbiota (Fig. 7) and many are

associated with disease and overwintering [43]. In both the KA and WW samples, blooms of Enterobacteriaceae and an undescribed *Gilliamella* spp. dominated the midgut microbiota of many individuals and colonies (Figs. 3 and 5). The hindgut (both ileum and rectum) of these same KA and WW samples also experienced a significant increase of the same two OTUs, but they were < 1% of the hindgut microbiome cell count [8]. The abundance of these two putative opportunists was strongly inter-correlated ($R_s = 0.78$, $p = 0.0001$), and both were strongly associated with significant overwintering increases of total fungi and bacterial diversity abundance throughout the entire gut[8]. The relationships of putative opportunists with host gene expression were similar to those for the pervasive midgut pathogen *Nosema* (Table S1) supporting the hypothesis of opportunism.

Our results suggest that the Enterobacteriaceae OTU detected by this study can survive at low abundance overwinter on the mouthparts of aging honey bees. It is readily transmitted, and present on 97% of worker mouthparts > 19 days of age at < 1% average abundance (Table S2). From the same individuals with midgut infections, Enterobacteriaceae also established in the hindgut, but at very low relative abundance [8]. This scenario suggests that infection starts in the midgut where microbial competition is low, then spreads to the hemolymph and/or hindgut. Similar to the five core-hindgut phylotypes, the abundance of Enterobacteriaceae in the midgut was correlated positively with GOX and SOD gene expression but the same host-microbe relationship on the mouth was modestly negative, indicating that the mouth and associated HPG secretions may act as a first line of defense against invaders (Table 2). We hypothesize that most microbes from the mouth and midgut are part of the core colony microbiota having evolved physiology to counter ROS, and strategies to exploit the hive and social environment (Fig. 7). Larger mouthpart microbiomes are associated with *P. apium*, which multiplies rapidly in response to royal jelly or propolis exposure [4, 32]. The queen mouth and midgut are exposed constantly to royal jelly, and are dominated by *P. apium* [33]. However, it seems that Enterobacteriaceae can evade honey bee defenses on the mouth and crop, then establish in the midgut (Figs. 3 and 7). Labeled the honey bee assassin [45], *Serratia marcescens* was abundant in the midguts of two 70-day-old worker bees. Like many other Enterobacteriaceae found in the colony environment, *Serratia marcescens* and *Enterobacter* spp. are chitinolytic[45], which may provide a growth advantage once they attain the midgut environment.

In early adult life, bacterial establishment in the midgut is low relative to the available surface area likely because midgut pollen digestion involves continuous shedding of peritrophic membrane from the epithelium creating an unstable microbial niche [114]. Following pollen digestion however, the production of peritrophic membrane slows or stops [101, 115], and it is unknown how the midgut regulates microbial growth with age. There is new evidence that the diutinus phenotype will continue to consume available pollen or artificial pollen substitutes overwinter (Randy Oliver pers. comm). The anterior midgut produces a peritrophic gel [116] that may be more vulnerable to opportunism than the posterior midgut, but this requires further study. The anterior midgut is significantly more acidic than the posterior midgut[27] favoring core hindgut bacteria. Our diutinus sample set began at day 19, when it is likely that all bees of this age have transitioned from nursing to either foraging or a diutinus state. As diutinus bees aged, bacterial load remained stable on the mouthparts, increased significantly in the midgut, remained stable in the ileum, and decreased in the rectum (Fig. 4), [8] suggesting decreased pollen volume in the rectum,

but we did not account directly for this variable. Regardless of age, we discovered that the midgut microbiota can be volatile in late winter harboring 10^5 - 10^6 bacterial cells when healthy, but 10^7 - 10^9 cells when dysbiotic (Fig. 6). These midgut microbiotas showed two general enterotypes; smaller microbiotas with somewhat even distributions of 5–9 OTUs and significantly larger microbiotas dominated by one or two OTUs. Collectively this suggests that the midgut of late winter bees may be more vulnerable to microbial opportunism following a transition in midgut physiology, and if not countered by host gene expression, bacterial and fungal opportunism may contribute to premature senescence of workers and colonies.

Conclusion/perspective

The evolution of the gut microbiome in corbiculate bees tracks social evolution [117], and the shift to a perennial lifestyle has refined the function of both hindgut and colony microbiota. Our findings for diutinus bees, microbiota and overwintering survival provides a novel perspective on health and disease in the social group context. Climate controlled indoor wintering was remarkably stable according to our metrics, suggesting that all sampled worker bees had effectively transitioned to the diutinus phenotype. In contrast, colonies kept outdoors in southern environments suffered dysbiosis that proliferates in the midgut. Workers born during the winter in southern environments were susceptible to opportunism, while workers funded by fall nutrition were expressing the diutinus phenotype (queen-like physiology) to some degree to finance an environment of individual and social immunity [102, 118, 119]. At the colony level, the cost/benefit ratio associated with this phenotypic expression may be a major factor in overwintering survival.

There are many potential explanations for the host-microbial interactions presented here (Table 1, Table S1). We have provided one parsimonious perspective evoking colony dynamics in response to environmental conditions. Our hypothesis of colony-microbiome interaction is supported by demonstrated host behavior and physiology [56, 119, 120] and a growing understanding of beneficial, opportunistic and pathogenic honey bee microbes in the gut and colony environment [7, 13, 121]. We note that host niches and associated microbiotas are far more complex than discussed, and that the confirmation of a core colony microbiota will require further iteration and experimentation. Given the variety of social behaviors and microbes throughout the social environment [122], the behavioral and physiological processes associated with microbial odors or microbe-associated molecular patterns is an open question [68]. Hygienic worker behavior is presently defined by response to death-associated larval odors, and removal behavior by adult workers [123, 124]. A few large loci were associated with recognizing and removing dead larvae according to quantitative trait loci analysis [125]. More recent work suggests the involvement 73 candidate genes, many under positive selection, consistent with rapidly evolving complex social behavior mitigating the colony microbiome [126].

While the honey bee is a generalist pollinator, it is a specialist at controlling microbial growth, often considered a distinguishing characteristic of sociality [4, 18, 32, 70, 127]. Opportunistic (non-communicable) disease states in honey bees remain a mystery, but the tendency for diseased individuals

to leave the hive in self-sacrifice may be an indication of their socially adaptive significance [128]. The relationship of colony and gut microbiota with both individual and social immune function highlights the range of host-microbial interaction associated with the superorganismal honey bee microbiota, and its potential influence on colony health, disease resistance and gut integrity [121]. While selection for the holobiont is considered weak in most organisms, highly cohesive host-microbial systems with strong group selection, overlapping generations, reliable resource abundance and built colony structures might better fit the criteria of a holobiont [1, 2, 121, 129–131].

Abbreviations

WW: Warm Winter; CW: Cold Winter; KA: Known Age; HPG: Hypopharyngeal Gland; GOX: Glucose Oxidase; H₂O₂: Hydrogen Peroxide; ROS: Reactive Oxygen Species; Redox: Reduction /oxidation; MnSOD: Manganese Superoxide Dismutase; CuZnSOD: Copper-Zinc Superoxide Dismutase; AMPs: Antimicrobial Peptides; Vg: Vitellogenin; 16S rRNA gene: 16S subunit of the ribosomal RNA gene; AWK: Aho, Weinberger, Kernighan; SED: Stream editor; ANOVA: Analysis of variance; AZ: Arizona; BLAST: Basic local alignment search tool; bp: Base pairs; CLR: Centered log ratio; DNA: Deoxyribonucleic acid; FDR: False discovery rate; GLM: General linear models; MANOVA: Multivariate analysis of variance; OTU: Operational taxonomic unit; PCA: Principal components analysis; qPCR: Quantitative polymerase chain reaction; RDP: Ribosomal Database Project; rRNA: ribosomal ribonucleic acid.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials Next gene sequencing libraries of the mouth and midgut were deposited in GenBank under Sequence Read Archive (SRA) accession PRJNA742765. Associated with the same KA WW and CW sample sets, next gene sequencing libraries for the ileum and rectum were deposited in GenBank under Sequence Read Archive (SRA) accessions PRJNA705676 (WW and CW samples) and PRJNA705672 (KA samples).

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Authors' contributions KEA and PM conceived of and designed the research. PM contributed new analytical tools. KEA and PM performed the experiments. KEA and PM analyzed the data. KEA wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Experimental Design

Cold Indoor Wintering

16 colonies/ 6+ frames of bees
 Sampled in early/late winter
 Random worker sampling
 DV = Midgut microbiota
 DV = Social gene expression



Warm Outdoor Wintering

16 colonies /6+ frames of bees
 Sampled in early/late winter
 Random worker sampling
 DV = Midgut microbiota
 DV = Social gene expression



Warm Outdoor Wintering

Four observation hives in greenhouses
 Targeted sampling of marked bees
 Sampled workers aged 19, 33, 50 and 70 days
 DV = mouth and midgut microbiota
 DV = Social gene expression: The secretion of antimicrobial peptides and enzymes that control oxidative state



Experiment 1 (KA)

Known Age
 19 days
 33 days
 50 days
 70 days
 Compare Statistically

Compare Deductively

(CW)
 Cold
 Winter

Experiment 2
 Compare statistically

(WW)
 Warm
 Winter

Hyp. 1: Overwintering environment alters the microbiota
 Hyp. 2: Host social gene expression is associated with microbiota / overwintering environment

Hyp. 1: Colonies express the diutinus phenotype
 Hyp. 2: Aging workers maintain a stable microbiota
 Hyp. 3: Host social gene expression is associated with aging / character of mouth and midgut microbiota

Figure 1

Experimental design, sampling details and hypotheses. The blue boxes describe the manipulations performed in this study.

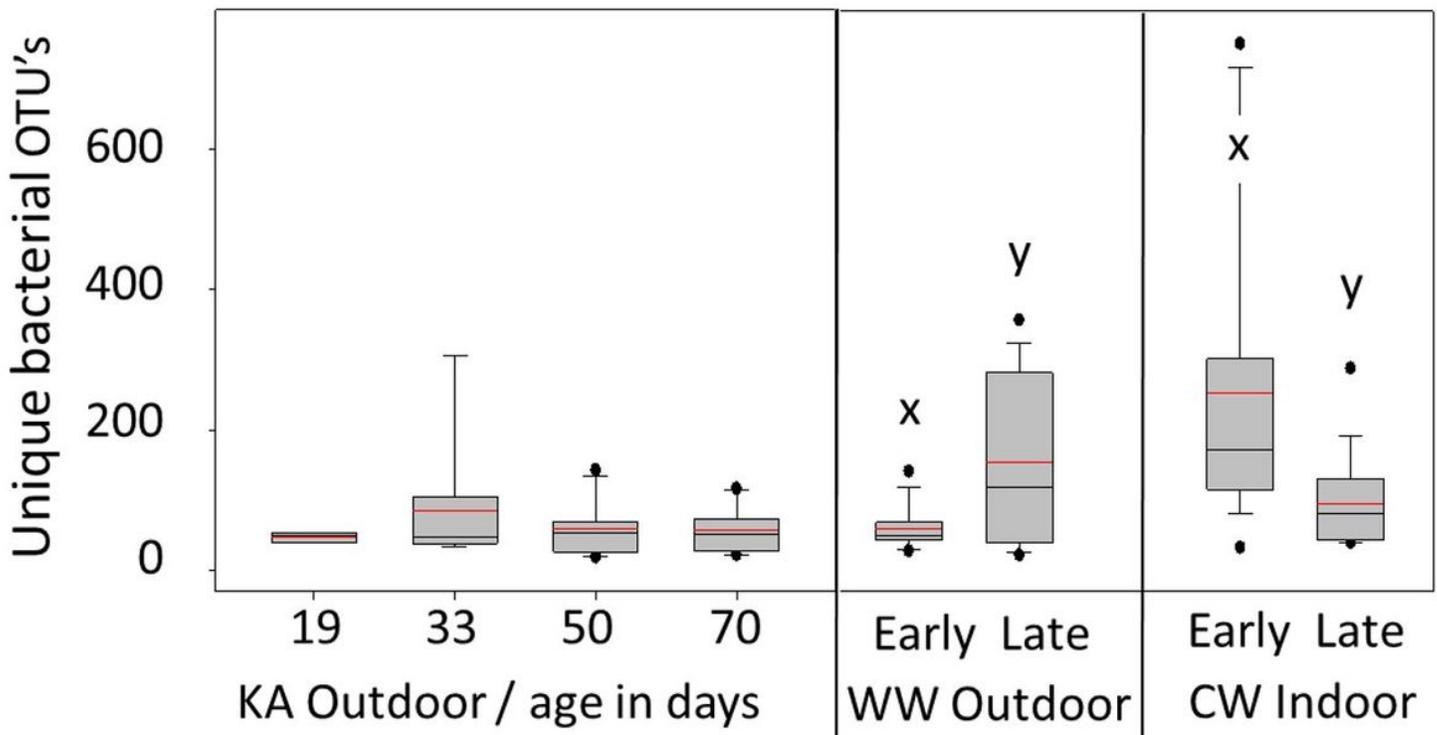


Figure 2

Bacterial diversity in midguts of overwintering bees depicted as the number of amplicon sequence variants or unique OTUs. Known age (KA) bees did not differ by age class overwinter. Diversity increased significantly in the warm winter (WW) outdoor environment ($t_{30} = 2.89$, $p = 0.007$) and decreased significantly in the cold winter (CW) indoor environment ($t_{32} = 2.89$, $p = 0.008$). Grey boxes contain 75% of the variation, whiskers contain 90%, and the dots are outliers. The horizontal red bar is the mean and black is the median.

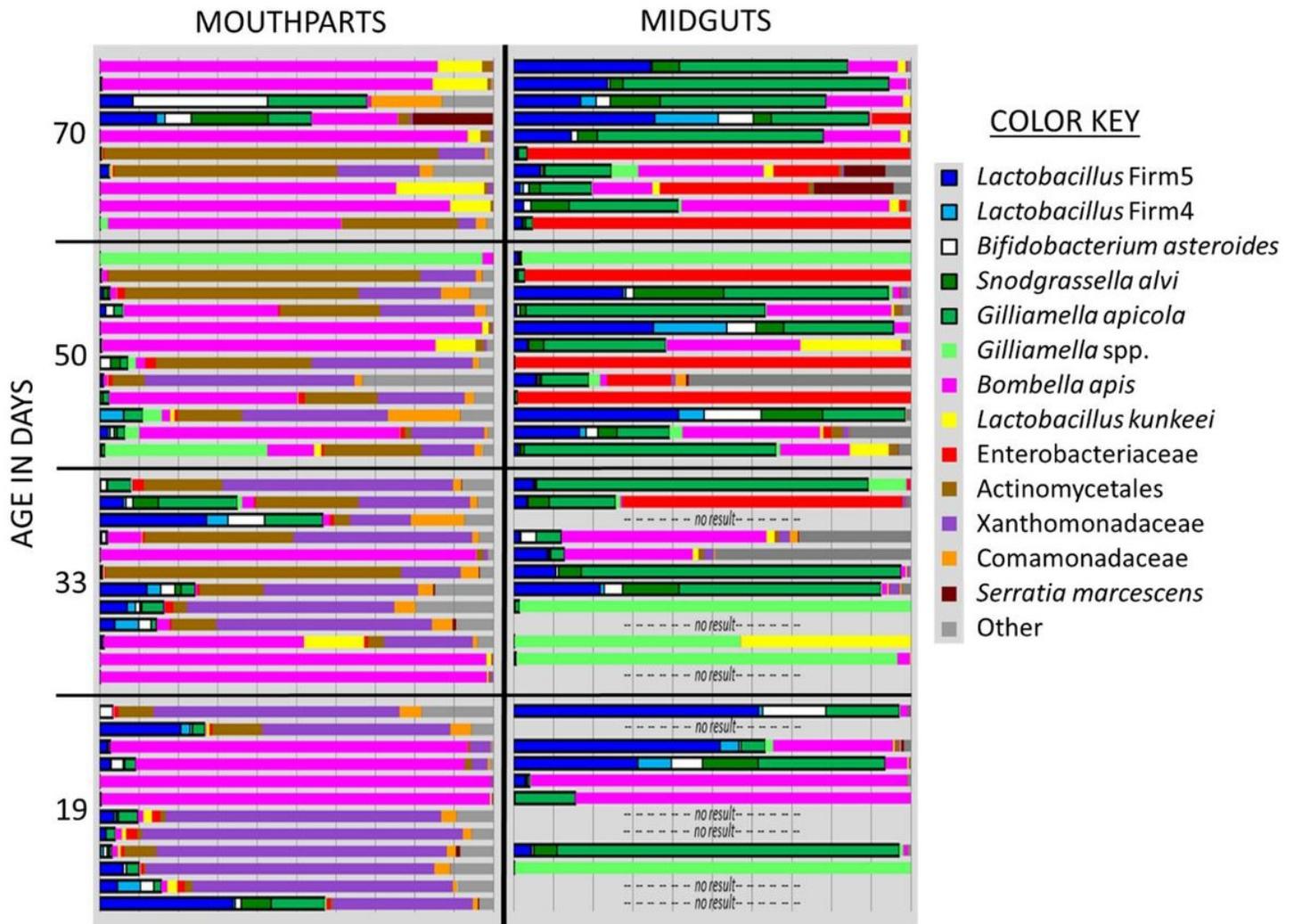


Figure 3

Relative OTU abundance for mouthpart and midgut microbiotas of diutinus worker bees overwintered in Tucson AZ, USA. The five core hindgut bacteria at the top of the key are outlined with a black border. Ages on the y-axis coincide with sampling dates; Newly emerged bees were marked and introduced to colonies in Early December, then sampled in late December at 19 days old, mid-January at 33 days old, late January at 50 Days old and mid-February at 70 days old. Significantly larger microbiotas are associated with *P. apium* (Pink) on the mouthparts, and Enterobacteriaceae (Red) and *Gilliamella spp.* (GFP green) in the midgut. See Table S2 for absolute abundance.

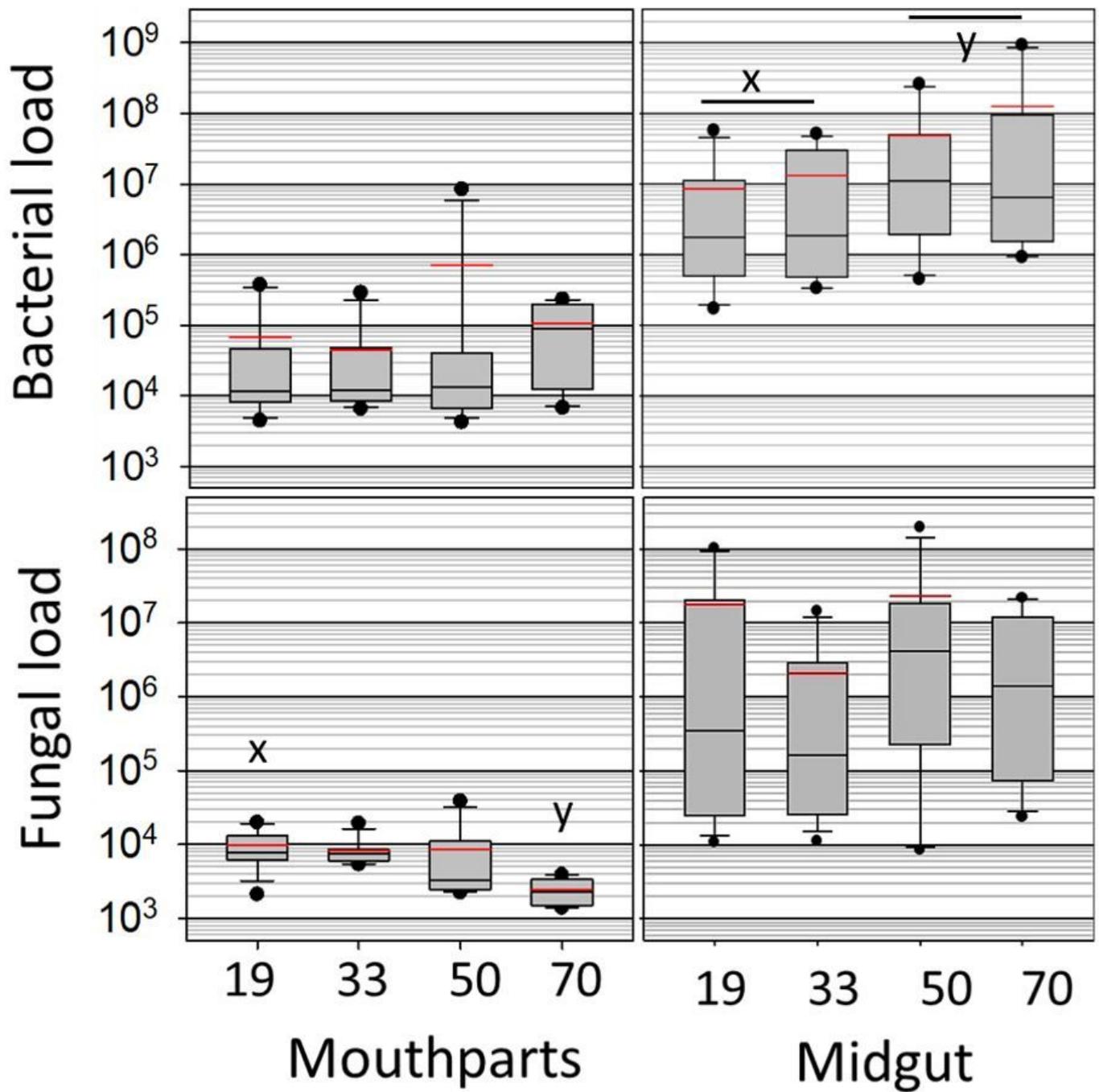


Figure 4

Change in microbial abundance on the mouthparts and in the midguts of diutinus worker bees. We collected known age KA samples at 19, 33, 50 and 70 days of age from early December to mid-February in Tucson AZ, USA. Fungal load decreased with age in the mouthparts ($W_{46} = 114$, $p = 0.0004$), and bacterial load increased with age in the midguts ($W_{38} = 166$, $p = 0.03$). The y-axis depicts mean 16S copy number (bacterial load) and mean 18S rRNA copy number (fungal load). Grey boxes contain 75% of the variation, whiskers contain 90%, and the dots are outliers. The horizontal red bar is the mean and black is the median. Bacterial load corresponds to Figure 3.

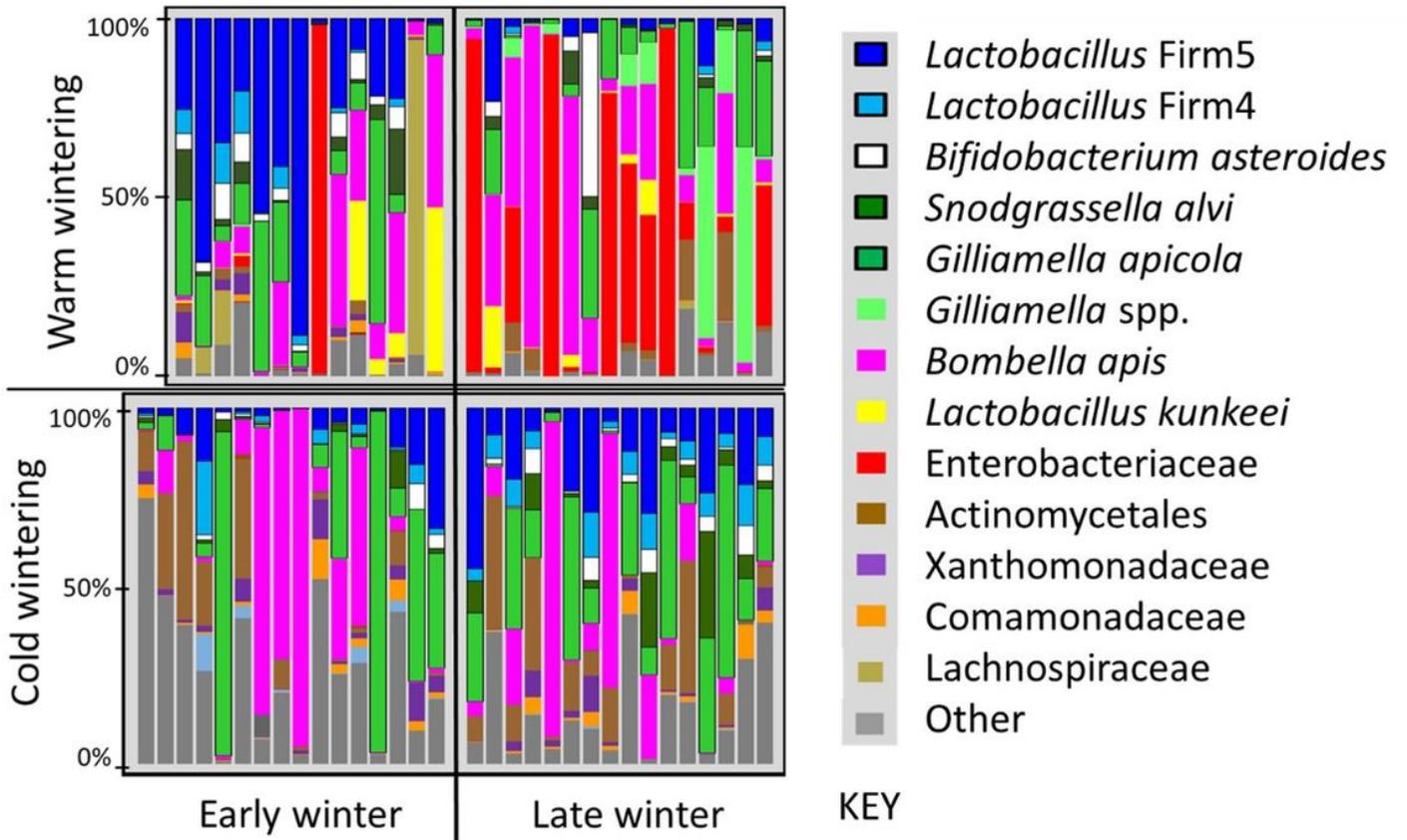


Figure 5

Relative abundance of midgut microbiotas (KEY = OTUs) of worker bees overwintered in an indoor climate controlled facility: “Cold wintering”, and an outdoor apiary in Southern Arizona, USA: “Warm wintering”. Each vertical bar is the midgut microbiota of a single bee from a different colony. Outlined in bold, the core five hindgut bacteria declined significantly in the warm outdoor environment, but increased significantly in the cold indoor environment. Significantly larger midgut microbiotas are associated with Enterobacteriaceae (RED) and *Gilliamella spp.* (GFP green). See Table S2 and results for more statistical details.

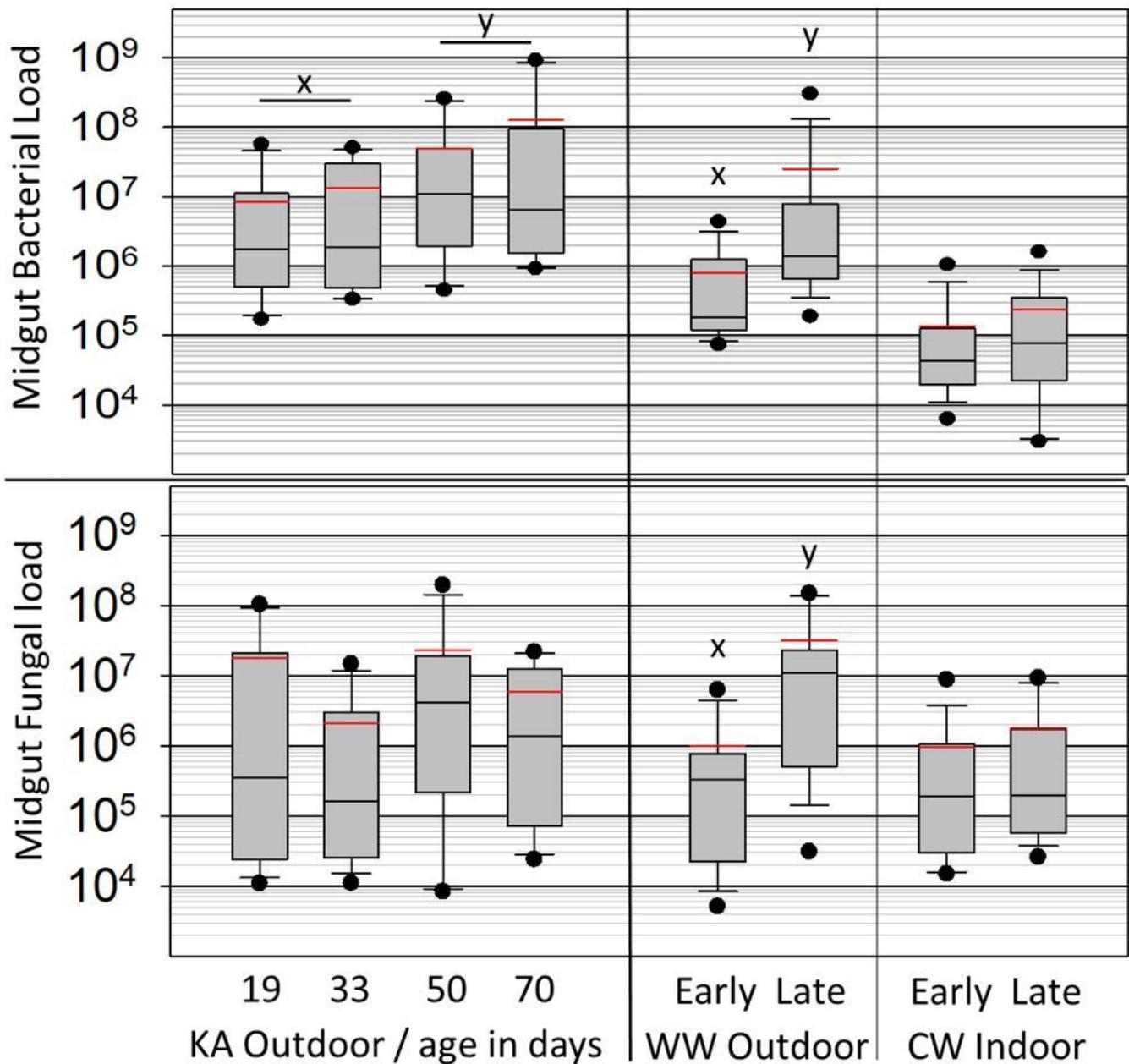


Figure 6

Bacterial and fungal load in the worker midgut of overwintering honey bees. The y-axis represents 16S rRNA copy number (bacterial load) and 18S rRNA copy number (fungal load). To provide reference, we repeat midgut microbial loads (from Fig. 4) of known age bee midguts. The WW samples differed significantly from early to late winter for both fungal load ($W_{32} = 42$, $p = 0.0008$) and bacterial load ($W_{30} = 43$, $p = 0.003$). See methods for more sampling details. Grey boxes contain 75% of the variation, whiskers contain 90%, and the dots are outliers. The horizontal red bar is the mean and black is the median. Bacterial load corresponds to microbiotas in Figure 3 (KA) and Figure 5 (WW/CW).

disease transmission and establishment. Note the distribution and abundance of other OTU's associated with mouthparts and midguts in the present study, including Xanthomonadales (D) and Actinomycetales (E) highlighted in grey.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.Hostmicrobialcorrelations.xlsx](#)
- [TableS2.Microbiotaabundanceandanalysis.xlsx](#)
- [TableS3.NosemaqPCR.xlsx](#)
- [TableS4.Immunegeneexpression.xlsx](#)