Gut and Colony Microbiota of Honey Bees: Social Immunity and Opportunism Overwinter

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Abstract

Overwintering is a major contributor to honey bee colony loss and involves factors that influence disease susceptibility. Honey bees possess a secretory head gland that interfaces with the extended social environment on many levels. 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.

Our results provide the first evidence that social immune gene expression is associated with diutinus bees, and highlight the midgut as a target of opportunistic disease overwinter. Host microbial interactions 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. The results are consistent with increased social immunity overwinter, including host associations with the colony microbiota, and a social immune response by long-lived diutinus workers to combat microbial opportunism. The cost/benefit ratio associated with limited expression of the diutinus phenotype may be a strong determinant of colony survival overwinter.

Introduction

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 environment³⁻⁶. The social context of disease susceptibility involves multiple factors that shape the evolution of life history, including resident microbial symbionts and resistance to opportunistic disease⁷⁻¹⁰. Many opportunistic disease states are associated with changes in core hindgut structure or the overgrowth of native colony microbiota, 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}. A primary function of the eukaryotic microbiome is protection from pathogens, and many factors may weaken the core microbiota rendering the host organism susceptible to disease¹²⁻¹⁶. In humans, the skin and nasal pharyngeal microbiome are considered the first line of defense against pathogen colonization and infection¹⁷. 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 social 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

various cocktails of 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 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 ^{31–34}. Similar to other model systems, the hindgut microbiota tracks the physiology of honey bee behavioral and reproductive phenotypes associated with age and nutritional state ^{30,35}. 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 ³⁶. Workers raised in stressed conditions 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 cocktails 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 ^{1,37–39}. 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 ^{40,41}. The HPG of workers can resume past physiological states associated with youth, or shift forward to assume the task of an older bee ⁴². The gland can also react more proximally to colony-level challenge associated with a variety of social processes like emergency queen rearing ^{43,44}. In addition to providing shared nutrition, jelly produced in the HPG may transmit immune training molecular patterns across generations, extend life expectancy, modulate microbiota structure and prevent opportunistic disease ^{44–46}. Gene expression associated with

individual and social immunity is costly, and only generated by well-nourished individuals^{38,47}. 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 resistance⁴⁸.

Honey bee colonies respond rapidly to environmental conditions. The proximal cue of artificial rainfall causes a shift to conservation physiology in a matter of hours⁴⁹. Similarly, forage dearth and 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^{50,51}. The presence of brood in the hive environment accelerates both behavioral and cellular senescence among worker bees³¹. After approximately 10 days of foraging, workers experience a sharp increase in mortality³¹.

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^{52,53}. 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 social immune status of diutinus workers overwinter^{54,55}. A recent investigation of the diutinus hindgut microbiota implied healthy overwintering physiology in cold, climate-controlled (7°C) indoor climates, but the proliferation of opportunistic bacteria associated with southern outdoor overwintering⁸. However, the putative 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. 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 and gut environment.

Results

Experiment 1: Known age cohort

To compliment previous results from the hindgut⁸, we deep-sequenced the mouthpart and midgut microbiota of aging worker bees overwintered in southern Arizona. As the winter progressed, we sampled known age (KA) diutinus workers 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). The mean number of unique OTUs detected on the mouth did not differ from that detected in the midgut and did not change with age ($n = 38$, mouth = 57, midgut = 62). The mouth and midgut environments contained many of the same bacteria, including all five of the core hindgut phylotypes (Fig. 3). 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. 3).

The mouthpart microbiota was comprised of *P. apium*, Actinomycetales, Xanthomonadaceae, *Delftia* (Comamonadaceae), an unknown *Gilliamella* spp., and *L. kunkeei*. Enterobacteriaceae occurred 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). The mouth microbiota of the aging bee (KA) differed from early to late winter. 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 significantly with age (Supplementary Table S1). 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 (Fig. 4).

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 (Fig. 3). 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 S1). 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 (Supplementary Table S1).

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. 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 (Supplementary Table S2).

Experiment 1: Known age cohort gene expression

To examine the hypothesis that social 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 (Table 1, see Supplementary Table S3 for details). 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 oxidative state and AMP production increased significantly with age (Table 1). ROS associated gene expression had a strong and significant negative association with both bacterial diversity abundance and fungal load on the mouth. 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 (see Supplementary Table S4 for details).

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

Change (Δ) overwinter	Warm Winter Known age*			Warm winter Random sample			Cold winter Random sample		
Gene	Z	p	Δ	Z	p	Δ	Z	p	Δ
Nutritional State									
Vitellogenin	0.69	0.49	NC	-2.00	0.05	↓	0.42	0.68	NC
Antimicrobial Peptides									
Hymenoptaecin	2.69	0.007	↑	-1.52	0.13	↓	1.34	0.18	↓
Defensin-1	-0.67	0.53	NC	1.62	0.11	↓	2.18	0.03	↓
Abaecin	2.87	0.004	↑	-2.21	0.03	↑	1.42	0.16	↓
Oxidative State									
Glucose Oxidase	2.80	0.005	↑	0.02	0.98	NC	2.05	0.04	↓
Cu/ZnSOD	5.14	0.0001	↑	-0.58	0.56	NC	-0.18	0.86	NC
MnSOD	5.57	0.0001	↑	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 S3 for details.									

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. The strongest positive relationship within this core group was *Snodgrassella alvi*, an obligate aerobe. 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 were strongly and positively correlated with the group of five core hindgut bacteria, as was *GOX*, a pro-oxidant widely considered the social immune gene (see Supplementary Table S4 for details).

Relative to *actin*, the expression of two of three measured AMPs increased significantly with age, *hymenoptaecin* and *abaecin* (Table S4). 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 midgut. *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 S4).

Experiment 2: Overwintering climate comparison

We sequenced the midgut microbiota of worker bees overwintered outdoors in southern Arizona or indoors in Idaho 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 manuscript⁸. 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. 5, 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 significantly overwinter in WW bees and decreased in CW bees.

Although separated by 1000 miles, WW and CW midgut environments were comprised of the same OTUs, including all five core hindgut phylotypes, *P. apium* = (*Bombella apis*), *L. kunkeei*, Actinomycetales, Xanthomonadaceae, and *Delftia* (Comamonadaceae). The latter three OTUs had 100% prevalence and relatively even abundance across both groups (Fig. 5). Core hindgut bacteria increased significantly in the midguts of CW bees overwinter, but decreased in WW bees (Fig. 5, Supplementary Table S1).

The midgut microbiota from early to late winter differed between WW and CW climates. The CW midguts did not differ from early to late winter when comparing OTU absolute abundance and correcting for multiple comparisons (Supplementary Table S1). 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. 6). Overwinter the CW colonies experienced 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, colonies overwintered in a warm outdoor environment changed drastically for both measures (Figs. 6 and 7).

Rare or absent in the CW samples, Enterobacteriaceae and an unknown *Gilliamella* spp. were abundant in the midguts of late WW samples (Fig. 7). 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 (Supplementary Table S1). Enterobacteriaceae and the *Gilliamella* spp. accounted for the vast majority of the bacterial increase in midgut microbiota size overwinter (Fig. 7). 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. 5), 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. 6). 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 absolute abundance, and relative to the total community (Supplementary Table S1).

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.

Levels of *Nosema ceranae* differed by overwintering climate. 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 (see Supplementary Table S2).

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). *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 (Supplementary Table S4). *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 (Supplementary Table S4).

In the WW outdoor environment, HPG expression differed from early to late winter for two of seven genes (Table 1, Supplementary Table S3). 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. *Abaecin* expression was negatively associated with Xanthomonadaceae abundance in the midgut overwinter, but positively associated with Enterobacteriaceae (Supplementary Table S4). *Hymenoptacin* and Defensin-1 expression decreased markedly, but not significantly from early to late winter (Table S3). Both were negatively correlated with the midgut increase in *Nosema ceranae* abundance. *Hymenoptacin* expression was negatively correlated with bacterial diversity, fungal abundance and Actinomycetales. 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 (Table 1 and Supplementary Table S4).

Discussion

We describe a novel relationship in honey bees involving the colony microbiota, the hindgut microbiota, and social gene expression overwinter. We suggest that the hypopharyngeal gland (HPG) performs a vital defensive role in diutinus (long-lived) honey bees, on par with its established role in providing shared nutrition for colony members during periods of growth. Our results reveal associations of microbiota and social immunity by overwintering environment and highlight the potential role of HPG secretions in mitigating the colony microbiota and shaping the gut microbiota overwinter. Our results are similar to host-microbial dynamics seen in other social insect species including host secretions that nurture defensive symbionts and reduce colony pathogens^{2,6,47,56}. We suggest one mechanistic hypothesis for the lack of immune genes in the honey bee genome⁵⁷; the introduced and resident microbiota may be mitigated in part by the redox potential of the individual and the group^{1,27}.

Our results correspond 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 here⁸. It is clear from these and other results that physiologically distinct gut niches are altered synchronously by opportunism or perturbation^{8,14}. Fungal abundance and bacterial diversity decreased significantly and collectively on the mouthparts, ileums and rectums of aging diutinus bees⁸, (Fig. 4), and showed a strong negative association with the expression of *GOX* and both *SOD* genes from the HPG. Resulting gene expression relative to the general microbial character and taxonomy of all four alimentary tract niches suggests a systemic host-microbial dynamic reflected throughout the alimentary tract.

All cold winter (CW) colonies overwintered successfully, and showed a broad collection of factors that distinguish them from the two warm winter apiaries. The entire CW gut microbiota (midgut, ileum, rectum) was either improved overwinter or not diminished (Fig. 5)⁸. Fungi remained low and constant overwinter throughout the entire alimentary tract (Fig. 6)⁸. Bacterial diversity throughout the gut decreased significantly (Fig. 2). 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 social environment and little microbial challenge (Table 1); similar to

other findings from healthy hives kept in cold winter conditions⁵⁸. The abundance of *Nosema ceranae*, a destructive midgut parasite, also remained low and constant in the midgut overwinter. The same two bacterial opportunists detected in warm winter apiaries also occurred in CW samples, but at negligible prevalence and abundance, decreasing overwinter in the worker hindgut⁸.

In contrast, colonies kept in warm wintering environments (KA and WW) experienced changes that suggest microbial opportunism and disease susceptibility that varied by age cohort. The midguts of both KA and WW treatment groups were overgrown with Enterobacteriaceae and *Gilliamella* spp. in late winter (Fig. 7). 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 bees⁸. In contrast, fungal load and bacterial diversity increased significantly overwinter in the midguts of randomly sampled WW bees (Figs. 2 and 5), concurrent with significant fungal increases recorded for the ileum and rectum⁸. According to our estimates of worker age, this randomly sampled cohort was < 30 days old. Based on these results we suggest that longevity-associated (diutinus) physiology and associated HPG gene expression suppresses fungal growth and bacterial diversity at the colony level, and may influence the hindgut microbiota over extended time scales.

Host immune response and various microbial metrics differed significantly between KA and WW samples (Table 1). The significant upregulation of *Vitellogenin* (*Vg*), *GOX*, *SODs* and *AMPs* in the HPG of diutinus bees is consistent with host response to a microbially challenging overwintering environment (Table 1). *Vitellogenin* is an indicator of nutritional state and a potent antioxidant⁵⁹, while superoxide dismutase (*MnSOD*, and *CuZnSOD*) are able to scavenge excess oxidants associated with metabolic demand or microbial challenge and convert them into less harmful molecules⁶⁰. Of these two, only *CuZnSOD* is secreted by the HPG⁶¹, while increased expression of *MnSOD* is an indication of increased mitochondrial function (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 response³⁸. *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. In general, however, the core hindgut bacteria 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 relationship²⁷ that likely facilitates hindgut microbiome establishment/maintenance and limits opportunism.

Social immunity overwinter differed by age and overwintering environment; *GOX* expression 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 dynamics 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 environment²⁷. We hypothesize that oxidative control of the gut is associated with the

increased abundance and structure of the core hindgut phylotypes in the midgut, as modeled for the ileum microbiota²⁷. In the cold indoor environment, *GOX* expression decreased significantly, while the abundance of core hindgut bacteria *S. alvi*, *G. apicola* and *B. asteroides* increased. In fact, all five of the core hindgut bacteria increased significantly as a correlated group in the CW midgut, while less abundant OTUs, and bacterial diversity decreased (Fig. 7). 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*^{62,63}. Several commensal bacteria, like *Lactobacillus* species secrete ROS into their surroundings, and this activity discourages the growth of other commensal organisms including fungi. The collective results indicate that CW colonies possessed a healthy microbiota, including low fungal abundance and low microbial diversity, and did not require a social immune response.

In the warm southern environment, *GOX* and *SOD* expression increased significantly overwinter in the HPG of older diutinus workers (KA samples), associated with a significant reduction of fungal abundance and bacterial diversity on the mouthparts. Older diutinus bees had a strong immune response and low levels of fungal infection and bacterial diversity, while younger bees (WW) sampled at the same time from the same environment appeared less resistant to opportunism. In the WW samples, comprised mostly of middle-aged bees born during the winter, *GOX* production was unchanged overwinter, concurrent with significant increases in *Nosema ceranae*, bacterial diversity and fungal abundance. Although both WW and KA were dysbiotic in late winter, the significant decrease in *Vg* expression in WW bees overwinter suggests they could not afford to mount an immune response. Although much older at 50-70 days of age, the long-lived diutinus (KA) workers possessed the molecular resources to mount a social immune response to bacterial and fungal opportunism (Table 1). We hypothesize that many of the microbial differences between older and younger cohorts overwinter can be explained by differences in social and individual immune expression, particularly the control of reactive oxygen species.

Concurrent with increased upregulation of *GOX*, *SOD*, *abaecin* and *hymenoptacin* in diutinus (KA) bees overwinter, the abundance of Xanthomonadaceae (an aerobe), fungal load, and bacterial diversity all decreased significantly on the mouthparts (Fig. 3, Table 1). Although *hymenoptacin* and *abecin* are active against a variety of gram negative and enteric pathogens including Enterobacteriaceae⁶⁴, their expression in the HPG was unassociated with the majority of bacteria in the midgut or mouth of diutinus bees. 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. *Abaecin* is highly effective against *Xanthomonas campestris*, a well-known plant pathogen⁶⁵. Known age diutinus bees showed a significant reduction of Xanthomonadaceae throughout the gut⁸ overwinter. 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 host response targeted towards a native colony opportunist but requires further study. 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 growth⁶⁶. There is a wide variety of native and relatively unknown Actinobacteria in the social environment of honey bee colonies²². 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.

The honey bee midgut is a likely target for opportunistic fungi and bacteria overwinter in southern climates. 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 (Fig. 7). 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 native colony microbiota, but are not core gut bacteria and many are associated with disease and overwintering⁶⁷. In both the KA and WW samples, blooms of Enterobacteriaceae and an undescribed *Gilliamella* spp. dominated the midgut microbiota of many individuals and colonies (Fig. 7). 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 gut fungi and bacterial diversity abundance throughout the entire gut⁸. 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 count⁸, suggesting the hindgut was largely uncompromised. The relationship of host gene expression with these two putative opportunists was similar to that for the pervasive midgut pathogen *Nosema*, supporting the hypothesis of opportunism.

The general relationship of oxidative stress with the honey bee gut microbiome merits further study. Oxidative stress can either deter or encourage opportunistic infection⁶⁸, and may become difficult to manage in the midgut of aging workers overwinter. Our results show that the Enterobacteriaceae OTU detected by this study can evade honey bee defenses on the mouth and crop, then proliferate in the aging worker midgut (Fig. 7). Labeled the honey bee assassin⁶⁹, *Serratia marcescens* was abundant in the midguts of two 70-day-old worker bees. While our Enterobacteriaceae OTU is undescribed, a variety of Enterobacteriaceae are found consistently throughout the colony environment. Among these, *Serratia marcescens* and *Enterobacter* spp. are demonstrated chitinolytic⁶⁹, which may provide a growth advantage once they attain the midgut environment. Similar to glucose oxidase GOX in honey bees, the NOX /DUOX enzyme system plays a key role in midgut mucosal immunity of *Drosophila* and *Anopheles* by generating ROS^{70,71}. This site-specific response prevents the overproliferation of midgut opportunists, and similar factors may influence gut microbial balance in the honey bee.

Conclusion

Our results suggests that the midgut of late winter bees are vulnerable to microbial opportunism following an age associated transition in physiology, and if not countered by host gene expression, such opportunism may contribute to premature senescence of workers and colonies. There are many potential explanations for the host-microbial interactions presented here. We have highlighted one parsimonious

perspective evoking colony dynamics in response to environmental conditions. Our hypotheses are supported by demonstrated host behavior and physiology^{32,72,73} and a growing understanding of beneficial, opportunistic and pathogenic honey bee microbes in the gut and colony environment^{7,13,74}. We note that host niches and associated microbiotas are far more complex than discussed, and that detailed experimentation is required to test hypotheses generated by this study.

Our findings provide 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 warm winter environments suffered dysbiosis that proliferates primarily in the midgut. Workers born during the winter in southern environments were susceptible to opportunism, while older 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^{73,75,76}. At the colony level, the cost/benefit ratio associated with this phenotypic expression may be a major factor in overwintering survival. Ultimately, our findings suggest that plasticity of lifespan evolution in honey bees was a major factor shaping host-microbial interactions at both the individual and colony level.

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 HPG 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 samples⁸. 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. 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 frames 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.

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. 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 spring/summer. In the companion study, we estimated that only 10% of introduced bees survived until 50 days of age⁸. 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 HPG 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 hindgut⁸.

Experiment 2: Overwintering climate comparison

In a second experiment, we hypothesized 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. 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^{50,51}. 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. 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 Enterobacteriaceae⁸.

DNA analysis

We performed microbial DNA analysis on 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 ^{77,78}. We amplified the 16s gene template using forward primer 27F (5'-AGAGTTTGATCCCTCAG-3') and reverse primer 1522R (5'-AAGGAGGTGATCCAGCCGCA-3'). 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 pCRTM2.1 TOPOTM 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 (acCMGGATTAGATACCKG) 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. ⁷⁹. Bioinformatic commands are available in the companion study⁸.

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 RNAlaterTM 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 ⁸⁰ for primer details], 3ul of molecular grade water and 2ul of template). Cycle conditions were a 95°C for 30 s and then 40x of 95°C for 20 s and 60°C for 30 s ⁸⁰. 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 ⁸¹. 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 ⁸¹ 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 ⁸², we converted bacterial abundances to ratio abundances among all OTUs ⁸³ using the software CoDaPack's centered log-ratio (CLR) transformation ⁸⁴. 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. The remaining OTUs (14-6294) were summed, and assigned a gene copy number of 4.2, the average for all bacteria⁸⁵. 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 across the entire data set.

Data availability

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).

Declarations

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Contributions

K.E.A. designed the experiment, performed the experiment, analyzed the data, and wrote the paper. P.M. performed the experiment, analyzed the data, and approved the final manuscript.

Ethics declarations

Competing interests

The authors declare no competing interests.

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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

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

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

Compare
Deductively

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

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 4.

Figure 4

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.

Figure 5

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. 3) of known age bee midguts. The WW samples differed significantly from early to late winter for both fungal load (W32 = 42, $p = 0.0008$) and bacterial load (W30 = 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 4 (KA) and Figure 6 (WW/CW).

Figure 6

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 Supplementary Table S1 and results for more statistical details.

Figure 7

Mean absolute abundance of the midgut microbiota by overwintering environment and sample set. Worker bees were sampled in early or late winter. Random samples are in-hive worker bees of unknown age selected from around the brood nest. Known age samples are aged 19/33 days (early) and 50/70 days (late). Values for each y-axis are presented in millions and differ significantly by sample set. See Methods for detailed information.

Supplementary Files

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

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