Journal of Insect Science, (2022) 22(2): 6; 1–5
https://doi.org/10.1093/jisesa/ieac016
Short Communication

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Classic Hoarding Cages Increase Gut Bacterial Abundance and Reduce the Individual Immune Response of Honey Bee (*Apis mellifera*) Workers

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Subject Editor: Julie Urban

Received 7 September 2021; Editorial decision 8 February 2022

Abstract

Laboratory experiments have advanced our understanding of honey bee (*Apis mellifera)* responses to environmental factors, but removal from the hive environment may also impact physiology. To examine whether the laboratory environment alters the honey bee gut bacterial community and immune responses, we compared bacterial community structure (based on amplicon sequence variant relative abundance), total bacterial abundance, and immune enzyme (phenoloxidase and glucose oxidase) activity of cohort honey bee workers kept under laboratory and hive conditions. Workers housed in the laboratory showed differences in the relative abundance of their core gut taxa, an increase in total gut bacterial abundance, and reduced phenoloxidase activity, compared to bees housed in hives.

Key words: microbiome, honey bee, immunity

Honey bees *Apis mellifera* L. (Hymenoptera: Apidae) harbor distinct gut bacterial communities that consist of 8–10 core bacterial taxa (Engel et al. 2012). The honey bee gut bacterial community, commonly referred to as the gut microbiome, contributes substantially to honey bee health and fitness (Raymann and Moran 2018). Due to the highly conserved nature of the honey bee gut microbiome, as well as the global importance of honey bees as managed pollinators, significant efforts have been made to understand how the honey bee gut bacterial community interacts with its host and the environment (Motta et al. 2018, Zheng et al. 2018, Castelli et al. 2020, Zhang et al. 2020).

Honey bee physiology and behavior are commonly studied in laboratory settings using hoarding cages (ventilated, translucent containers) (Williams et al. 2013). This method has been extended to analyze the effect of pesticides and pathogens on the abundance and composition of the honey bee gut microbiome (Li et al. 2017, Motta and Moran 2020). Honey bees naturally live in complex eusocial groups within highly regulated hive environments. The diet, social interactions, and behavior of honey bees housed in hoarding cages differ from honey bees under normal hive conditions (Brodschneider et al. 2017, Bosua et al. 2018). Therefore, it is possible that the laboratory environment alone could alter the composition and structure of the honey bee gut microbiome.

While laboratory studies are typically followed by field studies, it is valuable to know how representative laboratory studies are of field conditions, especially since laboratory housing alters the gut communities of other insects (Martinson et al. 2017, Tinker and Ottesen 2021). To our knowledge, the gut bacterial communities of honey bees kept under laboratory and hive conditions have not been previously compared. To fill this knowledge gap, we compared the structure (16S rRNA amplicon sequencing (Caporaso et al. 2011)) and abundance (qPCR for total bacterial abundance (Fierer et al. 2005)) of honey bee gut bacterial communities from cohort honey bee workers housed in laboratory and field conditions. Since the gut microbiome can regulate the honey bee immune response, we also measured phenoloxidase (POX) and glucose oxidase (GOX) activity, indicators of individual and social immunity respectively (White et al. 1963, Lovallo and Cox-Foster 1999). Our goal in measuring indicators of immune activity was to explore potential variation in host physiology that may be associated with microbiome shifts. We predicted differences in the structure and abundance of the gut bacterial communities, as well as variation in immune activity, of honey bees maintained under laboratory and field conditions, and tested this prediction in a series of two experimental trails in 2014 and 2016.

Materials and Methods

In two separate trials (September 2014 and 2016), frames of capped brood were collected from colonies maintained by Virginia Tech's

Department of Entomology, Blacksburg VA USA. Frames were collected from 4 hives in 2014 and 5 hives in 2016, and held overnight at 31°C with 50–80% humidity. After incubating for less than 24 hr, newly emerged bees were marked on the thorax with Testors enamel paint, and randomly divided into cage and hive treatment groups. Bees in the hive treatment were returned to their respective hives for 7 d. We collected newly emerged bees and analyzed gut microbiome structure after 7 d to ensure the bees had an established microbiome (Powell et al. 2014) and control for age-related differences in the gut communities (Dong et al. 2020).

Bees in the cage treatment were separated based on hive into 'classic' 9 × 9 × 8 cm hoarding cages (Williams et al. 2013) with approximately 30 marked bees per cage. Cages were maintained in a dark incubator at 31°C with 50–80% humidity for 7 d, and were fed 50% (w:v) sucrose solution and Ultra Bee Pollen Substitute from Mann Lake Ltd (as a pollen paste) in 1.5 ml centrifuge tubes every 2–3 d. After 7 d, marked bees from the hives and cages were euthanized with CO_2 and frozen for gut community DNA extraction and immune assays.

Five whole guts were dissected per hive per treatment using a sterile technique, and pooled in individual tubes containing 180 µl DNA lysis buffer (20 mM Tris-HCL pH 8, 2 mM EDTA pH 8, and 1.2% Triton-x-100) and lysozyme (20 mg lysozyme/1 ml lysis buffer). Pooled samples were homogenized using sterile plastic pestles and then incubated at 37°C for 1 hr. The Qiagen DNeasy Kit (Qiagen, Germantown, MD) protocol was used to extract DNA, with final elution in 100 µl molecular grade water. Gut bacterial community structure was assessed with 16S rRNA gene amplicon sequencing of the V4 region using primers 515F and barcoded 806R (Caporaso et al. 2011). Samples were pooled at equimolar concentrations after quantification with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA), and 100 µl of the pooled sample was cleaned using the Qiagen QIAquick PCR purification kit (Qiagen, Germantown, MD). The multiplexed sample was sequenced as part of a run on an Illumina MiSeq platform using a 250 bp single-end strategy at the Dana Farber Cancer Institute of Harvard University. Sequences are available through the Virginia Tech Data Repository.

The single-end 16S rRNA amplicon sequences were demultiplexed and quality filtered in QIIME2 (v.2019.1) (Bolyen et al. 2019). The default filtering parameters in DADA2 (Callahan et al. 2016) were used to trim reads to 250 bp, correct read errors, and filter out chimeric and PhiX sequences, except that we truncated reads with *q*-score <11 and used 10,000 reads to build the error distribution, which we have found is adequate for our high-quality sequence data. We then filtered out any amplicon sequence variants (ASVs) that comprised less than 0.005% of the total reads. We assigned taxonomy to the remaining ASVs with the SILVA database (v.13.2) (Quast et al. 2013), and removed ASVs classified as chloroplast and mitochondria or that were unassigned. The data were then rarefied to 55,000 reads/sample to standardize read depth across the samples. The final ASV table used for our analysis contained 103 unique ASVs across 18 samples.

Total gut bacterial abundance was estimated in 2014 using a 16S rRNA quantitative PCR (qPCR) assay similar to Fierer et al. (2005). Assays were run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Labs, Hercules, CA) with 15 µl reactions containing 3 µl PCR water, 7.5 µl SSoAdvanced Universal SYBR Green Supermix (Bio-Rad Labs), 0.75 µl of each primer (10 µM), and 3 µl template DNA. PCR conditions were 10 min at 95°C, and 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Samples were run in duplicate, and no template controls were run in triplicate. A standard curve was generated with triplicate reactions of 10-fold dilutions of plasmid DNA [10^8–10^2 gene copies; gene fragment inserted in pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA)]. Sample gene copy numbers were calculated from the standard curve, and replicate values were averaged.

GOX and POX assays were conducted to assess immune activity. POX assay methods were modified from Laughton and Siva-Jothy (2011), and previously performed by Reeves et al. (2018) and O'Neal et al. (2019). The bees used for the POX and GOX assays were different individuals than those used in the gut microbiome analyses.

POX assays were performed on hemolymph of ten bees from each experimental group (i.e., ten bees from the five individual hives and five individual hoarding cages). Perfusion bleeds were performed on live bees using microcaps inserted under the dorsal abdominal sclerites near the dorsal vessel. We measured the change in optical density over time at 490 nm in 15-s intervals for 60 min using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA), and standardized using the total protein concentration of each sample determined from a bovine serum albumin standard curve. Mean optical density of the ten samples was calculated for each group, and used as an indicator of individual immune activity for the respective group.

Similarly, GOX assays were performed on pools of ten worker heads from each treatment group. Methods for the GOX assays were modified from Alaux et al. (2010), and previously performed by Reeves et al. (2018) and O'Neal et al. (2019). GOX activity was measured at 430 nm in 15-s intervals for 90 min using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA), and standardized using the total protein concentration for each bee head, determined from a bovine serum albumin standard curve. Mean optical density of the ten samples was calculated, and used as an indicator of social immunity for the respective group.

Statistical analyses were performed in R (v.1.2.1335) (R Core Team 2020) using the Phyloseq package (v.1.30.0) (McMurdie and Holmes 2013). We used an alpha level of 0.05 for statistical significance, while values slightly higher were considered trends. We used two metrics of alpha diversity to compare the gut bacterial communities of bees across the two treatments: richness (the number of ASVs) and the effective number of species (Hill numbers, which are calculated from the Shannon Index as [exp(Shannon)], Jost 2006). Differences in richness and Hill numbers among treatment groups, study years, and their interactions were assessed using a two-way analysis of variance (ANOVA).

We also compared community structure between caged and hive bees using two metrics of beta diversity: Bray–Curtis dissimilarity (to assess differences in ASV relative abundance) and Jaccard dissimilarity (to assess differences based on presence or absence of ASVs). Before analysis, we normalized our data by converting raw ASV counts to proportions to obtain relative abundance values. Bray–Curtis and Jaccard dissimilarity matrixes were generated, and the PERMANOVA function (adonis2; vegan package v2.5-6 (Dixon 2003); based on 999 permutations) was used to assess differences across treatment groups, study year, and their interaction.

Total gene copy numbers, POX, and GOX group means were compared using an independent samples *t*-test. Total gene copy numbers were log transformed before analysis.

Results

The bacterial communities were dominated by ASVs in genera that make up the core honey bee gut microbiome (Raymann and Moran 2018), including *Lactobacillus* (Lactobacillales: Lactobacillaceae),

Snodgrassella (Neisseriales: Neisseriaceae), *Bifidobacterium* (Bifidobacteriales: Bifidobacteriaceae), *Gilliamella* (Orbales: Orbaceae), *Frischella* (Orbales: Orbaceae), and *Fructobacillus* (Lactobacillales: Lactobacillaceae) (Fig. 1). To assess whether the laboratory environment impacted the within sample bacterial diversity, we analyzed bacterial richness (number of ASVs), and the effective number of species (Hill numbers) based on treatment, year, and their interaction. Richness did not vary based on treatment (ANOVA, $p = 0.942$; Fig. 2A), but differed between years (ANOVA, $p = 0.003$); there was no interaction between the variables ($p = 0.14$). There was a trend for the effective number of species to be lower in caged bees (ANOVA, $p = 0.051$: Fig. 2B). Year also had a significant effect on the effective number of species (ANOVA, $p = 0.001$), and there was no significant interaction between treatment and year ($p = 0.350$).

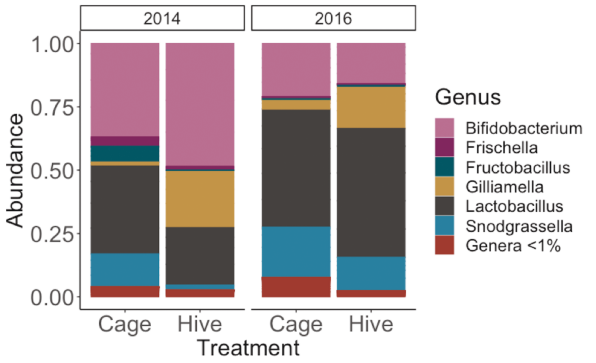

Fig. 1. Relative abundance of bacterial genera across cage (mean relative abundance: *Bifidobacterium* = 28.1%, *Frischella* = 2.13%, *Fructobacillus* = 2.78%, *Gilliamella* = 2.67%, *Lactobacillus* = 41.6%, *Snodgrassella* = 16.9%, Genera <1% = 5.82%) and hive (mean relative abundance: *Bifidobacterium* = 30.4%, *Frischella* = 1.12%, *Fructobacillus* <1%, *Gilliamella* = 19.4%, *Lactobacillus* = 38.3%, *Snodgrassella* = 8.15%, Genera <1% = 2.6) treatments and sampling year. Communities were dominated by six taxa known to be part of the core honey bee gut microbiome. Genera whose relative abundance comprised less than 1% were grouped together for visualization.

To assess whether gut bacterial community structure varied between cage and hive bees and between years, we used two distance metrics: Bray–Curtis, which considers the relative abundance of taxa (ASVs), and Jaccard, which only relies on taxa (ASV) presence/absence. We used permutational multivariate analysis of variance (PERMANOVA) to assess differences based on treatment, year, and their interaction. While the composition of the bacterial gut community was not significantly different between cage and hive bees (Jaccard PERMANOVA, $p = 0.206$; Fig. 2C), there were trends toward a shift in the relative abundances of the taxa (Bray–Curtis PERMANOVA $p = 0.053$; Fig. 2D). Year explained significant variation for both distance metrics (Jaccard PERMANOVA, $p = 0.002$, Fig. 2C; Bray–Curtis PREMANOVA, $p = 0.002$ Fig. 2D), and there was a trend for an interaction between year and treatment for Bray–Curtis ($p = 0.058$), but not Jaccard ($p = 0.410$).

There were significantly more bacteria in the guts of caged bees (16S qPCR; Two sample *t*-test, $p = 0.034$; Fig. 2E), presumably due to the reluctance of the bees to defecate in the cages, as they would normally only defecate on cleansing flights outside the hive (Winston 1991). For our immune parameters, GOX activity (~social immunity) was not significantly different between cage and hive bees (Two-sample *t*-test, $p = 0.447$), but POX activity (~individual immunity) was significantly reduced in the caged bees (Two-sample *t*-test, $p = 0.002$; Fig. 3).

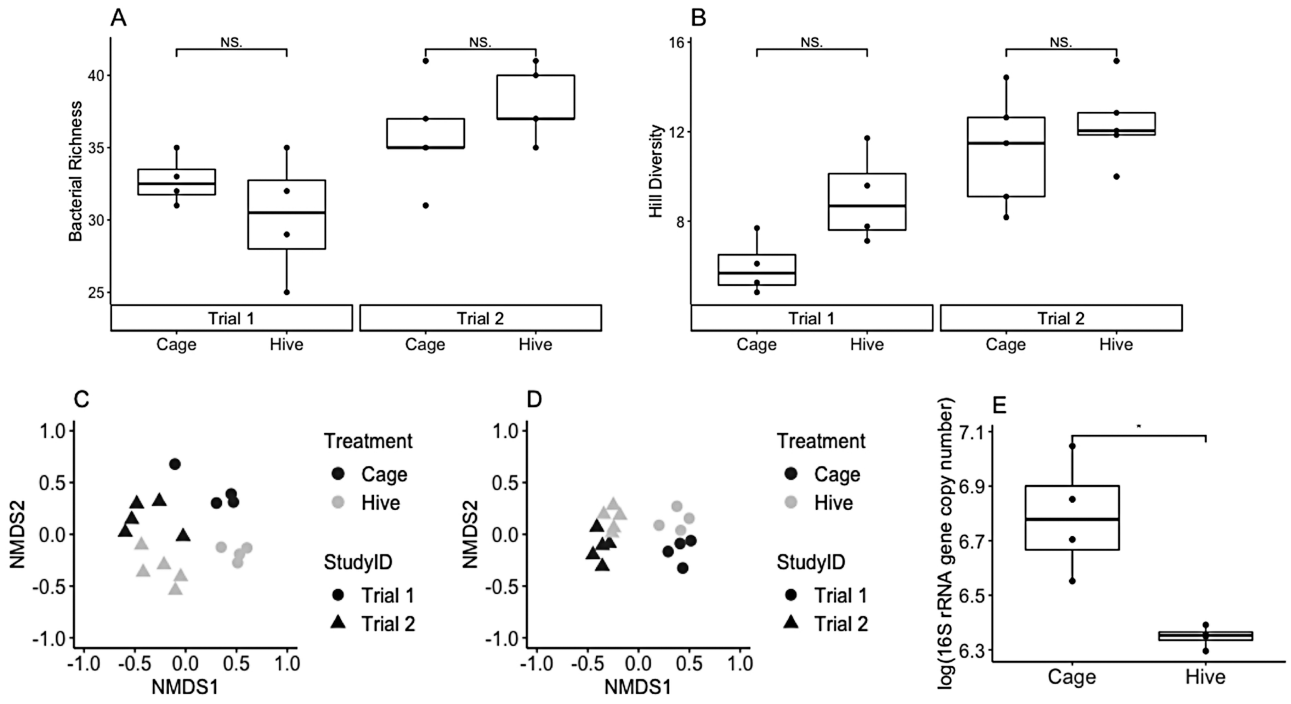

Fig. 2. Comparisons of gut bacterial community alpha diversity (A–B), beta diversity (C–D), and total bacterial abundance (E) between honey bee workers kept under normal field conditions (hive) or in classic hoarding cages (cage). A) Bacterial richness did not differ between bees from the cage and hive environments. B) Hill diversity was suggestive of a trend towards higher diversity in bees from hives. C) There was no difference in the presence/absence of bacterial taxa (Jaccard metric) in the gut communities of workers kept in cages or hives, but varied between experimental year. D) Relative-abundance based bacterial communities (Bray–Curtis metric) tended to differ between the cage and hive treatment, and varied between experiment year. E) Total gut bacterial abundance was higher in caged bees compared to bees from the hive. * $p < 0.05$.

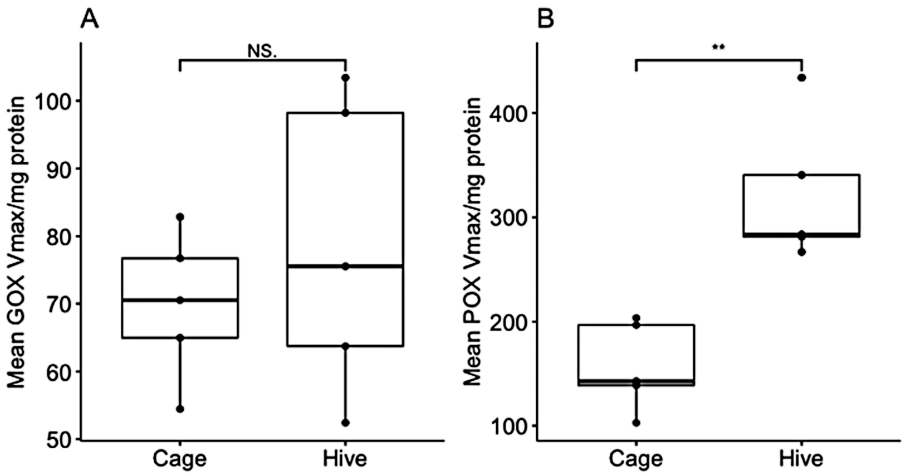

Fig. 3. A) Glucose oxidase (GOX) activity did not differ between cage and hive housed bees. B) Phenoloxidase (POX) expression was lower in caged bees compared to hive housed bees. ** $p < 0.001$.

Discussion

Our analysis revealed minimal differences in the gut bacterial community structure of cohort honey bee workers housed in laboratory and field conditions, although it is possible that there were some differences among gut regions that we did not detect with our whole gut analysis. We observed trends towards laboratory bees having lower effective number of species and different relative abundance of bacterial taxa compared to their field cohort, but neither of these trends reached statistical significance.

We did, however, observe that total gut bacterial abundance was significantly higher in the laboratory bees relative to the field bees. Honey bees typically will not defecate in hoarding cages (Winston 1991). The accumulation of feces in the hindgut likely caused the disparity in total bacterial abundance, and may have contributed to the compositional trends observed in the gut communities along with dietary differences, stress, and a lack of trophallactic food exchange with hive bees.

Our results also showed a significant reduction in the individual immune response of caged bees, represented by lower POX activity. Average GOX activity, an indicator of social immunity, was also lower in caged bees, but was not significantly different from the field average. Since we did not observe significant shifts in gut microbiome structure, it is unlikely that the reduction in POX activity was related to regulation by the gut microbiome. The presence of the gut microbiome stimulates aspects of the honey bee immune response (Kwong et al. 2017); therefore, it is also unlikely that the increase in total bacterial abundance in the caged bees was related to gut microbiome modulation.

Overall, our results suggest that the gut microbiomes of laboratory maintained honey bees may provide a reasonable representation of the community structure of honey bees under field conditions, but only when using metrics that do not directly account for total bacterial abundance. These data also suggest that isolated studies on the immune response of honey bees in laboratory settings may not be representative of field conditions. Although laboratory studies followed by or in conjunction with field studies produce the most reliable data, it is beneficial to understand the baseline variation that results under laboratory conditions, and should be considered to improve the applicability of laboratory results to field conditions.

Acknowledgments

We would like to thank Philene Vu for performing the immunological assays, and Hannah Miko for assisting with lab and field work. This work was supported by the Virginia Agricultural Foundation (Project number 677). We thank Virginia Tech's Open Access Subvention Fund for assistance with open access fees.

Author Contributions

RDF, LKB, and JBW designed the study. JBW and RDF set up the study and collected the data. JBW prepped samples for sequencing and completed qPCR. LKB processed the sequencing data. CLG performed the data analysis and prepared the first draft of the manuscript. CLG, RDF, LKB, and JBW edited the manuscript.

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