Journal of Medical Entomology, 59(2), 2022, 648–658
https://doi.org/10.1093/jme/tjab184
Advance Access Publication Date: 8 November 2021
Research

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Vector/Pathogen/Host Interaction, Transmission

Bacterial Communities of Lab and Field Northern House Mosquitoes (Diptera: Culicidae) Throughout Diapause

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Subject Editor: Theodore Andreadis

Received 13 August 2021; Editorial decision 6 October 2021

Abstract

Diapause is a hormonally driven response which is triggered by environmental cues that signal impending adverse conditions and prompts metabolic, developmental, and behavioral changes to allow survival until the return of favorable conditions. Microbial symbionts have been shown to influence the metabolism, development, and behavior of their host organisms, all of which are common diapause-associated characteristics. Surveys of bacterial components in relation to diapause have been examined in few systems, of which the species are usually inactive during dormancy, such as eggs or pupae. This is specifically intriguing as adult female diapause in *Culex pipiens* (Diptera: Culicidae) can last between 4 and 7 mo and females remain mobile within their hibernacula. Furthermore, it is unknown how microbiota changes associated with prolonged dormancy are different between the lab and field for insect systems. This study aims to characterize how the microbiota of *C. pipiens* changes throughout diapause under both field and lab settings when provided identical food and water resources. Based on these studies, *C. pipiens* microbiota shifts as diapause progresses and there are considerable differences between field and lab individuals even when provided the same carbohydrate and water sources. Specific bacterial communities have more association with different periods of diapause, field and lab rearing conditions, and nutritional reserve levels. These studies highlight that diapausing mosquito microbiota studies ideally should occur in field mesocosms and at multiple locations, to increase applicability to wild *C. pipiens* as prolonged exposure to artificial rearing conditions could impact metrics related to diapause-microbiome interactions. Additionally, these findings suggest that it would be worthwhile to establish if the microbiota shift during diapause impacts host physiology and whether this shift is critical to diapause success.

Key words: diapause, microbiome, *Culex pipiens*

Members of the plant and animal kingdoms are not considered to be stand-alone as they are in constant contact with microorganisms including bacteria, fungi, and viruses (Kiers and Denison 2008, Rodriguez et al. 2009, Mcfall-Ngai, 2013, Rey and Schornack 2013). Some organisms are associated with obligate symbionts that are essential for the host's survival; others harbor non-obligate symbionts that nonetheless improve or are critical to their host's status (Marshall et al. 2006, Neufeld et al. 2011, Ridley et al. 2012, Coon et al. 2014, Tapia et al. 2016, Didion et al. 2021). Within mosquitoes, bacterial residents have been implicated as important modulators of various life traits such as vector capability and larval development (Shin et al. 2011, Boissière et al. 2012, Chu et al. 2013, Nieuwdorp et al. 2014, Scully et al. 2014, Coon et al. 2016). Additionally, the microbiota has been linked to nutritional factors including lipid metabolism (Valzania et al. 2018a, Valzania et al. 2018b, Didion et al. 2021). As mosquitoes act as vectors for disease-causing pathogens, understanding how the microbiota influences their ability to survive, interact with the environment, and transmit these disease-causing agents is of the utmost importance (Cansado-Utrilla et al. 2021, Guégan et al. 2018). Development of axenic or mosquitoes with reduced microbial communities has proven useful in establishing the role of bacterial residents in relation to mosquito biology (Correa et al. 2018, Steven et al. 2021, Romoli et al. 2021).

Unfortunately, these relationships are difficult to establish without a baseline understanding of bacterial communities that are typically present for a given physiological state. Diapause is an alternative biological state that is commonly entered by certain mosquito species, and many other insects, to survive adverse environmental conditions

(Denlinger and Armbruster 2014). Undergoing diapause allows specific mosquito species to survive in temperate regions which are unfavorable for year-round habitation, leading to rapid repopulation upon the return of favorable conditions (Denlinger and Armbruster 2016). Diapause can be entered during the egg, larval, and adult stages in mosquitoes, though it is typically restrained to one life stage per species (Denlinger and Armbruster 2016). The link between diapause and the microbiota has not been well characterized in mosquitoes, or invertebrates in general, with few studies focusing on this subject and largely investigating early diapause (Almada et al. 2015, Liu et al. 2016, Ferguson et al. 2018, Didion et al. 2021), with the only full surveys of microbial changes during larval dormancy in the parasitoid wasp, *Nasonia vitripennis* (Hymenoptera: Pteromalidae), and during seasonal changes in adults for the cricket, *Gryllus veletis* (Orthoptera: Gryllidae) (Ferguson et al. 2018, Dittmer and Brucker 2021). Studies have shown that bacteria can influence host overwintering physiology and bacterial communities can be influenced by cold temperatures, whereas for mosquitoes little is known about how cold or overwintering may impact their microbiome. In some freeze-intolerant insects, bacteria that act as ice nucleators are ejected before overwintering (Zachariassen 1985, Olsen and Duman 1997) to reduce the risk of freezing such as in the pyrochroid beetle larvae, *Dendroides canadensis* (Coleoptera: Pyrochroidae) (Olsen and Duman 1997). The supercooling point of insects can be directly influenced by bacteria, including ladybird beetles (Lee et al. 1991), and stored grain pests (Lee et al. 1992). Microbiota transplants from cold reared *Drosophila melanogaster* (Diptera: Drosophilidae) to high temperature reared conspecifics led to increased cold tolerance, suggesting a direct role of the insect microbiota in cold tolerance (Moghadam et al. 2018).

This study aims to enhance our understanding of the microbiome-diapause relationship in the mosquito, *Culex pipiens*, throughout adult female diapause by monitoring the bacterial communities in lab-derived mosquitoes reared in both lab and field-mesocosm conditions. This comparison will provide valuable information on whether maintaining diapausing females within a lab colony influences their microbial composition as compared to a sister cohort in a field mesocosm even when provided the same sugar and water resources. Studies investigating the microbiome and diapause to date have largely focused on early diapause, in lab settings. One long-term lab study on diapausing wasp larvae, *Nasonia vitripennis* (Dittmer and Brucker 2021), recently revealed that wasps early in diapause had generally higher bacterial titers than under prolonged diapause and that the microbial composition changed as diapause progressed. *Nasonia vitripennis* diapause however is markedly different from that of *C. pipiens* as their diapause is maternally programmed, they overwinter as immobile fourth instar larvae that cannot imbibe food or water and are typically exposed to colder temperatures than occurs for *C. pipiens*. Microbial community changes are typical in aging animals (Hopkins et al. 2001, Guo et al. 2014, Langille et al. 2014), and despite microbial communities changing in diapausing *N. vitripennis* larvae over time, it remains unclear whether the extension of life via adult diapause in *C. pipiens* will change similarly. Our previous studies have confirmed that that the microbiome is critical to lipid accumulation in early diapause for *C. pipiens* (Didion et al. 2021), but this only examined microbiome differences in the first two weeks of diapause preparation in *C. pipiens*. Age-associated microbial community changes can be important because they have been associated with morbidities and can lead to increased pathogen susceptibility in both vertebrate and invertebrate hosts (Tamboli et al. 2004, Turnbaugh et al. 2006, Lee and Lee 2014, Hegde et al. 2015). Additionally, it is clear that microbial communities are impacted by rearing location (Akorli et al. 2019) and abiotic factors such as temperature (Guégan et al. 2018) in nondiapausing mosquitoes but whether similar trends exist within the context of diapause remains unknown. Understanding how lab versus field rearing of mosquitoes, even when provided the same food and water resources, can impact microbiota composition is important as the underlying interest of most of these studies is to better understand and identify targets to more efficiently impact the natural world. This study is the first to assess how the microbiota of *Culex pipiens pipiens* females undergoing diapause in both field and lab conditions change throughout diapause.

Methods

Mosquito Rearing

Larvae were reared in water collected from a stream at the University of Cincinnati Center for Field Studies (39°17′07.5″ N, 84°44′36.4″ W) where *C. pipiens* larvae had previously been observed. Larvae were held in an 18°C incubator with a 9:15 light: dark cycle to induce dormancy. Upon pupation in early November, the pupae were randomly assigned to either lab or field conditions. The lab-reared mosquitoes were stored under similar conditions as used to induce dormancy in the larvae. Lab and field-reared mosquitoes were provided the same water and sugar sources (10% sucrose) that were replenished twice per week, so that difference between the mosquitoes was not due to variation in the materials ingested and examining microbiome differences between water sources would provide very little insight. The field destined larvae were transported to the basement of the University of Cincinnati field station (Harrison, Ohio). Diapausing female *C. pipiens* have been observed at this location in 2016–2017 and 2018–2019 in early (December) and late (March) winter, suggesting that this site represents a natural field location. To prevent escape, prevent predation, and limit water and sugar sources to only those provided, field mosquitoes were kept in a 12- × 12-inch cage within a 6- × 6-foot cage (Bioquip). Temperature and humidity data at the field station were recorded using an Onset HOBO MX101 (Supp Fig. S1 [online only]).

Mosquito Sampling

To determine how the microbiota changed over time in the lab versus field-reared mosquitoes, 20 diapausing females were sampled from each group at 2 wk, 2 mo, 3 mo, 4 mo, and after exposure to conditions to terminate diapause. Diapause break in the lab was unable to be assessed as insufficient females were remaining at the end for the collection of these samples at 4 mo. Upon sampling at the specific time points mentioned, females were surface sterilized with 70% ethanol for 5 min in a sterilized fume hood before placement into a 1.5 ml Eppendorf tube, which was then stored at −70°C until DNA extraction.

16S rRNA Sequencing and Bioinformatic Analysis

Samples were thawed before the addition of 200 µl sterile 1% PBS and sterilized Zirconium beads, which were then used to homogenize the samples using a BeadBlaster. Twenty microliters of ProtK was added to the sample, vortexed, and then allowed to incubate at 56°C for 2 h to ensure complete lysation of the sample. DNA was then extracted using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen) using a standard protocol. The concentration and quality of the extracted DNA were determined using a nanodrop2000 and amplified via PCR with bacteria-specific primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) which target the 16S rRNA

V4 hypervariable region to ensure the presence of bacterial DNA (Caporaso et al. 2010, Apprill et al. 2015). Samples were sequenced by SeqMatic (Fremont, California) which utilized the Illumina MiSeq platform to generate 25 million overlapping, paired 251bp reads that were used in the downstream analyses. 16S rRNA datasets are available through NCBI (Accession: PRJNA742055)

Data Analysis

Sequences were analyzed by SeqMatic through the QIIME pipeline using Greenegenes as the reference database. In addition, raw sequences acquired from SeqMatic Illumina Miseq were also processed using QIIME 16S FASTQ Paired end run type (v1.9.1) through Nephele (v2.6.0) with the following pipeline parameters (max_bad_run: 3, max_n: 0, min_overlap: 10, otu_strategy: open, perc_max_diff: 25, phred_offset: 33, phred_quality: 19, picrust: False, ref_db: sv99 (Caporaso et al. 2010, Kozich et al. 2013, Weber et al. 2018). Briefly, paired-end reads were combined using the join_paired_ends.py command and then trimmed with screen.seqs to ensure each read was between 250bp and 263bp long. The command pick_open_reference_otus.py was then used to classify the sequences as operational taxonomic units (OTUs) followed by core_diversity_analyses.py to perform the analyses of core diversity. Assignment as Gram − and Gram + bacteria was determined by searching for representative at the bacterial family level through Google Scholar and if a specific family had members that were split (Gram + and Gram −) search was conducted at the genus level. To further examine differences between samples, Non-Metric Multidimensional Scaling (NMDS) plots were created using the metaMDS function from the R package vegan (Dixon 2003, Oksanen et al. 2017) based on the relative abundances of the specific OTUs. Analysis of similarities (ANOSIM, using the anosim from the R package vegan) tests was performed to test for significant differences in the community assemblages between field and lab samples. Post-hoc comparison between samples was conducted by ANOSIM between individual samples to establish the specific location and times that are significantly different.

Weighted Gene Co-Expression Network Analysis (WGCNA)

The WGCNA package in R (Langfelder and Horvath 2007) was used to create a correlation network in which the “expression,” or more accurately, the abundance of specific OTUs, was correlated with microbiota changes related to specific traits found in previous studies (Tong et al. 2013, Leite-Mondin et al. 2021). As insufficient mosquitoes were available to directly measure nutrition levels in this study, nutritional abundance data were used from a previous study on adult female *C. pipiens* (Benoit and Denlinger 2007). This incorporated proteins, lipids, and carbohydrates levels into discrete groups, high (2 wk), moderate (2–3 mo), or low (4 mo, diapause break) nutritional reserve amounts that are associated with specific time points during *C. pipiens* dormancy. Based on our recent studies, the nutritional metrics from this previous study is similar to diapausing mosquitoes used in this study (Didion et al. 2021), which are the same strain and reared under nearly identical conditions. These data were incorporated into an unsupervised WGCNA to assess whether nutritional availability correlated with microbial composition. Any OTUs with less than five detections were removed before analyses. The WGCNA includes network construction, detection of modules, and their correlation to the expression data was implemented as described previously (Zhang and Horvath 2005, Langfelder and Horvath 2007). A scale-free topology threshold of 0.8 was used to identify the soft power of 12. Adjacency matrix was calculated for signed network construction to identify the specific modules of OTUs that correlated with specific treatment. Significance was denoted based on high levels of correlation with the specific trait to the module eigengene (ME), yielding a group of OTUs with similar detected levels. A correlation test *P*-value below $P < 0.05$ was considered to be significant between the trait and the specific ME that consists of the OTUs of interest.

Results

Microbial composition changes with rearing location and diapause length. Two analyses pipelines were used with trends being similar between methods of analysis. At the Phylum level, both pipeline/sequence database combinations (SeqMatic/Greengenes and Nephele/SILVA) revealed similar compositions (Fig. 1). Proteobacteria dominated field-reared mosquitoes throughout diapause, increasing marginally over time, peaking in mosquitoes exposed to diapause-breaking conditions. Conversely, while 2-wk and 2-mo-old lab individuals were similar to field-reared, Proteobacteria abundance precipitously dropped with each subsequent time-point (Fig. 1). At 2 mo of age, lab-reared mosquitoes saw an increase in Actinobacteria, whereas 3- and 4-mo-old individuals were dominated by Firmicutes.

The family level revealed differences between the two pipeline/sequence database combinations, though the overall trends remained similar (Fig. 2). The Seqmatic/Greengenes (S/G) combination had Enterobacteriaceae within field-reared mosquitoes remaining consistent throughout diapause representing on average between 30 and 58% of the reads at each time point while QIIME/SILVA (Q/S) averaged 21–39%. Lab-reared mosquitoes on the other hand had less Enterobacteriaceae overall, starting at ≈27% and ≈22% (S/G) or ≈12% and ≈20% (Q/S) in months 1 and 2, and dropping to ≈2% and ≈0.3% (S/G) and ≈1% and ≈0.01% (Q/S) in subsequent time points. Bacillaceae were prevalent in late diapause (S/G—59%, 55%, Q/S—68%, 85%) for lab-reared mosquitoes while Acetobacteraceae_Asaia started high and decreased over diapause (S/G—36%, 50%, 16%, 18%, Q/S—76%, 73%, 28%, 5%). In field-reared mosquitoes, an opposite trend was seen, where Acetobacteraceae composed only (S/G—5% and 0.4%, Q/S—2% and 20%) in 2-wk and 2-mo samples and increased in subsequent time points (S/G—36%, 38%, 37%, Q/S—1%, 54%, 75%), of which most OTUs were associated with *Asaia*. The differences seen in microbial composition between the pipeline/database methods are likely due to the reference database, but are useful to ensure thorough analyses. Greengenes is a smaller database and is not updated as frequently as SILVA; therefore, it is likely that the QIIME/SILVA analysis is more accurate (Schloss et al. 2009).

Most bacteria associated with the field-reared mosquitoes were gram-negative and became more so as diapause progressed, peaking when the mosquitoes were exposed to diapause breaking conditions. In lab-reared mosquitoes, most bacteria were gram-negative when sampled at 2 wk and 2 mo of age. This changed significantly at 3- and 4-mo time-points where gram-positive bacteria represented ≈90% and ≈99% of the community, respectively (Table 1). This suggest that the number of observed species and alpha diversity decreased over diapause in both lab and field-reared mosquitoes though neither were significantly different (Fig. 3; Supp Data S2 [online only]). Weighted UniFrac distance metrics which consider the phylogenetic relationship of the microbiota present within each group were used to assess beta diversity similarities within and between groups. Two sample t-tests were used to make these pairwise comparisons and were Bonferroni corrected. When considering the phylogenetic origin of the present bacteria the beta diversity (Supp Data S2 [online only]) between samples within a group was

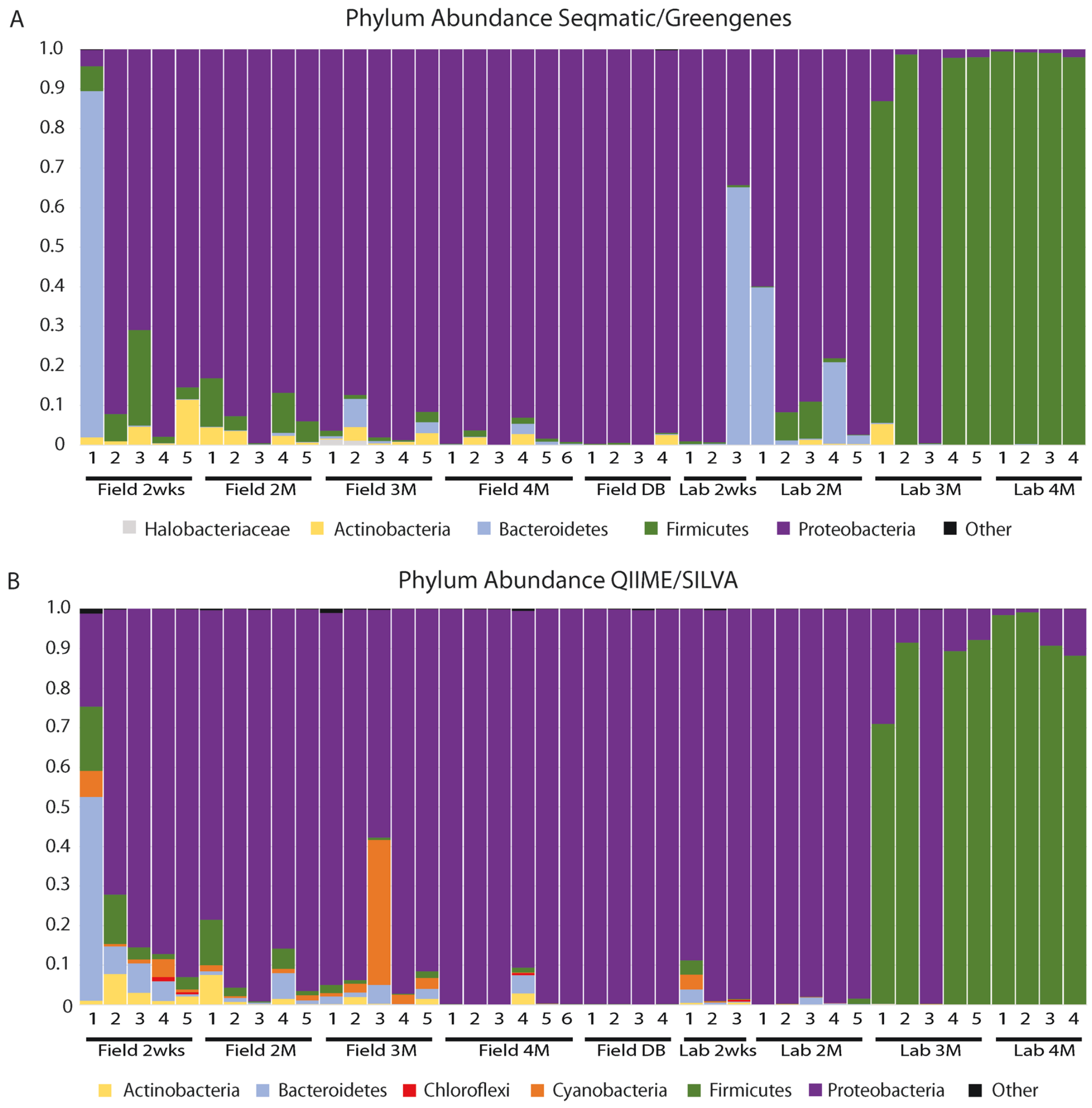


Fig. 1. Whole body microbial composition at the phylum level of diapausing *C. pipiens* in lab and field conditions at different time points. Relative abundances of bacterial phyla associated with each sample (N = 3–6 per group). Midgut microbiota phyla distribution for field (F) and lab (L) samples at 2 wk (2wks), 2 mo (2M), 3 mo (3M), 4 mo (4M), and diapause break (DB). Data were assessed through two different pipeline/reference datasets: (A) SeqMatic-Greenegenes and (B) QIIME/SILVA. Both methods returned similar results at the phylum level (Supp Data S3 [online only]). See online version for color figure.

more diverse than between the groups (t = –6.1, P = 1.43E-06). The diversity measured within 2-mo-old field-reared mosquitoes was significantly greater than their lab-reared counterparts (t = 5.74, P = 0.02). This extends to when comparing the beta diversity between early diapause field-reared mosquitoes (2 wk and 2 mo) and early lab-reared diapause mosquitoes (t = 4.86, P = 0.022). The phylogeny informed beta diversity between early time point lab mosquitoes was also greater than that between late timepoints (3 and 4 mo; t = 11.8, P = 2.34E-10). Overall, the comparison between early field samples yielded the highest beta diversity and was significantly different from the late field derived samples. Within the lab samples, the late timepoints were so profoundly different in phylogeny informed beta diversity that the early time points clustered with late field samples rather than within their environmental treatment group, which was driven by the massive increase in *Bacillus* reads in the late lab samples.

When compared through the use of NMDS, there was a significant variation between both the field and lab samples (Fig. 4; ANOSIM, R = 0.859, P = 1.43E-06). When field and lab samples were analyzed individually, significant differences were noted community composition between early (2 wk) and late (4 mo) diapause under both treatments, but intermediate points showed no significance. Of interest, the composition at two weeks was not significantly different between field and lab reared mosquitoes (ANOSIM, R = 0.4128, P = 0.1698), but all samples at 2, 3, and 4 mo had noted composition differences (ANOSIM, P < 0.05 in all cases). Following the break in diapause, there was not a noted difference compared to field mosquitoes under extended diapause (4 mo, ANOSIM. R = –0.01984, P =0.408). These results highlight that even when a single mosquito strain is provided the same water and carbohydrate resources, significant divergence in bacterial composition occurs as diapause progresses.

WGCNA Reveals Putative Correlation With *C. pipiens* Diapause Traits and Microbial Communities

WGCNA supported our other methods of analysis by revealing significant differences (student asymptotic P-value for correlation)

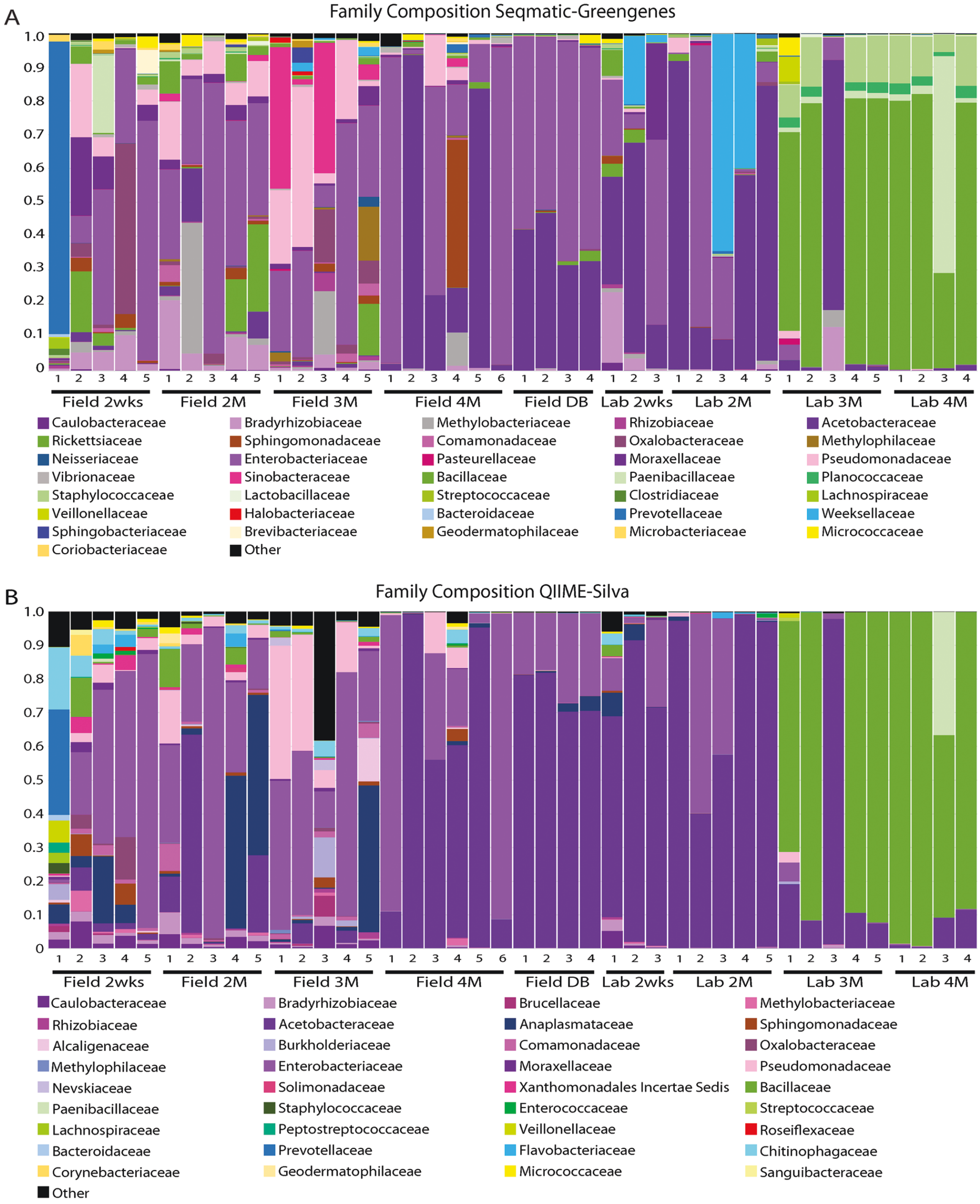


Fig. 2. Midgut family composition of diapausing *C. pipiens* at the family level of diapausing *C. pipiens* in lab and field conditions at different time points. Relative abundances of bacterial families associated with each sample (*N* = 3–6 per group). Midgut microbiota familial distribution for field (F) and lab (L) samples at 2 wk (2wks), 2 mo (2M), 3 mo (3M), 4 mo (4M), and diapause break (DB). Families within a single phylum are assigned shades of the same color. Data were assessed through two different pipeline/reference datasets: (A) SeqMatic-Greenegenes and (B) QIIME/SILVA. Both methods returned relatively similar results at the phylum level (Supp Data S3 [online only]). See online version for color figure.

between lab and field-reared mosquitoes (Fig. 5; Supp Data S4 [online only]). Many slight differences between lab and field-reared mosquito microbial communities were noted, but the biggest underlying separation was the abundance of *Bacillus* in the 3- and 4-mo-old lab-reared mosquitoes. Late lab samples were specifically dominated by *Bacillus* sp. CPSM8 ($P \le 0.01$), levels of this bacteria were so high that late diapause was also significantly associated with this phylum ($P \le 0.01$) and was negatively correlated with both field ($P \le 0.01$) and early ($P = 0.01$) diapause mosquitoes. Several modules were associated with 2-wk field samples, which were particularly diverse. The brown ($P \le 0.01$), red ($P \le 0.01$), purple, and turquoise modules included highly expressed OTUs including

Table 1. Proportion of Gram + and Gram – bacteria throughout the course of diapause in *Culex pipiens*

	Field 2 Weeks	Field 2 Months	Field 3 Months	Field 4 Months	Field Breaks	Lab 2 Weeks	Lab 2 Months	Lab 3 Months	Lab 4 Months
Gram –	0.925	0.927	0.968	0.985	0.989	0.989	0.97	0.13	0.015
Gram +	0.075	0.073	0.032	0.015	0.011	0.011	0.03	0.87	0.985

Summary of gram – and gram + bacteria.

families Oxalobacteraceae, Chitinophagaceae, and Prevotellaceae, of which several modules were significantly associated with 2-wk-old and high nutrient reserve groups (red: $P = 0.01$, brown: $P \leq 0.01$, turquoise: $P \leq 0.01$).

Two-month-old field mosquitoes were significantly associated with the pink module ($P \leq 0.01$) which included families Bradyrhizobiaceae, Microbacteriaceae, and Comamonadaceae, with a particularly high abundance of Bradyrhizobiaceae. Three-month-old field mosquitoes were significantly associated with blue ($P \leq 0.01$), magenta ($P \leq 0.01$), and green modules ($P \leq 0.01$), of which Enterobacteriaceae and Pseudomonadaceae, were most highly abundant. Magenta was also significantly correlated with all field samples ($P \leq 0.01$), while blue was associated with the three-month group ($P = 0.01$). Supp Data S4 (online only) provides a complete table of module-associated OTUs, as well as correlation and *P*-values for all group-module correlations. Overall, specific modules correlated bacterial OTUs with specific rearing conditions and dormancy duration.

Discussion

Understanding microbial community composition and structure has become a topic of interest as researchers try to understand the relationship of microbial communities, host physiology, and environmental influence (Spor et al. 2011, Parfrey et al. 2018, Brinker et al. 2019). Foundational studies, such as the current study, provide a general survey of microbial communities in various host physiological states. These surveys provide valuable information for future studies looking to understand dynamics between microbial community, host, and environmental factors in diapausing adult female *C. pipiens*. The interplay between the microbiota, their host organism, and abiotic factors is intricate and often difficult to establish, specifically mosquitoes which do not have consistent microbial communities. Abiotic factors that can influence host microbial communities include breeding/rearing site, season, and temperature (Akorli et al. 2016, Krajacich et al. 2018, Akorli et al. 2019, Thapa et al. 2019, Lemoine et al. 2020, Sepulveda and Moeller 2020), while important host factors include species, host DNA, age, sex, and diet (Gusmão et al. 2007, Rani et al. 2009, Dillon et al. 2010, Zouache et al. 2011, Minard et al. 2013, Wu et al. 2019). Insects that overwinter in temperate regions are exposed to long periods of cold and show drastic changes to their feeding habits, behavior, and physiology (Denlinger and Armbruster 2014, Denlinger and Armbruster 2016, Gill et al. 2017). The reduction of temperature seen during diapause likely not only impacts host physiology but may impact bacterial associations and community composition (Webster et al. 2008, Lokmer and Wegner 2015). Notably, diapause extends their lifespans by several months and the microbiota tends to vary as organisms age. It is essential to characterize these host-microbe community shifts with host and environmental changes as they impact host phenotypes (Douglas et al. 2011, Douglas et al. 2015, Ferguson et al. 2018), and the uniqueness of mobile dormancy in *C. pipiens* necessitates a thorough study.

The current study revealed a high prevalence of Proteobacteria which increased throughout diapause, except for lab-reared mosquitoes in late diapause (months 3 and 4) which became dominated by the phylum Firmicutes. Proteobacteria has been repeatedly associated with the diapause state in other organisms including wasp, *Nasonia vitripennis* (Dittmer and Brucker 2021), cricket, *G. veletis* (Ferguson et al. 2018), where these bacteria increased with diapause duration and in the cabbage beetle, and *Colaphellus bowringi* (Coleoptera: Chrysomelidae) (Liu et al. 2016), in which it was associated with early diapause. It is of note that Proteobacteria increased in between nascent and seventeen day old *Aedes albopictus* (Diptera: Culicidae) (Wang et al. 2018), suggesting that Proteobacteria may simply form stronger associations in mosquitoes as mosquitoes age. Similar to what was found in the cabbage beetle (Liu et al. 2016), Firmicutes had a higher prevalence earlier in diapause at 2 wk of age (17%) in field-reared mosquitoes than at later time-points (0%). Lab-reared mosquitoes alternatively had a relatively low prevalence of Firmicutes in early diapause, increasing steadily with each subsequent time point, becoming the dominant species towards the end of diapause. *Wolbachia* abundance during diapause thus far has been mixed with the prevalence within the cricket microbiota (Ferguson et al. 2018) increasing over time while a marked decrease was seen in the wasp (Dittmer and Brucker 2021). *Wolbachia* in *C. pipiens* diapause was similar to that of the wasp, decreasing in prevalence as diapause progressed. In *Drosophila*, *Wolbachia* has been shown to increase with a sucrose diet (Serbus et al. 2015) which may explain why it was found in higher abundance in early diapause samples and dwindled throughout diapause, as sucrose consumption ceased, and mosquitos were relying on their lipid reserves. As intracellular bacteria with critical roles in relation to the biology of mosquitoes and others insects (Kaur et al. 2021), the specific impact of this reduction throughout diapause may be of interest or only reflect a general decline due to lack activity in specific tissues.

Diapausing insects including wasps (Dittmer and Brucker. 2021), crickets (Ferguson et al. 2018), and now *C. pipiens* in the current study have all shown changes in response to diapause duration. Alpha diversity and the overall number of OTUs present generally decreased with diapause length in both field and lab samples, even though these changes were not significant. This lack of decrease may be from the fact that *C. pipiens* will continually drink water during diapause (Benoit and Denlinger 2007), which could allow for recolonization of new bacteria as others die off. Importantly, the water and sugar sources provided to both the lab and field mosquitoes were consistent and from the same source throughout diapause, indicating the microbiome differences between field- and lab-reared mosquitoes are not solely due changes occurs in water between the locations due to prolonged cold exposure. When incorporating the bacterial lineage of the OTUs into diversity using a weighted-UniFrac, a general

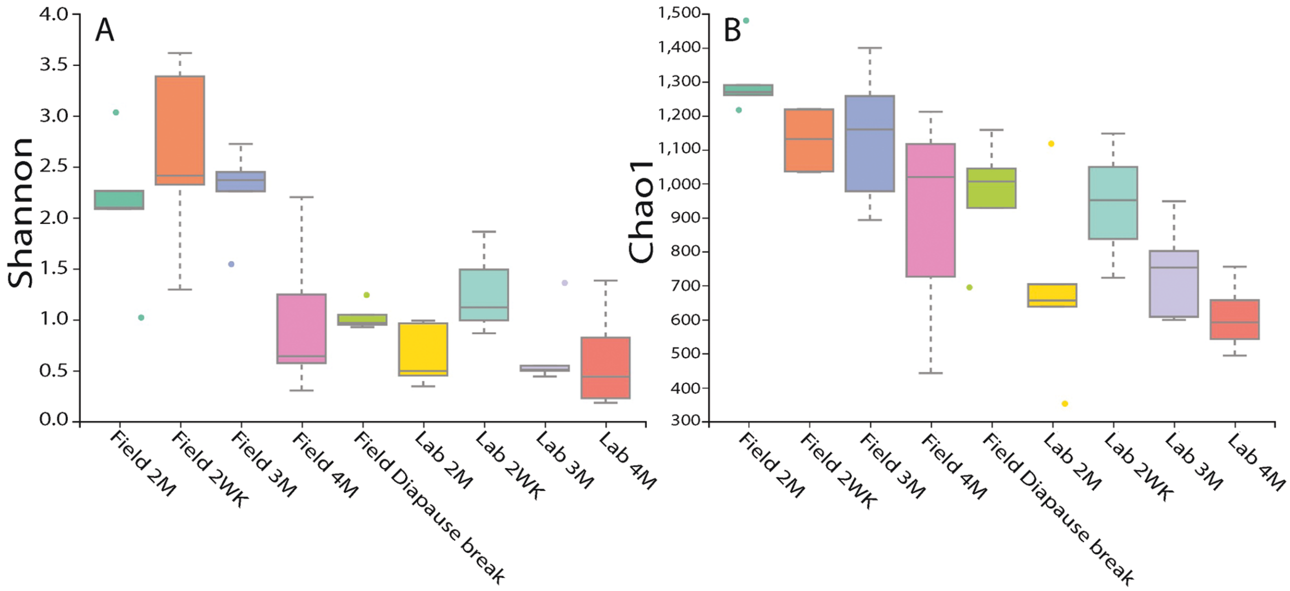


Fig. 3. Alpha diversity metrics of diapausing *C. pipiens* in lab and field conditions at different time points. (A) Chao diversity and (B) Shannon diversity. Despite a general downward trend as diapause progressed, no significant differences were seen between groups (see Supp Data S1 [online only] for statistics). See online version for color figure.

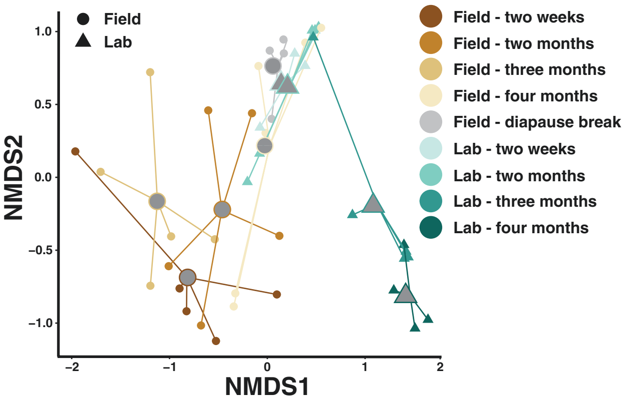
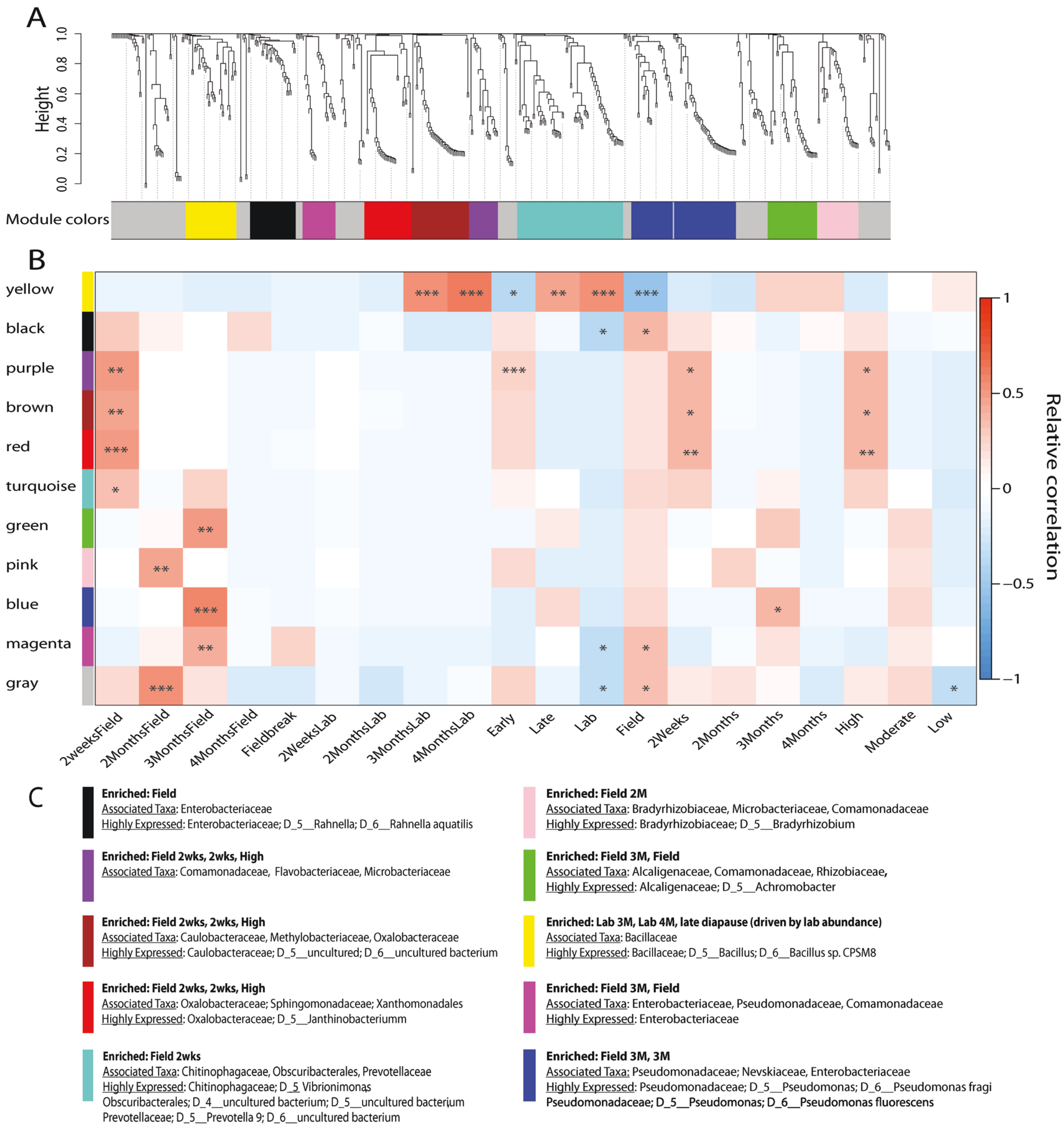


Fig. 4. Comparison of bacterial communities between field and lab mosquitoes and across diapause. Nonmetric multidimensional scaling analysis (NMDS) of proportional OTU abundance data using the Bray–Curtis dissimilarity matrix. The symbols indicate field and lab and coloration is based on duration within diapause. Large symbols represent mean of all samples. Small symbols represent each individual sample. See online version for color figure.

decrease in beta diversity was seen as diapause progressed in both lab and field cohorts. In addition, NMDS analyses indicate significant differences between early (2 wk) and late (4 mo) diapause. This decrease in beta diversity and other general changes late in diapause suggests that although new species are may be introduced, most likely via water consumption, the bacteria could phylogenetically similar or are unable to supplant current bacterial resident.

The roles of specific bacteria on host physiology are of great interest. The impact of the microbiota in nutrient acquisition, storage, and utilization is well supported in both vertebrate and invertebrate systems (Akman et al. 2002, Bäckhed et al. 2004, Dillon and Dillon 2004, Feldhaar et al. 2007, Hosokawa et al. 2010, Nieuwdorp et al. 2014, Salem et al. 2014, Douglas 2017, Didion et al 2021). Identifying how specific microbial associates impact host physiology is complicated. Bacterial communities may change due to an inability of some members to survive in the new host state while others may be inadvertently selected for due to benefits conferred to the host (Spor et al. 2011). It has been suggested that Proteobacteria may play a role in lipid accumulation during diapause preparation (Liu et al. 2016) and a specific Proteobacteria, *Escherichia coli*, has been shown to impact lipid accumulation in larval fat bodies (Valzania et al. 2018a), due to induced hypoxia in the larval gut initiating insulin/insulin growth factor signaling (Valzania et al. 2018b). Similarly, some *Asaia* strains within the phylum Proteobacteria are speculated to assist in nutrient digestion or produce a metabolite to increase larval growth in mosquitoes (Chouaia et al. 2012, Mitraka et al. 2013) and are known to alter the expression of lipid metabolism genes in adults (Mancini et al. 2020). *Asaia* reads were present in the current study and increased in abundance as diapause progressed in field-reared mosquitoes which bring into question whether they may be functioning similarly in diapausing *C. pipiens*. Proteobacteria, in general, were highly abundant in all field samples and some lab samples (2 wk and 2 mo), and several were associated with high nutritional reserve levels. WGCNA did not identify this association however because many different OTUs were associated with the genera *Asaia*. It is still possible that some of these significantly correlated Proteobacteria are involved in *C. pipiens* metabolism early on in diapause. This is supported by research showing the impact of the removal of the microbiota on diapause preparation, specifically impaired sugar metabolism and lower accumulation of lipids that leads to reduced survival (Didion et al. 2021).

After *C. pipiens* undergoes diapause for 2 mo or longer in the field, microbial community composition does not seem to be significantly impacted by general host nutritional reserve status. The Bacillaceae species associated with late lab diapause are not generally found in mosquito species and are more typically associated with sources such as riverbeds (Maitra et al. 2014) or



Enriched: Field
Associated Taxa: Enterobacteriaceae
Highly Expressed: Enterobacteriaceae; D_5__Rahnella; D_6__Rahnella aquatilis

Enriched: Field 2wks, 2wks, High
Associated Taxa: Comamonadaceae, Flavobacteriaceae, Microbacteriaceae

Enriched: Field 2wks, 2wks, High
Associated Taxa: Caulobacteraceae, Methylobacteriaceae, Oxalobacteraceae
Highly Expressed: Caulobacteraceae; D_5__uncultured; D_6__uncultured bacterium

Enriched: Field 2wks, 2wks, High
Associated Taxa: Oxalobacteraceae; Sphingomonadaceae; Xanthomonadales
Highly Expressed: Oxalobacteraceae; D_5__Janthinobacteriumm

Enriched: Field 2wks
Associated Taxa: Chitinophagaceae, Obscuribacterales, Prevotellaceae
Highly Expressed: Chitinophagaceae; D_5 Vibrionimonas
Obscuribacterales; D_4__uncultured bacterium; D_5__uncultured bacterium
Prevotellaceae; D_5__Prevotella 9; D_6__uncultured bacterium

Enriched: Field 2M
Associated Taxa: Bradyrhizobiaceae, Microbacteriaceae, Comamonadaceae
Highly Expressed: Bradyrhizobiaceae; D_5__Bradyrhizobium

Enriched: Field 3M, Field
Associated Taxa: Alcaligenaceae, Comamonadaceae, Rhizobiaceae,
Highly Expressed: Alcaligenaceae; D_5__Achromobacter

Enriched: Lab 3M, Lab 4M, late diapause (driven by lab abundance)
Associated Taxa: Bacillaceae
Highly Expressed: Bacillaceae; D_5__Bacillus; D_6__Bacillus sp. CPSM8

Enriched: Field 3M, Field
Associated Taxa: Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae
Highly Expressed: Enterobacteriaceae

Enriched: Field 3M, 3M
Associated Taxa: Pseudomonadaceae; Nevskiaceae, Enterobacteriaceae
Highly Expressed: Pseudomonadaceae; D_5__Pseudomonas; D_6__Pseudomonas fragi
Pseudomonadaceae; D_5__Pseudomonas; D_6__Pseudomonas fluorescens

Fig. 5. WGCNA reveals OTUs associated with diapausing *C. pipiens* in lab and field conditions at different time points and nutritional reserve levels. (A) OTU dendrogram of co-expressed OTU modules. (B) Heat map depicts the OTU modules positively (red) or negatively (blue) correlated with specific treatments. (C) Summary of group type, taxa, and highly expressed tax associated with each module color. The associated taxa listed were the three most abundant families associated with each module while highly expressed is taxa information for OTUs that had an average read total over 1000. Not all OTUs within a specific taxon were similarly expressed. See Supp Data S4 (online only) for all OTU-module associations. * denotes significance at 0.05, ** denotes significance at 0.01, and *** denotes significance at 0.001. See online version for color figure.

plant rhizomes (Gutiérrez-Mañero et al. 2001). They are facultatively anaerobic, can form spores (Rooney et al. 2009), perform environmental nutrient cycling (Maitra et al. 2014), and have been implicated in plant growth (Gutiérrez-Mañero et al. 2001). Their functionality, if any, within the late lab samples is unknown. A more focused look into specific nutritional reserves such as lipid, sugar, and protein content may reveal associations not revealed by the current study. Future studies should incorporate additional field and lab sites to ensure that the findings of this study are not idiosyncratic and provide additional insight into the impact the microbiota has on host metabolism and survival throughout diapause in *C. pipiens*.

Conclusions

Rearing location and diapause duration impact the microbial composition of *C. pipiens*, even when provided the same food and water sources. Like in other investigated diapausing species, Proteobacteria increased within mosquitoes undergoing diapause in the field which was different from lab-reared individuals that showed an increase in Firmicutes. Alpha diversity experienced a general decrease as diapause progressed, while beta diversity, which incorporated phylogenetic distance, decreased significantly. Through 2 wk of age, lab and field-reared mosquitoes maintained similar community compositions which diverged significantly after 2 mo old, even when provided the same water and sugar sources. These findings are similar to results from some aging studies, though it is unclear whether *C. pipiens* undergoing diapause show signs of an aging microbiota. Future studies should take a more focused look at the factors causing the microbial community changes seen as diapause progresses in *C. pipiens*, as they do not appear to be significantly impacted by general host nutritional reserve status.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

This work was funded by the University of Cincinnati Faculty Development Research Grant (to J.B.B.). Partial funding for reusable equipment was provided by the National Science Foundation (DEB-1654417 to J.B.B.), National Institutes of Health (1R01AI48551-01A1 to J.B.B.), and Weiman Wendell Benedict award from the University of Cincinnati Biological Sciences (E.M.D.).

References Cited

Akman, L., A. Yamashita, H. Watanabe, K. Oshima, T. Shiba, M. Hattori, and S. Aksoy. 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, Wigglesworthia glossinidia. Nat. Genet. 32: 402–407.

Akorli, J., M. Gendrin, N. A. Pels, D. Yeboah-Manu, G. K. Christophides, and M. D. Wilson. 2016. Seasonality and locality affect the diversity of *anopheles gambiae* and *anopheles coluzzii* midgut microbiota from Ghana. PLoS One. 11: e0157529.

Akorli, J., P. A. Namaali, G. W. Ametsi, R. K. Egyirifa, and N. A. P. Pels. 2019. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (sensu lato) during colonization in the laboratory. Parasit. Vectors. 12: 27.

Almada, A. A. 2015. Interactions between calanoid copepod hosts and their associated microbiota. Doctoral dissertation, Massachusetts Institute of Technology.

Apprill, A., McNally, S., Parsons, R., & Weber, L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat. Microb. Ecol. 75(2): 129–137.

Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. U. S. A. 101: 15718–15723.

Benoit, J. B., and D. L. Denlinger. 2007. Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. J. Exp. Biol. 210: 217–226.

Boissière, A., M. T. Tchioffo, D. Bachar, L. Abate, A. Marie, S. E. Nsango, H. R. Shahbazkia, P. H. Awono-Ambene, E. A. Levashina, R. Christen, et al. 2012. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. PLoS Pathog. 8: e1002742.

Brinker, P., M. C. Fontaine, L. W. Beukeboom, and J. Falcao Salles. 2019. Host, symbionts, and the microbiome: the missing tripartite interaction. Trends Microbiol. 27: 480–488.

Cansado-Utrilla, C., S. Y. Zhao, P. J. McCall, K. L. Coon, and G. L. Hughes. 2021. The microbiome and mosquito vectorial capacity: rich potential for discovery and translation. Microbiome. 9: 111.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7: 335–336.

Chouaia, B., P. Rossi, S. Epis, M. Mosca, I. Ricci, C. Damiani, U. Ulissi, E. Crotti, D. Daffonchio, C. Bandi, et al. 2012. Delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. BMC Microbiol. 12 Suppl 1: S2.

Chu, H., and S. K. Mazmanian. 2013. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nat. Immunol. 14: 668–675.

Coon, K. L., K. J. Vogel, M. R. Brown, and M. R. Strand. 2014. Mosquitoes rely on their gut microbiota for development. Mol. Ecol. 23: 2727–2739.

Coon, K. L., M. R. Brown, and M. R. Strand. 2016. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol. Ecol. 25: 5806–5826.

Correa, M. A., D. E. Brackney, and B. Steven. 2018. Generation of *axenic Aedes* aegypti demonstrate live bacteria are not required for mosquito development. Nature communications. 9(1): 1–10.

Denlinger, D. L., and P. A. Armbruster. 2014. Mosquito diapause. Annu. Rev. Entomol. 59: 73–93.

Denlinger, D. L., and P.A. Armbruster. 2016. Molecular physiology of mosquito diapause. Advances in insect physiology. 51, 329–361.

Didion, E. M., Z. L. Sabree, L. Kenyon, G. Nine, R. W. Hagan, S. Osman, and J. B. Benoit. 2021. Microbiome reduction prevents lipid accumulation during early diapause in the northern house mosquito, *Culex pipiens pipiens*. J. Insect Physiol. 134: 104295.

Dillon, R. J., and V. M. Dillon. 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49: 71–92.

Dillon, R. J., G. Webster, A. J. Weightman, and A. Keith Charnley. 2010. Diversity of gut microbiota increases with aging and starvation in the desert locust. Antonie Van Leeuwenhoek. 97: 69–77.

Dittmer, J., and R. M. Brucker. 2021. When your host shuts down: larval diapause impacts host-microbiome interactions in *Nasonia vitripennis*. Microbiome. 9: 85.

Dixon, P. 2003. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science. 14: 927–930.

Douglas, A. E. 2011. Lessons from studying insect symbioses. Cell Host Microbe. 10: 359–367.

Douglas, A. E. 2015. Multiorganismal insects: diversity and function of resident microorganisms. Annu. Rev. Entomol. 60: 17–34.

Douglas, A. E. 2017. The B vitamin nutrition of insects: the contributions of diet, microbiome and horizontally acquired genes. Curr. Opin. Insect Sci. 23: 65–69.

Feldhaar, H., J. Straka, M. Krischke, K. Berthold, S. Stoll, M. J. Mueller, and R. Gross. 2007. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. BMC Biol. 5: 48.

Ferguson, L. V., P. Dhakal, J. E. Lebenzon, D. E. Heinrichs, C. Bucking, and B. J. Sinclair. 2018. Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. Funct. Ecol. 32(10): 2357–2368.

Gill, H. K., G. Goyal, and G. Chahil. 2017. Insect diapause: a review. J. Agric. Sci. Technol. 7: 454–473.

Guégan, M., K. Zouache, C. Démichel, G. Minard, V. Tran Van, P. Potier, P. Mavingui, and C. Valiente Moro. 2018. The mosquito holobiont: fresh insight into mosquito-microbiota interactions. Microbiome. 6: 49.

Guo, L., J. Karpac, S. L. Tran, and H. Jasper. 2014. PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell. 156: 109–122.

Gusmão, D. S., A. V. Santos, D. C. Marini, E. d. e. S. Russo, A. M. Peixoto, M. Bacci Júnior, M. A. Berbert-Molina, and F. J. Lemos. 2007. First isolation of microorganisms from the gut diverticulum of *Aedes aegypti* (Diptera: Culicidae): new perspectives for an insect-bacteria association. Mem. Inst. Oswaldo Cruz. 102: 919–924.

Gutiérrez-Mañero, F. J., B. Ramos-Solano, A. N. Probanza, J. Mehouachi, R. Tadeo F, and M. Talon. 2001. The plant-growth-promoting rhizobacteria

Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins. Physiol. Plant. 111: 206–211.

Hegde, S., J. L. Rasgon, and G. L. Hughes. 2015. The microbiome modulates arbovirus transmission in mosquitoes. Curr. Opin. Virol. 15: 97–102.

Hopkins, M. J., R. Sharp, and G. T. Macfarlane. 2001. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut. 48: 198–205.

Hosokawa, T., R. Koga, Y. Kikuchi, X. Y. Meng, and T. Fukatsu. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proc. Natl. Acad. Sci. U. S. A. 107: 769–774.

Kaur, R., J. D. Shropshire, K. L. Cross, B. Leigh, A. J. Mansueto, V. Stewart, S. R. Bordenstein, and S. R. Bordenstein. 2021. Living in the endosymbiotic world of *Wolbachia*: a centennial review. Cell Host Microbe. 29: 879–893.

Kiers, E. T., and R. F. Denison. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. Annu. Rev. Ecol. Evol. Syst. 39: 215–236.

Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79: 5112–5120.

Krajacich, B. J., D. L. Huestis, A. Dao, A. S. Yaro, M. Diallo, A. Krishna, J. Xu, and T. Lehmann. 2018. Investigation of the seasonal microbiome of *Anopheles coluzzii* mosquitoes in Mali. PLoS One. 13: e0194899.

Langfelder, P., and S. Horvath. 2007. Eigengene networks for studying the relationships between co-expression modules. BMC Syst. Biol. 1: 54.

Langille, M. G., C. J. Meehan, J. E. Koenig, A. S. Dhanani, R. A. Rose, S. E. Howlett, and R. G. Beiko. 2014. Microbial shifts in the aging mouse gut. Microbiome. 2: 50.

Lee, K. A., and W. J. Lee. 2014. *Drosophila* as a model for intestinal dysbiosis and chronic inflammatory diseases. Dev. Comp. Immunol. 42: 102–110.

Lee R. E. Jr, J. M. Strong-Gunderson, M. R. Lee, K. S. Grove, and T. J. Riga. 1991. Isolation of ice nucleating active bacteria from insects. J. Exp. Zool. 257(1): 124–127.

Lee, R. E. Jr, J. M. Strong-Gunderson, M. R. Lee, and E. C. Davidson. 1992. Ice-nucleating active bacteria decrease the cold-hardiness of stored grain insects. J. Econ. Entomol. 85(2): 371–374.

Leite-Mondin, M., M. J. DiLegge, D. K. Manter, T. L. Weir, M. C. Silva-Filho, and J. M. Vivanco. 2021. The gut microbiota composition of *Trichoplusia* ni is altered by diet and may influence its polyphagous behavior. Sci. Rep. 11: 5786.

Lemoine, M. M., T. Engl, and M. Kaltenpoth. 2020. Microbial symbionts expanding or constraining abiotic niche space in insects. Curr. Opin. Insect Sci. 39: 14–20.

Liu, W., Y. Li, S. Guo, H. Yin, C. L. Lei, and X. P. Wang. 2016. Association between gut microbiota and diapause preparation in the cabbage beetle: a new perspective for studying insect diapause. Sci. Rep. 6(1): 1–8.

Lokmer, A., and K. Mathias Wegner. 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. Isme J. 9: 670–682.

Mancini, M. V., C. Damiani, S. M. Short, A. Cappelli, U. Ulissi, A. Capone, A. Serrao, P. Rossi, A. Amici, C. Kalogris, et al. 2020. Inhibition of *Asaia* in adult mosquitoes causes male-specific mortality and diverse transcriptome changes. Pathogens. 9(5): 380.

Marshall, K., I. Joint, M. E. Callow, and J. A. Callow. 2006. Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. Microb. Ecol. 52: 302–310.

Maitra, N., W. B. Whitman, S. Ayyampalayam, S. Samanta, K. Sarkar, C. Bandopadhyay, M. Aftabuddin, A. P. Sharma, and S. K. Manna. 2014. Draft genome sequence of the aquatic phosphorus-solubilizing and-mineralizing bacterium Bacillus sp. strain CPSM8. Genome Announ. 2: e01265-13.

McFall-Ngai, M., M. G. Hadfield, T. C. Bosch, H. V. Carey, T. Domazet-Lošo, A. E. Douglas, N. Dubilier, G. Eberl, T. Fukami, S. F. Gilbert, et al. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Minard, G., P. Mavingui, and C. V. Moro. 2013. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit. Vectors. 6: 146.

Mitraka, E., S. Stathopoulos, I. Siden-Kiamos, G. K. Christophides, and C. Louis. 2013. Asaia accelerates larval development of *Anopheles gambiae*. Pathog. Glob. Health. 107: 305–311.

Moghadam, N. N., P. M. Thorshauge, T. N. Kristensen, N. de Jonge, S. Bahrndorff, H. Kjeldal, and J. L. Nielsen. 2018. Strong responses of *Drosophila melanogaster* microbiota to developmental temperature. Fly (Austin). 12: 1–12.

Neufeld, K. M., N. Kang, J. Bienenstock, and J. A. Foster. 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. J. Neurogastroenterol. Motil. 23(3): 255–e119.

Nieuwdorp, M., P. W. Gilijamse, N. Pai, and L. M. Kaplan. 2014. Role of the microbiome in energy regulation and metabolism. Gastroenterology. 146: 1525–1533.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, et al. 2017. *vegan: Community Ecology Package*. Available at: https://CRAN.R-project.org/package=vegan

Olsen, T. M., and J. G. Duman. 1997. Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of ice nucleators and antifreeze proteins. J. Comp. Physiol. B. 167(2): 114–122.

Parfrey, L. W., C. S. Moreau, and J. A. Russell. 2018. Introduction: the host-associated microbiome: pattern, process and function. Mol. Ecol. 27: 1749–1765.

Rani, A., A. Sharma, R. Rajagopal, T. Adak, and R. K. Bhatnagar. 2009. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. BMC Microbiol. 9: 96.

Rey, T., and S. Schornack. 2013. Interactions of beneficial and detrimental root-colonizing filamentous microbes with plant hosts. Genome Biol. 14: 121.

Ridley, E. V., A. C. Wong, S. Westmiller, and A. E. Douglas. 2012. Impact of the resident microbiota on the nutritional phenotype of *Drosophila melanogaster*. PLoS One. 7: e36765.

Rodriguez, R. J., J. F. White, Jr, A. E. Arnold, and R. S. Redman. 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182: 314–330.

Romoli, O., J. C. Schönbeck, S. Hapfelmeier, and M. Gendrin. 2021. Production of germ-free mosquitoes via transient colonisation allows stage-specific investigation of host–microbiota interactions. Nat. Commun. 12(1): 1–16.

Rooney, A. P., N. P. Price, C. Ehrhardt, J. L. Swezey, and J. D. Bannan. 2009. Phylogeny and molecular taxonomy of the Bacillus subtilis species complex and description of Bacillus subtilis subsp. inaquosorum subsp. nov. Int. J. Syst. Evol. Microbiol. 59: 2429–2436.

Salem, H., E. Bauer, A. S. Strauss, H. Vogel, M. Marz, and M. Kaltenpoth. 2014. Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host. Proc. Biol. Sci. 281: 20141838.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75: 7537–7541.

Scully, E. D., S. M. Geib, J. E. Carlson, M. Tien, D. McKenna, and K. Hoover. 2014. Functional genomics and microbiome profiling of the Asian longhorned beetle (*Anoplophora glabripennis*) reveal insights into the digestive physiology and nutritional ecology of wood feeding beetles. BMC Genomics. 15: 1096.

Sepulveda, J., and A. H. Moeller. 2020. The effects of temperature on animal gut microbiomes. Front. Microbiol. 11: 384.

Serbus, L. R., P. M. White, J. P. Silva, A. Rabe, L. Teixeira, R. Albertson, and W. Sullivan. 2015. The impact of host diet on *Wolbachia* titer in Drosophila. PLoS Pathog. 11: e1004777.

Shin, S. C., S. H. Kim, H. You, B. Kim, A. C. Kim, K. A. Lee, J. H. Yoon, J. H. Ryu, and W. J. Lee. 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science. 334: 670–674.

Spor, A., O. Koren, and R. Ley. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat. Rev. Microbiol. 9: 279–290.

Steven, B., J. Hyde, J. C. LaReau, and D. E. Brackney. 2021. The axenic and gnotobiotic mosquito: emerging models for microbiome host interactions. Front. Microbiol. 12: 714222.

Tamboli, C. P., C. Neut, P. Desreumaux, and J. F. Colombel. 2004. Dysbiosis as a prerequisite for IBD. Gut. 53: 1057.

Tapia, J. E., B. González, S. Goulitquer, P. Potin, and J. A. Correa. 2016. Microbiota influences morphology and reproduction of the brown alga *ectocarpus* sp. Front. Microbiol. 7: 197.

Thapa, S., Y. Zhang, and M. S. Allen. 2019. Effects of temperature on bacterial microbiome composition in *Ixodes scapularis* ticks. Microbiologyopen. 8: e00719.

Tong, M., X. Li, L. Wegener Parfrey, B. Roth, A. Ippoliti, B. Wei, J. Borneman, D. P. McGovern, D. N. Frank, E. Li, et al. 2013. A modular organization of the human intestinal mucosal microbiota and its association with inflammatory bowel disease. PLoS One. 8: e80702.

Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444: 1027–1031.

Valzania, L., V. G. Martinson, R. E. Harrison, B. M. Boyd, K. L. Coon, M. R. Brown, and M. R. Strand. 2018a. Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. PLoS Negl. Trop. Dis. 12: e0006638.

Valzania, L., K. L. Coon, K. J. Vogel, M. R. Brown, and M. R. Strand. 2018b. Hypoxia-induced transcription factor signaling is essential for larval growth of the mosquito *Aedes aegypti*. Proc. Natl. Acad. Sci. U. S. A. 115: 457–465.

Wang, X., T. Liu, Y. Wu, D. Zhong, G. Zhou, X. Su, J. Xu, C. F. Sotero, A. A. Sadruddin, K. Wu, et al. 2018. Bacterial microbiota assemblage in *Aedes albopictus* mosquitoes and its impacts on larval development. Mol. Ecol. 27: 2972–2985.

Weber, N., D. Liou, J. Dommer, P. MacMenamin, M. Quiñones, I. Misner, A. J. Oler, J. Wan, L. Kim, M. Coakley McCarthy, et al. 2018. Nephele: a cloud platform for simplified, standardized and reproducible microbiome data analysis. Bioinformatics. 34: 1411–1413.

Webster, N. S., R. E. Cobb, and A. P. Negri. 2008. Temperature thresholds for bacterial symbiosis with a sponge. Isme J. 2: 830–842.

Wu, C. S., J. Y. Noh, E. Tuchaai, S. Devaraj, Z. Chen, and Y. Sun. 2019. Aging gut microbiome profile and ghrelin signaling in microbiome homeostasis. The FASEB J. 33(S1): 794–817.

Zachariassen, K. E. 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65: 799–832.

Zhang, B., and S. Horvath. 2005. A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. 4.

Zouache, K., F. N. Raharimalala, V. Raquin, V. Tran-Van, L. H. Raveloson, P. Ravelonandro, and P. Mavingui. 2011. Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti,* from different geographic regions of Madagascar. FEMS Microbiol. Ecol. 75: 377–389.