FEMS Microbes, 2024, 5, xtae002
DOI: 10.1093/femsmc/xtae002
Advance access publication date: 12 February 2024
Research Article – Microbes & Multicellular Life

Culex pipiens and *Culex restuans* larval interactions shape the bacterial communities in container aquatic habitats

Teresia M. Njoroge [1,2,3,*], May R. Berenbaum[2], Christopher M. Stone[3], Chang-Hyun Kim[3], Christopher Dunlap[4], Ephantus J. Muturi [4]

[1]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Raclin- Carmichael Hall, 1234 N. Notre Dame Ave, South Bend, IN 46617, United States
[2]Department of Entomology, University of Illinois at Urbana-Champaign, 505 S. Goodwin Ave, Urbana, IL 61801, United States
[3]Illinois Natural History Survey, Prairie Research Institute, University of Illinois at Urbana-Champaign, 1816 S. Oak St, Champaign, IL 61820, United States
[4]Crop Bioprotection Research Unit, Agricultural Research Service, US Department of Agriculture, 1815 N. University St., Peoria, IL 61604, United States
*Corresponding author. Department of Medical and Molecular Genetics, Indiana University School of Medicine, Raclin- Carmichael Hall, 1234 N. Notre Dame Ave, South Bend, IN 46617, United States. E-mail: tenjorog@iu.edu; tnjoroge83@yahoo.co.uk
Editor: [Kimberly Kline]

Abstract

Container aquatic habitats host a community of aquatic insects, primarily mosquito larvae that browse on container surface microbial biofilm and filter-feed on microorganisms in the water column. We examined how the bacterial communities in these habitats respond to feeding by larvae of two container-dwelling mosquito species, *Culex pipiens* and *Cx. restuans*. We also investigated how the microbiota of these larvae is impacted by intra- and interspecific interactions. Microbial diversity and richness were significantly higher in water samples when mosquito larvae were present, and in *Cx. restuans* compared to *Cx. pipiens* larvae. Microbial communities of water samples clustered based on the presence or absence of mosquito larvae and were distinct from those of mosquito larvae. *Culex pipiens* and *Cx. restuans* larvae harbored distinct microbial communities when reared under intraspecific conditions and similar microbial communities when reared under interspecific conditions. These findings demonstrate that mosquito larvae play a major role in structuring the microbial communities in container habitats and that intra- and interspecific interactions in mosquito larvae may shape their microbiota. This has important ecological and public health implications since larvae of the two mosquito species are major occupants of container habitats while the adults are vectors of West Nile virus.

Keywords: larvae; *Culex pipiens*; *Culex restuans*; container aquatic habitats; microbiota

Introduction

In nature, most animals have complex life cycles where the juvenile and adult stages are separated by metamorphosis and exploit different niches. Adaptive decoupling hypothesis postulates that metamorphosis is beneficial as it may decouple juvenile and adult stages and allow each stage to independently adapt to their respective environments (Moran 1994). However, this hypothesis is frequently breached in nature as demonstrated by numerous reports showing that the environment experienced by the juveniles often affects later adult life-history traits. Developmental plasticity of this type is often referred to as a "carryover effect" (Dickson et al. 2017, Moore and Martin 2019) and can impact a variety of adult traits such as survival, reproduction, body size, immunity to pathogens, and behavior (Relyea 2001, Watkins 2001, Alto et al. 2008, Muturi et al. 2011, Schmidt et al. 2012, Muller and Muller 2015, Collet and Fellous 2019). Therefore, accurate knowledge of the ecology of juvenile stages of organisms is critical to the understanding of how traits evolve in biological systems.

For many aquatic insects (e.g. mosquitoes, dragon flies, caddisflies, mayflies, and stoneflies), the juvenile stages develop in aquatic habitats while the adults are terrestrial. Water-filled containers such as tree holes, bamboo stumps, discarded cans, and waste tires are among a variety of aquatic habitats that are used by juveniles of aquatic insects, primarily mosquitoes. These systems are fueled by detritus from terrestrial environments, mainly leaf litter, with occasional input of invertebrate carcasses and stemflow (Carpenter 1982, Daugherty et al. 2000, Kitching 2000). The detritus is colonized by microbial communities which initiate the decomposition process and provide a critical food resource for mosquito larvae and other invertebrate consumers. Some of the microbes ingested by mosquito larvae colonize the midgut and other internal tissues and support larval growth and development (Coon et al. 2014, Coon et al. 2016,Valzania et al. 2018). A portion of these microbes is transstadially transmitted to the adult stage and contribute to host reproduction, resistance to toxicants, defense against pathogens, and other essential biological functions (Cirimotich et al. 2011, Coon et al. 2016, Soltani et al. 2017). Therefore, container-dwelling mosquitoes serve as excellent models for studying larval nutritional ecology as well as the interactions between invertebrate consumers and microbial communities in container habitats. This knowledge could inform formulation of hypotheses to test how the specific microbes encountered by juvenile insects contribute to adult performance and fitness.

The main mosquito species found in water-filled container habitats in mid-western USA include some important vectors such as *Aedes albopictus*, *Ochlerotatus triseriatus*, *Oc. japonicus*, *Culex pipiens*, and *Cx. restuans* (Yee et al. 2010, Bara and Muturi 2015, Bara et al. 2016). However, most studies on the interactions between mosquito larvae and the microbial communities in container habitats have mainly focused on *Oc. triseriatus*. These stud-

Received 22 August 2023; **revised** 30 January 2024; **accepted** 9 February 2024

ies reported that the presence of *Oc. triseriatus* larvae can affect the microbial abundance and composition in container habitats (Kaufman et al. 1999, Kaufman et al. 2001, 2008, Xu et al. 2008). Although *Cx. pipiens* and *Cx. restuans* larvae are major occupants of container habitats, and are important vectors of West Nile virus, little is known about their impact on microbial communities in container aquatic habitats (Muturi et al. 2020). The two *Culex* species tend to differ in their seasonal peaks in abundance, but often overlap during parts of the summer and early fall and can co-occur in the same container habitats (Harbison et al. 2017). The larvae of both species are filter feeders and are thought to essentially be ecological homologues with *Cx. restuans* as the slightly superior competitor (Reiskind and Wilson 2008).

In this study, we investigated how the microbial communities in container habitats respond to the feeding activity of *Cx. pipiens* and *Cx. restuans* larvae under intra- and interspecific rearing conditions. We also investigated how intra- and interspecific interactions among larvae of the two mosquito species affect their microbial composition and structure. This was accomplished using laboratory microcosms that mimicked the container aquatic habitats that are frequently utilized as larval habitats by these mosquito species. We predicted that the microbial communities of container-aquatic habitats would shift in response to larval feeding activity and that interactions among larvae of the two mosquito species would influence the microbial communities that colonize their bodies. The findings of this study improve our understanding of how invertebrate consumers interact with their prey (microbial communities) in container aquatic habitats and provide valuable knowledge on the larval ecology of two important West Nile virus vectors.

Materials and methods

Experimental set up

Culex egg rafts were collected from South Farms, an agriculture experimental farm of the University of Illinois located in Urbana, IL, USA. Nineteen-liter white cylindrical buckets (30 cm diameter) with overflow holes about 18 cm from the base were used as oviposition traps. Each bucket was baited with 3 L of 5-day-old grass infusion that was prepared by mixing 600 g of fresh grass with 50 L of tap water. The collections were done in May to late June 2021, a time associated with large populations of *Cx. restuans*, while *Cx. pipiens* are typically less abundant in central Illinois during this period (Westcott et al. 2011). The egg rafts were collected using a paint brush, placed in moist filter papers, and transported to the laboratory for hatching. More than 300 *Culex* egg rafts were collected for this study. Each egg raft was hatched individually in 12-well cell culture plates containing 3 mL of deionized water and a single first instar larva identified to species morphologically based on the presence of a clear scale anterior to the sclerotized egg-breaker that is present in *Cx. restuans* but not *Cx. pipiens* (Crabtree et al. 1995). First instar larvae for each species were pooled and rinsed three times with deionized water before they were added to the experimental microcosms.

The experimental microcosms included fifty 400-mL tri-pour beakers filled with 350 mL of grass infusion prepared as described above. The grass infusion was filtered with a screen mesh to exclude large debris before it was dispensed into the experimental containers without dilution. The 50 containers were divided into five experimental groups of 10 containers (10 replicates per group). Fifteen milliliter of grass infusion from group 1 containers were drawn immediately on day 0 for the processing and analysis of initial microbiota. This group is comprised of water initial (WI) samples. Groups 2 and 3 containers were each stocked with 40 first instar larvae of *Cx. pipiens* (PIP) and *Cx. restuans* (RES), respectively, while group 4 containers (BOTH) were stocked with 20 first instar larvae of each species (20 *Cx. pipiens* and 20 *Cx. restuans*). Group 5 containers were incubated without mosquito larvae (WNLV). The experimental containers were maintained in the environmental chamber (incubator) at 26°C, 70% relative humidity, and 14:10 h (light:dark cycle) and monitored daily for larval development. On day 5 when most larvae had matured to fourth instars, 15 mL of water samples from containers with and without mosquito larvae were collected after agitating the content and labeled by treatment (WI, WNLV, WPIP, WRES, and WBOTH). The larvae from all treatments (PIP, RES, and BOTH) were collected and preserved in a −80°C freezer in pools of five larvae. Three larval pools replicated five times for each of the 10 replicates per group were processed for microbiome analysis.

DNA extraction, library preparation, and sequencing

The water samples were centrifuged at 5000 × *g* for 20 min and DNA was isolated from the resulting pellet using DNeasy Power Soil Pro Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. For larval samples, each larval pool was surface-sterilized in 3% bleach solution for 3 min, transferred to 70% ethanol for 5 min, and then rinsed three times in 0.8% sterile phosphate-buffered saline solution for 10 s (Coon et al. 2014). DNA was extracted using the same kit used for the water samples. Before DNA extraction, the fourth instar *Cx. pipiens* and *Cx. restuans* larvae that were reared together (BOTH) were each identified morphologically and separated based on the number of hairs on the siphon with *Cx. restuans* bearing a single hair, whereas *Cx. pipiens* has three or more hairs (Ross and Horsfall 1965). This was further confirmed using a previously described TaqMan assay (Sanogo et al. 2007). Briefly, DNA extracted from the pooled samples was assayed as described in the reference with the following exceptions. The polymerase chain reaction (PCR) master mix was AmpliTaq gold 360 (Thermo Fisher Scientific, Waltham, MA, USA) and each of the three primer/probe sets was run as a single reaction using fluorescein as the probe dye. Three primer/probes sets were tested, *Cx. pipens*, *Cu. restuans*, and *Cx. quinquefasciatus*.

PCR amplification of the V3–V4 region of the 16S rRNA gene was accomplished using PCR primers 341f and 806r (Muyzer et al. 1993, Caporaso et al. 2011). Thermocycling conditions were 95°C, 10 min, 35 cycles of 95°C, 30 s; 58°C, 30 s; and 72°C, 60 s. After normalization of the PCR amplicons using the SequalPrep™ normalization plate (Thermofisher inc, Waltham, MA, USA), the sample libraries were pooled, quantified with a Kapa Library Quantification Kit (Kapa Biosystems Willington, MA, USA) and sequenced using an Illumina MiSeq system with a MiSeq V3 2 × 300 bp sequencing kit. Using CLC Bio Microbial Genomics module (Qiagen Inc., Valencia, CA, USA), demultiplexed reads were quality trimmed to Q30 and paired-end reads were merged, trimmed to fixed length, and clustered into operational taxonomic unit (OTU). Taxonomic affiliation was assigned at 97% sequence similarity using the SILVA database (Quast et al. 2013).

Statistical analyses

Data were imported into R version 4.1.0 statistical software and manipulated using the *phyloseq* package (McMurdie and Holmes 2013). Samples with <2397 sequences and OTUs with <10 sequences were discarded. For alpha diversity, samples were rar-

Table 1. Number of samples that were processed for each treatment.

Treatment	# of replicates	# of DNA samples
WI	10	10
PIP	10	50
RES	10	50
BOTH	10	60
WNLV	10	10
WBOTH	10	10
WPIP	10	10
WRES	10	10

efied to 2397 reads per sample to avoid biases arising from different sampling depths across sample types. Conversely, beta diversity was estimated based on log-normalized nonrarefied data using the *phyloseq* package. Alpha diversity metrics included Shannon diversity index and observed OTUs (richness) and were computed using *vegan* package in R (Oksanen et al. 2016). The nonparametric Kruskal–Wallis test was used to determine whether intra- and interspecific larval interactions had significant effects on microbial communities. Significant means were separated using Wilcoxon rank sum test with Bonferroni correction. Beta diversity ordination was conducted using the *microbiome* package and was achieved by applying principal component analysis (PCA) to the centered log-ratio transformed counts. Permutational multivariate analysis of variance (PERMANOVA) with 999 random permutations was used to test whether different treatments had a significant effect on bacterial OTU abundance. Pairwise adonis with Bonferroni corrections for multiple testing was used to perform pairwise comparisons between treatments. The multivariate homogeneity of group dispersions test was used to measure the overall variation in microbiome composition within treatments.

Results

In total, 210 samples including 50 water samples and 160 larval samples (Table 1) were sequenced generating 1 993 976 high-quality reads belonging to the domain bacteria. After discarding 11 samples with <2397 reads and OTUs with <10 reads, a total of 1 967 077 reads were retained (mean ± SE = 12763.49 ± 402.17). OTU diversity and richness differed significantly among treatments (Shannon diversity index: Kruskal–Wallis test, $\chi^2 = 129.6$, df = 8, $P < 0.001$; richness: Kruskal–Wallis test, $\chi^2 = 126.7$, df = 8, $P < 0.001$; Fig. 1). Pairwise comparisons revealed that water samples with mosquito larvae had significantly higher OTU diversity and richness relative to water samples that were incubated without mosquito larvae. When reared separately, *Cx. restuans* larvae had significantly higher bacterial OTU diversity and richness compared to *Cx. pipiens* larvae, but this effect was not observed when the two species were reared together. When reared together, *Cx. pipiens* OTU richness was increased, whereas that of *Cx. restuans* decreased. On bacterial OTU diversity, *Culex* larvae reared in the presence of the other species have elevated diversity, strongly for *Cx. pipiens* and weakly for *Cx. restuans*.

A total of six bacterial phyla were identified across all treatments (Fig. 2). Proteobacteria (41.98%) consisting of Gammaproteobacteria (30.53%), Alphaproteobacteria (4.96%), Epsilonproteobacteria (2.53%), Betaproteobacteria (2.21%), and Deltaproteobacteria (1.76%) was the most dominant phylum followed by Firmicutes (29.63%), and Bacteroidetes (22.23%). The other bacterial phyla identified included Actinobacteria (4.53%), TM7 (0.89%), and Verrucomicrobia (0.76%). All bacterial phyla were identified in all sample types but at varying proportions. Irrespective of larval presence or absence, Proteobacteria (33.50%–75.72%) and Bacteroidetes (35.49%–60.32%) were the most abundant bacterial phyla in water samples. Gammaproteobacteria was the most abundant Class of Proteobacteria in the water samples. Gammaproteobacteria, Alphaproteobacteria, Epsilonbacteria, and Verrucomicrobia were less abundant in water samples incubated without mosquito larvae (WNLV) than in water samples with mosquito larvae (WRES, WPIP, and WBOTH). In contrast, Bacteroidetes and Betaproteobacteria were more abundant in water samples incubated without mosquito larvae (WNLV) than in water samples with mosquito larvae (WRES, WPIP, and WBOTH).

In mosquito larvae, the dominant bacteria phyla varied markedly depending on whether the larvae of the two species were reared together or separately (Fig. 2). When reared together, both species had similar bacterial communities dominated by Firmicutes (81.46%–86.43%) and Actinobacteria (12.34%–15.79%). In contrast, the two species had different bacterial communities when reared separately. The most dominant bacterial phyla in *Cx. pipiens* larvae were Gammaproteobacteria (61.46%), Firmicutes (21.99%), and Alphaproteobacteria (11.38%), while the dominant bacterial phyla in *Cx. restuans* were Firmicutes (58.48%), Gammaproteobacteria (16.5%), Actinobacteria (7.44%), and Bacteroidetes (6.79%). In terms of changes in the trajectory of bacterial composition between groups, Gammaproteobacteria, the most abundant subphylum in water initial infusion (WI) reported a decrease in abundance compared to water samples incubated without mosquito larvae (WNLV), whereas Bacteroidetes abundance increased in the latter. Firmicutes and Alphaproteobacteria were more abundant in the *Cx. pipiens* larvae (PIP) samples compared to WI. The abundance of Gammaproteobacteria and Bacteroidetes was low in *Cx. restuans* larvae (RES) samples compared to WI treatment, whereas the Firmicutes were higher in the larvae samples. Gammaproteobacteria was more abundant in *Cx. pipiens* larvae samples compared to *Cx. restuans* larvae samples, whereas the Firmicutes were more abundant in *Cx. restuans* larvae samples. Compared to WNLV, the WBOTH treatment reported a higher Gammaproteobacteria abundance, whereas the Bacteroidetes decreased in abundance. Both Gammaproteobacteria and Firmicutes reported a higher abundance in *Cx. pipiens* (PIP) larvae reared separately compared to water samples containing both species (WBOTH); however, Bacteroidetes were more abundant in the water samples compared to the larvae samples. When reared together, both larvae samples had identical bacterial community composition that was dominated by Firmicutes and Actinobacteria (Fig. 2).

A total of 545 OTUs belonging to 171 genera were detected. The top 21 genera accounted for 80.29% of the total sequences (Table 2; Supplementary Fig. S1). The 10 most abundant genera included unclassified *Clostridium* (17.56%), unclassified Enterobacteriaceae (12.34%), *Thorsellia* (8.04%), *Bacteroides* (7.25%), *Pseudomonas* (5.0%), *Fluviicola* (4.72%), *Lucobacter* (3.52%), *Arcobacter* (2.52%), Bacillus (2.32%), and *Pedobacter* (1.9%). The same bacterial genera were detected in all water samples, but their relative abundance differed among treatments. *Cloacibacterium*, *Pseudomonas*, and unclassified Porphyromonadaceae were more abundant in water samples that were incubated without mosquito larvae (WNLV) compared to water samples with mosquito larvae (WRES, WPIP, and WBOTH). In contrast, unclassified Enterobacteriaceae, *Pedobacter*, *Enterobacter*, *Phenylobacterium*, *Acinetobacter*, *Arcobacter*, and unclassified Bacteriovoracaceae were more abundant in water samples with mosquito larvae (WRES, WPIP, and

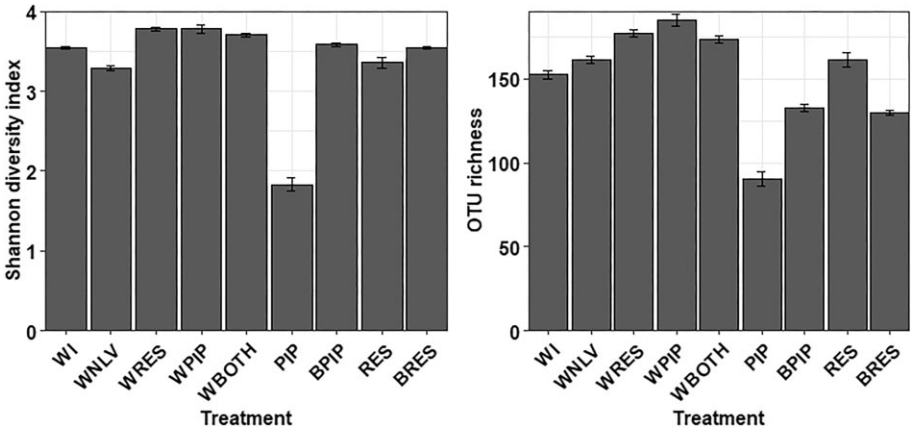

Figure 1. Bacterial OTU diversity and richness for *Culex pipiens* and *Culex restuans* larvae and water samples from the microcosms. Initial water infusion (WI), water infusion without larvae (WNLV), water infusion with *Cx. restuans* larvae (WRES), water infusion with *Cx. pipiens* larvae (WPIP), water infusion with both *Cx. pipiens* and *Cx. restuans* larvae (WBOTH), *Cx. pipiens* larvae (PIP), *Cx. pipiens* from containers with larvae of both species (BPIP), *Cx. restuans* larvae (RES), and *Cx. restuans* from containers with larvae of both species (BRES).

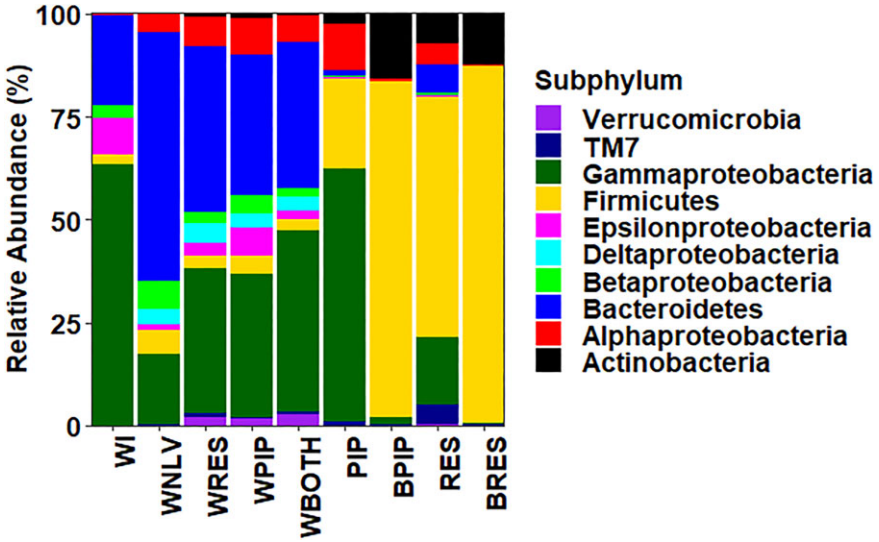

Figure 2. Relative abundance of bacterial phyla associated with different treatments. Initial water infusion (WI), water infusion without larvae (WNLV), water infusion with *Cx. restuans* larvae (WRES), water infusion with *Cx. pipiens* larvae (WPIP), water infusion with both *Cx. pipiens* and *Cx. restuans* larvae (WBOTH), *Cx. pipiens* larvae (PIP), *Cx. pipens* larvae from containers with larvae of both species (BPIP), *Cx. restuans* larvae (RES), and *Cx. restuans* larvae from containers with larvae of both species (BRES).

WBOTH) than in water samples without mosquito larvae (WNLV). Compared to WI samples, WNLV treatment reported more diverse bacteria genera (Supplementary Fig. S1) indicating that some bacteria were acquired in succession. Similarly, this was observed in water samples containing mosquito larvae (WRES, WPIP, and WBOTH) where more diverse bacteria genera were reported compared to the initial water infusion (WI). All microbial genera found in the larval samples were also present in the water samples. The dominant bacterial genera in the two mosquito species varied markedly depending on whether they were reared together or separately. When reared together (BPIP and BRES), the two species had identical bacterial genera dominated by *Clostridium* (41.57%–43.98%), followed by *Leucobacter* (10.09%–12.95%), unclassified Leuconostocacea (6.09%–9.18%), and *Bacillus* (6.01%–6.25%). Furthermore, the water samples containing both larvae species (WBOTH) reported more diverse genera compared to individual larvae samples which was dominated by *Enterobacteriaceae*, *Bacteroides*, and *Fluviicola*. In contrast, the two mosquito species were dominated by different bacterial genera when reared separately. The most abundant bacterial genus in *Cx. pipiens* (PIP) larvae was *Thorsellia* (59.85%), followed by *Clostridium* (18.36%) and then *Wolbachia* (10.66%). For *Cx. restuans* (RES), *Clostridium* (49.50%) was the most abundant bacterial genus followed by *Thorsellia* (10.01%), *Leucobacter* (5.12%), and unclassified Enterobacteriaceae (4.92%).

We conducted PCA and PERMANOVA to determine the extent of similarity between bacterial OTU abundance among treatment groups. PCA demonstrated the separation and clustering of both water and larvae samples by treatment groups. Bacteria communities in water samples (WI, WPIP, and WNLV) were clearly separated and distinct from WBOTH and WRES groups that clustered together (Fig. 3A). In larval samples, the bacteria communities in *Cx. restuans* and *Cx. pipiens* demonstrated a clear separation; the two groups were also distinct from BPIP and BRES treatments that clustered together (Fig. 3B). PERMANOVA analysis revealed that the difference in bacterial community composi-

Table 2. Percentage abundance of top 21 bacteria genera in different treatment groups.

Genus	Phylum	Sum	Percentage abundance of top 21 bacteria genera in each treatment								
			WI	WNLV	WRES	WPIP	WBOTH	PIP	BPIP	RES	BRES
Leucobacter	Actinobacteria	3.52	0.02	0.13	0.35	1.04	0.25	1.75	12.95	5.12	10.09
Bacteroides	Bacteroidetes	7.25	8.71	13.91	15.06	12.22	13.42	0.25	0.02	1.69	0.01
Fluviicola	Bacteroidetes	4.72	0.18	12.20	10.05	7.84	9.86	0.62	0.01	1.74	0.00
Pedobacter	Bacteroidetes	1.90	0.04	1.39	5.57	4.72	4.53	0.08	0.01	0.77	0.01
Porphyromonadaceae	Bacteroidetes	1.90	6.71	7.88	0.58	1.22	0.63	0.05	0.00	0.05	0.00
Cloacibacterium	Bacteroidetes	1.43	0.07	12.20	0.01	0.57	0.00	0.01	0.00	0.00	0.00
Dysgonomonas	Bacteroidetes	1.07	2.93	1.51	1.67	1.42	1.51	0.10	0.03	0.42	0.04
Clostridium	Firmicutes	17.56	0.71	1.26	0.66	1.55	0.42	18.36	43.99	49.50	41.57
Bacillus	Firmicutes	2.32	0.07	1.44	1.72	1.19	1.23	1.03	6.25	1.98	6.01
Leuconostocaceae	Firmicutes	1.72	0.01	0.01	0.01	0.02	0.02	0.04	6.09	0.06	9.18
Staphylococcus	Firmicutes	1.23	0.06	0.02	0.02	0.03	0.02	0.04	4.45	0.06	6.40
Clostridiales	Firmicutes	1.02	0.05	0.03	0.03	0.06	0.03	0.19	4.05	0.25	4.51
Enterobacteriaceae	Proteobacteria	12.34	26.31	1.93	24.25	20.57	32.12	0.79	0.06	4.92	0.14
Thorsellia	Proteobacteria	8.04	0.06	0.01	0.30	0.17	0.14	59.85	1.66	10.01	0.18
Pseudomonas	Proteobacteria	5.01	19.81	13.19	2.42	6.54	2.08	0.56	0.00	0.45	0.00
Arcobacter	Proteobacteria	2.52	8.96	1.51	3.13	6.72	2.15	0.14	0.00	0.10	0.00
Acinetobacter	Proteobacteria	1.63	6.71	0.74	2.02	2.32	2.34	0.10	0.02	0.39	0.02
Bacteriovoracaceae	Proteobacteria	1.53	0.01	2.12	4.64	3.38	3.41	0.09	0.00	0.14	0.00
Wolbachia	Proteobacteria	1.24	0.01	0.01	0.00	0.00	0.00	10.66	0.37	0.15	0.00
Enterobacter	Proteobacteria	1.18	3.47	0.19	2.17	1.75	2.72	0.04	0.00	0.23	0.00
Phenylobacterium	Proteobacteria	1.14	0.01	0.98	3.47	1.84	3.14	0.09	0.01	0.74	0.01

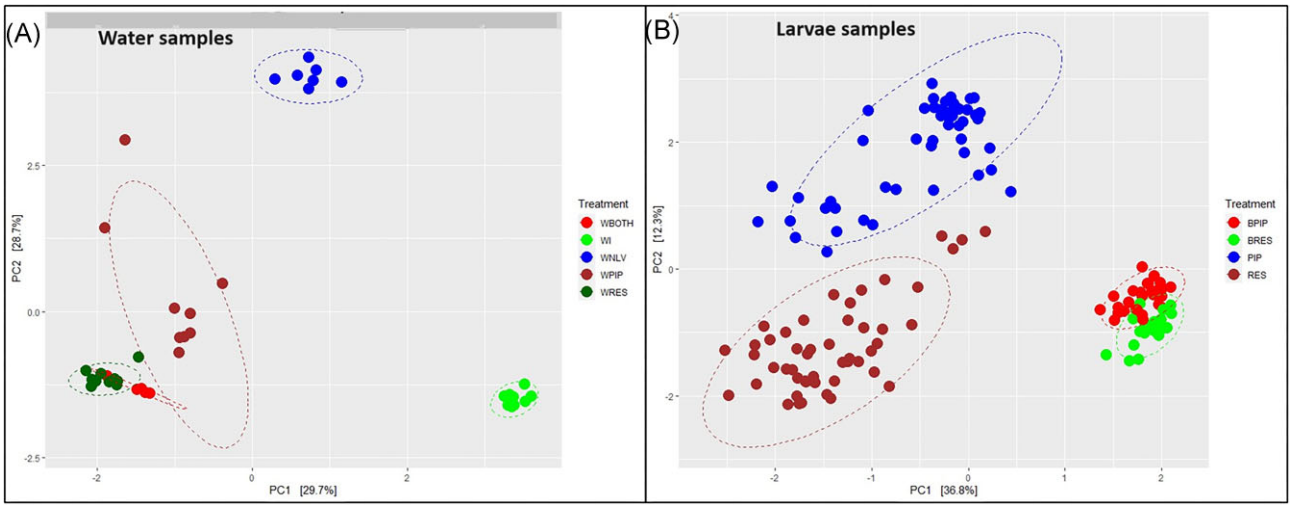

Figure 3. PCA of bacterial community of water samples: (A) initial water infusion (WI), water infusion without larvae (WNLV), water infusion with *Cx. restuans* larvae (WRES), water infusion with *Cx. pipiens* larvae (WPIP), water infusion with both *Cx. pipiens* and *Cx. restuans* larvae (WBOTH); and larvae samples: (B) *Cx. pipiens* larvae (PIP), *Cx. pipiens* from containers with larvae of both species (BPIP), *Cx. restuans* larvae (RES), and *Cx. restuans* from containers with larvae of both species (BRES).

tion was significant ($F = 44.98$, df = 8, 183, $R^2 = 0.66$, $P = 0.001$). Pairwise comparisons with Bonferroni adjustments for multiple comparisons revealed that all treatments were significantly different from each other. Test for multivariate dispersion was also significant ($F = 7.67$, df = 8, 163, $P = 0.001$). The average distance to median was 14.95 (WI), 15.40 (WNLV), 20.75 (WPIP), 16.50 (WRES), 12.68 (WBOTH), 19.62 (PIP), 21.33 (RES), 18.22 (BPIP), and 18.55 (BRES). Post-hoc tests revealed that dispersion was significantly greater in water samples with *Cx. pipiens* larvae (WPIP) relative to initial water samples (WI) and water samples with larvae of both species (WBOTH). However, none of these samples differed significantly from water samples with *Cx. restuans* (WRES) or water samples that were incubated without larvae (WNLV). Dispersion was not significantly different between *Cx. pipiens* (PIP) and *Cx. restuans* (RES) larvae that were reared separately but was significantly higher in *Cx. restuans* reared under intraspecific condition compared to *Cx. pipiens* from interspecific treatment.

Discussion

In this study, we investigated how intra- and interspecific interactions among larvae of two closely related container-dwelling mosquito species (*Cx. pipiens* and *Cx. restuans*) affect the microbial communities of container aquatic habitats. We also evaluated how these interactions affect the larval microbiota of the two mosquito species. Overall, we found that larval feeding and activity alters the microbial community composition and richness in container aquatic habitats, and that the two mosquito species

harbored similar microbial communities when sharing the larval environment and distinct microbial communities when reared separately under a common food resource. These findings are consistent with our previous findings where the presence of *Cx. restuans* larvae altered the bacterial community composition, abundance, diversity, and richness in the water column (Muturi et al. 2020). Similar findings were reported in a study by Coon et al. (2014) where the relative abundance of different bacterial families differed among *Aedes aegypti*, *Anopheles gambiae*, and *Georgecraigius atropalpus* larvae that were reared separately under the same food resource and laboratory conditions. Interestingly, Saab et al. (2020) reported that *An. gambiae* and *Aedes albopictus* adults derived from a shared larval environment harbored distinct gut microbiota which is inconsistent with our study. However, unlike our study, which focused on larval microbiota, Saab and colleagues' study focused on the impact of larval environment on gut microbiota of adult mosquitoes. Thus, the two studies are not directly comparable since many microbes are lost during metamorphosis and adult emergence (Moll et al. 2001).

Larvae of both mosquito species suppressed the abundance of *Cloacibacterium*, *Pseudomonas*, and unclassified Porphyromonadaceae and enhanced the abundance of unclassified Enterobacteriaceae, *Pedobacter*, *Enterobacter*, *Phenylobacterium*, *Acinetobacter*, *Arcobacter*, *Phenylobacterium*, and unclassified Bacteriovoracaceae. Kaufman et al. (1999) reported the suppression of Pseudomonadaceae and enhancement of Enterobacteriaceae by *Oc. triseriatus* larvae, which is consistent with our findings. A similar study by the same research group reported the suppression of Alphaproteobacteria and Bacteroidetes and enhancement of Betaproteobacteria by *Oc. triseriatus* larvae (Kaufman et al. 2008). In the current study, mosquito larvae suppressed Bacteroidetes, Betaproteobacteria, and Firmicutes, and enhanced Gammaproteobacteria, Alphaproteobacteria, Epsilonproteobacteria, and Verrucomicrobia in the simulated larval container aquatic habitats. Thus, only the suppression of Bacteroidetes mirrors the findings of Kaufman and colleagues (Kaufman et al. 2008). However, the pattern observed at the phylum level did not hold at the genus level where there were some genera from Bacteroidetes that were enhanced (e.g. *Pedobacter*) and some genera from Gammaproteobacteria that were suppressed (e.g. *Pseudomonas*) in water samples with mosquito larvae. While the enhancement of Verrucomicrobia and the suppression of *Pseudomonas* is consistent with our previous study with *Cx. restuans* larvae, we also find discrepancies between the two studies in which Gammaprotebacteria was enhanced in this study and suppressed in the previous study (Muturi et al. 2020). Collectively, these findings demonstrate that the interaction between the mosquito larvae and bacterial communities in container habitats is complex and can vary markedly within and between species. Larval feeding activity and/or larval excretions are some of the likely mechanisms through which the mosquito larvae may alter the microbial composition of their larval habitats (Kaufman et al. 2008, Saab et al. 2020), as some bacterial taxa may be more susceptible than others to digestion, redox potential, and the alkaline gut condition of mosquito larvae (Austin and Baker 1988, Sota and Kato 1994, Vallet-Gely et al. 2008).

Differences in microbial composition and diversity between *Cx. pipiens* and *Cx. restuans* larvae could partly be due to species differences in their physiology and/or feeding behavior. *Culex pipiens* larvae filter-feed on microbial communities on the water surface but may modify this behavior in response to resource availability (Yee et al. 2004). While *Culex restuans* larvae have similar mouthpart morphology as *Cx. pipiens* larvae, their feeding behavior is poorly understood. If larvae of the two species filter-feed at different water depths, they may be exposed to different bacterial communities (Gimonneau et al. 2014). Additionally, the larvae of the two mosquito species may also have different physiologies that may control the type of microbes that colonize and establish in their bodies. Further studies are needed to establish the contribution of larval physiology and larval feeding behavior to species differences in microbial composition and structure. Differences in bacteria composition and abundance may also have been caused by interactions between bacterial communities in both water and larvae treatments. Interbacterial interactions can be either symbiotic or competitive, which may promote growth and survival or deprivation of nutrients in others (Kern et al. 2021). Antagonistic interactions between bacteria communities such as exploitation and interference competition result in competition for, or alteration of a shared niche and nutrients (Hedge et al. 2018, Coyte and Rakoff-Nahoum 2019, Kozlova et al. 2021). Exploitation competition for a shared food resource can limit the resource for the competing bacterial community. Interference competition on the other hand may alter the niche or harm other bacteria taxa through the release of toxins or molecules, leading to replacement of some communities (Jakubovics 2015).

It is unclear why *Cx. pipiens* and *Cx. restuans* larvae harbored identical bacterial communities when reared together in the same containers. Given the small size of the containers used in this study, it is possible that individuals of the two species frequently came into contact and physically transferred the bacteria from one individual to another. We surface-sterilized the larvae before DNA isolation but it is possible that the sterilization was not perfect.

Thorsellia was detected from larvae of both mosquito species but was more abundant in *Cx. pipiens* than in *Cx. restuans* larvae, and among intraspecific treatment than in interspecific treatment. This bacterium was also present in water samples in low abundance suggesting that mosquito larvae may have acquired it from the larval environment. It is unclear why the abundance of *Thorsellia* was less abundant in larvae samples from the treatment where both species were reared together, but as alluded earlier, it is possible that interspecific interactions may have exposed the larvae of both mosquito species to other microbes that were antagonistic to *Thorsellia*. In *Cx. tarsalis*, *Thorsellia* was detected across all stages and accounted for 31.0%, 52.3%, 93.0%, and 3.3% of the total sequences, in early instar larvae, late instar larvae, pupae, and adults from field collected larvae, respectively (Duguma et al. 2015). In *Cx. nigripalpus* larvae, *Thorsellia* accounted for 46.5% of the total sequences (Duguma et al. 2017). *Thorsellia* has also been isolated in malaria vectors, including *An. gambiae*, *An. arabiensis*, *An. stephensis*, and *An. culifacies* (Lindh et al. 2005, Briones et al. 2008, Rani et al. 2009, Wang et al. 2011, Chavshin et al. 2014). It was also isolated from the water surface microlayer of *An. gambiae* habitats (Briones et al. 2008). The ability of *Thorsellia* to tolerate high pH and to utilize blood as a carbon source have been cited as potential explanations for its ability to thrive in the midguts of some mosquito species (Briones et al. 2008).

Clostridium was detected in larvae of both mosquito species but was less abundant in *Cx. pipiens* larvae that were reared separately from *Cx. restuans*. Previous studies reported *Clostridium* to account for 72.9% of the total sequences in *Cx. restuans* larvae (Muturi et al. 2020), 22.5% of the total sequences in *Cx. nigripalpus* larvae, and 5.7% of the total sequences in *Cx. colonator* larvae (Duguma et al. 2017). Thus, it appears to be a common bacterium among larvae of *Culex* mosquitoes that have been examined so far. Unsurprisingly, *Wolbachia*, a maternally inherited endosymbiont that infects numerous invertebrates was abundant in *Cx. pipiens* larvae and rare

in *Cx. restuans* larvae, as observed in our previous studies (Muturi et al. 2016, 2020).

The high abundance of *Thorsellia* in *Cx. pipiens* larvae and *Clostridium* in *Cx. restuans* larvae in our study warrants further investigation to determine their role in mosquitoes, focusing on mosquito life-history traits and vector competence. For the simulated larval habitats, *Cloacibacterium* and *Fluviicola* abundance was very high in water incubated without mosquito larvae (WNLV) compared to the initial grass infusion (WI), indicating that these bacteria taxa abundance may have increased over time due to either exploitative or interference competition among the bacterial community. Conversely, the abundance of *Enterobacteriaceae*, *Arcobacter*, and *Acinetobacter* was very low in water incubated without mosquito larvae compared to the initial grass infusion, an indication that these pioneer bacteria genera were lost over time.

In summary, we found that *Cx. pipiens* and *Cx. restuans* larval feeding and activity alters the microbial community composition, diversity, and richness in container aquatic habitats and that intra- and interspecific interactions among larvae of the two mosquito species strongly impacts the microbial communities that colonize their bodies. These findings demonstrate that mosquito larvae play a vital role in structuring the community of microbes in container aquatic habitats and that biotic interactions in aquatic habitats are key determinants of *Cx. pipiens* and *Cx. restuans* larval microbiota. Some microbial communities are known to enhance or suppress pathogen transmission and thus, the observed within and between species differences in microbial communities may account for individual and population variation in vector competence that is commonly reported among wild caught mosquitoes. Further studies are needed to examine whether the results of this study carryover to the adult stage and to assess how the distinct bacterial communities detected among larvae that were reared under intra- and interspecific treatments affect vector competence for West Nile virus and other pathogens that are transmitted by the two mosquito species. Additional studies should also investigate which of the detected microbes are consumed by mosquito larvae and assess how larval feeding activity affects litter decomposition in container aquatic habitats.

Acknowledgments

The authors would like to thank Leta Chesser, Edna Alfaro, and Millon Blackshear at Illinois Natural History Survey and Heather Walker and Madeleine Adolf at the US Department of Agriculture for their technical support. This work was supported by the Used Tire Management and Emergency Public Health Funds from the State of Illinois, and the US Department of Agriculture, Agricultural Research Service. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the US Department of Agriculture. The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned. USDA is an equal opportunity provider and employer.

Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

Conflict of interest: None declared.

References

Alto BW, Lounibos LP, Mores CN *et al.* Larval competition alters susceptibility of adult *Aedes* mosquitoes to dengue infection. *Proc Biol Sci* 2008;**275**:463–71.

Austin DA, Baker JH. Fate of bacteria ingested by larvae of the freshwater mayfly, *Ephemera danica*. *Microb Ecol* 1988;**15**:323–32.

Bara JJ, Muturi EJ. Container type influences the relative abundance, body size, and susceptibility of *Ochlerotatus triseriatus* (Diptera: culicidae) to La Crosse Virus. *J Med Entomol* 2015;**52**: 452–60.

Bara JJ, Parker AT, Muturi EJ. Comparative susceptibility of *Ochlerotatus japonicus*, *Ochlerotatus triseriatus*, *Aedes albopictus*, and *Aedes aegypti* (Diptera: culicidae) to La Crosse Virus. *J Med Entomol* 2016;**53**: 1415–21.

Briones AM, Shililu J, Githure J *et al. Thorsellia anophelis* is the dominant bacterium in a Kenyan population of adult *Anopheles gambiae* mosquitoes. *ISME J* 2008;**2**:74–82.

Caporaso JG, Lauber CL, Walters WA *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *P Natl Acad Sci USA* 2011;**108**:4516–22.

Carpenter S. Stem flow chemistry: effects on population dynamics of detrivorous mosquitoes in tree hole ecosystems. *Oecologia* 1982;**53**:1–6.

Chavshin AR, Oshaghi MA, Vatandoost H *et al.* Isolation and identification of culturable bacteria from wild *Anopheles culicifacies*, a first step in a paratransgenesis approach. *Parasit Vectors* 2014;**7**:419.

Cirimotich CM, Ramirez JL, Dimopoulos G. Native microbiota shape insect vector competence for human pathogens. *Cell Host Microbe* 2011;**10**:307–10.

Collet J, Fellous S. Do traits separated by metamorphosis evolve independently? Concepts and methods. *P Roy Soc B Biol Sci* 2019;**286**:20190445.

Coon KL, Brown MR, Strand MR. Gut bacteria differentially affect egg production in the anautogenous mosquito *Aedes aegypti* and facultatively autogenous mosquito *Aedes atropalpus* (Diptera: culicidae). *Parasit Vectors* 2016;**9**:375.

Coon KL, Vogel KJ, Brown MR *et al.* Mosquitoes rely on their gut microbiota for development. *Mol Ecol* 2014;**23**:2727–39.

Coyte KZ, Rakoff-Nahoum S. Understanding competition and cooperation within the mammalian gut microbiome. *Curr Biol* 2019;**29**:R538–44.

Crabtree MB, Savage HM, Miller BR. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. *Am J Trop Med Hyg* 1995;**53**:105–9.

Daugherty MP, Alto BW, Juliano SA. Invertebrate carcasses as a resource for competing *Aedes albopictus* and *Aedes aegypti* (Diptera: culicidae). *J Med Entomol* 2000;**37**:364–72.

Dickson LB, Jiolle D, Minard G *et al.* Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. *Sci Adv* 2017;**3**:e1700585.

Duguma DM, Hall W, Rugman-Jones P *et al.* Developmental succession of the microbiome of *Culex* mosquitoes. *BMC Microbiol* 2015;**15**:140.

Duguma DM., Hall W, Smartt CT *et al.* Temporal variations of microbiota associated with the immature stages of two Florida *Culex* mosquito vectors. *Microb Ecol* 2017;**74**:979–89.

Harbison JE, Hulsebosch B, Buczek J *et al.* Reduced productivity of *Culex pipiens* and *Cx. restuans* (Diptera: Culicidae) mosquitoes in parking area catch basins in the northeast Chicago metropolitan area. *J Vector Ecol* 2017;**42**:148–54.

Hedge S, Khanipov K, Albayrak L *et al.* Microbiome interaction networks and community structure from laboratory-reared and field-collected *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquito vectors. *Front Microbiol* 2018;**9**:2160.

Jakubovics NS. Inter-microbial interactions as a driver for community composition and stratification of oral biofilms. *J Mol Biol* 2015;**427**:3662–75.

Kaufman MG, Bland SN, Worthen ME *et al.* Bacterial and fungal biomass responses to feeding by larval *Aedes triseriatus* (Diptera: culicidae). *J Med Entomol* 2001;**38**:711–9.

Kaufman MG, Chen S, Walker ED. Leaf-associated bacterial and fungal taxa shifts in response to larvae of the tree hole mosquito, *Ochlerotatus triseriatus*. *Microb Ecol* 2008;**55**:673–84.

Kaufman MG, Walker ED, Smith TW *et al.* Effects of larval mosquitoes (*Aedes triseriatus*) and stemflow on microbial community dynamics in container habitats. *Appl Environ Microb* 1999;**65**:2661–73.

Kern L, Abdeen SK, Kolodziejczyk AA *et al.* Commensal inter-bacterial interactions shaping the microbiota. *Curr Opin Microbiol* 2021;**63**:158–71.

Kitching RL. *Food Webs and Container Habitats: the Natural History and Ecology of Phytotelmata*. London: Cambridge University Press, 2000.

Kozlova EV, Hegde S, Roundy CM *et al.* Microbial interactions in the mosquito gut determine *Serratia* colonization and blood-feeding propensity. *ISME J* 2021;**15**:93–108.

Lindh JM, Terenius O, Faye I. 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiae* sensu lato and *A. funestus* mosquitoes reveals new species related to known insect symbionts. *Appl Environ Microb* 2005;**71**:7217–23.

McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013;**8**:e61217.

Moll RM, Romoser WS, Modrzakowski MC *et al.* Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: culicidae) metamorphosis. *J Med Entomol* 2001;**38**:29–32.

Moore MP, Martin RA. On the evolution of carry-over effects. *J Anim Ecol* 2019;**88**:1832–44.

Moran NA. Adaptation and constraint in the complex life-cycles of animals. *Annu Rev Ecol Syst* 1994;**25**:573–600.

Muller T, Muller C. Behavioural phenotypes over the lifetime of a holometabolous insect. *Front Zool 12 Suppl* 2015;**1**:S8.

Muturi EJ, Dunlap C, Caceres CE. Microbial communities of container aquatic habitats shift in response to *Culex restuans* larvae. *FEMS Microbiol Ecol* 2020;**96**:fiaa112.

Muturi EJ, Kim CH, Alto BW *et al.* Larval environmental stress alters *Aedes aegypti* competence for Sindbis virus. *Trop Med Int Health* 2011;**16**:955–64.

Muturi EJ, Kim CH, Bara J *et al.* *Culex pipiens* and *Culex restuans* mosquitoes harbor distinct microbiota dominated by few bacterial taxa. *Parasit Vectors* 2016;**9**:18.

Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* 1993;**59**:695–700.

Oksanen J, Blanchet G, Friendly M *et al.* *vegan: community ecology package*. R package version 2.3-5, 2016. https://CRAN.R-project.org/package=vegan. (25 July 2023, date last accessed).

Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;**41**:D590–6.

Rani A, Sharma A, Rajagopal R *et al.* 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* 2009;**9**:96.

Reiskind M, Wilson ML. Interspecific competition between larval *Culex restuans* Theobald and *Culex pipiens* L. (Diptera: culicidae) in Michigan. *J Med Entomol* 2008;**45**:20–27.

Relyea RA. The lasting effects of adaptive plasticity: predator-induced tadpoles become long-legged frogs. *Ecology* 2001;**82**:1947–55.

Ross HH, Horsfall WR. A synopsis of the mosquitoes of Illinois (Diptera, Culicidae). *Biol Notes* 1965;**052**.

Saab SA, Dohna HZ, Nilsson LKJ *et al.* The environment and species affect gut bacteria composition in laboratory co-cultured *Anopheles gambiae* and *Aedes albopictus* mosquitoes. *Sci Rep* 2020;**10**:3352.

Sanogo YO, Kim CH, Lampman R *et al.* A real-time TaqMan polymerase chain reaction for the identification of Culex vectors of West Nile and Saint Louis encephalitis viruses in North America. *Am J Trop Med Hyg* 2007;**77**:58–66.

Schmidt BR, Hodl W, Schaub M. From metamorphosis to maturity in complex life cycles: equal performance of different juvenile life history pathways. *Ecology* 2012;**93**:657–67.

Soltani A, Vatandoost H, Oshaghi MA *et al.* The role of midgut symbiotic bacteria in resistance of *Anopheles stephensi* (Diptera: culicidae) to organophosphate insecticides. *Pathog Glob Health* 2017;**111**:289–96.

Sota T, Kato K. Bacteria as diet for the mosquito larvae *Aedes* (Stegomyia) (Diptera, Culicidae)-preliminary experiments with *Pseudomonas fluorescens*. *Appl Entomol Zool* 1994;**29**:598–3600.

Vallet-Gely I, Lemaitre B, Boccard F. Bacterial strategies to overcome insect defences. *Nat Rev Micro* 2008;**6**:302–13.

Valzania L, Martinson VG, Harrison RE *et al.* Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. *PLoS Negl Trop Dis* 2018;**12**:e0006638.

Wang Y, Gilbreath TM, Kukutla P *et al.* Dynamic gut microbiome across life history of the malaria mosquito *Anopheles gambiae* in Kenya. *PLoS One* 2011;**6**:e24767.

Watkins TB. A quantitative genetic test of adaptive decoupling across metamorphosis for locomotor and life-history traits in the Pacific tree frog, *Hyla regilla*. *Evolution* 2001;**55**:1668–77.

Westcott NE, Hilberg SD, Lampman RL *et al.* Predicting the seasonal shift in mosquito populations preceding the onset of the West Nile virus in central Illinois. *Bull Am Meteorol Soc* 2011;**92**:1173–80.

Xu Y, Chen S, Kaufman MG *et al.* Bacterial community structure in tree hole habitats of *Ochlerotatus triseriatus*: influences of larval feeding. *J Am Mosq Control Assoc* 2008;**24**:219–27.

Yee DA, Kesavaraju B, Juliano SA. Larval feeding behavior of three co-occurring species of container mosquitoes. *J Vector Ecol* 2004;**29**:315–22.

Yee DA, Kneitel JM, Juliano SA. Environmental correlates of abundances of mosquito species and stages in discarded vehicle tires. *J Med Entomol* 2010;**47**:53–62.

Received 22 August 2023; **revised** 30 January 2024; **accepted** 9 February 2024