Preprint

Geographic heterogeneity in *Anopheles albimanus* microbiota is lost within one generation of laboratory colonization

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Abstract

Research on mosquito-microbe interactions may lead to new tools for mosquito and mosquito-borne disease control. To date, such research has largely utilized laboratory-reared mosquitoes that may lack the microbial diversity of wild populations. To better understand how mosquito microbiota may vary across different geographic locations and upon laboratory colonization, we characterized the microbiota of F_1 progeny of wild-caught adult *Anopheles albimanus* from four locations in Guatemala using high throughput 16S rRNA amplicon sequencing. A total of 132 late instar larvae and 135 2-5day old, non-blood-fed virgin adult females were reared under identical laboratory conditions, pooled (3 individuals/pool) and analyzed. Larvae from mothers collected at different sites showed different microbial compositions (p=0.001; F = 9.5), but these differences were no longer present at the adult stage (p=0.12; F =1.6). This indicates that mosquitoes retain a significant portion of their field-derived microbiota throughout immature development but shed them before or during adult eclosion. This is the first time the microbiota of F_1 progeny of wild-caught mosquitoes has been characterized in relation to parental collection site, and our findings provide evidence that geographically associated heterogeneity in microbiota composition persists for a single generation, but only until the end of the larval stage. These findings advance our understanding of how the mosquito microbiota is altered upon first laboratory colonization, and raises considerations for how mosquito microbiome research may be extended beyond the laboratory to field settings.

Keywords

mosquito microbiota, *Anopheles albimanus*, metabarcoding, next generation sequencing, 16S rRNA amplicon sequencing, microbiome

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Introduction

Mosquitoes contain microbes that inhabit various tissues, such as the alimentary canal, reproductive organs, and cuticle surface (1). These microbes are thought to be principally obtained from the mosquito habitat during larval development, and from food sources at the adult stage (1). In addition to acquisition from larval habitats and/or adult food sources, transovarial bacterial transmission from adult females to their eggs, and transstadial transmission across different immature stages, and into the adult stage, have been demonstrated (2, 3). As a key component of the mosquito microbiota, environmentally-acquired microbes have been shown to affect mosquito life history traits such as the rate of pupation and adult body size (4). The mosquito microbiota has also been shown to affect mosquito immunity to human pathogens (5), reproduction (6), insecticide resistance (7, 8), and ultimately vector competence—the mosquito's ability to acquire, maintain and transmit pathogens (5). These effects on multiple physiological characteristics are being leveraged to develop novel approaches to fight mosquito-borne diseases (9).

The use of next generation molecular biology tools has resulted in extensive characterization of mosquito microbiota, with the initial focus on bacterial and archaeal components now expanding to eukaryotic microbes (10, 11) and viruses (12, 13). These advances in mosquito microbiota research have led to field applications of mosquito symbionts for mosquito control. For *Aedes aegypti*, the principal vector of dengue, Zika, chikungunya and yellow fever viruses, mosquito-derived symbionts are now being used to suppress mosquito populations (14) and also being considered to control the spread of pathogens (15). However, studies exploring mosquito symbionts for malaria control have largely remained at the laboratory stage (16, 17). Similarly, the microbiota of mosquito vectors in some geographical regions are well characterized and studied compared to those from other regions. In malaria vectors for example, studies on the microbiota have largely focused on Sub-Saharan African species—in particular, *Anopheles gambiae*—and to a lesser extent on those from Southeast Asia (18). In contrast, the microbiota of Latin American malaria vectors have only recently been comprehensively characterized (7, 8, 19-21), with these studies describing associations between *An. albimanus* microbiota and insecticide resistance (7, 8), and the factors that shape the composition of *An. darlingi*, *An. albimanus*, *An. nunetzovari*, *An. rangeli,* and *An. triannulatus* microbiota (19-21).

To exploit the mosquito microbiota for malaria and malaria vector control, research must successfully advance from laboratory to field settings, a transition which can be fraught with challenges. For example, some malaria vectors such as *An. darlingi, An. vestitipennis,* and *An. gambiae,* breed in sites that are small, temporary and often difficult to find and/or access (22-26), making it hard to obtain sufficient immature field mosquitoes for experiments. Where larval habitats are plentiful and easy to find and/or access, the subsequent rearing of field-collected mosquitoes to obtain uniform characteristics can pose additional challenges (27, 28). Additionally, some malaria vectors belong to species complexes whose members are morphologically indistinguishable (29-32), constituting another layer of complexity that needs to be considered in elucidating mosquito-microbe interactions in malaria vectors.

These challenges, which are common to research on mosquito ecology and control, are often not reported or discussed in mosquito microbiome studies. Several failed attempts at collecting and rearing sufficient immature mosquitoes from the field for our previous study on the role of mosquito microbiota in insecticide resistance resulted in ultimately using either wild-caught adults (7) or F_1 progeny derived from field-collected adult mosquitoes (8, 33). While field-caught adult mosquitoes or their F_1 progeny may offer insights into mosquito-microbe interactions in field scenarios, obtaining adult field-collected mosquitoes with uniform and/or controlled physiological characteristics is not feasible. Although geographically associated heterogeneity in microbiota of field-collected mosquitoes has been previously described (34, 35), there is limited information on the fate of

field-acquired microbiota after laboratory colonization of field-collected mosquitoes. However, it has been previously observed that upon eclosion, newly-emerged laboratory-reared adult mosquitoes show a reduction in bacterial diversity in contrast to earlier developmental stages (36). In addition, a recent study on the fate of field-acquired microbiota in laboratory-colonized *An. gambiae s.l.* showed a reduction in bacterial diversity of the F_5 progeny (the first point of measurement) that were reared in dechlorinated tap water in contrast to F_0 (37). Another study of *Ae. aegypti* from different geographical locations showed no geographically associated heterogeneity microbiota composition after several generations of laboratory colonization (38). At what point the microbiota of laboratory-colonized mosquitoes become homogenous and whether the microbiota of F_1 laboratory progeny represent their parental origin—and thus could be used for symbiont-based translational studies—remains largely undescribed.

Here, we present findings on the microbiota of laboratory-reared F_1 progeny from field-caught adult *An. albimanus*. We studied whether, upon laboratory colonization, F_1 larvae and adults retained their location- and/or maternally-derived microbiota in both internal and cuticle surface microbial niches. We discuss implications or our results for advancing mosquito microbiota research applications from the laboratory to the field.

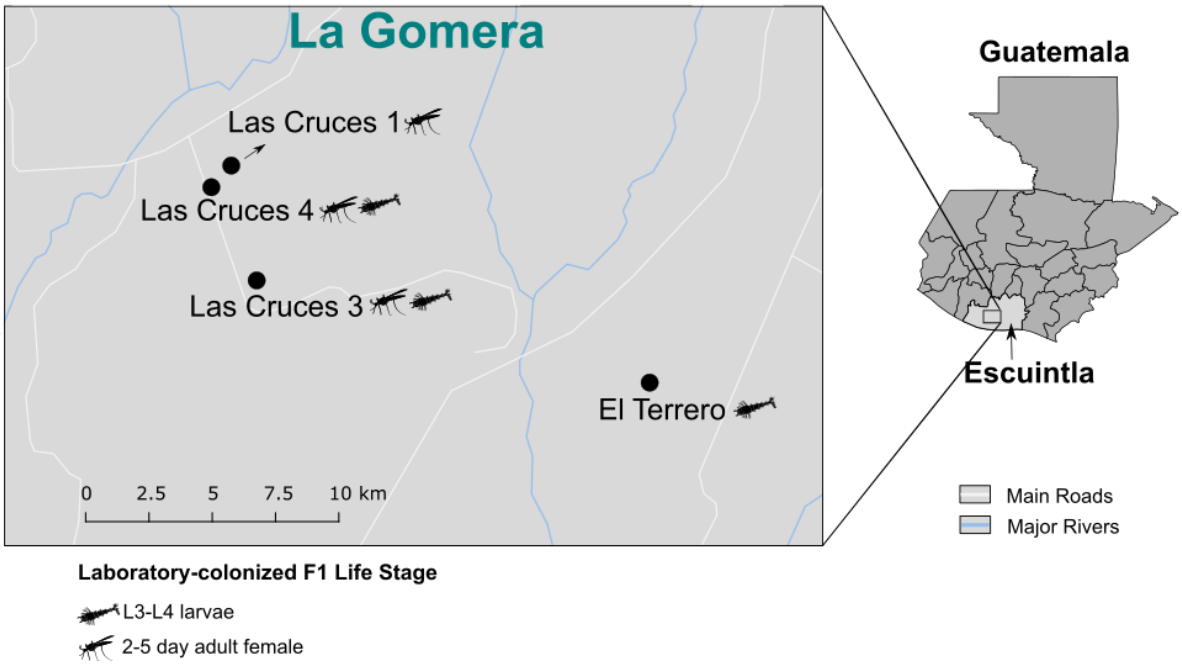


Figure 1: Map showing the geographical origins of F_1 *Anopheles albimanus*. F_1 laboratory-colonized mosquitoes were derived from gravid and/or blood fed females collected from each location. Mosquito icons show the geographical origin of the F_1 life stages studied.

Results

Using high throughput deep sequencing of the universal bacterial and archaeal 16S rRNA gene, we characterized the microbiota of the internal and cuticle surface microbial niches of laboratory reared *An. albimanus* F_1 larvae (n=132) and adult (n=135) progeny originating from mothers collected at four locations in south central Guatemala (Fig 1). Mosquitoes were processed as pools (3 individuals per pool), resulting in a total of 44 larval and 45 adult pools. F_1 larvae were from mothers collected in El Tererro, Las Cruces 3 and Las Cruces 4, while F_1 adults were from mothers collected in Las Cruces 1, Las Cruces 3 and Las Cruces 4 (Table 1). Following quality control of the resulting sequencing reads, amplicon sequence variants (ASVs) were identified and used in downstream analysis. Since

adult mosquito microbiota is distinct from that of immature stages (39, 40), ASVs from larvae and adults were analyzed separately, as were ASVs from each microbial niche.

Table 1. Descriptive statistics of laboratory colonized F_1 *An. albimanus* processed, per life stage and maternal collection site.

Maternal collection site	Number of mosquitoes (number of pools)	
	L3-L4 larvae	Adult ♀s
El Terrero	45 (15)	-
Las Cruces 1	-	27 (9)
Las Cruces 3	42 (14)	45 (15)
Las Cruces 4	45 (15)	63 (21)

Internal and cuticle surface microbiota of laboratory-colonized F_1 An. albimanus larvae differed by maternal collection site

Table 2. Pairwise alpha and beta diversity comparisons of laboratory colonized F_1 *An. albimanus* microbiota from different maternal collection sites. a. Pairwise beta (Bray-Curtis) diversity comparison showed significant differences in larval internal and cuticle surface microbiota between maternal collection sites. In contrast, only adult cuticle surface but not internal microbiota were significantly different across maternal collection sites. **b.** Pairwise alpha (Shannon) diversity comparison showed significant differences in larval internal but not cuticle surface microbiota between maternal collection sites (two of the three pairs). In contrast, there was no significant difference in adult internal or cuticle surface microbiota between maternal collection sites. Pairwise alpha and beta diversity comparisons were conducted using Kruskal-Wallis and PERMANOVA (999 permutations) tests respectively, with Benjamini-Hochberg FDR correction (q-value). Significance was determined at $q < 0.05$.

a.

			Internal			Cuticle surface		
	Group 1	Group 2	pseudo-F	p-value	q-value	pseudo-F	p-value	q-value
Adults	LasCruses1	LasCruses3	2.21	0.088	0.161	**5.80**	**0.001**	**0.003**
	LasCruses1	LasCruses4	1.92	0.107	0.161	**4.97**	**0.004**	**0.005**
	LasCruses3	LasCruses4	0.85	0.443	0.443	**3.07**	**0.005**	**0.005**
Larvae	ElTerrero	LasCruses3	**9.81**	**0.001**	**0.001**	**4.48**	**0.001**	**0.001**
	ElTerrero	LasCruses4	**7.98**	**0.001**	**0.001**	**11.43**	**0.001**	**0.001**
	LasCruses3	LasCruses4	**10.88**	**0.001**	**0.001**	**11.37**	**0.001**	**0.001**

b.

			Internal			Cuticle surface		
	Group 1	Group 2	H	p-value	q-value	H	p-value	q-value
Adults	LasCruses1	LasCruses3	0.00	0.98	0.98	1.09	0.30	0.41
	LasCruses1	LasCruses4	0.49	0.48	0.72	1.23	0.27	0.41
	LasCruses3	LasCruses4	1.77	0.18	0.55	0.67	0.41	0.41
Larvae	ElTerrero	LasCruses3	**6.63**	**0.01**	**0.02**	4.45	0.03	0.10
	ElTerrero	LasCruses4	**6.94**	**0.01**	**0.02**	1.87	0.17	0.26
	LasCruses3	LasCruses4	0.49	0.48	0.48	1.10	0.29	0.29

Non-pairwise Bray-Curtis distance comparison showed significant differences in internal ($p=0.001$) and cuticle surface ($p=0.001$) microbiota between F_1 larvae from different maternal collection sites. Thus, irrespective of microbial niche, the microbial community structure (composition and relative abundance of ASVs) of F_1 laboratory-colonized larvae differed based on maternal collection location. Pairwise PERMANOVA comparison of

Bray-Curtis distances further showed significant differences in microbial community structure in larval internal ($q<0.01$) and cuticle surface microbiota ($q<0.01$) between every pair of maternal collection sites (Table 2a). This location-driven heterogeneity in microbial community structure was further demonstrated by principal coordinate analysis (PCoA), where F_1 larval internal and cuticle surface microbiota clustered distinctly by maternal collection site (Fig. 2).

Non-pairwise Shannon diversity comparisons showed significant differences in internal ($p=0.009$) but not cuticle surface ($p=0.09$) microbiota of F_1 laboratory-colonized larvae from different maternal collection sites, indicating that there was inter-sample variation in the diversity of internal but not cuticle surface microbiota of larvae when all maternal collection sites were taken into consideration. A pairwise Kruskal-Wallis comparison of Shannon diversity indices showed that the inter-sample variation in diversity of larval internal microbiota held true when every pair of maternal collection sites was considered except between Las Cruces 3 and 4 (Table 2b and Suppl. 1). Larvae originating from Las Cruces 3 had the highest internal microbiota diversity, followed by Las Cruces 4 and El Terrero (Suppl. 1)

Cuticle surface, but not internal, microbiota of laboratory-colonized F_1 adult An. albimanus differed by maternal collection site

Non-pairwise Bray-Curtis diversity comparisons showed significant differences in cuticle surface ($p=0.001$), but not internal microbiota ($p=0.12$) between adult F_1 mosquitoes from different maternal collection sites, indicating that location-driven heterogeneity in microbial community structure was lost in internal but not cuticle surface microbial niche of laboratory-colonized F_1 adults. Pairwise PERMANOVA comparisons of Bray-Curtis distances also showed significant differences in microbial community structure of F_1 adult cuticle surface microbiota ($q<0.01$) between every pair of maternal collection sites (Table 2a). These results were corroborated by PCoA which showed that F_1 adult cuticle surface microbiota, but not internal microbiota, clustered distinctly by maternal collection site (Fig. 2).

Non-pairwise Shannon diversity comparisons showed no differences in the internal ($p=0.42$) or cuticle surface ($p=0.4$) microbiota of F_1 adults from different maternal collection sites, indicating that there was no inter-sample variation in diversity of F_1 adult microbiota when all maternal collection sites were taken into consideration. Pairwise Kruskal-Wallis comparisons of Shannon diversity indices also detected no inter-sample variation in diversity of F_1 adult cuticle surface or internal microbiota when every pair of maternal collection sites was considered (Table 2b and Suppl. 1).

Laboratory-colonized F_1 An. albimanus larvae comprised a rich and diverse microbiota that differed by maternal collection site

Overall, ASVs from larval internal microbiota were assigned to 180 bacterial taxa, and cuticle surface microbiota to 194 bacterial taxa (suppl 2.). A majority of these taxa across all locations (ranging from 118-139 taxa) were shared between the internal and cuticle surface microbiota (Fig 3a), as well as across maternal collection sites (n=110 for cuticle surface and n=117 for internal microbiota) (Fig 3b). While a majority of the identified microbial taxa were shared between both microbial niches, their abundance was generally higher in internal (Fig 4a) compared to cuticle surface (Fig 4b) microbiota. Although a majority of identified microbial taxa in both internal and cuticle surface microbiota were shared across all locations, their abundance differed by maternal location (Fig 4a, 4b and 5).

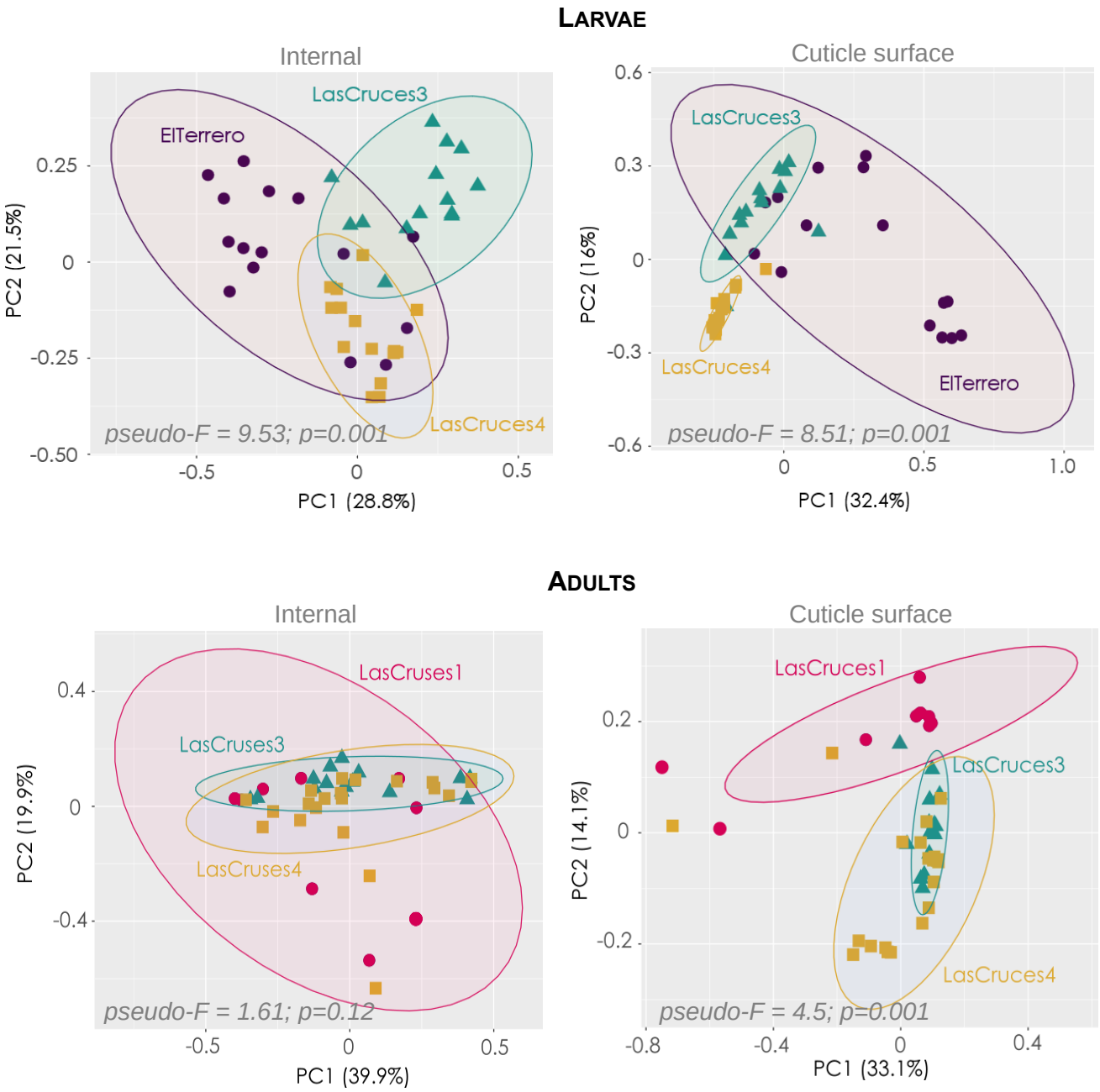


Figure 2. Principal coordinate analysis (PCoA) ordinations of internal and cuticle surface microbiota from F_1 laboratory-colonized *An. albimanus*. The PCoA plots are based on Bray-Curtis distances between the microbiota of mosquitoes with differing maternal collection sites. Each point on the plot represents the microbial composition of a pool of three individuals, and mosquito pools are color-coded by their origin/maternal location. For larvae, the first two principal component (PC) axes captured 50% (internal) and 48% (cuticle surface) of the variance in the data, with both internal and cuticle surface microbiota clustering distinctly by maternal collection site. For adults, the first two PC axes captured 59% (internal) and 47% (cuticle surface) of the variance in the data, with cuticle surface but not internal microbiota clustering distinctly by maternal collection site. PERMANOVA statistics are presented at the bottom of each plot.

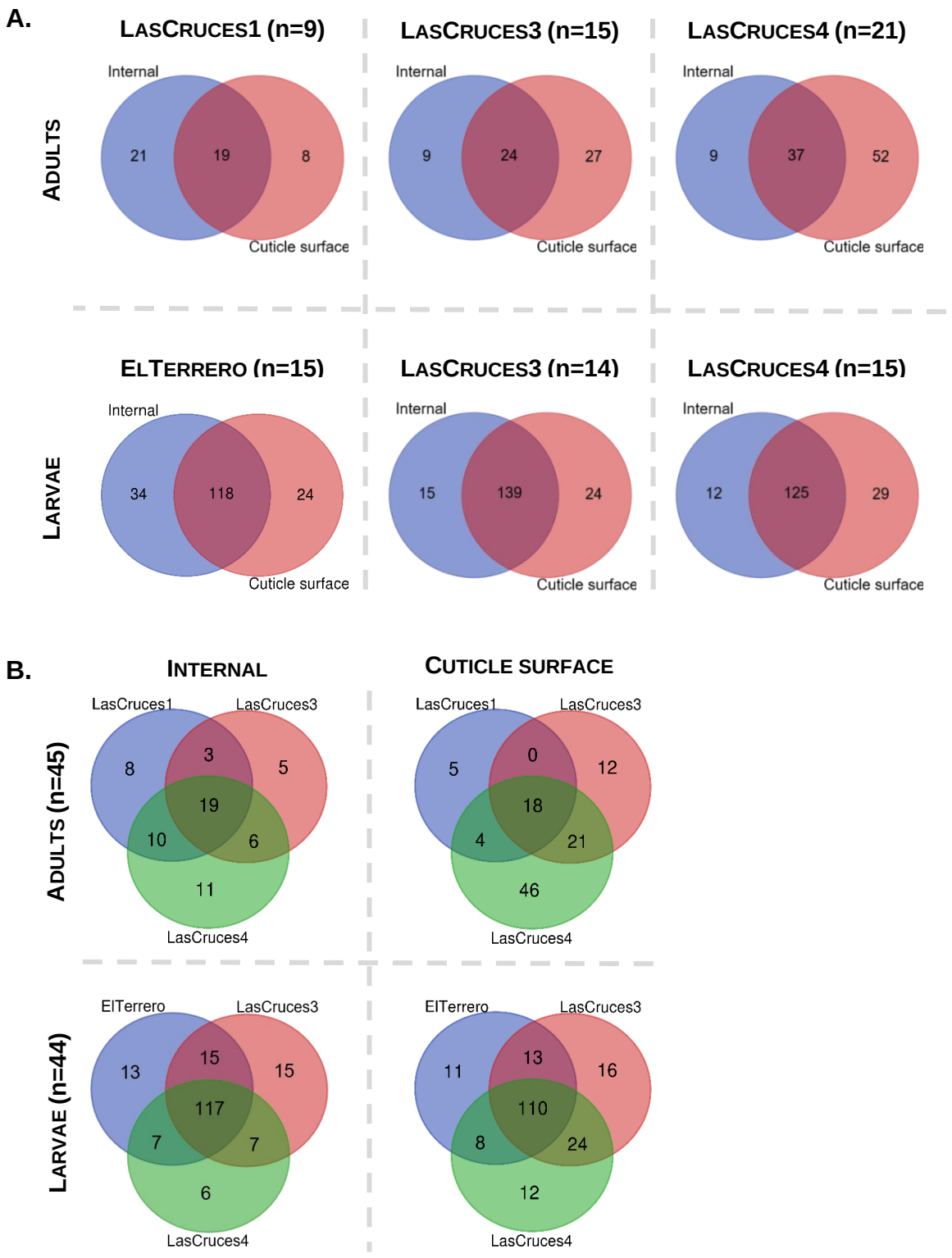


Figure 3. Number of unique and shared microbial taxa between microbial niches (A); number of unique and shared microbial taxa between maternal collection sites (B). The number of taxa shown in the Venn diagram represent bacterial taxa except in the cuticle surface microbiota of adults from Las Cruces 4, where two archaeal taxa were present. n = pools of mosquito samples analyzed per location or microbial.

In general, larval internal microbiota was dominated by ASVs identifed as an uncharacterized *Enterobacteriaceae, Leucobacter, Thorsellia,* and *Chryseobacterium* (Fig 4a), together making up over 50% of ASVs (Suppl. 2). In contrast, *Acidovorax,* unchracterized *Comamonadaceae,* and *Paucibacter* (Fig 4b) made up over 50% of ASVs detected on the larval cuticle surface (Suppl. 2).

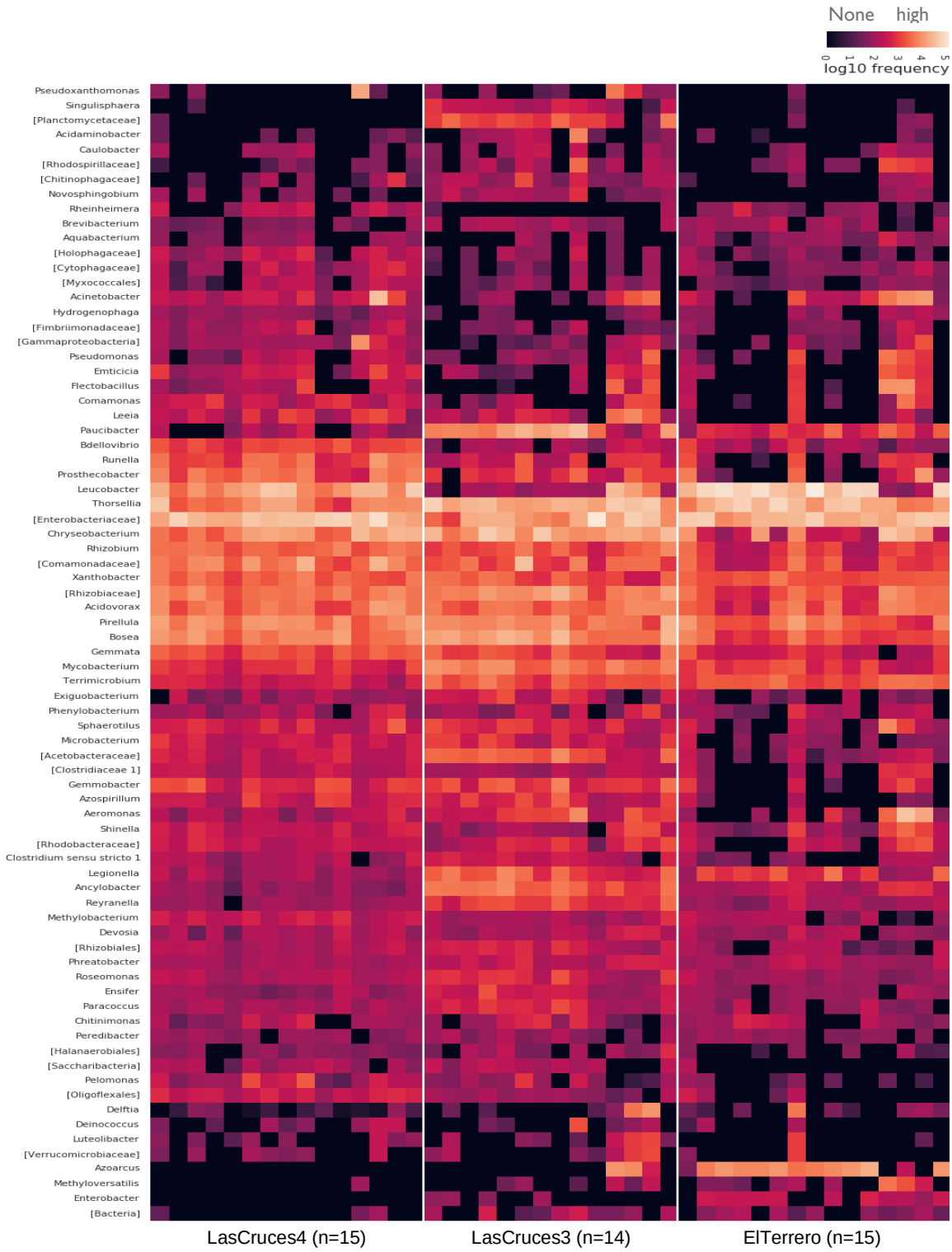
A few predominant bacterial taxa were present in the larval internal microbial niche across all three maternal collection sites: unclassified *Enterobacteriaceae, Thorsellia, Rhizobium, Xantobacter, Acidovorax* and *Pirellula* (Fig 4a.), with remaining taxa showing different patterns of abundance between collection sites (Fig 5 and Suppl. 2). For example, ASVs assigned to the genus *Azoracus* were predominant in larvae from El Terrero, while *Singulisphaera, Paucibacter, Ancylobacter, Gemmobacter,* and *Rayranella* were predominant in those from Las Cruces 3. Predominant in larvae from Las Cruces 3 and Las Cruces 4 were *Azospirillum, Bosea* and *Microbacterium*; and in those from El Terrero and Las Cruces 3 were *Terrimicrobium* and *Legionella* (Fig 5). Although outside of the cut off limit set for differential abundance, ASVs assigned to the bacterial genera *Leucobacter* were predominant in the internal microbiota of larvae from Las Cruces 4 and El Terrero, but predominant in only three of the 14 sample replicates from Las Cruces 3 (Fig 4a). Similarly, *Chryseobacterium* was predominant in Las Cruces 4 and 3, but only predominant in six of the 16 pools of larvae from El Terrero. Bacterial taxa that were unique to each location comprised <8% of all taxa in larval internal microbiota (Suppl. 3b), and were below the threshold for inclusion in the heatmap and differential abundance testing (Suppl. 2).

Unlike the internal microbial niche, no microbial taxa was predominant in larval cuticle surface microbiota across all three collection sites. However, some taxa showed notable patterns of abundance between locations (Fig 5). These included the genus *Azoarcus,* which was detected at low to moderate frequencies in 13 of 15 pools of larvae from El Terrero, at low frequency in a single pool of larvae from Las Cruces 3, and was not detected at all in Las Cruces 4 (Fig 4b and 6). Similarly, ASVs assigned to the genus *Spirosoma* were detected at moderate frequencies in all pools of larvae from Las Cruces 4, but only in a few pools from the other two locations. ASVs assigned to the genus *Paucibacter* were present at relatively higher abundance in larvae from both Las Cruces 3 and El Terrero compared to those from Las Cruces 4. Those assigned to the genus *Acidovorax* were predominant in larvae from Las Cruces 3 and Las Cruces 4 in contrast to El Terrero. ASVs assigned to *Microbacterium, Bdellovibrio* and *Pelomonas* were present at moderate frequencies in larvae from both Las Cruces 3 and Las Cruces 4 but were not detected in El Terrero(Fig. 5). Bacterial taxa that were unique to each maternal collection site comprised <8% of larval cuticle surface microbiota (Fig 3b), and were below the threshold for inclusion in the heatmap and differential abundance testing (Suppl. 2).

Laboratory-colonized adult F_1 An. albimanus were comprised of sparse internal and cuticle surface microbiota that were dominated by ASVs assigned to the genus Asaia

ASVs from adult internal microbiota were assigned to 62 microbial taxa and cuticle surface microbiota were assigned to 106 microbial taxa. Two of these ASVs which were only present in the cuticle surface microbiota were classified as archaea, while all other remaining ASVs were classified as bacteria (Suppl. 2). Unlike larval microbiota, less than half of the assigned taxa across all locations (ranging from 19-37 taxa) were shared between internal and cuticle surface microbiota (Fig 3a), and only 18 taxa on the cuticle surface and 19 internal taxa were shared across all maternal collection sites (Fig 3b).

a.


None
high
0 1 2 3 4 5
log10 frequency
Pseudoxanthomonas
Singulisphaera
[Planctomycetaceae]
Acidaminobacter
Caulobacter
[Rhodospirillaceae]
[Chitinophagaceae]
Novosphingobium
Rheinheimera
Brevibacterium
Aquabacterium
[Holophagaceae]
[Cytophagaceae]
[Myxococcales]
Acinetobacter
Hydrogenophaga
[Fimbriimonadaceae]
[Gammaproteobacteria]
Pseudomonas
Emticicia
Flectobacillus
Comamonas
Leeia
Paucibacter
Bdellovibrio
Runella
Prosthecobacter
Leucobacter
Thorsellia
[Enterobacteriaceae]
Chryseobacterium
Rhizobium
[Comamonadaceae]
Xanthobacter
[Rhizobiaceae]
Acidovorax
Pirellula
Bosea
Gemmata
Mycobacterium
Terrimicrobium
Exiguobacterium
Phenylobacterium
Sphaerotilus
Microbacterium
[Acetobacteraceae]
[Clostridiaceae 1]
Gemmobacter
Azospirillum
Aeromonas
Shinella
[Rhodobacteraceae]
Clostridium sensu stricto 1
Legionella
Ancylobacter
Reyranella
Methylobacterium
Devosia
[Rhizobiales]
Phreatobacter
Roseomonas
Ensifer
Paracoccus
Chitinimonas
Peredibacter
[Halanaerobiales]
[Saccharibacteria]
Pelomonas
[Oligoflexales]
Delftia
Deinococcus
Luteolibacter
[Verrucomicrobiaceae]
Azoarcus
Methyloversatilis
Enterobacter
[Bacteria]
LasCruces4 (n=15)
LasCruces3 (n=14)
ElTerrero (n=15)

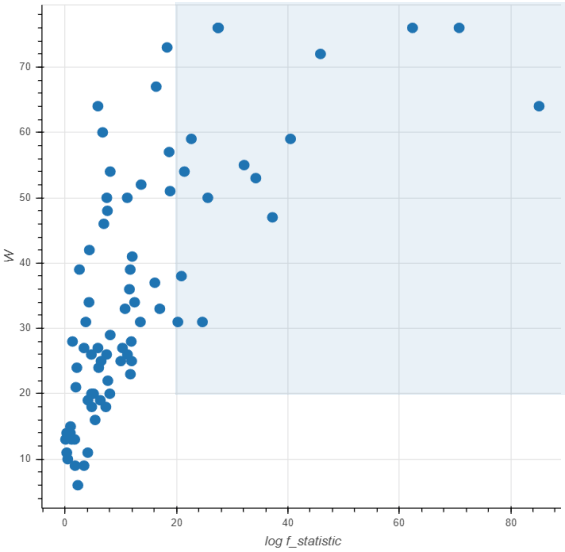
b.

Figure 4. Frequency of ASVs from the internal (a) and cuticle surface (b) microbiota of laboratory-colonized *An. albimanus* F_1 larvae originating from different maternal collection sites. ASVs were annotated to the genus level or the lowest possible taxonomic level (in square brackets) and are clustered by the average nearest-neighbors chain algorithm. Only taxonomically annotated ASVs with frequencies ≥2000 are presented.

Overall, ASVs assigned to the bacterial genus *Asaia* dominated both adult internal and cuticle surface microbiota (Fig 6), constituting at least 70% of taxa in each microbial niche (Suppl. 2). A majority of identified taxa in adult internal, but not cuticle surface, microbiota was detected across all three maternal collection sites, with a few of these taxa present in high abundance across all collection sites (Fig. 6). Across all three maternal collection sites, ASVs assigned to the genera *Acinetobacter, Gluconobacter, Pantoea* and *Pseudomonas* were present in moderate to high abundance in adult internal microbiota in addition to *Asaia* (Fig 6).

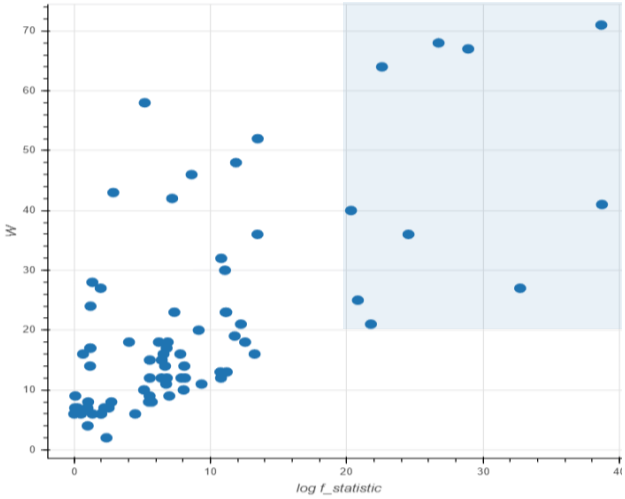
After excluding low abundance taxa (Suppl. 2), the number of remaining taxa did not meet the requirements for identifying collection site-specific microbial taxa in adult cuticle surface microbiota. This was compounded by dominance (>70%) of ASVs that were assigned to the bacterial genus *Asaia* (Suppl. 2). In addition, the cuticle surface microbiota of adults originating from Las Cruces 4 comprised 43% of all adult cuticle surface microbial taxa (Fig 3b), although a majority were of low abundance.

LARVAL INTERNAL MICROBIOTA



Significantly different taxa	W	ElTerrero	LasCruces3	LasCruces4
Singulisphaera	76		✓	
[Planctomycetaceae]	76		✓	
Paucibacter	76		✓	
Azoarcus	76	✓		
Azospirillum	72		✓	✓
Ancylobacter	64		✓	
Gemmobacter	59		✓	
Terrimicrobium	59	✓	✓	
Legionella	54	✓	✓	
[Oligoflexales]	55		✓	✓
Reyranella	53		✓	
[Acetobacteraceae]	50		✓	
Mycobacterium	47		✓	
[Saccharibacteria]	38		✓	✓
Bosea	31		✓	✓
Microbacterium	31		✓	✓

LARVAL CUTICLE SURFACE MICROBIOTA



Significantly different taxa	W	ElTerrero	LasCruces3	LasCruces4
Azoarcus	71	✓		
Paucibacter	68	✓	✓	
[Planctomycetaceae]	67		✓	
Ancylobacter	64		✓	
Microbacterium	41		✓	✓
Spirosoma	40			✓
Bdellovibrio	36		✓	✓
[Oligoflexales]	27			✓
Pelomonas	25		✓	✓
Acidovorax	21		✓	✓

Figure 5. Volcano plots of differentially abundant bacterial taxa in F_1 laboratory colonized *An. albimanus* larvae originating from three different maternal collection sites. The plots show results

of analysis of composition of microbiomes (ANCOM) tests for differentially abundant microbial taxa between maternal collection site, with an effect size set to log F≥20, and a cut-off of differential abundance set to W≥20 (i.e. a taxon was differentially abundant across maternal collection sites if the ratio of its abundance to those of at least 20 other taxa (25% of all included taxa) differed significantly across sites). Differentially abundant taxa are highlighted (blue shaded area) and the taxa names and locations in which they were most abundant are presented in the adjoining tables.

Figure 6. Frequency of ASVs from the internal and cuticle surface microbiota of laboratory-colonized F_1 adult *An. albimanus* originating from different locations. ASVs were annotated to the genus level or the lowest possible taxonomic level (in square brackets) and are clustered by the average nearest-neighbors chain algorithm. Only taxonomically annotated ASVs with frequencies ≥1000 are presented.

Discussion

The scientific community is increasingly investigating the role of mosquito microbiota in fighting mosquito-borne diseases (9). The successful transition of mosquito microbiome research from laboratory to field requires a comprehensive understanding of the

dynamics underlying the composition of the microbiota of field-collected mosquitoes and their progeny. We provide evidence of the fate of field-acquired microbiota following laboratory colonization in F_1 progeny of field-collected *An. albimanus*. Our results show that while location-driven heterogeneity in the microbial community structure of both internal and cuticle surface microbiota was present in laboratory-colonized F_1 larvae, this heterogeneity was only evident in the cuticle surface microbiota of adult progeny from the same generation. This suggests that field-acquired mosquito microbiota can be lost in as early as the first generation of laboratory colonization.

Previous studies on other mosquito species have also shown a loss of field-acquired internal mosquito microbiota following several generations of laboratory colonization (37, 38). Until now, however, the laboratory generation at which this loss occurs was largely undescribed. Our study fills this knowledge gap and provides further details regarding the dynamics of changes in both the internal and cuticle surface microbial niches. This loss of field-acquired internal microbiota by the adult stage within the first generation of laboratory colonization could pose an additional set of challenges for field or semi-field mosquito microbiome studies—particularly studies that rely on laboratory progeny in lieu of field populations to circumvent the challenges involved (25, 27, 28) in obtaining sufficient numbers of wild mosquitoes of uniform physiological characteristics. Previous findings showed that rearing mosquitoes in water from the field could preserve the field-derived internal microbiota in laboratory-colonized adult progeny of *An. gambiae s. l.* for several generations (37). In addition, other studies, have shown that the microbiota in larval habitat water significantly influences the internal microbiota of emerging adult mosquitoes (40, 41). Thus, to preserve wild-type microbiota, care should be taken to ensure that the first laboratory generation are reared in water collected from larval habitats in the field. This first generation would be most suitable for studies that require progeny from field populations.

Maternal egg-smearing has been proposed as a mechanism through which adult female mosquitoes and other insects transfer microbes to their progeny (42-45). This egg-smearing could explain the location-driven heterogeneity observed in both internal and cuticle surface microbiota of larval progeny in this study, since laboratory progeny were generated by forced oviposition using blood-fed and/or gravid adult female mosquitoes collected from different field sites. Subsequent proliferation of these maternally derived microbiota in larval rearing trays thus resulted in the presence of collection site-derived microbes in the water where the respective larval progeny hatched and grew. A lack of this location-driven heterogeneity in the internal microbiota of adult progeny could be attributed to the physiological changes that occur during metamorphosis and adult eclosion, whereby elimination of the larval meconial peritrophic membrane and meconium (midgut and midgut content), along with ingestion of exuvial fluid—which is said to be bactericidal—results in sterile or nearly sterile midguts in newly emerged adults (39, 46). This, in addition to mechanisms that regulate the composition of adult mosquito internal microbiota (47-49), could explain the observed homogeneity in F_1 adult internal microbiota. Conversely, the collection site-driven heterogeneity in cuticle surface microbiota of adult progeny suggests that collection site-derived microbes in the rearing trays may have colonized adult cuticle surfaces during emergence. The mechanisms underlying the assemblage of the mosquito cuticle surface microbiome are largely undescribed, and thus require further investigation.

The low inter-sample variation in microbial diversity observed in this study has largely been described in laboratory mosquito colonies (38, 50), and contrasts starkly to the high inter-sample variations in microbial diversity reported in wild mosquitoes (51, 52). This low inter-sample variation in microbial diversity likely arises due to rearing mosquitoes under controlled, laboratory conditions and analyzing samples with uniform physiological characteristics for this study. The microbial composition of laboratory-reared larvae is typically less diverse (50, 53) compared to those of field-derived larvae, but our laboratory-reared larvae exhibited a rich microbial composition that was comparable to those

of field populations (39, 40). In contrast, our adult progeny had a less diverse microbial composition that was reflective of typical laboratory-reared adult mosquitoes (36, 54). These findings further show that field-acquired microbiota, although transferred to laboratory progeny, can be lost within one generation of laboratory colonization—particularly at the adult stage.

In this study, we detected microbial taxa that have previously been identified in *Anopheles* and other mosquito genera (7, 41, 55, 56). While a majority of the taxa in larval laboratory progeny were shared between both the internal and cuticle surface microbial niches, a greater abundance of microbial taxa was detected in the internal microbial niche compared to the cuticle surface. The cuticle surface microbiota of mosquitoes and other hematophagous insects are largely uncharacterized and the mechanisms underlying their assemblage remain unknown. As such, we hypothesize that although both internal and cuticle surface niches are exposed to the same water from which the microbiota is derived, a more conducive and protected internal environment allows for greater proliferation of colonizing bacteria. In the adult progeny however, less than half of the detected microbial taxa were shared between the internal and cuticle surface microbial niches, suggesting differences in physiological conditions that favor microbial colonization, and corroborating findings that suggest the presence of microbial regulatory mechanisms within the mosquito midgut (47-49). This minimal overlap of microbial taxa between internal and cuticle surface microbial niches also underpins the contrasts in the fate of maternally acquired microbes between both niches. Although a few microbial taxa overlapped between adult internal and cuticle surface microbial niches and the most abundant taxa were shared, many of the unshared taxa have been previously detected in adult mosquitoes including *Anopheles* (1, 56, 57), indicating that the cuticle surface microbiota characterized in this study are inherently associated with mosquitoes. Like the larval microbiota, a greater abundance of microbial taxa was detected in the adult internal microbial niche compared to the cuticle surface, further supporting our hypothesis of a more conducive and protected internal environment permitting greater proliferation of colonizing bacteria.

With the exception of the adult cuticle surface microbial niche, a majority of all detected microbial taxa were shared across maternal collection sites in both larval and adult laboratory progeny, albeit with differing abundances. This reflects how the controlled laboratory environment contributed to shaping the mosquito microbiota. In both microbial niches of both larvae and adults, microbial taxa that were specific to maternal collection sites were low in abundance, compared to the moderate to high abundance of those that were shared across all locations. This was particularly true for *Asaia*—notorius for rapidly colonizing laboratory mosquitoes (58)—which constituted at least 70% of both adult internal and cuticle surface microbiota from progeny across all maternal collection sites. These results reflect how field-derived microbes persist upon initial laboratory colonization of mosquitoes, but measures beyond regular laboratory rearing conditions are required to maintain them.

We recognize that not having the microbial community profiles of the mothers from which the F_1 progeny were derived is a limitation of this study. However, these findings represent an unanticipated discovery that arose out of a separate study whose focus was on field-derived F_1 progeny reared under controlled settings (8). The findings herein advance our understanding of the effects of laboratory colonization on field-derived mosquito microbiota and provides novel insight regarding the mosquito cuticle surface microbiota. A comprehensive understanding of the fate of field-derived microbes upon laboratory colonization is critical for advancing mosquito microbiome studies and their applications beyond laboratory settings.

Materials and methods

Laboratory generation of F_1 progeny and 16S rRNA amplicon sequencing

This study was conducted as part of a larger project aimed at determining how insecticide exposure affects mosquito internal and cuticle surface microbiota (8). Thus, the mosquito collection, processing and sequencing procedures have previously been described in detail (8). Briefly, gravid and/or blood-fed adult female *An. albimanus* were sampled across four field sites in La Gomera, Escuintla, Guatemala (Figure 1).

Field-collected mosquitoes were brought to the insectary at Universidad del Valle de Guatemala in Guatemala City for species identification, oviposition and subsequent rearing of F_1 progeny. Eggs from field-collected females that were identified as *An. albimanus* were pooled by collection site and reared separately under identical laboratory conditions. A total of 132 third to fourth instar larvae (L3-L4) were processed for microbial community characterization. The remaining larvae were reared until the pupal stage, then separated by sex to obtain virgin adults. The microbial communities of 135 non-blood fed, 2-5 day old females were characterized. Larval and adult samples were preserved in RNALater® solution (Applied Biosystems, Foster City, CA), shipped on dry ice to the US Centers for Disease Control and Prevention (CDC) in Atlanta, USA, and stored at -80 ^{o}C until further processing.

Mosquitoes were pooled (3 individuals per pool), resulting in 44 pools of larvae and 45 pools of adults for microbial community characterization. Table 1 shows the number of pools (replicates) processed per life stage and maternal collection site.

Following the procedures outlined in (8), genomic DNA was extracted from the cuticle surface and from surface sterilized (subsequently referred to as 'internal microbiota') whole larvae and adults using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). The resulting DNA was used as template for PCR targeting the V3-V4 region of the universal bacterial and archaeal 16S rRNA gene. The resulting 16S rRNA gene amplicons were sequenced on an Illumina HiSeq 2500, using 2x250 cycle paired-end sequencing kits. All mosquito handling and processing for microbial community characterization were performed under sterile conditions with blanks (negative controls) included for each processing step and then sequenced.

Preprocessing of sequencing reads

Sequencing outputs were demultiplexed and converted to the fastq format for downstream analysis using the bcl2fastq (v2.19) conversion software (Illumina®). A total of 115,250,077 demultiplexed paired-end sequencing reads, with a maximum length of 250 bp were initially imported into the 'quantitative insights into microbial ecology' pipeline, QIIME2 v.2017.7.0 (59), and further sequencing read processing and analysis were performed in v.2018.2.0 of the pipeline. Following the sequencing read quality control and filtering process described in (8), sequencing reads were 'denoised' and 'dereplicated' using QIIME2 DADA2 (60). The resulting amplicon sequence variants (ASVs; n=30,956,883) were further filtered to remove ASVs with <100 counts and ASVs that were associated with blanks. This last filtering step resulted in 17,225,776 ASVs, ranging from 3,277 to 223,222 per sample (mean 96,774), that were used for downstream comparison of bacterial composition and taxonomic analysis. Suppl. 3. shows sequencing reads and ASV summary statistics.

Diversity indices

Analysis of microbial diversity within (alpha diversity) and between (beta diversity) samples were performed in QIIME2 using the Shannon diversity index and Bray-Curtis dissimilarity index, respectively. The Shannon diversity indices were calculated using rarefied ASVs counts per sample, in which ASVs per sample were selected randomly without

replacement at an even depth (Suppl. 4) for ten iterations. The resulting average Shannon indices are presented and were compared between samples using pairwise Kruskal-Wallis tests with Benjamini-Hochberg false discovery rate (FDR) corrections for multiple comparisons.

The Bray-Curtis dissimilarity indices were computed with or without rarefaction, and resulting indices were compared between samples using pairwise PERMANOVA tests (999 permutations) with FDR corrections. There were no discernable differences between results of rarefied and non-rarefied data. Thus, results of Bray-Curtis dissimilarity indices using non-rarefied data were visualized by Principal Co-ordinates Analysis (PCoA) plots in R (61) using the phyloseq R package (62).

Significance for both pair-wise analyses was set to $q < 0.05$ (i.e. post FDR p-value corrections).

Taxonomic analysis and differentially abundant microbial taxa

Taxonomic analysis of ASVs was performed using QIIME2's pre-trained Naïve Bayes classifier (63) and q2-feature-classifier plugin (64). Prior to analysis, the classifier was trained on the QIIME-compatible 16S SILVA reference (99% identity) database v.128 (65), and using the extract-reads command of the q2-feature-classifier plugin, the reference sequences were trimmed to the v3-v4 region (425 bp) of the 16S rRNA gene. The relative abundance of annotated ASVs across samples were subsequently visualized using the qiime feature-table heatmap plugin based on Bray-Curtis distances with the plugin's default clustering method.

Differentially abundant microbial taxa across locations were identified using QIIME2's analysis of composition of microbiomes (ANCOM) (66) plugin. The cut-off for differential abundance was set to an effect size of log F≥20 and W≥20, i.e. a taxon was differentially abundant across maternal collection sites if the ratio of its abundance to those of at least 20 other taxa (25% of all included taxa) differed significantly across sites.

Only annotated ASVs with counts ≥ 2000 (larvae) or ≥ 1000 (adults) were included in the heatmaps and ANCOM analysis. Prior to each analysis, ASV frequency data was normalized by $\log_{10}$ transformation following the addition of pseudocounts of 1.

The outputs of data analyses were edited using Inkscape (67).

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The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the US Centers for Disease Control and Prevention (CDC) or the American Society for Microbiology (ASM).

Data Accessibility Statement

The raw sequencing reads generated from this project, including those from negative controls (blanks), have been deposited in the National Center for Biotechnology Information (NCBI), Sequence Read Archive under the BioProject PRJNA512122.

Author Contributions

ND† & AL conceptualized and designed the study; NP facilitated and provided facilities for field work; ND†, ACB, FL & JCL performed mosquito collections, and mass rearing; ND† & MS performed molecular analysis and sequencing; ND† analyzed the data; ND† and ND performed the data visualizations; ND† drafted the manuscript; all authors reviewed and approved the final version of the manuscript.

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Supporting Information

Suppl. 1. Shannon alpha diversity plots
Suppl. 2. Frequency and relative abundance of microbial taxa detected in internal and cuticle surface microbiota of F_1 laboratory colonized *An. albimanus* larvae and adults
Suppl. 3. Summary statistics of sequencing reads and amplicon sequence variants (ASVs) used for downstream analysis
Suppl. 4. Rarefaction depth and plots of sequencing reads

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