Aedes aegypti gut transcriptomes respond differently to microbiome transplants from field-caught or laboratory-reared mosquitoes.

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Abstract

The mosquito microbiome is critical for host development and plays a major role in many aspects of mosquito biology. While the microbiome is commonly dominated by a small number of genera, there is considerable variation in composition among mosquito species, life stages, and geography. How the host controls and is affected by this variation is unclear. Using microbiome transplant experiments, we asked whether there were differences in transcriptional responses when mosquitoes of different species were used as microbiome donors. We used microbiomes from four different donor species spanning the phylogenetic breadth of the Culicidae, collected either from the laboratory or field. We found that when recipients received a microbiome from a donor reared in the laboratory, the response was remarkably similar regardless of donor species. However, when the donor had been collected from the field, many more genes were differentially expressed. We also found that while the transplant procedure did have some effect on the host transcriptome, this is likely to have had a limited effect on mosquito fitness. Overall, our results highlight the possibility that variation in mosquito microbiome communities are associated with variability in host-microbiome interactions and further demonstrate the utility of the microbiome transplantation technique for investigating host-microbe interactions in mosquitoes.

Keywords: Microbiome, RNA-Seq, Transplant, Transcriptome, Mosquito, *Aedes aegypti*, Insect.

Background

The collection of microorganisms associated with an organism (*i.e.*, its microbiome) has profound effects on its host biology. The mosquito microbiome in particular is critical for larval development (Coon et al., 2014), plays a profound role in host fitness (Sharma et al., 2013, Schmidt and Engel, 2021, Giraud et al., 2022), and, importantly, can affect the mosquito's

ability to transmit pathogens such as dengue and Zika viruses (Ramirez et al., 2012, Carlson et al., 2020, Cansado-Utrilla et al., 2021). As such, manipulating the mosquito microbiome has the potential to reduce transmission of globally important mosquito-borne pathogens.

Traditionally, manipulating the microbiome has involved treating mosquitoes with antibiotics that alter microbiome composition, but can also affect mosquito physiology (Chabanol et al., 2020, Ha et al., 2021). However, approaches rearing axenic (germ-free) mosquito larvae followed by supplementation with defined bacterial assemblages to produce gnotobiotic mosquitoes have since proven to be an excellent way to interrogate host-microbe interactions without using antibiotics, thus removing effects of the antibiotic and the 'original' microbiome. This gnotobiotic approach has largely been used to investigate the role of the microbiome in mosquito development (Coon et al., 2016, Correa et al., 2018). More recently, this approach has been exploited to perform interspecies microbiome transfers thereby enabling further studies to dissect the mechanisms underpinning microbial symbiosis in mosquitoes (Romoli et al., 2021, Coon et al., 2022).

The ability to rear axenic/gnotobiotic mosquitoes also provides an opportunity to understand how the presence or absence of gut microbial communities affects host gene expression. Previously, in a comparison of axenic, gnotobiotic and conventionally-reared *Aedes aegypti*, over a thousand host transcripts were differentially expressed in the guts of both axenic gnotobiotic mosquito larvae and conventionally-reared controls (Vogel et al., 2017). Another study found a much smaller effect in adult *Ae. aegypti*, with only 170 genes differentially expressed between axenic and conventionally-reared mosquitoes (Hyde et al., 2020). These studies demonstrate the utility of the axenic/gnotobiotic system for investigating mosquito-microbiome interactions, and furthermore point to larval stages being key for understanding how the host reacts to the microbiome.

Recently, we developed an interspecies microbiome transplantation technique in mosquitoes and showed that we could successfully recapitulate microbial composition in the recipient host

(Coon et al., 2022). This novel approach allowed us to manipulate the microbiome and to investigate the impact of complex heterogeneous communities on mosquito gene expression.

Here we sought to address two questions: (1) Do *Ae. aegypti* experience transcriptomic changes associated with the transplantation procedure itself? and (2) How does the *Ae. aegypti* transcriptome change upon receiving a microbiome transplant when a different mosquito species is used as a microbiome donor? To address the first question, we transplanted microbiomes isolated from four donor species (*Ae. aegypti*, *Aedes taeniorhynchus*, *Culex tarsalis* and *Anopheles gambiae*) into recipient germ-free *Ae. aegypti* larvae, whilst rearing an additional group of *Ae. aegypti* larvae conventionally as a no-transplant control. We then performed RNA-Seq analysis on guts dissected from recipients and compared transcriptional profiles of each of the *Ae. aegypti* treatment groups that had received a microbiome transplant to Ae. aegypti reared conventionally in the same system (*i.e*., without a microbiome transplant). To address the second question, we compared transcriptional profiles of recipients of a microbiome transplant from *Ae. taeniorhynchus*, *Cx. tarsalis*, and *An. gambiae* to that of *Ae. aegypti* recipients transplanted with their original microbiome. We also considered whether microbiomes derived from field-caught or laboratory-reared *Ae. aegypti* and *Ae. taeniorhynchus* mosquitoes affect recipient host transcriptomes differently. Using mosquito microbiome transplants to unravel the intricacies of how mosquitoes are affected by their microbiomes is relevant for both mosquito biology and our understanding of host-microbiome interactions more broadly.

Methods

Experimental setup

The experimental setup comprised seven treatments, each with three replicates (Figure 1): (*i*) *Ae. aegypti* receiving a transplant isolated from conspecific individuals of the same laboratory-maintained Galveston line and of the same generation (*i.e.*, their original microbiome); *Ae.*

aegypti receiving a transplant from one of five different donor pools from varying locations and phylogenetically distinct species (henceforth termed 'extraneous donors'); these included (*ii*) field-caught *Ae. aegypti*, (*iii*) field-caught *Ae. taeniorhynchus,* (*iv*) laboratory-reared *Ae. taeniorhynchus*, (*v*) laboratory reared *Cx. tarsalis*, and (*vi*) laboratory-reared *An. gambiae*; and (*vii*) *Ae. aegypti* Galveston line, again of the same line and generation, reared under aseptic conditions without egg sterilization to retain their original microbiome (conventionally reared control).

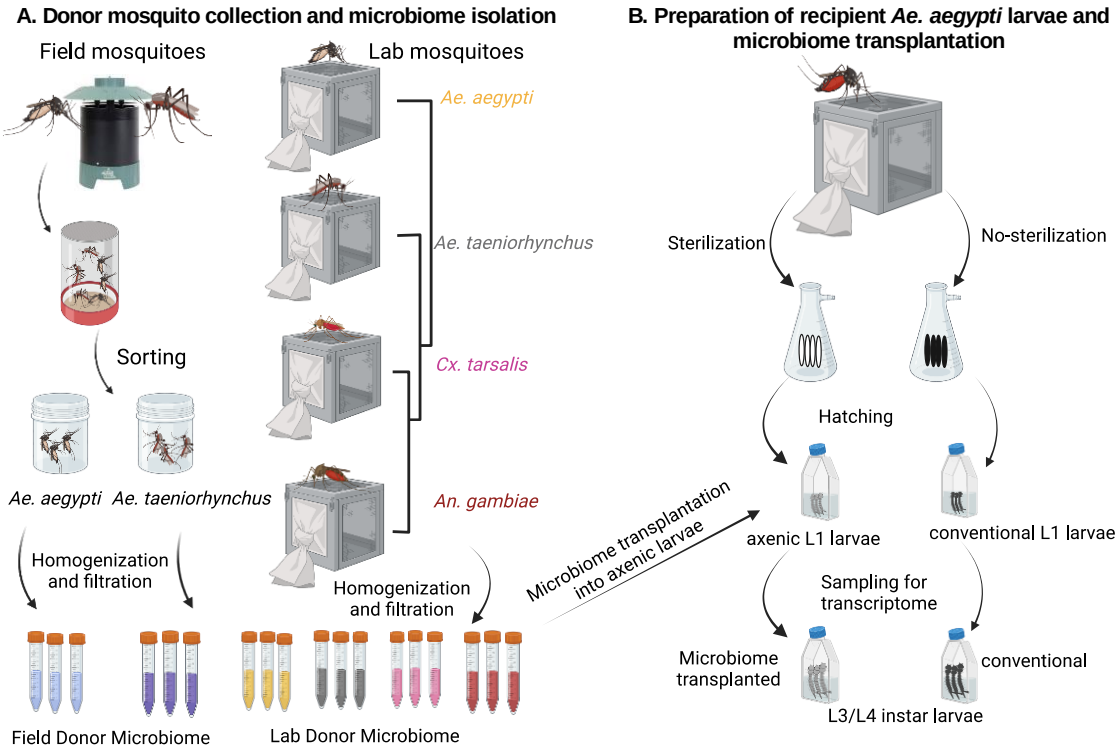

Figure 1. Microbiome transplantation from field-collected and laboratory-reared mosquitoes into recipient laboratory-reared mosquitoes. **A.** Adult mosquitoes from field populations of *Ae. aegypti* or *Ae. taeniorhynchus* were trapped using BG sentinel traps in Galveston, Texas and sorted according to species and sex. Three replicate pools of 20 adult females were then used to isolate donor microbiomes from each species. Donor microbiomes were also isolated from three replicate pools of 20 laboratory-reared *Ae. aegypti*, *Ae. taeniorhynchus*, *Cx. tarsalis*, and *An. gambiae* adult females. The cladogram adjacent to the cages indicates the phylogenetic relationship of the laboratory reared mosquitoes used as microbiome donors**.** Laboratory-reared *Ae. aegypti* were used as recipient hosts for all transplants. In brief, eggs were surface sterilized using ethanol and bleach before vacuum hatching to obtain 1st instar axenic larvae. As a control for the transplantation process, we also vacuum hatched a batch of non-sterilized eggs from the same colony and generation. These were maintained conventionally in closed

conditions to retain their original microbiome. Axenic larvae were transferred into T75 tissue culture flasks at 20 larvae per flask with three replicates per treatment. Here they were inoculated with the donor microbiome through supplementation of the larval water. Flasks were maintained at 28 °C and fed with sterile fish food on alternative days. Once larvae had reached the 4th instar they were harvested, their guts dissected and RNA-Seq was carried out using pools of five guts for each of three replicate flasks per treatment. Figure created using Biorender.

Donor mosquito collections

Microbiome transplantations were carried out by first isolating donor microbiomes from one of four mosquito species (*Ae. aegypti*, *Ae. taeniorhynchus*, *Cx. tarsalis*, or *An. gambiae*), which had either been laboratory-reared or field-caught (Figure 1). Laboratory colonies of all four species had been continually maintained at the University of Texas Medical Branch following standard conditions, at 28 °C with 12 hr light/dark cycles, fed defibrinated sheep's blood to allow egg production and provided with 10% sucrose solution *ad libitum.* The laboratory colony of *Ae. aegypti* (Galveston line) were the F3 generation, whereas all other laboratory-reared mosquito colonies had been maintained for approximately ten years. For each species, three pools of 20 three-to-four-day old sugar fed adult females from one colony were, collected from the same generation at the same time, used for microbiome isolations. We also collected individuals belonging to two of these species, *Ae. aegypti* and *Ae. taeniorhynchus* from field populations. Collections were made in 2018 in Galveston, Texas using Biogents sentinel (BG) traps. Adult mosquitoes were collected and immediately sorted morphologically according to species and sex. Three pools of 20 adult females belonging to each of the two species were used for microbiome isolations.

Preparation of recipient mosquitoes and microbiome transplantation

Microbiome isolation and transplantation was carried out using our recently developed methodology (Coon et al., 2022). Briefly, recipient mosquitoes were prepared by surface sterilising *Ae. aegypti* eggs using 70% ethanol and vacuum hatching under sterile conditions

to generate axenic 1st instar larvae. The larvae were then transferred to T75 tissue culture flasks in sterile water at the rate of 20 larvae per flask (three replicate flasks per treatment). The same generation of the laboratory-reared *Ae. aegypti* (Galveston line) colony as used for microbiome donation was used as the source of recipient hosts for all transplants. For each of the six donor types (four laboratory-reared and two field-caught), three replicate pools of 20 mosquitoes were surface sterilised using 70% ethanol and bleach washes followed by homogenisation and filtration. Resulting donor microbiome aliquots were directly transplanted into recipient, without prior freezing, larvae by inoculating the larval water, with one aliquot per replicate flask. Recipient larvae were maintained in a closed environment at 28 °C with 12 hr light/dark cycles and supplemented with sterile fish food on alternative days until they reached the 4th instar. Since *Ae. aegypti* larvae require bacteria for their development (Coon et al., 2014), only those individuals that had been successfully inoculated with the donor microbiota developed. The axenic larvae, which did not receive a microbiome failed to reach to the 4th instar.

Sample preparation, RNA extraction and preparation of cDNA libraries for RNA-Seq

When recipient mosquitoes reached the 4th instar, five larvae were collected from each flask, surface sterilised, and their whole guts excluding Malpighian tubules were dissected. We focussed on larvae because they harbour higher microbial diversity compared to other life stages (Strand, 2018, Coon et al., 2022), and guts, given this is a relevant tissue for host-microbe interactions. The five guts were then pooled to obtain sufficient RNA for cDNA library preparation and RNA-Seq. RNA was extracted using the PureLink RNA mini kit (Thermo Fisher Scientific), then using between 100ng-1ug total RNA, polyA+ RNA transcripts were isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Non-directional libraries were created using the NEBNext Ultra II RNA Library Prep Kit (New England biolabs) and Next Generation Sequencing was carried out using the Illumina NextSeq

550 platform to generate 75bp paired end reads at the University of Texas Medical Branch Core Next Generation Sequencing Facility.

Data analysis

Sequence data were obtained in fastq format and quality checked using FASTQC v0.11.5 (Andrews, 2016). All samples had an average phred score of > 30, with no adapter sequences present so no trimming was performed. FeatureCounts v2.0.1(Liao et al., 2014) was used to obtain raw count data from the sequencing files using default parameters and the *Ae. aegypti* reference genome (Genome version GCA_002204515.1, Annotation version AaegL.5.3) to determine feature locations. The resulting feature count table was then imported into RStudio v1.4.1106 and filtered to remove any genes which did not have at least ten reads present in all three replicates of at least one treatment group before continuing with subsequent analyses.

Firstly, we investigated how the recipient host transcriptome was affected by the transplantation procedure itself. We compared the transcriptional profiles of recipients of a microbiome transplant to that of conventionally reared, no-transplant controls. Differential expression (DE) analysis was carried out using DESeq2 v1.30.1 (Love et al., 2014) using default parameters. DESeq2 takes as input raw read counts from programs such as FeatureCounts, using the DESeqDataSetFromMatrix command. As part of its internal workflow, DESeq2 automatically normalizes gene expression data based on the input raw count data. Thresholds were applied to the resulting list of differentially expressed genes (DEGs) to retain only those with an adjusted p value of < 0.05 and an absolute $\log_2$ fold change of ≥ 1.5. An upset plot was created using the UpsetR package v1.4.0 (Conway et al., 2017) to visualise the number of DEGs in each pairwise comparison between recipients of a transplant and the conventionally reared control, as well as to identify DEGs that were common to every transplant recipient group. The ComplexHeatmap package v2.12.0 (Gu, 2022) was then used

to visualise the log_2 fold changes of DEGs identified in each transplant recipient group relative to the conventionally reared control. Finally, a Gene Ontology (GO) enrichment analysis was performed using the VectorBase Gene Ontology Enrichment Analysis tool with default parameters to identify functions of commonly enhanced and suppressed DEGs (VectorBaseIDs), retaining those terms (within the ontology categories biological process, molecular function and cellular component) that passed a threshold of Bonferroni adjusted p value < 0.05.

To address the question of how *Ae. aegypti* responded to receiving a microbiome transplant from an extraneous donor, we compared gene expression in each recipient group that had received a microbiome from an extraneous donor belonging to a different species or collected from a different environment (laboratory or field) to recipients that had received a transplant of their 'original' microbiome isolated from a conspecific donor, from the same generation. To focus on the gene expression in transplant-recipients and remove any transplant-effect, for this analysis we removed the conventionally reared control mosquitoes. Again, we used DESeq2 to identify differentially expressed genes using the same thresholds, identified sets of DEGs that were unique or common to multiple transplant recipient groups using the UpsetR package, and visualised the log_2 fold changes of DEGs in each recipient treatment group compared to the 'original' microbiome control using the ComplexHeatmap package. We further investigated those DEGs identified as enhanced or suppressed in recipients of extraneous donor-derived microbiomes by using the VectorBase Gene Ontology enrichment analysis tool to identify enriched GO terms in the enhanced or suppressed DEGs.

Sequencing reads were deposited in the National Centre for Biotechnology Information Sequence Read Archive under the accession PRJNA941184. All R codes used in analyses, as well as raw counts table and metadata are available at https://github.com/laura-brettell/microbiome_transplant_RNASeq

Results and Discussion

A core set of genes were consistently affected when conducting a microbiome transplantation.

To assess whether mosquitoes respond differently to varied mosquito-derived microbiomes, we performed transplantations using donors spanning the phylogenetic diversity of the Culicidae and a combination of laboratory-reared and field-caught samples. All microbiomes were transplanted into recipient laboratory reared *Ae. aegypti* (Galveston line) from the same generation (Figure 1). With the exception of the axenic control larvae that failed to develop, larvae in all experimental treatments successfully developed to the 4th instar, indicating that each of the mosquito microbiomes used in this experiment facilitated larval development and corroborating previous findings indicating that mosquito larvae require microbes for their development (Correa et al., 2018; Vogel et al., 2017) . Furthermore, no differences were observed in either growth rate or size of 4th instar larvae upon sampling of transplant recipients, irrespective of donor species or collection environment. This consistency of larval development is in agreement with the findings of several previous studies that looked at the impact of altered larval microbiomes on mosquito development (Correa et al., 2018; Vogel et al., 2017). Given the axenic larvae that had been surface sterilized and maintained in a microbe-free environment failed to develop, it was not possible to disentangle the impacts of the sterilization or transplantation procedures individually under our experimental settings.

To maximise the potential of microbiome transplantation experiments, it is important to determine whether the transplant technique itself influence the host. Prior to this study, we knew that transplant recipients successfully develop to adulthood (Coon et al., 2022), but we did not know if recipients experience transcriptomic changes associated with the experimental procedure. We addressed this here by using RNA-Seq analysis and comparing the gut transcriptomes of *Ae. aegypti* larvae which received a microbiome transplant (either using their original microbiome or a microbiome isolated from an extraneous donor) to the gut transcriptomes of *Ae. aegypti* larvae from the same laboratory population and generation that

had not received a transplant to look for commonalities between responses (Figure 1, 2). Across the entire dataset, we obtained an average of 23.6M reads per sample (range 16.1M – 30.8M) with an average of 74% of reads (range: 70.4% – 76.3%) mapping uniquely to the *Ae. aegypti* genome (Supplementary Table 1).

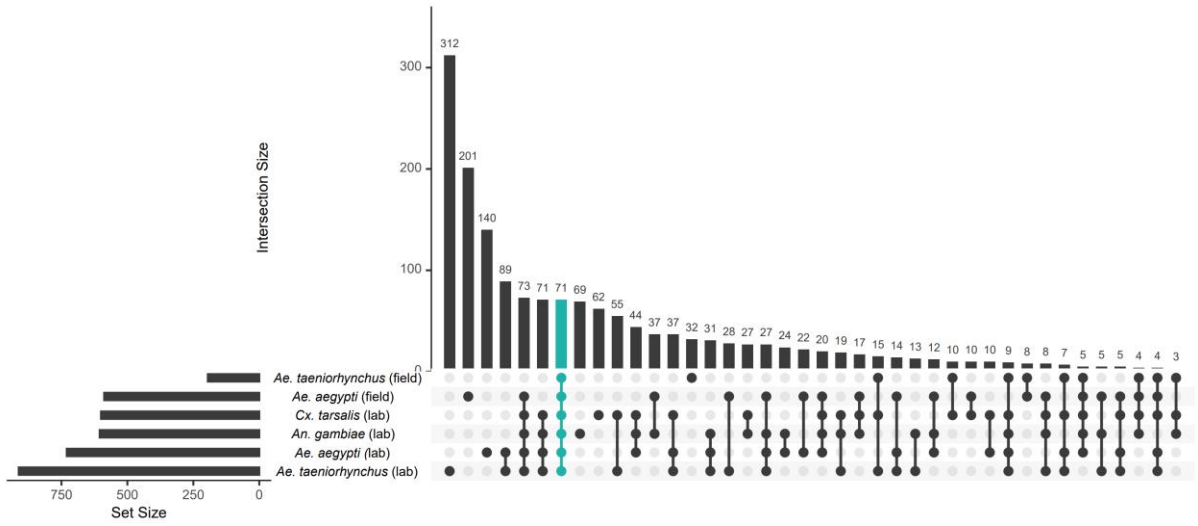

Figure 2. Upset plot showing the number of differentially expressed genes (DEGs) in recipients of each of the microbiome transplant treatments relative to the conventionally reared control. The plot shows the numbers of genes as a matrix, with the rows corresponding to sets and the columns showing intersections between sets. The horizonal bar chart shows the set size (number of DEGs) in microbiome transplant recipients relative to conventionally reared control mosquitoes, *i.e.,* recipients of a microbiome transplant from field-caught *Ae. taeniorhynchus* showed 195 DEGs relative to conventionally reared mosquitoes. Balls and sticks represent intersections where DEGs were identified in multiple groups, with vertical bars showing the number of DEGs in each intersection, *i.e.,* there were 312 DEGs unique to recipients of microbiome transplant from a laboratory-reared *Ae. taeniorhynchus* donor, and there were 71 DEGs identified in every transplantation group (highlighted in teal).

We conducted a differential expression analysis to compare gene expression in each of the microbiome transplant recipient groups individually to conventionally reared control larvae (Supplementary Table 2). We found 1680 DEGs in at least one transplantation group relative to the conventionally reared control (Figure 2, Supplementary Table 2). This number ranged from 614 DEGs in the comparison between conventionally reared larvae and recipients of a field-caught *Ae. taeniorhynchus* donor microbiome, up to 1269 genes in the comparison with recipients of a laboratory-reared *Ae. taeniorhynchus* donor microbiome. We further identified 71 genes that were differentially expressed in all recipients of a microbiome transplant, and thus could be a conserved response to the technique itself. Interestingly, and further supporting this assertion, these genes all showed the same direction of change in all comparisons, with 50 genes consistently enhanced when a transplant was performed, and 21 genes consistently suppressed (Supplementary Table 3). Of the DEGs that were enhanced in the transplant recipients, one gene showed substantially higher differential expression than any other, a threonine dehydratase/deaminase gene (AAEL003564) involved in ammonia transport and detoxification (Durant et al., 2021). Among the most strongly suppressed DEGs in the transplantation groups were two glucosyl/glucuronosyl transferases genes (AAEL008560 and AAEL010381) previously found to be enriched in the 3rd and 4th instars (Matthews et al., 2016).

Given that the 71 genes identified in every comparison with conventionally reared controls were affected in the same manner, we next asked whether other genes that had been identified in multiple comparisons were also affected in the same direction. We looked at all genes that passed our differential expression thresholds (adjusted p value of < 0.05 and an absolute $\log_2$ fold change of ≥ 1.5) at least one transplant recipient group. We saw that, of the 1680 DEGs, all but 26 showed the same direction of change when they were identified in multiple comparisons (Figure 3a, Supplementary Table 2). Thus, while only a small number of genes were identified in every comparison (and are therefore likely those most impacted by the transplant technique itself), there were general similarities in transcriptomic responses to

a transplant overall, similar to our previous study where interspecies microbiome transplantation did not impact mosquito growth (Coon et al., 2022). However, the magnitude of differential gene expression differed between treatment groups. Interestingly, the treatment group that showed the most similar transcriptome to the conventional controls were the recipients of donor microbiomes isolated from field-caught *Ae. taeniorhynchus* (Figure 2, 3a). As a different mosquito species collected from a different environment, this presumably harboured a substantially different microbiome composition to the *Ae. aegypti* control mosquitoes that were conventionally reared in the laboratory.

To investigate whether the transplantation process impacted biological functions of the recipients, we performed GO enrichment analysis using the genes that were consistently enhanced or suppressed in at least one transplant group across the dataset. The GO terms are classified as either biological process, cellular component, or molecular function. Among the 45 GO terms identified, 21 were biological processes, 20 were molecular functions and four correspond to cellular components (Figure 3b, Supplementary Table 4). The genes that were suppressed when a transplant was carried out were largely those with roles in metabolism and RNA processing (Figure 3b, Supplementary Table 4), all processes that typically occur in the gut (Vogel et al., 2017, Hixson et al., 2022). Furthermore, one of the GO terms identified in the DEGs that were suppressed when a transplant was performed (ribonucleoprotein complex biogenesis) has previously been found to be affected by blood meal digestion (Hixson et al., 2022).

Overall, these results support a lack of any strong, consistent physiological response to the transplant technique. While there were numerous DEGs identified amongst all different transplant groups compared to conventionally reared controls, most of these genes were only identified in a subset of comparisons. Additionally, while other studies have shown alterations to the transcriptome when carrying out microbiome manipulations, there does not appear to be a consistent pattern. Hyde et. al. (2020) reported minimal effects on gut transcriptomes when comparing adult *Ae. aegypti* that had either received their native microbiome or had

been reared axenically. In contrast, Vogel et al (2017) reported a larger difference in the gut transcriptomes of 1st instar larvae that had been axenically or gnotobiotically reared compared to conventionally reared larvae. It should be noted that in both studies, these differences were likely attributable in large part to starvation stress associated with the developmental arrest of axenic larvae and are therefore not directly comparable to other studies, including this one, which sampled later life stages. Overall, we can speculate that while the transplant technique is likely having some minor effect, it is largely transient and not severely detrimental to the recipient host. Nevertheless, it is known that exposure of larvae to *Bacillus* and *Enterobacteriaceae* can affect biological traits in adulthood (Dickson et al., 2017, Carlson et al., 2020), warranting further work to identify whether the transplant technique affects recipients as they develop into adulthood. Additionally, given the microbiome donors in this study were all non-blood fed adults, it would be interesting to conduct further studies to determine whether using donor microbiomes derived from other life stages including larvae or blood fed adults will generate similar results.

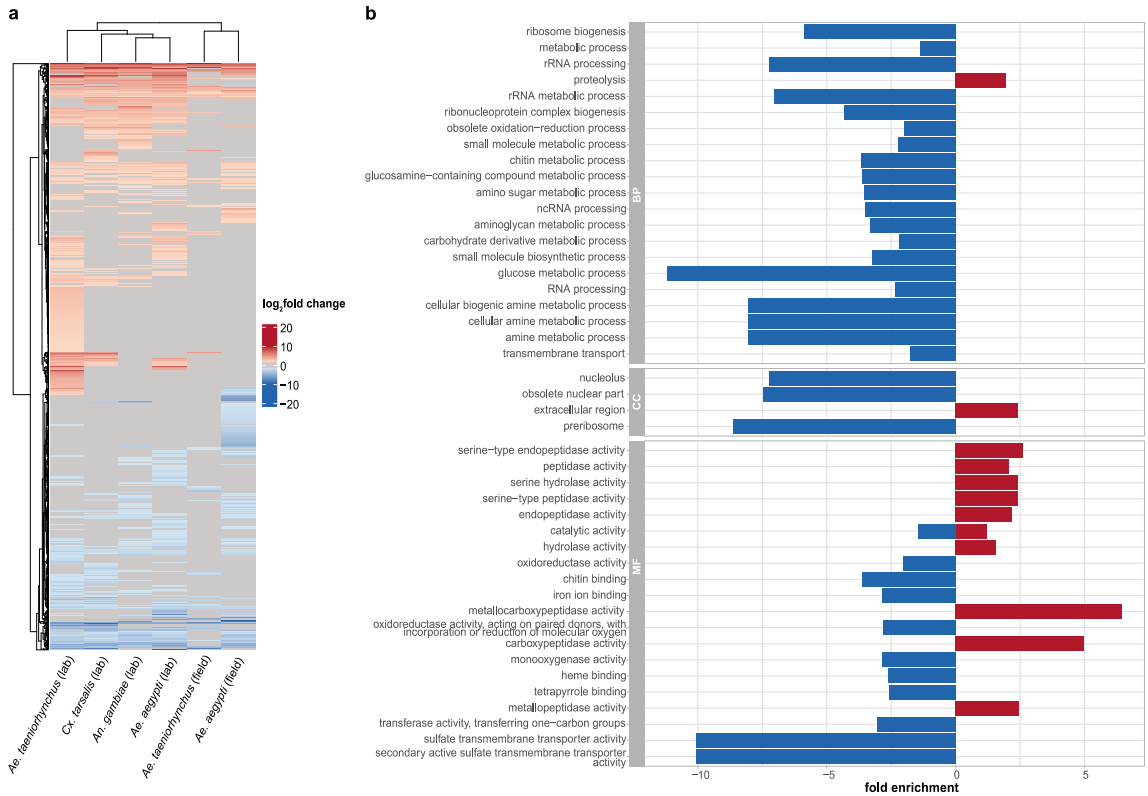

Figure 3. a. Heatmap showing the log_2 fold change of each of the 1680 genes identified as differentially expressed in at least one comparison between a transplant treatment group and conventionally reared mosquitoes. Red cells indicate when gene expression was enhanced in the transplant group and blue cells indicate when gene expression was suppressed. Grey denotes where a gene did not pass the differential expression threshold (absolute log_2 fold change ≥1.5, adjusted p value < 0.05). The microbiome donor is shown on the *x*-axis, with each row on the *y*-axis corresponding to a differentially expressed gene (DEG). The dendrograms represent clustering of similar responses as determined through the *hclust* function within the *ComplexHeatmap* package. **b**. Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in at least one recipient condition of a microbiome transplant, relative to the conventionally reared control (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP), cellular component (CC) and molecular function (MF). Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced/suppressed DEGs, divided by the percentage of genes with this term in the background. GO terms identified in the enhanced genes are shown by red bars with positive values. The suppressed GO terms are shown with blue bars are negative values.

Host gene expression differs based on field-caught versus laboratory-reared microbiome donors.

Microbiome transplantation experiments provide a unique opportunity to investigate how the host interacts with a selection of diverse microbiomes in a controlled environment. While mosquito microbiomes are commonly dominated by a small number of bacterial genera (Coon et al., 2014), microbiome composition varies amongst host species (Kozlova et al., 2020), even when reared under identical insectary conditions (Hegde et al., 2018, Accoti et al., 2023), geography (Zouache et al., 2011, Coon et al., 2016), and across individuals (Osei-Poku et al., 2012, Coon et al., 2022), raising the question how mosquitoes respond to these varied microbiomes.

Here, we sought to determine whether transplantation with different microbiomes alter gene expression in host guts. We conducted differential expression (DE) analysis comparing gene expression in recipients of a microbiome transplant using an extraneous donor, belonging to a different species, or collected from a different environment to control recipients of a transplant using their original microbiome. This revealed a striking difference between recipients inoculated with laboratory-reared versus field-caught donor microbiomes. When

recipients received a transplant from a donor reared in the same laboratory, there was little change to the gut transcriptome regardless of which donor species was used (Figure 4). Transplants using microbiomes derived from laboratory-reared *Ae. taeniorhynchus, Cx. tarsalis,* and *An. gambiae* donors resulted in 55, 49, and 19 DEGs, respectively (Figure 4, Supplementary Table 5). In contrast, transplantation using microbiomes derived from field-caught donors resulted in far more DEGs, with microbiomes from field-caught *Ae. aegypti* resulting in 447 DEGs and those from field-caught *Ae. taeniorhynchus* resulting in 448 DEGs.

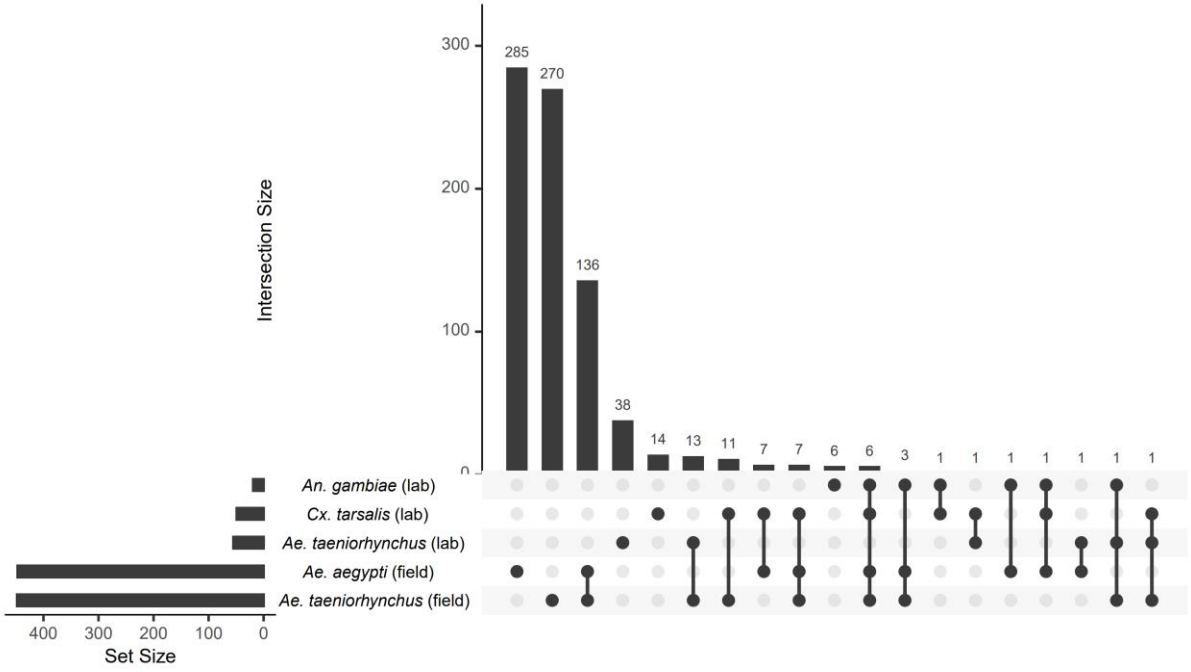

Figure 4. Upset plot showing the number of differentially expressed genes (DEGs) in each of the microbiome transplant recipients relative to the control recipients that had received their original microbiome. Set size refers to the number of DEGs in the recipient when transplanted with microbiomes from each of five donor types (*An. gambiae, Cx. tarsalis,* and *Ae. taeniorhynchus* reared in the laboratory, and *Ae. aegypti* and *Ae. taeniorhynchus* collected from the field). The number of DEGs identified in each treatment group relative to control recipients is demonstrated by the horizontal bars (ie recipients of a field-derived *Ae. taeniorhynchus* microbiome showed 448 DEGs). Intersections where the same DEGs were identified in multiple transplantation recipient groups are denoted by the ball and stick diagram, with vertical bars showing the number of DEGs in each intersection, *i.e.,* 285 DEGs were seen only in recipients of a field-derived *Ae. aegypti* microbiome and 136 DEGs were seen in recipients of both a field-derived *Ae. aegypti* microbiome and a field-derived *Ae. taeniorhynchus* microbiome.

While we did not characterize the composition of the different donor microbiomes in our study, the consistency in response, or lack thereof, of recipient hosts to laboratory-reared donor microbiomes suggests some level of similarity in composition between the different laboratory-derived donor microbiomes we isolated. The overall stronger differences in responses we observed across recipients of field-caught donor microbiomes also suggests that field-caught mosquitoes harbour more variable microbial communities that differ in composition from those present in laboratory-reared mosquitoes. This is also consistent with previous studies comparing the microbiomes of *Ae. aegypti* and other animals maintained in captivity to their free-living counterparts (Eichmiller et al., 2016, Lemieux-Labonté et al., 2016). Collectively, this suggests that microbiome composition is generally affected more by environment than host species, although it is not always the case (Hegde et al., 2018, Accoti et al., 2023), suggesting that factors governing microbiome assembly are complex.

In each of the groups receiving a transplant from a field-caught donor, approximately one quarter of DEGs compared to the original microbiome control were common to both comparisons (136/447 when field-caught *Ae. aegypti* was used as a donor and 136/448when field-caught *Ae. taeniorhynchus* were used as a donor) (Figure 4, 5, Supplementary Table 6). Amongst the many differences between the two recipient groups, an adenylate cyclase gene (AAEL001047), which has previously been shown to be important for mosquito growth in the absence of a microbiome(Romoli et al., 2021), was suppressed in recipients of a microbiome from field-caught *Ae. aegypti* but was not affected in recipients of a microbiome from field-caught *Ae. taeniorhynchus*. We assume that the two field-derived microbiomes were different from one another, given we have previously seen that different species harbour distinct microbiomes (Hegde et al., 2018). However, the overlap in DEGs suggests some level of commonality in response, or that divergent field bacterial elicit similar transcriptional effects. Furthermore, of the DEGs common to both field-derived transplants, all but one DEGs showed the same direction of change (Supplementary Table 5). Nine genes were enhanced when a transplantation was performed using a field-caught donor: a putative cytochrome b5 gene

(AAEL004450), a ubiquitin-conjugating enzyme (AAEL001208), transcription initiation factor RRN3 (AAEL012265), a sterol o-acyltransferase (AAEL009596), and five for which the product is unknown. The same sterol o-acyltransferase has previously been found to be enhanced in gnotobiotic and axenically reared larvae compared to conventionally reared individuals (Vogel et al., 2017). Of the 126 genes that were suppressed in both field-caught donor groups, 62 are of unknown function. However, the genes showing the strongest levels of suppression across the two field-transplant samples included three metalloproteases (AAEL011540 and AAEL011559, and the zinc metalloprotease AAEL008162). Zinc metalloproteases have previously been implicated as contributors to gut microbiome homeostasis in mice (Rodrigues et al., 2012). We did not identify any immune signal associated with receiving a microbiome transplant from an extraneous donor. Therefore, while immune function is affected by particular gut functions *i.e.,* blood meal digestion (Hyde et al., 2020), it does not appear to be affected by the presence of different transplanted mosquito-derived microbiomes in accordance with previous observations (Vogel et al., 2017, Romoli et al., 2021).

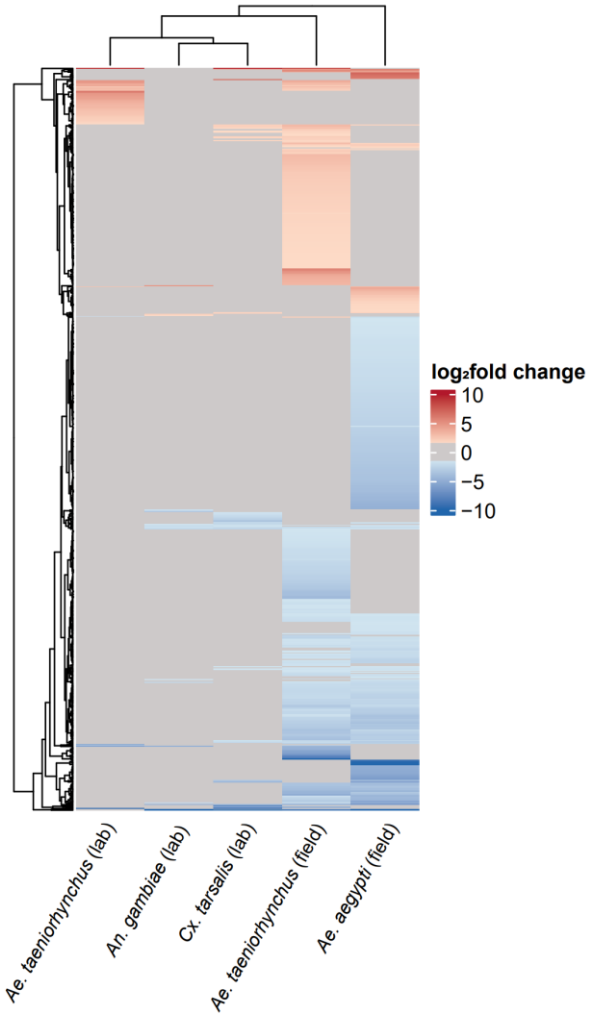

Figure 5. Heatmap showing differential gene expression between recipients of microbiome transplants using extraneous donors relative to recipients of transplants using their original microbiome. Red cells represent when gene expression was enhanced in the transplant treatment (absolute $\log_2$ fold change ≥ 1.5, adjusted p value < 0.05). Blue cells represent a suppression of gene expression, using the same thresholds. Grey denotes where a gene did not pass the differential expression threshold (absolute $\log_2$ fold change ≥ 1.5, adjusted p value < 0.05). The microbiome donor is shown on the *x*-axis, with each row on the *y*-axis corresponding to a DEG. The dendrograms represent clustering of similar responses as determined through the *hclust* function within the *ComplexHeatmap* package.

It is notable that when field-caught *Ae. taeniorhynchus* was used as the extraneous microbiome donor, similar numbers of genes were enhanced as suppressed compared to the original microbiome control (Figure 5, Supplementary Table 6). However, when using field-caught *Ae. aegypti* as the extraneous microbiome donor, recipients showed far greater numbers of suppressed than enhanced genes compared to the original microbiome control (Figure 5, Supplementary Table 6). That we did not observe a more profound effect when

using field-caught *Ae. taeniorhynchus* donor microbiomes over field-caught *Ae. aegypti* donor microbiomes may be related to the inherent variability of using pools of field-caught mosquitoes.

Given that the majority of DEGs were different between recipients of the two field-caught microbiome donor groups, we also looked at each of the two groups separately to identify whether any of the same functions/processes may be implicated across both groups. We used Gene Ontology Enrichment analysis to identify GO terms that were enriched in the enhanced or suppressed DEGs in recipients of each of the microbiomes from field-caught donors. Considering the suppressed genes, four biological processes were identified in recipients of both field-caught *Ae. aegypti* and field-caught *Ae. taeniorhynchus* microbiomes (Supplementary Figure 1 & 2, Supplementary Table 7 & 8). These included carbohydrate metabolic process, a dominant process of the anterior midgut and proventriculus (Hixson et al., 2022), transmembrane transport, obsolete oxidation-reduction process, and small molecule catabolic process. In keeping with the gene-level results, which showed only a small number of enhanced genes in the recipients of field-caught *Ae. aegypti* donor microbiomes, no GO terms were significantly enriched (Supplementary Figure 1 and Supplementary Table 7). The recipients of field-caught *Ae. taeniorhynchus* donor microbiomes however, showed an enrichment of GO terms related to translation, including ribosome. biogenesis, rRNA processing, and rRNA metabolic process in their enhanced genes (Supplementary Figure 2 and Supplementary Table 8).

We next considered enrichment of GO terms in recipients of a microbiome from a laboratory-reared donor. The same nine GO terms within the molecular function category were associated with the suppressed genes in recipients of both laboratory-reared *An. gambiae* and laboratory-reared *Cx. tarsalis* microbiomes (Supplementary Figure 3, 4 and Supplementary Table 9, 10). Interestingly, these molecular functions which were largely related to protein degradation and included metallocarboxypeptidase activity and exopeptidase activity were also affected in recipients of microbiomes from field-caught mosquitoes. All nine were affected

in recipients of field-caught *Ae. aegypti* microbiomes (Supplementary Figure 1 and Supplementary Table 7) and eight out of nine were affected in recipients of *Ae. taeniorhynchus* microbiomes (Supplementary Figure 2 and Supplementary Table 8), potentially suggesting some commonality of functional response to an extraneous donor microbiome. Recipients of laboratory-reared *Cx. tarsalis* microbiomes showed enhancement of some of the same biological processes related to translation which had also been seen in recipients of field-caught *Ae. taeniorhynchus* microbiomes, the only other treatment group that showed any enrichment of GO terms in their enhanced genes. Contrastingly, only one GO term was affected in the recipients of laboratory-reared *Ae. taeniorhynchus* microbiomes, the biological process O−acyltransferase activity (Supplementary Figure 5 and Supplementary Table 11), demonstrating the variability of functional responses to different microbiomes.

Conclusions

The gut transcriptome of *Ae. aegypti* responded differently to a microbiome transplant from a field-caught compared to a laboratory-reared donor, regardless of donor species. Microbiomes isolated from different field-caught species showed divergent expression patterns when transplanted into the recipient, but a more subtle effect was seen when microbiomes were derived from laboratory-reared species. While the transcriptional changes across the transplants were varied, generally, DEGs involved in gut functions such as metabolism were commonly altered in the recipients. Importantly, the responses seen here to the transplantation process itself were minimal, and combined with other findings suggest the approach is not severely detrimental to the recipient mosquito. Taken together, these findings demonstrate the utility of the mosquito microbiome transplantation technique in dissecting the molecular basis of mosquito-microbiome interactions and underscores how mosquito larval life history has generally relaxed the dependence of larvae on any particular microbiome, at least under ideal diet/nutrient conditions. Future studies should focus on studying such interactions under variable diet/nutrient conditions that mimic field conditions and determining effects on adults.

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Conflict of interest

The authors declare no conflict of interest.

Ethical statement

Ethical considerations are not applicable as mosquitoes are not classified as animals requiring ethical approval.

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Supplementary Information

Supplementary Figure S1: Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in recipients of a microbiome transplant using field-caught *Aedes aegypti* as the donor, relative to larvae that had received a microbiome transplant using their original microbiome (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP), cellular component (CC) and molecular function (MF). Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced or suppressed DEGs, divided by the percentage of genes with this term in the background. There were no GO terms identified in the enhanced DEGs with a Bonferroni adjusted p value < 0.05. Suppressed GO terms are shown with blue bars and negative values.

Supplementary Figure S2: Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in recipients of a microbiome transplant using field-caught *Aedes taeniorhynchus* as the donor, relative to larvae that had received a microbiome transplant using their original microbiome (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP), cellular component (CC) and molecular function (MF). Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced or suppressed DEGs, divided by the percentage of genes with this term in the background. GO terms identified in the enhanced genes are shown by red bars with positive values and the suppressed GO terms are shown with blue bars and negative values.

Supplementary Figure S3: Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in recipients of a microbiome transplant using laboratory-

reared *Aedes taeniorhynchus* as the donor, relative to larvae that had received a microbiome transplant using their original microbiome (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP) and molecular function (MF). There were no GO terms identified in the enhanced DEGs with a Bonferroni adjusted p value < 0.05, or in the suppressed DEGS within the category of cellular function. The suppressed GO terms are shown with blue bars and negative values. Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced or suppressed DEGs, divided by the percentage of genes with this term in the background.

Supplementary Figure S4: Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in recipients of a microbiome transplant using laboratory-reared *Culex tarsalis* as the donor, relative to larvae that had received a microbiome transplant using their original microbiome (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP), cellular component (CC) and molecular function (MF). Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced or suppressed DEGs, divided by the percentage of genes with this term in the background. GO terms identified in the enhanced genes are shown by red bars with positive values and the suppressed GO terms are shown with blue bars and negative values.

Supplementary Figure S5: Bar charts show results of Gene Ontology enrichment analysis of enhanced and suppressed DEGs in recipients of a microbiome transplant using laboratory-reared *Aedes taeniorhynchus* as the donor, relative to larvae that had received a microbiome transplant using their original microbiome (passing a threshold of Bonferroni adjusted p value < 0.05). The GO terms identified are separated into biological process (BP) and molecular function (MF). There were no GO terms identified in the enhanced DEGs with a Bonferroni adjusted p value < 0.05, or in the suppressed DEGS within the category of cellular function. The suppressed GO terms are shown with blue bars and negative values. Fold enrichment is calculated as the percentage of DEGs with this term in the total lists of enhanced or suppressed DEGs, divided by the percentage of genes with this term in the background.

Supplementary Table S1: Summary of RNA-Seq data obtained, showing total number of paired reads for each sample with the proportion mapping to the *Ae. aegypti* reference genome (GCA_002204515.1), both singly and with multiple matches and the proportion of unmapped reads.

Supplementary Table S2: All differentially expressed genes that were identified in recipients of a microbiome transplant relative to control larvae that were conventionally reared in the laboratory (passing thresholds of padj < 0.05 and absolute $\log_2$fold change ≥ 1.5). VectorBase IDs are given alongside $\log_2$ fold change when a microbiome transplant was performed with each donor.

Supplementary Table S3: Differentially expressed genes that were commonly identified across all transplant groups relative to the conventionally reared control larvae (passing thresholds of padj <0.05 and $\log_2$fold change >1.5). VectorBase IDs, VectorBase product descriptions and Kegg functional descriptions are given for each of the 71 genes, alongside

log_2 fold change in expression when larvae received microbiome transplants from each donor group.

Supplementary Table S4: GO terms enriched in the DEGs identified in all transplant recipients compared to the conventionally reared, no transplant control, calculated using the VectorBase Gene Ontology enrichment analysis tool.

Supplementary Table S5: All differentially expressed genes that were identified in recipients of a microbiome transplant from an extraneous donor, relative to control larvae that had received their original microbiome (passing thresholds of padj < 0.05 and absolute log_2 foldlog$_2$fold change ≥ 1.5). VectorBase IDs are given alongside log_2 fold change in recipients of a transplant from each extraneous donor.

Supplementary Table S6: Differentially expressed genes that were identified in recipients of a microbiome transplantation using a field-caught donor relative to control larvae that had received their original microbiome (passing thresholds of padj <0.05 and log_2 foldchange >1.5). VectorBase IDs, VectorBase product descriptions and Kegg functional descriptions are given for each gene, alongside log_2 fold change in expression when larvae received microbiome transplants from each donor group (field-caught *Ae. aegypti* or field-caught *Ae. taeniorhynchus*).

Supplementary Table S7: GO terms enriched in the DEGs identified in recipients of a microbiome transplantation using a donor of field-caught *Aedes aegypti* relative to larvae that had received a transplantation using their original microbiome, calculated using the VectorBase Gene Ontology enrichment analysis tool.

Supplementary Table S8: GO terms enriched in the DEGs identified in recipients of a microbiome transplantation using a donor of field-caught *Aedes taeniorhynchus* relative to larvae that had received a transplantation using their original microbiome, calculated using the VectorBase Gene Ontology enrichment analysis tool.

Supplementary Table S9: GO terms enriched in the DEGs identified in recipients of a microbiome transplantation using a donor of laboratory-reared *Anopheles gambiae* relative to larvae that had received a transplantation using their original microbiome, calculated using the VectorBase Gene Ontology enrichment analysis tool.

Supplementary Table S10: GO terms enriched in the DEGs identified in recipients of a microbiome transplantation using a donor of laboratory-reared *Culex tarsalis* relative to larvae that had received a transplantation using their original microbiome, calculated using the VectorBase Gene Ontology enrichment analysis tool.

Supplementary Table S11: GO terms enriched in the DEGs identified in recipients of a microbiome transplantation using a donor of laboratory-reared *Aedes taeniorhynchus* relative to larvae that had received a transplantation using their original microbiome, calculated using the VectorBase Gene Ontology enrichment analysis tool.