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Predaceous *Toxorhynchites* mosquitoes require a living gut microbiota to develop

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Most species of mosquitoes are detritivores that feed on decaying plant and animal materials in their aquatic environment. Studies of several detritivorous mosquito species indicate that they host relatively low diversity communities of microbes that are acquired from the environment while feeding. Our recent results also indicate that detritivorous species normally require a living gut microbiota to grow beyond the first instar. Less well known is that some mosquitoes, including those belonging to the genus *Toxorhynchites*, are predators that feed on other species of mosquitoes and nektonic prey. In this study, we asked whether predaceous *Toxorhynchites amboinensis* larvae still require living microbes in their gut in order to develop. Using the detritivorous mosquito *Aedes aegypti* as prey, we found that *T. amboinensis* larvae harbour bacterial communities that are highly similar to that of their prey. Functional assays showed that *T. amboinensis* first instars provided axenic (i.e. bacteria-free) prey failed to develop, while two bacterial species present in gnotobiotic (i.e. colonized by one or more known bacterial species) prey successfully colonized the *T. amboinensis* gut and rescued development. Axenic *T. amboinensis* larvae also displayed defects in growth consistent with previously identified roles for microbe-mediated gut hypoxia in nutrient acquisition and assimilation in *A. aegypti*. Collectively, these results support a conserved role for gut microbes in regulating the development of mosquitoes with different feeding strategies.

1. Introduction

Dietary shifts are common drivers of life-history evolution in animals [1], but how such shifts impact interactions between animal hosts and their microbial associates is not well understood. The most abundant animals on Earth are insects, which as a group are unrivalled in species richness and trophic diversity [2]. The evolutionary success of insects has, in part, been facilitated by their associations with beneficial microbes, many of which inhabit the digestive tract [3]. Much attention has been given to the role of gut microbiota in facilitating the adaptive radiation of insects to use plant-based food resources (i.e. herbivory and detritivory), which are nutrient-poor and hard to digest [3–6]. By contrast, relatively little is known about the diversity and function of gut microbiota in insect predators, whose prey are of high quality and readily digested and assimilated.

Mosquitoes are an ideal system in which to examine the effects of dietary lifestyle on gut microbiota. All mosquitoes are aquatic as larvae and moult through four instars before pupating and emerging as adults [7,8]. Adults of both sexes are terrestrial and feed on sugar sources, while adult females of most species take a blood meal from a vertebrate host to produce eggs, which is how mosquitoes acquire and transmit disease-causing pathogens [8,9]. The mosquito family Culicidae is divided into two subfamilies: the Anophelinae and the Culicinae (figure 1). These two subfamilies diverged approximately 217 Ma from a common blood-feeding ancestor [10,11]. Previous studies also strongly support that detritivory is the ancestral state of larval mosquitoes, with all species within the basal subfamily Anophelinae having
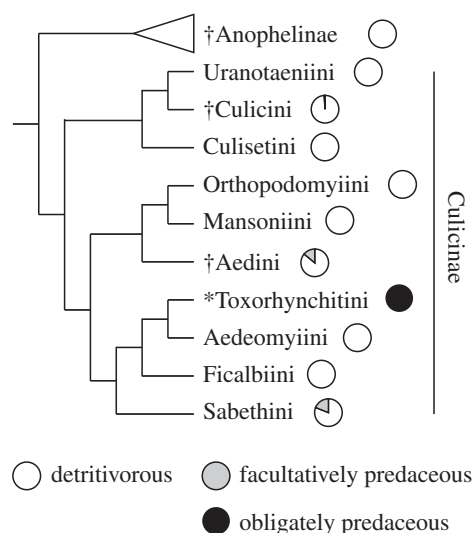
Figure 1. Phylogenetic overview of larval feeding habits in mosquitoes (Culicidae). A cladogram is modified from Reidenbach *et al.* [10] for illustrative purposes. Pie charts indicate the proportion of species belonging to a given subfamily or tribe that are classified in the literature as detritivorous, facultatively predaceous or obligately predaceous. Branches marked with a dagger (†) represent clades in which at least one species is known to normally require a living gut microbiota for development. The clade of interest in this study (Toxorhynchitini) is marked with an asterisk (*).

specialized mouthparts adapted for browsing and filter feeding [7] (figure 1). The vast majority of species within the Culicinae are also detritivores as larvae [7] (figure 1). However, several species have evolved to be predators with prehensile mouthparts adapted for catching prey [12]. A small number of facultatively predaceous species occur in genera otherwise comprised of detritivorous mosquitoes, while all species of *Toxorhynchites* (tribe Toxorhynchitini) are obligately predaceous and feed exclusively on other aquatic invertebrates, including larvae of other culicine mosquitoes [13,14] (figure 1). *Toxorhynchites* spp. are frequently found throughout tropical and subtropical regions of the world and are known to prey upon vector species of mosquitoes such as *Aedes aegypti*, which has stimulated interest in their potential for use as biological control agents in endemic areas of disease [12,13,15]. The success of *Toxorhynchites* mosquitoes as predators is owing, in part, to their large size, with larvae achieving body lengths that are more than triple the length of the detritivorous mosquitoes they feed upon. Adult *Toxorhynchites* mosquitoes are also much larger than adults from detritivorous species and females do not feed on vertebrate blood, as sufficient teneral reserves are carried over from the larval stage for egg development (termed autogeny) [8,16].

Studies of several detritivorous species indicate that they host relatively low diversity communities of microbes that are acquired from the environment while feeding [17–19]. Our previous results also indicate that detritivorous species under normal rearing conditions require living microbes in their gut to grow beyond the first instar [18–20] (figure 1). However, our previous results argue against bacteria providing a specific nutrient because larvae fail to moult in the presence of dead bacteria or diet conditioned by living bacteria [18,21]. Several bacterial species including *Escherichia coli* can individually support the development of detritivorous *A. aegypti* larvae into adults that are comparable in size

and fecundity to conventional mosquitoes reared under non-sterile conditions [18,20], which indicates that mosquitoes do not rely on a particular species or community of bacteria. More recent work using *E. coli* as a model bacterium has demonstrated that bacteria induce a hypoxia response in the gut that modulates growth-related signalling pathways associated with achieving the critical size necessary for moulting [22,23].

Here, we address the fundamental question of whether a predaceous lifestyle has relaxed the requirement for living microbes in *Toxorhynchites* spp. that prior studies of detritivorous mosquito species indicate are required for growth. We first used Illumina sequencing of 16S rRNA gene amplicons to compare the microbiota in *Toxorhynchites amboinensis* larvae to *A. aegypti* larvae that served as prey. We then used previously established methods to assess the development of *T. amboinensis* larvae provided axenic (i.e. bacteria-free), gnotobiotic (i.e. colonized by one or more known bacterial species), or conventional (i.e. non-sterile) prey under sterile rearing conditions. We report that *T. amboinensis* and *A. aegypti* harbour very similar communities of bacteria and also show that this predaceous mosquito only grows under normal rearing conditions when fed gnotobiotic or conventional prey.

2. Material and methods

(a) Mosquito colony maintenance and egg collection

Toxorhynchites amboinensis and *A. aegypti* were conventionally reared in an insectary at 28°C, approximately 60% relative humidity and a 16 L : 8 D photoperiod [24]. *Aedes aegypti* larvae were fed a standard, nutritionally complete diet consisting of rat chow, lactalbumin and dead torula yeast (1 : 1 : 1) in open aluminium rearing pans containing distilled water. The resulting adult females were blood fed on an anesthetised rat to obtain eggs. *Toxorhynchites amboinensis* larvae were similarly maintained in plastic rearing trays and were provided an excess (more than 100) of *A. aegypti* larvae of the same instar for feeding daily (i.e. first instar prey larvae were provided to first instar predator larvae). *Toxorhynchites amboinensis* adults were maintained on 10% sucrose and water-soaked raisins, and females laid eggs in black plastic cups beginning 4–6 days post emergence. For both species, eggs were collected within 24 h of being laid and either stored in humidified containers (*A. aegypti*) or immediately surface-sterilized and hatched (*T. amboinensis*) for use in experimental assays.

(b) Comparison of microbiota in conventionally reared *Toxorhynchites amboinensis* larvae and their prey

To make informed decisions regarding what bacterial species to use in our experimental assays, we first assessed whether the bacterial community present in conventionally reared *T. amboinensis* larvae is similar to or different from their *A. aegypti* prey. To accomplish this, we prepared and sequenced 16S rRNA gene libraries from six individual *T. amboinensis* and *A. aegypti* fourth instars collected from replicate rearing trays in the laboratory. Our own previous studies identified a strong role for the aquatic environment in shaping bacterial communities in larvae of detritivorous mosquito species [18,19]. We therefore also prepared sequencing libraries from water samples collected from the same rearing trays. Larvae were surface washed with 70% EtOH to eliminate most bacteria from their cuticle, dried and homogenized in liquid nitrogen. DNA from the water and larval samples was then isolated using the Gentra Puregene

Yeast/Bacteria Kit (Qiagen). PCR amplifications of the V3–V4 regions of the 16S rRNA gene were performed in triplicate using the universal primers 341F and 785R as described previously [19]. Reaction products were purified with AMPure XP Beads (Beckman Coulter). The resulting amplicons were then subjected to Illumina sequencing on the MiSeq platform (2 × 300 bp sequencing run) at the University of Georgia Genomics Facility.

Illumina sequence reads were trimmed at any site receiving a Phred quality score of <Q30, and forward and reverse reads were joined using PEAR [25]. Merged reads were then processed in QIIME [26]. Briefly, chimeric sequences were removed using the UCHIME detection method [27]. Operational taxonomic units (OTUs) were clustered at 99% sequence similarity using the USEARCH 6.1 algorithm [28]. To eliminate sequencing errors, all OTUs present in less than 0.005% abundance were removed [29]. Taxonomic assignment was performed against the latest GREENGENES database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) using the QIIME-based RDP Bayesian classifier with a 0.80 confidence threshold. Downstream analyses including alpha and beta diversity estimates were conducted using the QIIME workflow core_diversity_analysis.py, with a sampling depth of 17 432 reads per sample and default parameters.

(c) Growth and development of *Toxorhynchites amboinensis* larvae provided axenic, gnotobiotic or conventional prey

(i) Preparation of axenic, gnotobiotic and conventional *Aedes aegypti* prey

We generated axenic, gnotobiotic and conventional *A. aegypti* prey by sterilizing the surface of eggs collected from our conventionally reared colonies and hatching first instars in sterile water [18] (electronic supplementary material). First instars were subsequently maintained in 150 × 20 mm Petri dishes containing either sterile water and 6 mg of the standard larval diet sterilized by exposure to cobalt 60 gamma irradiation (axenic prey), water inoculated with 10^8 cells of a single bacterial isolate plus food (gnotobiotic prey) or water inoculated with the mixed community present in rearing pans under non-sterile conditions in our facility plus food (conventional prey). Gnotobiotic prey were colonized by one of two bacterial isolates: a *Comamonas* (Proteobacteria: Comamonadaceae) sp. or the K-12 MG1655 strain of *E. coli*. We selected the former because a *Comamonas* sp. (OTU17) was present in all of the *T. amboinensis*, *A. aegypti* and water samples we sequenced in this study (electronic supplementary material, table S2), while prior studies likewise identified *Comamonas* as a common member of the microbiota in other species of mosquitoes we rear [18,20]. Prior studies had also shown that adding *Comamonas* to water containing axenic *A. aegypti* larvae or several other species of detritivorous mosquitoes produces monoxenic, gnotobiotic larvae that develop into adults [18,20]. We selected the latter because *E. coli* is absent in our laboratory cultures but is a known gut community member in several field populations of *A. aegypti* and other mosquito species [18,19,30]. Like *Comamonas*, this strain of *E. coli* also rescues growth of *A. aegypti* and several detritivorous species when added to water containing axenic larvae by persisting in the midgut [18,22].

The presence or absence of bacteria in conventional or axenic prey was confirmed by culture-based methods and PCR using universal 16S rRNA primers as previously described [18] (electronic supplementary material). The same methods were also used to confirm the presence of a particular bacterial isolate in gnotobiotic prey using previously designed taxon-specific primers [18,20] (electronic supplementary material). All prey larvae were rinsed with sterile water prior to feeding to avoid any transfer of diet from *A. aegypti* dishes to *T. amboinensis* plates.

(ii) *Toxorhynchites amboinensis* development assays

Axenic *T. amboinensis* larvae were produced using the same egg sterilization protocol. To avoid cannibalism, newly hatched *T. amboinensis* larvae from surface-sterilized eggs were reared individually in six-well culture plates (Corning) containing only sterile water. Each larva was provided 3–10 prey (either axenic, gnotobiotic or conventional) larvae daily until death or pupation, and the number of larvae moulting to the subsequent instar was recorded. As a control, plates containing conventional *T. amboinensis* larvae from our standard rearing colony were also assayed for the ability of larvae to develop in the absence of any prey. The presence or absence of bacteria (or a particular bacterial isolate) in axenic, gnotobiotic and conventional *T. amboinensis* larvae was determined using the same methods as described above for their *A. aegypti* prey (electronic supplementary material).

(iii) *Toxorhynchites amboinensis* prey consumption and body size measurements

We hypothesized that an inability of *T. amboinensis* larvae to moult under axenic conditions could be explained by (i) differences in feeding behaviour that reduce nutrient intake by axenic larvae, and/or (ii) differences in nutrient assimilation and growth after feeding, such that axenic *T. amboinensis* larvae fail to achieve the critical size necessary to trigger moulting. To address these questions, we first characterized the feeding behaviour of *T. amboinensis* larvae fed conventional or gnotobiotic prey. We then assessed whether larvae fed on similar numbers of prey under axenic conditions. *Toxorhynchites amboinensis* larvae were reared individually in plates as described above and provided 3–10 prey larvae daily. The number of consumed prey was then tracked each day, and the moulting of *T. amboinensis* larvae to the subsequent instar was recorded.

Within each instar, larvae feed and grow until they achieve a critical size, which stimulates a cessation of feeding and growth and release of the hormone ecdysone, which stimulates moulting to the next instar [31]. We recently described several morphological characters associated with larval growth and moulting, including body length and the ratio of prothorax width to head capsule width [32]. Using these characters, we assessed growth of *T. amboinensis* larvae under axenic conditions. Newly hatched *T. amboinensis* larvae were reared individually in plates containing sterile water and 10 axenic prey larvae. Groups of larvae were then destructively sampled every 2 h and their body length, prothorax width and head capsule width were recorded. Conventionally reared *T. amboinensis* larvae provided the same number of prey served as the control. Body length was measured from the anterior border of the head to the posterior border of the last abdominal segment, which precedes the siphon tube. The width of the head capsule and prothorax were measured from the dorsal side at their widest point. All measurements were made using a Leica stereomicroscope fitted with an ocular micrometer.

(iv) Gut hypoxia levels in axenic and conventional *Toxorhynchites amboinensis* larvae

To examine whether axenic *T. amboinensis* larvae display defects in hypoxia-induced transcription factor (HIF) stabilization, as previously shown in axenic *A. aegypti*, we monitored the abundance of the HIF-α subunit using an anti-HIF-α antibody [23]. This antibody targets highly conserved domains in HIF-α and cross-reacts with several mosquito species in addition to *A. aegypti*. Briefly, pools of five *T. amboinensis* larvae were homogenized in a protease/phosphatase inhibitor mixture and resuspended in Laemmli buffer containing mercaptoethanol (10 μM) prior to being electrophoresed on 4–20% Tris–HCl gels (Bio-Rad) and transferred to a polyvinyl difluoride membrane
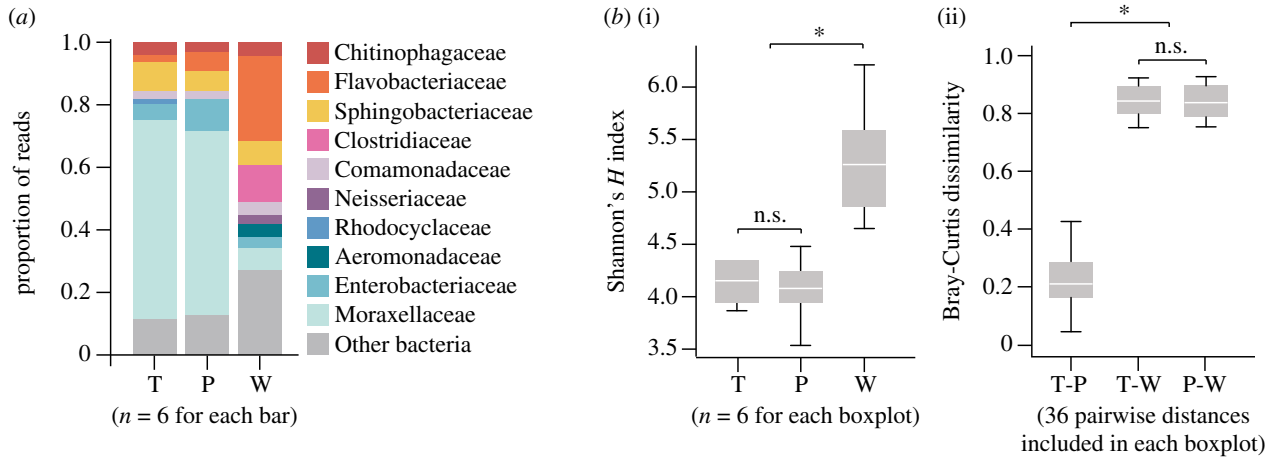
Figure 2. (*a*) Relative abundance of the 76 shared OTUs in *T. amboinensis* larvae (T), their *A. aegypti* prey (P) and water samples (W). Six biological replicates were pooled for the bar graphs presented. Each bar graph presents the proportion of sequencing reads assigned to a given bacterial family. Low abundance OTUs (less than 1%) or OTUs that were not present in all of the *T. amboinensis* larvae we sampled are represented by the 'other' category. (*b*) Alpha and beta diversity of larval and water samples. (i) The difference in alpha diversity between *T. amboinensis* larvae (T), their *A. aegypti* prey (P) and the water from which they were sampled (W) (measured by Shannon's *H* index) is shown. Box-and-whisker plots show high, low and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Alpha diversity in different sample sources was compared using a pairwise Wilcoxon rank sum test (*$p < 0.05$ and n.s., not significant). (ii) The average Bray–Curtis dissimilarity in bacterial communities between *T. amboinensis* larvae and their *A. aegypti* prey (T–P) versus between larvae and water (T–W and P–W) is shown. Box-and-whisker plots show high, low and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. Beta diversity between different sample sources was compared using a pairwise Wilcoxon rank sum test (*$p < 0.0001$ and n.s., not significant). All OTUs detected across all samples were included in the diversity analyses. (Online version in colour.)

(ThermoFisher). After blocking in 5% non-fat dry milk in PBS + 0.1% Tween 20 for 1 h, blots were probed with anti-HIF-α (1 : 5,000) or anti-actin (1 : 1,000, A2103; Sigma-Aldrich), which was used as a loading control. Samples were then washed and probed with a peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 5000; Jackson), followed by visualization using a chemiluminescent substrate (Clarity Western ECL Substrate; Bio-Rad) and the Syngene Imaging System. Three immunoblots using independently acquired samples were run for each treatment. HIF-α abundance for each sample was estimated by densitometry using the Syngene software, which calculated in arbitrary units the density of the HIF-α and actin loading control bands to generate a HIF-α/actin ratio for each replicate.

(d) Statistical analyses

Statistical analyses were performed using R v. 3.5.1 (http://www.r-project.org/). Alpha and beta diversity data were analysed by Bonferroni-corrected pairwise Wilcoxon rank sum tests. Development time, larval growth and feeding data were analysed by one-way analysis of variance (ANOVA) followed by post hoc Tukey–Kramer Honest Significant Difference (HSD) tests. Moulting data were analysed by Bonferroni-corrected pairwise Fisher's exact tests to compare axenic and gnotobiotic treatments to the conventional positive control. HIF-α abundance in axenic and conventional larvae was compared using a Student's *t*-test.

3. Results

(a) Comparison of microbiota in conventionally reared *Toxorhynchites amboinensis* larvae and their prey

We first characterized the bacterial community present in *T. amboinensis* larvae and compared it to the community present in their *A. aegypti* prey. Sequencing of 16S rRNA gene amplicons from water, *T. amboinensis* and *A. aegypti* fourth

instars collected from six replicate rearing trays in our standard rearing facility generated a total of 747 541 sequences after quality control filtering. Reads per sample ranged from 17 432 to 141 784 and grouped by percentage similarity into 452 OTUs at a cut-off threshold of 99% (electronic supplementary material, table S1). Rarefaction curves saturated at 5000 sequences, indicating that the number of sequence reads was sufficient to estimate alpha and beta diversity for all samples (electronic supplementary material, figure S1).

Only 76 OTUs were shared by all of the *T. amboinensis* larvae we sampled (electronic supplementary material, table S2). These OTUs belonged to one of four bacterial phyla (Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria) and included members of families previously detected in our laboratory cultures (e.g. Clostridiaceae, Microbacteriaceae, Flavobacteriaceae and Comamonadaceae) (figure 2*a*) [18,19]. These OTUs were also detected in *A. aegypti* prey larvae and water sampled from the same rearing trays, although there were dramatic shifts in their relative abundance between larval and water samples (figure 2*a*). While *T. amboinensis* and *A. aegypti* larvae contained a greater percentage of taxa within the Moraxellaceae (phylum Proteobacteria) (approx. 61%), water samples contained a higher percentage of Flavobacteriaceae (phylum Bacteroidetes) (approx. 27%) and Clostridiaceae (phylum Firmicutes) (approx. 12%) (figure 2*a*).

Taking into account both the presence/absence and relative abundance of all of the OTUs we detected across all samples, bacterial communities in *T. amboinensis* larvae and their *A. aegypti* prey exhibited no significant difference in alpha diversity as measured by Shannon's *H* index (figure 2*b*). By contrast, alpha diversity was significantly higher in water samples than in both *T. amboinensis* and *A. aegypti* samples. Beta diversity, measured as average Bray–Curtis dissimilarity, was also significantly higher between water and larval
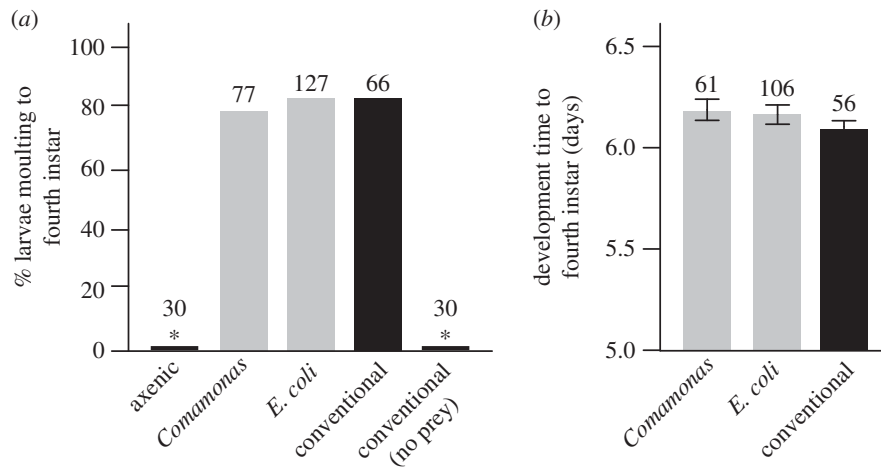
Figure 3. (a) Percentage of axenic first instars that developed into fourth instars when fed: axenic *A. aegypti* larvae, gnotobiotic *A. aegypti* larvae colonized by individual bacterial isolates or conventional *A. aegypti* larvae reared under non-sterile conditions (positive control). The percentage of conventionally reared first instars that developed into fourth instars in the absence of prey is also shown. The number above each bar represents the number of larvae assayed for a given treatment group. Asterisks (*) indicate a significant difference for a given treatment relative to the positive control as determined by pairwise Fisher's exact tests ($p < 0.0001$). (b) Development time of axenic *T. amboinensis* larvae to the fourth instar when fed gnotobiotic *A. aegypti* larvae colonized by different bacteria. *Toxorhynchites amboinensis* larvae fed conventional *A. aegypti* served as the positive control. Columns present mean values (±s.e.) for each treatment. ANOVA detected no differences between treatments ($F_{2,219} = 1.121$, $p = 0.328$). The number above each bar represents the number of larvae that moulted to the fourth instar and for which development time was recorded.

Table 1. Prey consumption by *T. amboinensis* larvae feeding on conventional or gnotobiotic *A. aegypti* larvae. The total number of *T. amboinensis* larvae assayed for each treatment group is listed in the first column. The number of prey consumed by each instar was tracked daily until at least one individual had pupated in a treatment cohort. Mean values (±s.e.) are shown. Separate ANOVA analyses were performed for each instar. Different letters indicate significant differences between treatments (Tukey–Kramer HSD test, $p < 0.05$). ANOVA detected no differences between treatments for either first ($F_{2,219} = 2.012$, $p = 0.136$) or fourth instars ($F_{2,97} = 1.062$, $p = 0.350$).

	N	first instar	second instar	third instar	fourth instar
Comamonas	200	10.00 ± 0.00	14.72 ± 0.21^A	18.87 ± 0.19^A	31.65 ± 1.23
E. coli	368	10.06 ± 0.03	14.93 ± 0.12^A	18.28 ± 0.15^A	29.92 ± 1.00
conventional	198	10.00 ± 0.00	14.00 ± 0.17^B	17.51 ± 0.24^B	29.21 ± 0.42

samples than between *T. amboinensis* larvae and their *A. aegypti* prey (figure 2b).

(b) *Toxorhynchites amboinensis* development assays

Next, we compared the development time and the percentage of *T. amboinensis* larvae that grew and moulted to the fourth instar when provided axenic, gnotobiotic or conventional *A. aegypti* prey. Results showed that 83% of *T. amboinensis* larvae from surface-sterilized eggs provided conventional *A. aegypti* larvae that were colonized by a mixed community of bacteria developed to the fourth instar at 6 days post-egg hatching (figure 3a,b). Axenic *T. amboinensis* fed gnotobiotic *A. aegypti* colonized by either *Comamonas* or *E. coli* similarly developed (figure 3a,b). However, axenic *T. amboinensis* fed axenic *A. aegypti* never grew beyond the first instar, while conventional *T. amboinensis* larvae from our standard rearing colony also failed to grow beyond the first instar when provided no prey (figure 3a,b).

PCR screening of bacterial 16S rDNA using universal primers and the template from individual *T. amboinensis* larvae confirmed the presence of bacteria in first instars that fed on conventional prey and the absence of bacteria in first instars

that fed on axenic prey (electronic supplementary material, figure S2). Screening with taxon-specific primers also confirmed that experimental cultures containing gnotobiotic prey produced gnotobiotic *T. amboinensis* fourth instars that contained the same bacterial isolate as their prey (electronic supplementary material, figure S2).

(c) *Toxorhynchites amboinensis* prey consumption and body size measurements

Prey consumption assays revealed that *T. amboinensis* larvae begin feeding within 1 h of egg-hatching and consume similar numbers of conventional and gnotobiotic prey before pupating and emerging as adults (table 1). However, while all conventionally reared *T. amboinensis* larvae moulted to the second instar after consuming only 10 prey larvae, no axenic *T. amboinensis* larvae moulted during the course of the experiment, even after consuming more than 100 prey larvae over the course of 10 days (electronic supplementary material, figure S3).

Body size measurements further revealed that axenic *T. amboinensis* larvae never achieve the critical size required for moulting. All *T. amboinensis* first instars hatched from
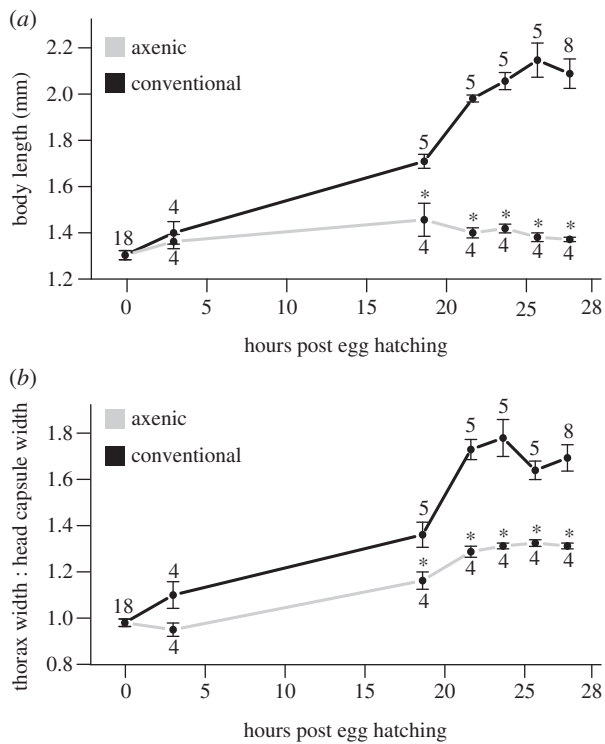
Figure 4. (a) Mean body length and (b) the ratio of thorax width : head capsule width for each treatment from the time of hatching (0 h) to 28 h. All larvae were provided 10 prey larvae during the duration of the experiment. Each data point represents the mean value (±s.e.), with the numbers above (conventional) or below (axenic) each data point representing the number of larvae that were destructively sampled to measure body size for each treatment group. A total of 18 newly hatched larvae were measured at 0 h (prior to the addition of axenic or conventional prey). Separate ANOVA analyses were performed for each time point. Asterisks (*) indicate time points where axenic larvae significantly differed from the conventional treatment ($p < 0.05$).

eggs and began feeding with an average head capsule width of 328.3 ± 16.0 (s.e.) µm. Conventionally reared larvae increased in body length for approximately 22 h before moulting to the second instar on average 26.9 ± 0.5 h after hatching (figure 4a). This increase in body length was accompanied by a corresponding increase in the ratio of prothorax width to head capsule width, which was approximately 1.0 at hatching (0 h) but increased to approximately 1.7 by 22 h after hatching (figure 4b). These data indicated that conventionally reared first instars achieved critical size at 22 h after hatching. By contrast, while axenic *T. amboinensis* larvae consumed their prey, they exhibited very minimal growth as measured by both body length and the ratio of prothorax width to head capsule width, which remained less than 1.4 (figure 4b).

(d) Gut hypoxia levels in axenic and conventional Toxorhynchites amboinensis larvae

Finally, we measured gut hypoxia in *T. amboinensis* first instars by estimating the abundance of the hypoxia-inducible transcription factor HIF-α, which is stabilized under low oxygen conditions and has previously been detected in *A. aegypti* larvae colonized by living bacteria [23]. Consistent with previous results in *A. aegypti*, immunoblot analysis detected HIF-α in conventionally reared, but not axenic

T. amboinensis larvae (electronic supplementary material, figure S4).

4. Discussion

Considerable attention has been given to characterizing microbial communities in vector species like *A. aegypti* because of results showing that the microbiota in mosquitoes affects both vector competency and development [33–35]. By contrast, little is known about the role of life history in the functional interactions between mosquitoes and their microbiota. We previously reported that larvae of several detritivorous mosquito species do not normally develop beyond the first instar in the absence of living microbes in their gut [18–21]. In this study, we asked whether this requirement is shared by larvae of predaceous species like *T. amboinensis*—a cultured species within the Toxorhynchitini, which as a group are obligate predators during the larval stage and no longer capable of vectoring vertebrate pathogens because adult females reproduce without blood feeding [13,14].

Our results show that axenic *T. amboinensis* larvae fed axenic *A. aegypti* prey under a standard photoperiod and sterile conditions fail to grow beyond the first instar but develop normally when fed conventionally reared or gnotobiotic *A. aegypti* prey colonized by a community member (Comamonas) present in our laboratory cultures that has previously been shown to support growth of gnotobiotic *A. aegypti* to levels consistent with conventionally reared individuals [20], or *E. coli*, which is absent in our laboratory cultures but also supports growth of gnotobiotic *A. aegypti* [18,19,22]. Our results also experimentally demonstrate that bacteria present in prey larvae can successfully colonize *T. amboinensis* larvae and persist to the fourth instar. The same Comamonas isolate has previously been shown to support the development and reproduction of gnotobiotic *A. aegypti* and *Aedes atropalpus* mosquitoes to levels consistent with conventionally reared individuals [20]. Several other bacterial isolates also support normal development and reproduction of *A. aegypti*, but only partially rescue development and reproduction by *A. atropalpus* [20]. A unique feature of the life history of *A. atropalpus* is that adult females produce a first clutch of eggs without blood feeding [8,16]. The ability to produce eggs without blood feeding is owing, in part, to enhanced nutrient acquisition by larvae, which provides resources for the first gonadotropic cycle after emergence [7,36–39]. Specific bacterial taxa like Comamonas have been shown to contribute differently to glycogen, protein and lipid stores in newly emerged *A. atropalpus* females, and gnotobiotic *A. atropalpus* females colonized by Comamonas produce more eggs than females colonized by other species of bacteria [20]. We did not measure nutrient levels or egg production by the gnotobiotic *T. amboinensis* mosquitoes we produced in this study. However, understanding how differences in gut community composition impact fitness of adult female *T. amboinensis* is of general interest, given that female *Toxorhynchites* mosquitoes never blood feed. Future studies evaluating egg production by *T. amboinensis* females colonized by different bacterial species, including the Comamonas sp. used in this study, could elucidate whether the same or different bacteria contribute to reproduction by *T. amboinensis* as for *A. atropalpus*.

The axenic *T. amboinensis* larvae generated in this study displayed defects in growth that were consistent with our own previous results, which showed that axenic *A. aegypti* larvae fail to achieve the critical size required for moulting past the first instar [32]. That HIF-α was stabilized in conventional but not axenic larvae is also consistent with microbe-associated gut hypoxia activating the insulin, target of rapamycin and multiple MAPK pathways in *T. amboinensis* larvae that were previously identified to be activated in *A. aegypti* [23,32]. We recently reported that a living yeast (*Saccharomyces cerevisiae*), alga (*Chlamydomonas reinhardtii*) and insect cell line (*Drosophila* S2) induce HIF-α stabilization and rescue growth and moulting of axenic *A. aegypti* larvae [21]. However, the same organisms fail to support moulting if heat-killed under the rearing conditions used in this study [21]. Previous results also demonstrate that axenic *T. amboinensis* larvae grow when fed living insect cells in a rich medium [40]. These results strongly suggest that, like detritivorous mosquitoes, predaceous *T. amboinensis* larvae do not *strictly* require bacteria for development, but rather rely more broadly on the different viable organisms they consume to serve the same function. Recent studies with *A. aegypti* also report that yeast extract and heat-killed bacteria can promote larval growth, but only when provided at very high doses with other nutrient-rich dietary components in darkness [41]. This suggests that yeast, bacteria and potentially other microbes produce photolabile nutrients or other factors that affect larval growth. It also suggests that viable microbes, even at the low densities present in aquatic habitats and the larval gut, are able to produce sufficient amounts of this factor under normal photoperiodic conditions while non-living microbes cannot.

A secondary goal of our study was to examine the bacterial community in laboratory-reared *T. amboinensis* larvae and compare it to the bacterial community in their prey, which in this study were *A. aegypti* larvae reared under the same environmental conditions in the same facility. Our results indicate that bacterial diversity in prey larvae strongly influences bacterial diversity in predaceous *T. amboinensis* and that the types of bacteria present in our laboratory culture of *T. amboinensis* are largely consistent with those previously identified in *A. aegypti* reared in the same facility [18,19]. These findings also provided a supportive rationale in our functional assays for using *Comamonas* as a representative community member in both species and *E. coli*, which is absent in our laboratory cultures but is present in other populations of *A. aegypti* and is also known to rapidly colonize the gut when fed to larvae [18,19,21]. We fully recognize that our microbiota data do not distinguish community members in *T. amboinensis* and *A. aegypti* larvae that reside in the gut versus other tissues since our sequencing libraries were prepared from whole body samples. This decision was driven in part by the very small size and fragility of these aquatic organisms, which makes explanting the gut with no possibility of contamination difficult. However, prior studies conducted in our laboratory and others experimentally show that *A. aegypti* and several other detritivorous mosquito species contain no bacteria as larvae before hatching from eggs, but rapidly acquire a gut microbiota from the aquatic environment in which they hatch by feeding (summarized in [35]). This, combined with the fact that (i) almost all of the OTUs we identified in our surface-sterilized larval samples were present in the water that larvae were reared

in and (ii) prior evidence shows that *Comamonas* and many of the other abundant community members we identified colonize the larval gut [18–20], strongly suggests that most of the bacteria we identified in *T. amboinensis* and *A. aegypti* larvae in the current study are gut community members.

Only a few other studies have examined the microbiota of insect predators in relation to their prey. Studies in lady beetles and certain species of crickets support a correlation between bacterial diversity in predators and the diversity of prey species on which they feed [42,43]. This suggests that, similar to the findings reported here, lady beetles and crickets harbour similar bacterial communities to their prey. However, unlike the present study, these studies did not characterize bacterial diversity in the prey species on which the predators they sampled fed. Thus, the extent to which the trends observed in these previous studies are associated with prey-derived microbes is unclear.

Our results differ from those in praying mantids, which harbour bacterial communities that are highly variable and dominated by bacteria that are present in low abundance or absent in their insect prey [44]. That praying mantids harbour a microbiota that differs from their prey could be owing, in part, to the spatial heterogeneity of terrestrial habitats, which may limit the co-occurrence of predators and prey in similar environments. By contrast, the aquatic lifestyle of mosquito larvae may provide a more reliable mechanism for maintaining prey-derived microbes, since predators and prey always co-occur in the same environment. That our results in *T. amboinensis* are more similar to those in lady beetles may also reflect the more specialized feeding patterns of these predators, which feed primarily on mosquito larvae and aphids, respectively. By contrast, praying mantids are generalist predators that capture any prey that falls within a specific size range.

The observation that the bacterial community in *T. amboinensis* and *A. aegypti* larvae differed so much from the water we sampled also suggests that, despite dramatic differences in their feeding habits, predaceous *T. amboinensis* and detritivorous *A. aegypti* larvae share features of digestive physiology that select for similar bacterial communities. However, whether these results are generalizable to field-collected *T. amboinensis*, which feed on other species in addition to *A. aegypti*, is currently unknown. Future studies could examine whether similar patterns in bacterial diversity exist when *T. amboinensis* larvae are provided different combinations of prey species, including close relatives outside of the Culicidae.

In summary, our results support a conserved role for a living gut microbiota in the development of mosquitoes, irrespective of their dietary lifestyle. They also strongly support that the requirement for living microbes is not obviated by added nutrients obtained by predaceous mosquitoes like *T. amboinensis* from the prey they consume. Rather, living microbes support the development of predaceous species by activating the same growth-related signalling pathways previously demonstrated to be essential for growth of detritivorous species. A fundamental question of interest moving forward is: what features of mosquito life history have selected for this dependency? The ever presence of bacteria and other microbes in aquatic environments has previously been hypothesized to select for greater dependence by aquatic animals on gut microbes for growth via the accumulation of mutations that are only deleterious in the unlikely absence of microbes [45]. Indeed, other aquatic organisms

have been shown to rely on their gut microbiota for survival, including zebrafish [46], *Daphnia* [47] and *Hydra* [48]. Whether microbe-mediated gut hypoxia or other factors play a role in regulating the growth of these and other aquatic animals is an important question for future study.

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