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1 **Larval habitat determines the bacterial and fungal microbiota of the mosquito vector**

2 *Aedes aegypti*

3

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29

30 **Abstract**

31 Mosquito larvae are naturally exposed to microbial communities present in a variety of larval  
32 development sites. Several earlier studies have highlighted that the larval habitat influences  
33 the composition of the larval bacterial microbiota. However, little information is available on  
34 their fungal microbiota, *i.e.* the mycobiota. In this study, we provide the first simultaneous  
35 characterization of the bacterial and fungal microbiota in field-collected *Aedes aegypti* larvae  
36 and their respective aquatic habitats. We evaluated whether the microbial communities  
37 associated with the breeding site may affect the composition of both the bacterial and fungal  
38 communities in *Ae. aegypti* larvae. Our results show a higher similarity in microbial  
39 community structure for both bacteria and fungi between larvae and the water in which larvae  
40 develop than between larvae from different breeding sites. This supports the hypothesis that  
41 larval habitat is a major factor driving microbial composition in mosquito larvae. Since the  
42 microbiota plays an important role in mosquito biology, unravelling the network of  
43 interactions that operate between bacteria and fungi is essential to better understand the  
44 functioning of the mosquito holobiont.

45

46 Key words: mosquito, microbiota, larva, breeding site, bacteria, mycobiota

47



## 48 **Introduction**

49 Like most living organisms, the mosquito microbiota includes resident (*i.e.*  
50 microorganisms that constantly live in or on the insect body) and transient (*i.e.*  
51 microorganisms that are only temporarily found in the insect body) microorganisms.  
52 Environmentally acquired microbes are thus part of the transient/flexible microbiota and are  
53 also considered as members of the mosquito microbiota. The recent and extensive scientific  
54 research on mosquito microbiota has led to the recognition that mosquito-microbe  
55 relationships are important for the host biology and pathogen transmission (Minard,  
56 Mavingui, Moro 2013; Guégan et al. 2018b; Dada et al. 2021). Regarding pathogen  
57 transmission, many examples demonstrated the ability of bacteria to affect vector competence  
58 for pathogens that infect humans (Dennison et al. 2014; van Tol and Dimopoulos, 2016).  
59 Some bacteria can negatively affect pathogen development and/or replication by activating  
60 immune response (Gabrieli et al. 2021) or prevent pathogen infection through competition for  
61 resources or the secretion of molecules against pathogens (Jupatanakul et al. 2014). In  
62 contrast, other bacteria as well as fungi can enhance mosquito host susceptibility to pathogen  
63 infection (Apte-Deshpande et al. 2014; Angleró-Rodríguez et al. 2017; Wu et al. 2019).  
64 Besides their influence in vector competence, bacteria were also shown to play important  
65 roles in mosquito development, reproduction and nutrition (Dong, Manfredini, Dimopoulos  
66 2009; Coon et al. 2014; Coon, Brown, Strand 2016a and 2016b; Dickson et al. 2017; Correa  
67 et al. 2018; Chen et al. 2020; Guégan et al. 2020; Scolari et al. 2021; Wang et al. 2021).

68 Bacterial microbiota of mosquitoes is a dynamic community that shifts across the insect  
69 biological cycle, as previously evidenced across several mosquito species (Gimonneau et al.,  
70 2014; Coon et al. 2014; Duguma et al. 2015; Alfano et al. 2019). In most observations, the  
71 microbiota diversity decreases during the development of mosquitoes from larvae to adults. A  
72 selective sweep occurs during the pupal stage when a renewal of the gut epithelial layers

73 induces a massive loss of bacterial communities (Alfano et al. 2019). Interestingly, a thorough  
74 study conducted in artificially reared *Ae. albopictus* mosquitoes under laboratory conditions  
75 highlighted that multiple factors including development stage, adult age and female feeding  
76 regimes determine the structure of bacterial microbiota from egg to adult stages (Chen et al.  
77 2020). Overall, multiple intrinsic and extrinsic factors may affect the acquisition and  
78 settlement of microbes during early stages. However, the breeding sites and habitats of  
79 mosquitoes were previously suggested as the main drivers of adult-associated bacterial  
80 communities (Guégan et al. 2018b; Dickson et al. 2017). As a matter of fact, an important part  
81 of the microbiota is environmentally acquired in mosquitoes (Coon et al. 2014). It was shown  
82 that larvae or newly emerged adults harbour microorganisms derived from their aquatic  
83 habitats (Guégan et al. 2018b; Mallasigné, Valiente Moro, Luis 2020). This means that  
84 environmental determinants strongly influence important traits in the biology of mosquitoes  
85 (Ranasinghe and Amarasinghe 2020; Dickson et al. 2017).

86 The yellow fever mosquito *Aedes aegypti* is a main vector for several medically  
87 important arboviruses, including dengue, Zika, yellow fever, and chikungunya viruses  
88 (Powell and Tabachnick 2013). Infections of humans with dengue and yellow fever viruses  
89 remain an important public health concern as they infect 390 million and 200,000 people each  
90 year, respectively (Barrett and Higgs 2007; Bhatt et al. 2013). To date, only few publications  
91 have explored the overlap between the microbiota of *Ae. aegypti* larvae and the microbial  
92 communities of their aquatic habitat (Dada et al. 2014; Ranasinghe and Amarasinghe 2020;  
93 Hery et al. 2021). Moreover, in comparison to bacteria, fungal communities (also referred to  
94 as mycobiota) were largely understudied across mosquito species including *Ae. aegypti*  
95 (Mallasigné, Valiente Moro, Luis 2020). A better understanding of the dynamics involved in  
96 microbial acquisition in this mosquito species is therefore needed. In particular, it remains  
97 unclear whether the microorganisms acquired from the water of breeding sites constitute a

98 major or negligible proportion of the larval microbiota and whether similar factors drive the  
99 acquisition of bacterial and fungal communities. Additional field studies comparing the  
100 microbial diversity including both the simultaneous analysis of bacteria and fungi of larvae  
101 and their aquatic habitats would provide important knowledge on the role of the environment  
102 in modulating the microbiota as a whole. In a previous study in central Gabon, we showed  
103 that the bacterial microbiota colonizing the gut of adult mosquitoes is highly correlated with  
104 variations in the microbiota of water they emerged from but markedly differed from water in  
105 terms of composition (Dickson et al. 2017). Indeed, bacteria found in adult guts are  
106 underrepresented in water. This result is consistent with the well-known microbiota  
107 reorganization that occurs between immature and adult stages (Alfano et al. 2019) and  
108 suggests that water may constitute a main source of microbes but that mosquitoes filter most  
109 of them out during their development. Considering this observation, we suggest that immature  
110 mosquito larvae that did not yet proceed to microbiota filtering during pupation may even be  
111 more influenced by the microbial composition of the water they are living in.

112       The objective of the current study was to investigate to which extent variations of the  
113 microbial communities in aquatic habitats influence *Ae. aegypti* larval microbiota  
114 composition and the paired structures of bacterial and fungal communities. For this, we  
115 collected larvae and water from various artificial containers within the Lopé village in Gabon.  
116 We hypothesized that similar environmental factors shape the composition of these two  
117 communities in the immature stages of mosquitoes. Such knowledge provides new insights  
118 into the environmental drivers of mosquito-associated bacterial and fungal communities.

119

## 120 **Material and Methods**

121

### 122 **Study site and sample collection**

123 Field sampling was performed in Gabon during the wet season in November - December 2017  
124 and April - May 2018 in Lopé village (latitude, -0.099221; longitude, 11.600330).  
125 Third- and fourth-instar larvae as well as water were sampled from artificial breeding sites  
126 such as small tanks and containers (Table S1). Samples were collected with a sterile plastic  
127 pipette into a sterile 50-ml falcon tube with filter-top lid. Water samples were agitated before  
128 sampling to mix detritus and the upper surface to a depth of 10cm was collected representing  
129 ~ 50 ml per breeding site. In the Station d'Etude des Gorilles et Chimpanzés field station,  
130 larvae were separated from the water for each breeding site under a laminar flow cabinet,  
131 transferred to a new sterile 50-ml falcon tube and both samples, water and larvae were then  
132 snap frozen in a liquid nitrogen tank and subsequently conserved at -80°C. All larvae  
133 collected from each sampled breeding site were selected for further sequencing analysis  
134 (Table S1).

135

### 136 **DNA extraction**

137 Water samples were homogenized after vortexing for 1 min and an aliquot of 1.5 mL was  
138 centrifuged for 10 min at maximum speed (13 400 rpm). The supernatant was discarded and a  
139 new aliquot of 1.5 mL was added. This step was repeated from 2 to 4 times depending on the  
140 volume of water available for each sample. Pooled pellets were resuspended in 500 µL of  
141 extraction buffer (10 mM tris-HCl [pH 8.0], 10 mM EDTA, disodium salt [pH 8.0], 100 mM  
142 sodium chloride, and 2% (v/v) SDS) and DNA was extracted as previously described  
143 (Dickson et al. 2017). The extractions were performed in 6 batches and a negative control  
144 (*i.e.*, extraction performed on an empty tube) was made for each batch. Prior to DNA  
145 extraction, larvae were surface-sterilized by three washes in sterile water, followed by 5 min  
146 soaking in 70% ethanol, and five rinses in sterile water. Each larva was crushed for 10 s using  
147 a Mini-beadbeaterTP apparatus (Biospec Products) into a 2-mL Eppendorf tube containing 1-

148 mm diameter beads (Biospec, 1 mm) and 250  $\mu$ L of CTAB buffer (2% hexadecyltrimethyl  
149 ammonium bromide, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris pH8, 0.2% 2- $\beta$   
150 mercaptoethanol). After incubation for 1h at 60°C on a shaker (300 rpm), 4  $\mu$ L of RNase (100  
151 mg/ml) was added and incubated at 37°C for 5 min. Mixtures of phenol:chloroform:isoamyl  
152 alcohol (25 :24 :1) and chloroform:isoamyl alcohol (24 :1) were added and after  
153 centrifugation for 30 min at 13 200 rpm, DNA was precipitated using isopropyl alcohol. DNA  
154 pellets were rinsed twice with 75% cold ethanol, air-dried under laminar flow and  
155 resuspended in 22 to 27  $\mu$ L of sterile RNase free water. DNA purity and concentration were  
156 measured using a NanoDrop™ spectrophotometer for both water and larvae samples.

157

#### 158 **Mitochondrial gene amplification**

159 DNA samples from larvae were used as templates to amplify a 597-base-pair (bp) region of  
160 the mitochondrial cytochrome c oxidase subunit I (COI) gene as previously described  
161 (Raharimalala et al. 2012). PCR products were sent to sequencing at BIOFIDAL-DTAMB  
162 (FR BioEnvironment and Health, Lyon, France). Sequences of each COI haplotype were  
163 deposited on European Nucleotide Archive (<https://www.ebi.ac.uk/ena/browser/home>) under  
164 the accession number PRJEB45388.

165

#### 166 **Library preparation and sequencing**

167 For amplicon sequencing, two-step Nextera PCR libraries were created following the Illumina  
168 protocol. First-step PCR were performed in triplicate on the hypervariable V5-V6 regions of  
169 the 16S rRNA gene (*rrs*) of ~ 280 bp and the nuclear ribosomal internal transcribed spacer  
170 (ITS2 region) for bacterial and fungal community identification, respectively. The modified  
171 primers 784F (5'-AGGATTAGATACCCTGGTA-3') and 1061R (5'-  
172 CRRCACGAGCTGACGAC-3') for bacteria and gITS7 (5'- GTG AAT CAT CGA RTC TTT

173 G -3') and ITS4 primers (5'- TCC TCC GCT TAT TGA TAT GC-3') for fungi were used.  
174 Each primer pair was associated with the two Illumina overhanging adapter sequences. Primer  
175 pair targeting the V5-V6 region was selected as these primers did not match mosquito 18S  
176 and mitochondrial 16S rDNA gene sequences and allowed the better coverage of the  
177 sequences belonging to the RDP Bacteria domain database (Minard et al. 2014). All PCR  
178 amplifications were carried out in triplicates on a Biorad C1000 thermal cycler (Biorad, CA,  
179 USA) in a 25 µl reaction. PCR reactions were performed using the 5X BioAmp® master mix  
180 (Biofidal, France) containing 2 µL sample DNA, 5 µL of Mix HotStarTaq 5X, 1X of GC rich  
181 Enhancer, 0.5 mg.mL<sup>-1</sup> of bovine serum albumin (New England Biolabs, Evry, France) and 1  
182 µM of each primer. For bacteria, amplifications were conducted for 10 min at 96°C followed  
183 by 5 cycles at 96°C for 20 s, 65°C for 30 s, 72°C for 60 s, 5 cycles at 96°C for 20 s, 61°C for  
184 30 s, 72°C for 60 s, 5 cycles at 96°C for 20 s, 57°C for 30 s, 72°C for 60 s, 25 cycles at 96°C  
185 for 20 s, 54°C for 30 s, 72°C for 60 s and a final extension at 72°C for 10 min. For fungi,  
186 amplifications were conducted with one cycle of 3 min at 94°C, followed by 40 cycles at  
187 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, and a final extension step of 5 min at 72°C. The  
188 three PCR product replicates from each sample (water or individual larvae) of each gene were  
189 pooled, purified and quantified with the Quant-iT Picogreen dsDNA Assay Kit (Life  
190 Technologies). A total of 148 amplicon libraries were constructed. A total of 21 (11 for  
191 bacteria, 10 for fungi), 115 (57 for bacteria and 58 for fungi) and 12 (6 for bacteria and 6 for  
192 fungi) amplicon libraries were generated for water samples, larvae and negative controls  
193 respectively. Subsequently, one Illumina MiSeq platform and a v3 600 cycles kit was used to  
194 sequence the PCR libraries (second-step PCR) at Biofidal (Vaulx-en-Velin, France). All  
195 FastQ files were deposited in the EMBL European Nucleotide Archive  
196 (<https://www.ebi.ac.uk/ena>) under the project accession number PRJEB45062.

197

198 **Bioinformatics analysis**

199 Total of 5,311,262 and 4,983,099 reads were obtained for the bacteria and fungi respectively  
200 and demultiplexed. Sequence quality control and analysis of sequence data were carried out  
201 using the FROGS pipeline (Escudie et al. 2016) with the previously described parameters  
202 (Guégan et al. 2018a; Guégan et al., 2020). Taxonomic affiliation was performed against the  
203 SILVA database 132 for bacteria (Yilmaz et al. 2014) and ITS UNITE database for fungi  
204 (Kõljalg et al. 2013) with the Mothur pipeline (Schloss et al. 2009) at 80% minimum  
205 bootstrap using a naïve Bayesian classifier (Wang et al. 2007). Contaminants were filtered out  
206 using negative control (blank extraction and PCR). OTUs were removed if they were detected  
207 in the negative control sample and their relative abundance was not at least 10 times greater  
208 than that observed in the negative control (Minard et al. 2015). Sequences were grouped into  
209 operational taxonomic units (OTUs) by clustering at 97% similarity. To compare samples,  
210 normalization was performed at 21,629 and 18500 sequences for the bacterial and fungal  
211 sequences, respectively. A total of 877 and 768 operational taxonomic units (OTUs) were  
212 obtained for bacteria and fungi, respectively. Venn diagrams were generated with the Venn  
213 Diagrams software from the Van de Peer Lab Bioinformatics and Evolutionary Genomics  
214 (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

215

216 **Statistical analysis**

217 Data analysis and statistical tests (Mann-Whitney-Wilcoxon and Adonis-ANOVA) were  
218 carried out using R software (R Core Team 2018) with phyloseq (McMurdie and Holmes  
219 2013) and vegan (Oksanen 2018) for diversity analysis as well as ggplot2 (Wickham 2018)  
220 for graphical representations. Bacterial and fungal within individual diversity also referred as  
221  $\alpha$ -diversity was estimated with the Shannon index ( $H'$ ). Community similarity between  
222 individuals also referred as  $\beta$ -diversity was estimated with the Bray-Curtis index.

223 Comparisons of  $\alpha$ -diversity between larvae and water were performed with an ANOVA.  
224 Correlations between larvae and water microbial communities (either bacteria and fungi)  
225 collected in the same habitats were performed with a Spearman rank-based analysis.  
226 Comparisons of microbial communities'  $\beta$ -diversity were performed with the *adonis*-ANOVA  
227 permutational analysis of variance with the package *vegan*. Spearman correlations between  
228 communities associated with water and larvae were computed with the *lineup2* package  
229 (Broman 2021) and heatmap graphs were represented with *complexHeatmap* (Gu 2021).

230

### 231 **Ethical statement**

232 Mosquito collections inside Lopé National Park were conducted under permit number  
233 AE17012/PR/ANPN/SE/CS/AFKP and the national research authorisation number  
234 AR0013/117/MESRSFC/CENAREST/CG/ CST/CSAR.

235

## 236 **RESULTS**

### 237 **Diversity and correlations between microbial communities of larvae and larval habitat**

238 **OTU diversity.** The microbiota of water and *Ae. aegypti* larvae samples were examined by  
239 sequencing of the bacterial 16S rRNA gene and ITS2 region, for bacterial and fungal  
240 communities respectively. Sequences of a total of 148 libraries (*i.e.*, 21 water samples; 115  
241 individual larval samples, 6 DNA extraction negative controls for each gene) resulted in  
242 5,311,262 sequence reads for bacteria, ranging from 57,562 to 116,144 (with an average of  
243 73,763 and 78,945 for water and larvae, respectively) and 4,983,099 sequence reads for fungi,  
244 ranging from 47,493 to 101,501 (with an average of 61,967 and 75,232 for water and larvae,  
245 respectively). Larvae and water exhibited a higher number of OTUs for bacteria than for fungi  
246 and the number of OTUs was higher in water than in larvae irrespective of the microbial  
247 community (Table S2 and S3). The mean number of OTUs detected in larvae sharing the



248 same breeding site varied from  $146 \pm 35$  to  $332 \pm 67$  for bacteria and from  $58 \pm 13$  to  $268 \pm$   
249  $62$  for fungi (Table S2 and S3). The average numbers of bacterial and fungal OTUs in water  
250 were  $202 \pm 40$  (range 138-266) and  $84 \pm 47$  (range 38-202), respectively (Table S2 and S3).

251 **Microbial communities comparison between water samples and larvae.** Larvae belonging  
252 to the same breeding site showed a relatively conserved microbiota for both bacteria (Fig 1A)  
253 and fungal communities (Fig 1B). Permutational analysis of variance showed that bacterial  
254 and fungal microbiota of larvae differed among individuals belonging to different breeding  
255 sites (*adonis*-ANOVA,  $F = 5.137$ ,  $R^2 = 0.678$ ,  $p = 0.001$ ;  $F = 7.249$ ,  $R^2 = 0.73$ ,  $p = 0.001$  for  
256 bacteria and fungi respectively) suggesting a strong impact of the larval breeding site on  
257 larval microbial communities. Water samples and larvae significantly differ from each other  
258 in terms of bacterial (*adonis*-ANOVA,  $F = 4.871$ ,  $R^2 = 0.033$ ,  $p = 0.001$ ) and fungal  
259 communities (*adonis*-ANOVA,  $F = 1.386$ ,  $R^2 = 0.02$ ,  $p = 0.001$ ) (Fig 2A and Fig 2B).

260 Comparison analysis performed with the Shannon index indicated no significant difference in  
261 the  $\alpha$ -diversity of bacterial communities between larvae and water (ANOVA,  $F = 0.305$ ,  $p =$   
262  $0.582$ ). However, significant differences were observed between larvae and water in the  
263 mycobiota diversity, the fungal microbiota being more diverse in larvae than in water  
264 (ANOVA,  $F = 4.345$ ,  $p = 0.04$ ). Furthermore a positive correlation was detected between the  
265 Shannon index of the water and the Shannon index of larvae for fungi (Spearman correlation  
266  $\rho = 0.74$ ,  $p = 3.379 \times 10^{-7}$ ; Fig. 3B) but not for bacteria (Spearman correlation  $\rho = -0.22$ ,  $p =$   
267  $0.18$ ; Fig. 3A). A total of 542 bacterial OTUs and 375 fungal OTUs (*i.e.* 61.8 % and 48.8 %  
268 of the total OTUs) were shared between larvae and water (Fig. 4A and Fig. 4B). Larvae-  
269 specific bacterial and fungal OTUs counted for 251 and 346, respectively while that of water-  
270 specific OTUs was lower with 27 and 21 respectively for bacteria (Fig. 4A) and fungi (Fig.  
271 4B).

272 **Correlation between the microbial composition of larvae and water.** A clear correlation of  
273 the microbiota of larvae and water was detected for individuals belonging to the same  
274 breeding site for both bacteria (Fig. S1) and fungi (Fig. S2). The same analysis performed at  
275 the scale of OTUs showed that OTUs split into different groups highlighting some patterns of  
276 positive correlations between larval and water microbiota composition (Fig. 5 and 6).  
277 Regarding bacteria, one group consisted of 5 OTUs in larval samples (*Clostridium sensu*  
278 *stricto*, *Camelimonas*, *Candidatus\_Proteochlamydia*, *Bosea* and *Vogesselia*) that showed  
279 correlated abundances with 4 OTUs in water samples (*Chryseobacterium*, *Legionella*,  
280 *Camelimonas* and *Bosea*) (Fig. 5). The identification of *Camelimonas* and *Bosea* OTUs in the  
281 two groups of both categories of samples, suggests that water of breeding sites was a major  
282 determinant of bacterial microbiota composition in larvae of these two taxa. Another group  
283 harbouring the OTUs *Chromobacterium*, *Arcicella* and one unclassified taxa in larvae  
284 samples was positively correlated with the OTUs *Novosphingobium*, uncultured *Chlamydiales*  
285 (*CvE6\_ge*) and uncultured *Beijerinckiaceae* (*FukuN57*) in water samples. Finally, a third  
286 group of OTUs composed of *Microbacterium*, *Phreatobacter* and 2 unclassified taxa were  
287 positively correlated with the OTUs *Leifsonia*, *Rudanella* and 5 other unclassified taxa.  
288 Regarding fungi, 5 groups of positive correlations were highlighted (Fig. 6). All together  
289 these results indicate that water of breeding sites is a strong driver of microbial assemblages  
290 in larvae, for both bacterial and fungal communities.

291

## 292 **Composition of microbial communities in *Aedes aegypti* larvae and their associated** 293 **aquatic habitats**

294 **Bacterial taxonomic composition.** A total of 877 OTUs belonging to 11 bacterial phyla and  
295 254 genera were identified across all the samples. The percentage of unclassified bacterial  
296 sequences represented less than 1% irrespective of the sample type except for one larval

297 sample, which exhibited 21% of unclassified sequences (Fig. 7). In larvae, 11 bacterial phyla  
298 were identified and 3 of them (Actinobacteria, Proteobacteria and Firmicutes) were prevalent  
299 and abundant (taken together range 47.07% - 99.97% of relative abundance, average 89.69%).  
300 Their relative abundance was variable among larval samples between breeding sites ranging  
301 from 3.34% to 88.11% for Actinobacteria, 10.25% to 56.54% for Proteobacteria and 1.19% to  
302 85.26% for Firmicutes. For water samples, only Proteobacteria and Actinobacteria were  
303 retrieved in all samples. Taken together, the mean abundance of the three phyla  
304 (Actinobacteria, Proteobacteria and Firmicutes) per larval habitat is more than 67.68 %  
305 (average 88.59%). The relative abundance of Proteobacteria ranged from 34.01% to 95.44%  
306 (average 76.73%) and was higher compared to that of Actinobacteria (range 0.82% - 35.49%,  
307 average 10.99%). When comparing water and larval samples, the proportion of Proteobacteria  
308 was more important in water compared to larvae ( $76.73 \pm 19.93$  vs  $32.32 \pm 16.79$ ) while  
309 higher proportions of Actinobacteria were reported in larvae compared to water ( $34.55 \pm$   
310  $25.78$  vs  $10.99 \pm 13.52$ ) (Fig. 7). A total of 62 bacterial genera out of 254 (24.4%) were  
311 specific to larvae (among which the most abundant were *Ralstonia* and  
312 *Candidatus\_Proteochlamydia*) and 8 genera (3.1%) were specific for water (among which the  
313 most abundant were unclassified Sporichthyaceae, *Pseudarcicella* and *Rudanella*). A  
314 combined analysis of prevalence and abundance was also performed. Prevalence refers to the  
315 overall infection rate of a given OTU overall samples (number of samples, i.e. larvae or  
316 water, in which the OTU was detected / total number of samples). Abundance refers to  
317 proportion occupied by an OTU within a sample among either the bacterial or fungal  
318 community (number of normalized reads assigned to the OTU in a given samples / total  
319 number of normalized reads). This analysis revealed the 5 OTUs *Mycobacterium*, *Clostridium*  
320 *sensu stricto*, unclassified *Peptostreptococcaceae*, unclassified *Burkholderiaceae* and  
321 unclassified *Microbacteriaceae* were the most prevalent (>75%) and abundant (>10<sup>2</sup>) across

322 larvae (Fig. 8A). In water, 2 OTUs identified as unclassified *Rhizobiales* were identified as  
323 the most prevalent (100%) with high abundance ( $>10^2$ ) (Fig. 8B).

324 **Fungal taxonomic composition.** A total of 668 OTUs belonging to 9 sub-phyla and 49 order  
325 were identified in *Ae. aegypti* larvae. Compared to bacterial communities, the percentage of  
326 unclassified fungal sequences was important and variable across samples. It was comprised  
327 between 6.65% and 99.79% in larvae or between 12.58% and 99.94% in water (Fig. 9).

328 Larvae and water samples exhibited similar communities dominated by the sub-phylum  
329 Pezizomycotina (from 9% to 91.59% in larvae and from 4.94% to 83.67% in water) and  
330 followed by the sub-phylum Agaromycotina (from 0,14% to 15,34% in larvae and from  
331 0,37% to 20,29% in water). The Zygomycota incertae sedis phylum was absent from water  
332 but present in 3 out of 17 samples of larvae while conversely the phylum Chytridiomycota  
333 was absent from larvae but identified in one sample of water. At the order level, similar  
334 fungal compositions were observed between larvae and their associated aquatic habitats  
335 within the phyla Ascomycota and Basidiomycota (Fig. S3 and S4). Within the phylum  
336 Ascomycota, the majority of reads of water samples was assigned to the order Pleosporales  
337 (33.73%) followed by Capnodiales (27.02%) and Hypocreales (10.04%) (Fig. S3). For larvae  
338 samples (at least one larva of the breeding site), it was Pleosporales (27.64%),  
339 Trichosphaeriales (13.46%), Eurotiales (13.39%) and Capnodiales (10.46%) (Fig. S3). Reads  
340 assigned to Pleosporales were detected in all of the water and mosquito samples (Fig. S3).

341 Within the Basidiomycota, the majority of reads were associated with the order Tremellales  
342 (46.87%) followed by Rhyzophydiales (20.19%) (Fig. S4). For larvae samples, it was  
343 Tremellales (32%), Polyporales (12.78%) and Wallemiales (11.92%) (Fig. S4). A combined  
344 analysis of prevalence and abundance revealed two OTUs (one unclassified *Trichosphaeriales*  
345 and another OTU identified as unclassified) as being the most prevalent and abundante ( $>60\%$   
346 and  $10^2$ , respectively) across larvae followed by the OTU *Penicillium citrinum* ( $>50\%$  and

347 10<sup>2</sup>) (Fig. 8C). In water, one OTU identified as unclassified Pleosporales was found to be the  
348 most prevalent (>80%) and abundant (5.10<sup>2</sup>) followed by the two OTUs *Toxidocladosporium*  
349 *irritans* and *Aureobasidium* (70%, 10<sup>1</sup>) (Fig. 8D).

350

## 351 **Discussion**

352 In a previous study, we demonstrated that larval exposure to different bacteria drive  
353 variation in *Ae. aegypti* vector competence (Dickson et al. 2017). Such a result underlines the  
354 importance to better characterize the microbial composition of mosquito larvae in relation  
355 with their breeding sites since larvae acquire the major part of their microbiota from water  
356 habitats (Xia et al. 2021). Despite growing information on the composition and diversity of  
357 bacterial communities associated with *Ae. aegypti* at the adult stage, in comparison limited  
358 data are available on larvae and their water habitats as well as on other associated microbes.  
359 To fill this gap, we used high-throughput sequencing to compare bacterial and fungal  
360 communities between water samples and *Ae. aegypti* larvae collected from various breeding  
361 sites in the Lopé village (Gabon).

362 Overall, our study showed that there was higher similarity in microbial structure, for  
363 both bacterial and fungal communities, between larvae and water in which larvae develop  
364 than between larvae and water from different breeding sites. This correlation in the microbial  
365 composition between larvae and their surrounding environment could reflect either an  
366 influence of larval microbiota on water microbial communities (through the release of  
367 microbial community from larvae feces or during molting as well as larval predation of  
368 microorganisms) or on the opposite, an influence of the water microbial communities on  
369 larval microbiota (through acquisition by filtration, nutrition, limited microbial vertical  
370 transmission). Interestingly, our results also support the fact that larval microbiota is highly  
371 similar to the microbial communities found in larval habitats contrary to what was observed

372 for adult mosquitoes. Since it is known that a massive loss of bacterial communities occurs  
373 during the pupal stage, this suggests that the mosquitoes select the microbiota in late stages  
374 (most likely between pupation and emergence) or that some bacteria are quickly able to re-  
375 colonize newly-emerged adult mosquitoes. The first hypothesis would explain why adults  
376 have a microbiota that varies symmetrically to the larval breeding site they originated from  
377 but have a different microbiota composed of sub-dominant species in water.

378         While no significant differences were detected for bacteria, a more diverse microbiota  
379 in larvae than in water was observed for fungal communities, associated with a positive  
380 correlation between the Shannon indices of the water and the larvae. This higher diversity  
381 could be explained by biotic and abiotic differences between both environments (*i.e.*  
382 difference in pH, oxygen, immunity ...) and / or differences in fungi acquisition mode (*i.e.*  
383 vertical transmission in insects, nutrition...). The hypoxia in the gut of larvae is a factor  
384 limiting the growth of particular aerobic bacteria but little is known for fungi (Coon et al.  
385 2017). Muturi et al (2020) also demonstrated that mosquito larvae are highly selective of the  
386 bacterial taxa from the larval environment that colonize their bodies. Interestingly, a recent  
387 study also demonstrated that amount of larval diet in the breeding water that proportionally  
388 increase with bacterial loads in breeding water, has a significant effect on *Ae. aegypti*  
389 mosquito microbiota size and composition and that these effects last into adulthood  
390 (MacLeod, Dimopoulos, Short 2021). Microbial interactions such as competition,  
391 amensalism, mutualism, cross-feeding or predation occur among microbial species in the  
392 mosquito microbiota and may also impact in structuring microbial communities (Gusmao et  
393 al. 2010). Thus, it is possible that bacteria or fungi already present in larvae limit the  
394 colonization by new microorganisms referred to as priority effects. This was observed in a  
395 recent study where the development of fungi was correlated with a reduction of bacterial  
396 numbers by antibiotics in field-caught mosquitoes (Hyde et al. 2019).

397 Moreover, previous studies also reported that water with or without mosquito larvae as well  
398 as larval density influence the bacterial community composition in water that in turn may  
399 have consequences on larvae microbiota composition (Nilsson et al. 2018; Muturi et al. 2020).  
400 However, such an impact remains to be investigated for fungal communities.

401 In agreement with other studies, Proteobacteria, Firmicutes and Actinobacteria were  
402 the most abundant phyla found in field-collected *Ae. aegypti* larvae representing on average  
403 89% of the total number of reads (Dada et al. 2014; Hery et al. 2021). These phyla were also  
404 very abundant and common in water habitats but contrary to what was previously observed  
405 (Hery et al. 2021), not all bacterial taxa identified in *Ae. aegypti* larvae were found in the  
406 corresponding breeding sites. This difference could potentially be due to the difficulty of  
407 sampling all microorganisms from a water source given that small volumes were sequenced  
408 or differences in the total biomass and the DNA extraction protocols between the two  
409 samples. Distinct patterns were observed for Proteobacteria and Actinobacteria. While the  
410 proportion of Proteobacteria was more important in water compared to larvae, the contrary  
411 was observed for Actinobacteria in which a higher proportion in larvae was obtained.  
412 However, at the genus level the bacterial composition diverged between larvae and water  
413 samples, with heterogeneity in the taxonomic profiles. The genus *Mycobacterium* was found  
414 to be the most prevalent and abundant in larvae while it was an OTU belonging to an  
415 unclassified *Rhizobiales* in water samples. Interestingly, the genus *Mycobacterium* was  
416 recently shown to dominate in *Ae. japonicus* larvae and this genus with an unclassified  
417 *Rhizobiales* occurred in disproportionally higher abundance in *Ae. japonicus* samples from  
418 used tires (Juma et al. 2021). Moreover, the distribution of the other most abundant and  
419 prevalent genera varied considerably among water and larvae except for an OTU belonging to  
420 an uncultured *Burkholderiaceae* which was highly abundant and prevalent in both samples.

421 Interestingly, *Wolbachia* was identified in 4 individual larvae belonging to different  
422 breeding sites with low number of sequences per larvae. Analysis of the V5-V6 sequences  
423 showed 99% identity GenBank accession number MN046789) with *Wolbachia* previously  
424 identified in field-collected *Ae. aegypti* mosquitoes in Philippines (Carvajal et al. 2019). This  
425 result confirms recent studies highlighting the presence of this bacterium in *Ae. aegypti*  
426 (Thongsripong et al. 2018; Balaji, Jayachandran and Prabakaran 2019; Carvajal et al. 2019;  
427 Kulkarni et al. 2019, Inacio da Silva et al. 2021) while the absence of natural infection of  
428 *Wolbachia* in *Ae. aegypti* mosquitoes had long been put forward (Gloria-Soria, Chiodo,  
429 Powell 2018). We can not exclude the possibility that the detection of the bacterium could be  
430 explained either by the infection of larvae by nematodes or parasitoids that carry *Wolbachia*  
431 themselves or the ingestion by larvae of arthropods infected with *Wolbachia*. However,  
432 further studies are needed to confirm the presence of naturally occurring *Wolbachia* in *A.*  
433 *aegypti* as the scientific consensus is that sequencing data alone is not enough to establish this  
434 (Chrostek et al. 2019; Ross et al. 2020). Screening on a larger number of individuals by using  
435 additional markers such as the *Wolbachia* outer surface protein gene *wsp* and the GroE operon  
436 would be performed in combination with a phylogeny of *Wolbachia* strains originating from  
437 other arthropod hosts and mosquitoes.

438 In fungal taxonomy and community ecology, the most studied DNA barcode is the  
439 nuclear ribosomal RNA intergenic spacer locus (Schoch et al. 2012). Due to a lack of  
440 references in public databases, many of those sequences remained unassigned. Despite this  
441 issue, we found that the sub-phyla Pezizomycotina and Agaromycotina were the most  
442 abundant phyla found in *Ae. aegypti* larvae and their associated aquatic habitats. Within the  
443 phylum Ascomycota, OTUs in the fungal orders Pleosporales were shared across all water  
444 and larvae samples as previously observed in *Ae. albopictus* (Tawidian et al. 2021). In  
445 addition, some specific patterns were observed between larvae and water as the majority of



446 reads was assigned to the orders Capnodiales and Hypocreales for water samples and  
447 Trichosphaeriales, Eurotiales and Capnodiales for larvae samples. Within the Basidiomycota,  
448 the majority of reads were associated with the order Tremellales that contrasts with previous  
449 observations that showed a dominance and prevalence of the order Agaricales in all of the  
450 water and *Ae. albopictus* mosquito samples (Tawidian et al. 2021). Interestingly, we showed  
451 that *P. citrinum* was one of the most abundant and prevalent species across larvae. Russel et al  
452 (2001) reported *P. citrinum* parasitizing eggs of *Ae. aegypti* in Australia by the production of  
453 mycotoxins that inhibit the complete development of the eggs during the dry season, thereby  
454 diminishing the incidence of mosquitoes during the rainy season. The fact that this species  
455 was highly prevalent and abundant in larvae raises the question of its impact on this mosquito  
456 species. Two OTUs belonging to the class Microbotryomycetes were found to be conserved  
457 fungal OTUs between adult female mosquito species *Coquillettidia perturbans* and  
458 *Ochlerotatus canadensis* collected at disparate sites (Hyde et al. 2019). OTUs belonging to  
459 this class were also detected in *Ae. aegypti* larvae and aquatic habitats but they were not  
460 among the most abundant and prevalent suggesting specific associations according to the  
461 mosquito species or the stage.

462         The strong homogeneity between the breeding sites samples did not allow to group  
463 them by type and to assess the role of differences in aquatic habitats on larval  
464 microbiota/mycobiota composition and structure. To date, there have been no studies on the  
465 influence of the type of habitat on both these communities among water and larvae. While  
466 different habitats may result in location-driven variability in microbial composition, further  
467 studies with replicates of habitats corresponding to each category would help to evaluate  
468 whether the type of habitat determine the presence of specific microbial communities in water  
469 and larvae. It means that the variation of some bacterial or fungal communities are linked  
470 between the water and the larvae. More globally, the nature of breeding sites as well as

471 physicochemical factors are important factors that need to take into account to explain the  
472 dynamics of microbial communities. Despite investigating the impact of spatial and temporal  
473 variability of abiotic properties of *Ae. aegypti* breeding sites on immature abundance and  
474 adult body size (David et al. 2021), no studies have yet disentangled their direct impact on the  
475 microbial communities and their indirect consequences on larvae development and  
476 physiological characteristics of the adult mosquito.

477         While many previous studies have examined the bacterial composition between larvae  
478 and the water in which they are developing, this study is the first to examine the bacterial and  
479 fungal composition of *Ae. aegypti* larval habitats in relation to that of their associated larvae.  
480 To our knowledge, this is the first characterization of the mycobiota from field-collected  
481 individual *Ae. aegypti* larvae and their associated aquatic habitats. We showed that larvae and  
482 water share microbial communities whose diversity and richness are driven by the breeding  
483 site. Further studies are needed to evaluate whether some fungi or bacteria detected in larvae  
484 are transstadially passed to the adult and how the natural variability of breeding sites shapes  
485 adult trait variation and vector competence.

486

#### 487 **Supplementary data**

488 Supplementary data are available at FEMSEC online.

489

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502

503 ***Conflicts of interest.*** None declared.

504

505

506 **References**

507

508 Alfano N, Tagliapietra V, Rosso F et al. Changes in microbiota across developmental stages  
509 of *Aedes koreicus*, an invasive mosquito vector in Europe: Indications for microbiota-Based  
510 Control Strategies. *Front Microbiol* 2019; 10:2832.

511

512 Angleró-Rodríguez YI, Talyuli OA, Blumberg BJ et al. An *Aedes aegypti*-associated fungus  
513 increases susceptibility to dengue virus by modulating gut trypsin activity. *Elife* 2017;  
514 6:e28844.

515

516 Apte-Deshpande AD, Paingankar MS, Gokhale MD, Deobagkar DN. *Serratia odorifera*  
517 mediated enhancement in susceptibility of *Aedes aegypti* for chikungunya virus. *IJMR* 2014;  
518 139(5):762-768.

519

520 Balaji S, Jayachandran S, Prabakaran SR. Evidence for the natural occurrence of *Wolbachia*  
521 in *Aedes aegypti* mosquitoes. *FEMS Microbiol Lett* 2019; 366(6):fnz055

522

523 Barrett AD and Higgs S. Yellow fever: a disease that has yet to be conquered. *Annu Rev*  
524 *Entomol* 2007; 52:209-29.

525

526 Bhatt S, Gething PW, Brady OJ et al. The global distribution and burden of dengue. *Nature*  
527 2013; 496: 504-7.

528

529 Broman KW. 2021. Lineup2: Lining up two sets of measurements.

530

531 Carvajal TM, Hashimoto K, Harnandika RK et al. Detection of *Wolbachia* in field-collected  
532 *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. *Parasit Vectors* 2019;  
533 *12(1)*:361.  
534

535 Chen S, Zhang D, Augustinos A et al. Multiple factors determine the structure of bacterial  
536 communities associated with *Aedes albopictus* under artificial rearing conditions. *Front.*  
537 *Microbio* 2020; *11*:605.  
538

539 Coon KL, Vogel KJ, Brown MR et al. Mosquitoes rely on their gut microbiota for  
540 development. *Mol. Ecol* 2014; *23*:2727–2739.  
541

542 Coon KL, Brown MR, Strand MR. Gut bacteria differentially affect egg production in the  
543 anautogenous mosquito *Aedes aegypti* and facultatively autogenous mosquito *Aedes*  
544 *atropalpus* (Diptera: Culicidae). *Parasit. Vectors* 2016a; *9*:1–12.  
545

546 Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential  
547 for development but vary greatly between local habitats. *Mol Ecol* 2016b; *25*:5806–26.  
548

549 Coon KL, Valzania L, McKinney DA et al. Bacteria-mediated development of mosquitoes.  
550 *PNAS* 2017 ; *114 (27)*:E5362-E5369.  
551

552 Correa MA, Matusovsky B, Brackney DE et al. Generation of axenic *Aedes aegypti*  
553 demonstrate live bacteria are not required for mosquito development. *Nat Commun* 2018;  
554 *9*:4464.  
555

556 Dada N, Jumas-Bilak E, Manguin S et al. Comparative assessment of the bacterial  
557 communities associated with *Aedes aegypti* larvae and water from domestic water storage  
558 containers. *Parasit Vectors* 2014; 7:391.

559

560 Dada, N, Jupatanakul, N, Minard, G et al. Considerations for mosquito microbiome research  
561 from the Mosquito Microbiome Consortium. *Microbiome* 2021; 9:36.

562

563 David MR, Dantas ES, Maciel-de-Freitas R et al. Influence of larval habitat environmental  
564 characteristics on Culicidae immature abundance and body size of adult *Aedes aegypti*. *Front*  
565 *Ecol Evol* 2021; 9:626757.

566

567 Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector  
568 competence for human pathogens. *Curr Opin Insect Sci* 2014 ; 3: 6–13.

569

570 Dickson LB, Jiolle D, Minard G et al. Carryover effects of larval exposure to different  
571 environmental bacteria drive adult trait variation in a mosquito vector. *Sci Adv.* 2017;  
572 3(8):e1700585.

573

574 Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the  
575 defense against malaria parasites. *PLoS Pathog* 2009; 5:e1000423.

576

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

579

580 Escudie, F, Auer L, Bernard M et al. FROGS: find rapidly OTU with galaxy solution.  
581 F1000Research 2016; 5:1287–1294.  
582  
583 Gabrieli P, Caccia S, Varotto-Boccazzi I, Arnoldi I et al. Mosquito trilogy: microbiota,  
584 immunity and pathogens, and their implications for the control of disease Transmission. *Front*  
585 *Microbiol* 2021; 12:630438.  
586  
587 Gloria-Soria A, Chiodo TG, Powell JR. Lack of evidence for natural *Wolbachia* Infections in  
588 *Aedes aegypti* (Diptera: Culicidae). *J Med Entomol* 2018; 55:1354-1356.  
589 Gu, Z. 2021. ComplexHeatmap: Make Complex Heatmaps; Bioconductor version: Release  
590 (3.12).  
591  
592 Guégan, M, Minard G, Tran FH et al. Short-term impacts of anthropogenic stressors on *Aedes*  
593 *albopictus* mosquito vector microbiota. *FEMS Microbiol Ecol* 2018a; 94:fiy188.  
594  
595 Guégan M, Zouache K, Démichel C et al. The mosquito holobiont: fresh insight into  
596 mosquito-microbiota interactions. *Microbiome* 2018b. 6:49.  
597  
598 Guégan M, Tran Van V, Martin E et al. Who is eating fructose within the *Aedes albopictus*  
599 gut microbiota? *Environ Microbiol* 2020; 22(4):1193-1206.  
600  
601 Gusmão DS, Santos AV, Marini DC et al. Culture-dependent and culture-independent  
602 characterization of microorganisms associated with *Aedes aegypti* (Diptera: Culicidae) (L.)  
603 and dynamics of bacterial colonization in the midgut. *Acta Trop* 2010; 115(3):275–281.  
604

605 Hery L, Guidez A, Durand AA et al. Natural variation in physicochemical profiles and  
606 bacterial communities associated with *Aedes aegypti* breeding sites and larvae on Guadeloupe  
607 and French Guiana. *Microb Ecol* 2021 81:93–109.

608

609 Hyde J, Gorham C, Brackney DE and Blaire S. Antibiotic resistant bacteria and commensal  
610 fungi are common and conserved in the mosquito microbiome. *Plos one* 2019 14(8):  
611 E0218907.

612

613 Inácio da Silva LM, Dezordi FZ, Paiva MHS et al. Systematic review of Wolbachia symbiont  
614 detection in mosquitoes: An Entangled Topic about Methodological Power and True  
615 Symbiosis. *Pathogens* 2021; 10:39.

616

617 Jupatanakul N, Sim S, Dimopoulos G. The insect microbiome modulates vector competence  
618 for arboviruses. *Viruses* 2014; 6(11): 4294–4313.

619

620 Gimonneau G, Tchioffo MT, Abate L et al. Composition of *Anopheles coluzzii* and *Anopheles*  
621 *gambiae* microbiota from larval to adult stages. *Infect Genet Evol* 2014 ; 28:715-24.

622

623 Juma EO, Allan BF, Kim CH et al. The larval environment strongly influences the bacterial  
624 communities of *Aedes triseriatus* and *Aedes japonicus* (Diptera: Culicidae). *Sci Rep* 2021; 11:  
625 7910.

626

627 Kõljalg U, Nilsson RH, Abarenkov K *et al.* Towards a unified paradigm for sequence-based  
628 identification of fungi. *Mol Ecol* 2013; 22:5271-5277.

629



630 Kulkarni A, Yu W, Jiang J et al. *Wolbachia pipientis* occurs in *Aedes aegypti* populations in  
631 New Mexico and Florida, USA. *Ecol Evol* 2019; 9:6148–6156.  
632  
633 MacLeod HJ, Dimopoulos G, Short SM. Larval diet abundance influences size and  
634 composition of the midgut microbiota of *Aedes aegypti* mosquitoes. *Front Microbiol*  
635 2021;12:645362.  
636  
637 Malassigné S, Valiente Moro C, Luis P. Mosquito mycobiota: an overview of non-  
638 entomopathogenic fungal interactions. *Pathogens* 2020; 9(7):564.  
639  
640 McMurdie PJ and Holmes S. Phyloseq: An R package for reproducible interactive analysis  
641 and graphics of microbiome census data. *PLoS One* 2013; 8:e61217.  
642  
643 Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the  
644 mosquito holobiont. *Parasit Vectors* 2013; 6:146.  
645  
646 Minard G, Tran F-H, Dubost A, et al. Pyrosequencing 16S rRNA genes of bacteria associated  
647 with wild tiger mosquito *Aedes albopictus*: a pilot study. *Front Cell Infect Microbiol* 2014;  
648 4:59.  
649  
650 Minard G, Tran FH, Van VT et al. French invasive Asian tiger mosquito populations harbor  
651 reduced bacterial microbiota and genetic diversity compared to Vietnamese autochthonous  
652 relatives. *Front Microbiol* 2015; 6:970.  
653

654 Minard G, Tran FH, Tran Van V et al. Shared larval rearing environment, sex, female size  
655 and genetic diversity shape *Ae. albopictus* bacterial microbiota. *PLoS One* 2018;13:e0194521.  
656

657 Muturi EJ, Dunlap C and Caceres CE. Microbial communities of container aquatic habitats  
658 shift in response to *Culex restuans* larvae. *FEMS Microbiol. Ecol* 2020; 96:fiaa112.  
659

660 Nilsson LKJ, Sharma A, Bhatnagar RK et al. Presence of *Aedes* and *Anopheles* mosquito  
661 larvae is correlated to bacteria found in domestic water-storage containers. *FEMS Microbiol*  
662 *Ecol* 2018; 94:fiy058.  
663

664 Oksanen J, Blanchet GF, Friendly M et al. Vegan: community ecology package. R package  
665 version 2.4-6. 2018; <https://CRAN.R-project.org/package=vegan>.  
666

667 Powell JR and Tabachnick WJ. History of domestication and spread of *Aedes aegypti* - A  
668 Review. *Mem Inst Oswaldo Cruz* 2013; 108:11-17.  
669

670 R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R  
671 Foundation for Statistical Computing. 2018; <https://www.R-project.org/>.  
672

673 Raharimalala FN, Ravaomanarivo LH, Ravelonandro P et al. Biogeography of the two major  
674 arbovirus mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae), in  
675 Madagascar. *Parasit Vectors*. 2012; 5:56.  
676

677 Ranasinghe AK and Amarasinghe LD. Naturally occurring microbiota associated with  
678 mosquito breeding habitats and their effects on mosquito larvae. *Biomed Res Int*. 2020;

679 4065315.  
680  
681 Russell BM, Kay BH, Shipton W. Survival of *Aedes aegypti* (Diptera: Culicidae) Eggs in  
682 Surface and Subterranean Breeding Sites During the Northern Queensland Dry Season. *J Med*  
683 *Entomol* 2001; 38(3): 441–445.  
684  
685 Schoch CL, Seifert KA, Huhndorf S et al. Nuclear ribosomal internal transcribed spacer (ITS)  
686 region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci.* 2012; 109: 6241–  
687 6246.  
688  
689 Schloss PD *et al.* 2009. Introducing mothur: Open-source, platform-independent, community-  
690 supported software for describing and comparing microbial communities. *Appl Environ*  
691 *Microbiol* 2009; 75(23):7537-41  
692  
693 Scolari F, Sandionigi A, Carlassara M et al. Exploring changes in the microbiota of *Aedes*  
694 *albopictus*: comparison among breeding site water, larvae, and adults. *Front. Microbiol.* 2021;  
695 12:624170.  
696  
697 Tawidian P, Coon KL, Jumpponen A et al. Host-environment interplay shapes fungal  
698 diversity in mosquitoes. *MSpehere* 2021; 6:5  
699  
700 Thongsripong P, Chandler JA, Green AB et al. Mosquito vector-associated microbiota:  
701 Metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic  
702 for dengue and other arthropod-borne diseases. *Ecol Evol* 2018; 8:1352–1368.  
703

704 van Tol and Dimopoulos G. Influences of the mosquito microbiota on vector competence.  
705 *Adv Insect Physiol* 2016; 51:243–291.  
706

707 Wang Q, Garrity GM, Tiedje JM et al. Naive Bayesian classifier for rapid assignment of  
708 rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; 73: 5261-  
709 5267.  
710

711 Wang X, Liu T, Wu Y et al. Bacterial microbiota assemblage in *Aedes albopictus* mosquitoes  
712 and its impacts on larval development. *Mol Ecol* 2018; 27:2972-2985.  
713

714 Wang Y, Hoon Eum J, Harrison RE et al. Riboflavin instability is a key factor underlying the  
715 requirement of a gut microbiota for mosquito development. *Proc Natl Acad Sci* 2021;  
716 118(15):e2101080118.  
717

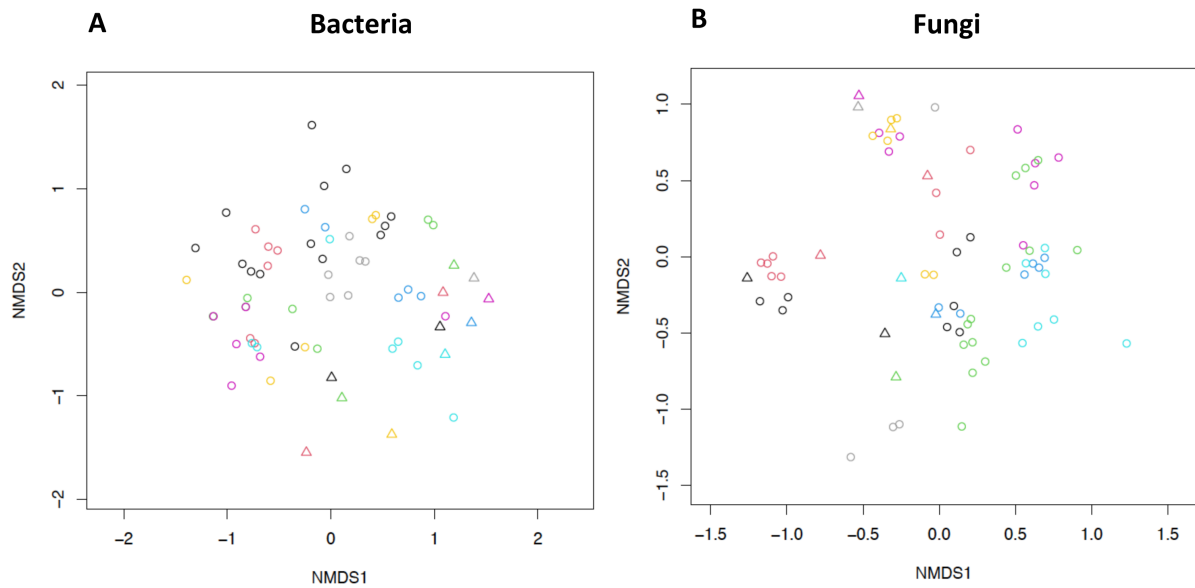
718 Wu P, Sun P, Nie K, et al. A gut commensal bacterium promotes mosquito permissiveness to  
719 arboviruses. *Cell Host Microbe* 2019; 25(1):101-112.e5.  
720

721 Wickham H, Francois R, Henry L et al. Dplyr: A grammar of data manipulation. URL  
722 <https://CRAN.Rproject.org/package=dplyr>.  
723

724 Xia S, Dweck HKM, Lutomiah J et al. Larval sites of the mosquito *Aedes aegypti formosus* in  
725 forest and domestic habitats in Africa and the potential association with oviposition evolution.  
726 *Ecol Evol* 2021; 11:16327-16343.  
727

728 Yilmaz P, Parfrey LW, Yarza P et al. The SILVA and “All-species Living Tree Project  
729 (LTP)” taxonomic frameworks. *Nucleic Acids Res* 2014; 42: D643–D648.  
730

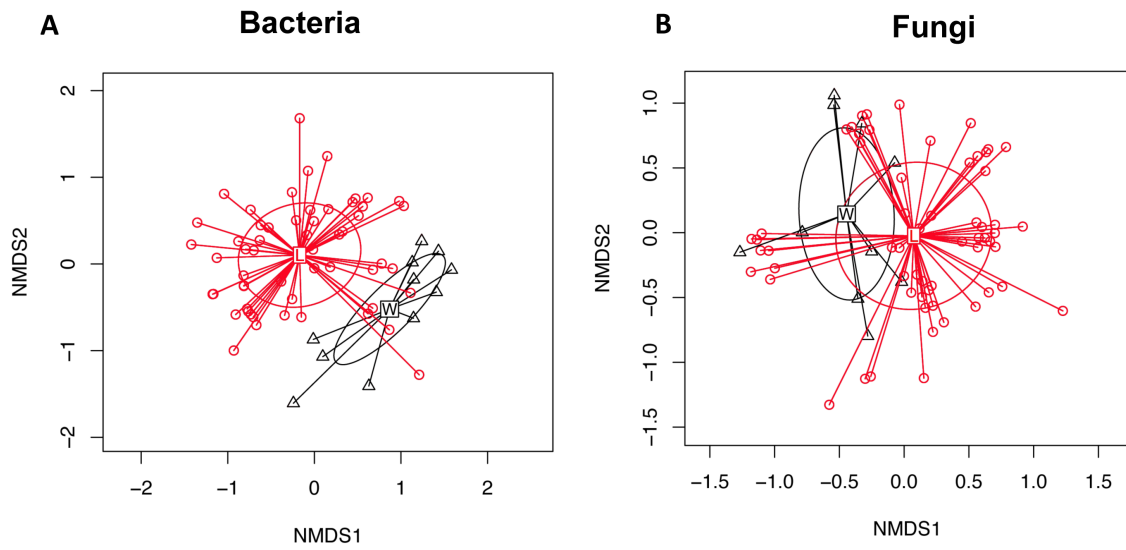
731 **Figures**



732

733 **Figure 1.** Non-metric multidimensional scaling (NMDS) ordination displaying bacterial (A)  
734 and fungal (B) Operational Taxonomic Units (OTUs) composition across larvae and water  
735 habitats originated from different breeding sites. NMDS ordination of samples was based on a  
736 Bray-Curtis dissimilarity matrix of the square root-transformed abundance data obtained from  
737 read counts. To better distinguish the larvae (indicated by a circle) collected from the same  
738 water habitat (indicated by a triangle), the shade of the corresponding colour is slightly  
739 different from a breeding site to another.

740



741

742 **Figure 2.** Non-metric multidimensional scaling (NMDS) ordination displaying bacterial (A)

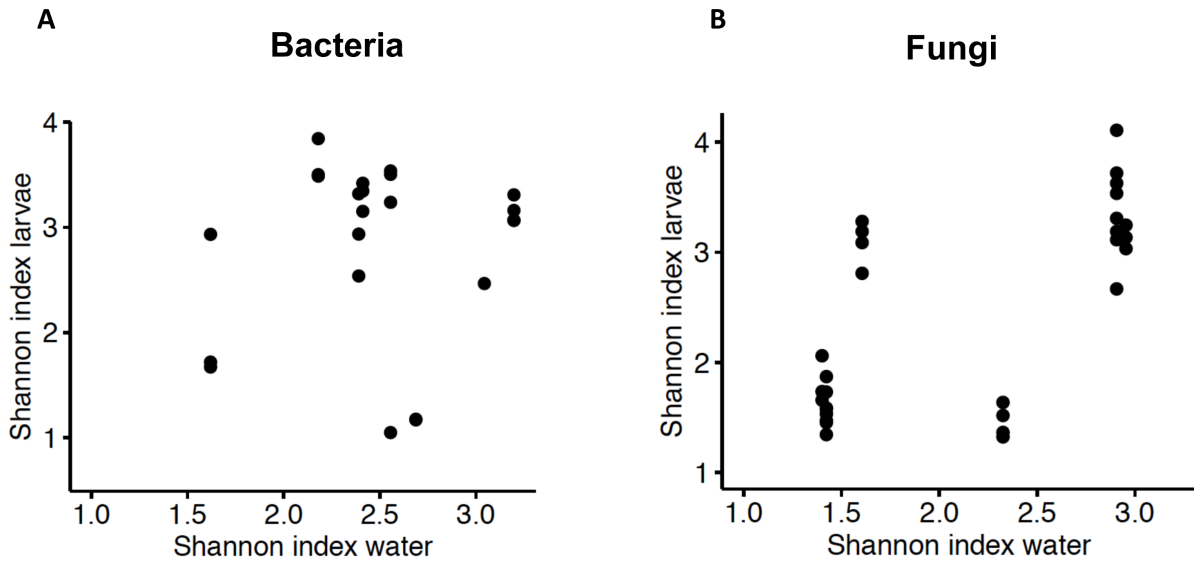
743 and fungal (B) Operational Taxonomic Units (OTUs) composition between larvae (L) and

744 aquatic habitats (W) indicated in red and black colour, respectively. The ovals refer to the

745 standard error of the centroid and the vectors refer to the distance of each sample from the

746 centroid of its group.

747

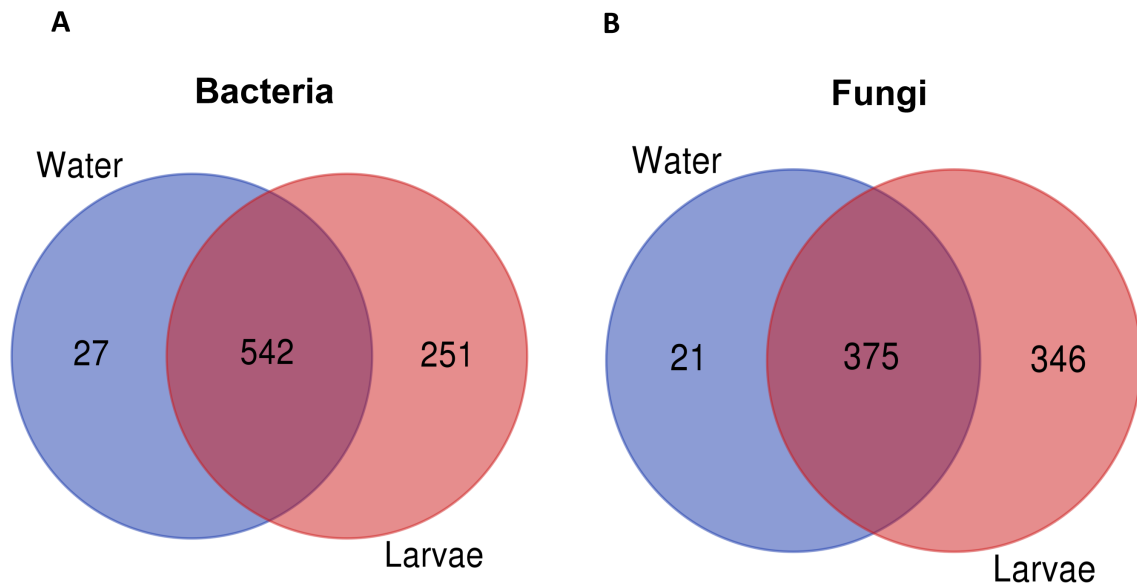


748

749 **Figure 3.** Correlation between Shannon indices of water and larval samples. The Shannon  
 750 indices for water and larval samples are positively correlated for fungi ( $\rho = 0.74$ ,  $p = 3.379 \times$   
 751  $10^{-7}$ ) (B) nor for bacteria ( $\rho = -0.22$ ,  $p = 0.18$ ) (A).

752





753

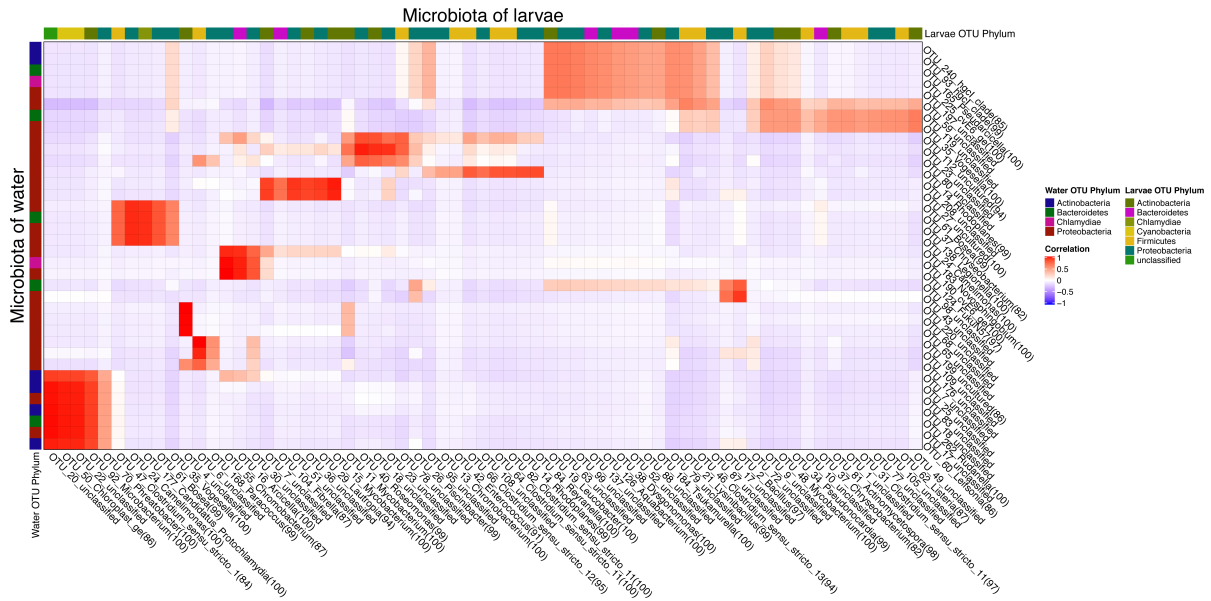
754 **Figure 4.** Venn diagrams showing the numbers of shared or unique bacterial (A) and fungal

755 (B) OTUs among larvae and water of breeding sites. A total of 542 and 375 bacterial and

756 fungal OTUs respectively (based on the sequencing of the V5-V6 and ITS-2 regions) were

757 detected among the two samples.

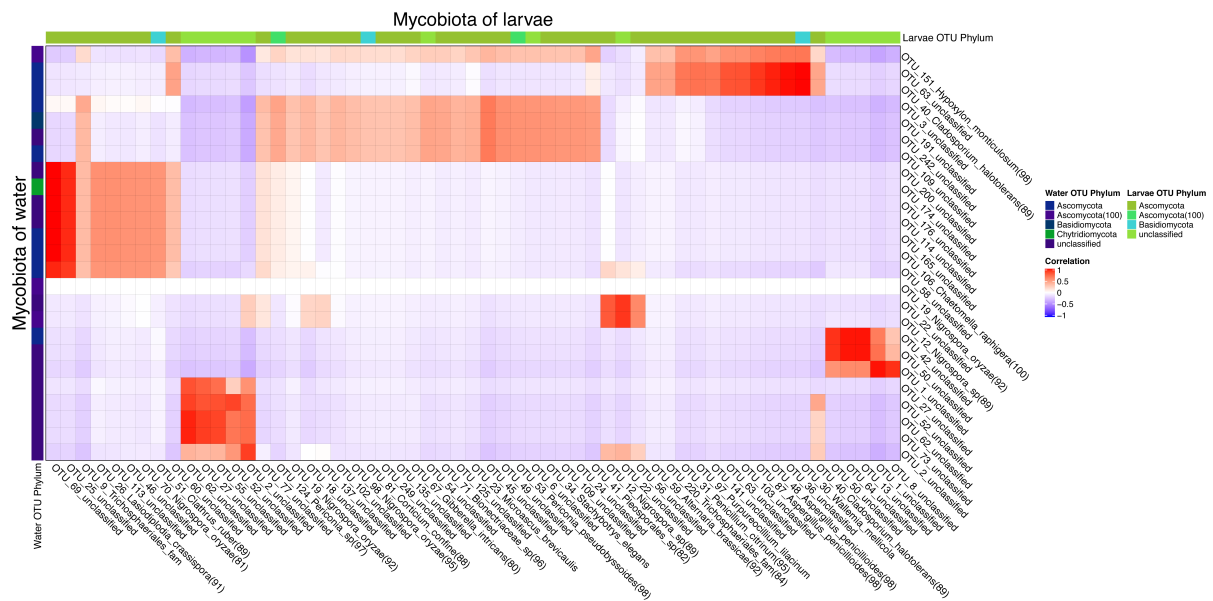
758



759

760 **Figure 5.** Correlation analysis between bacterial communities associated with the breeding  
 761 site and larval bacterial microbiota. OTU pairs for which the residual correlation was  
 762 estimated to be positive (respectively, negative) with 95% credibility level are shown in red  
 763 (respectively, green) colour.

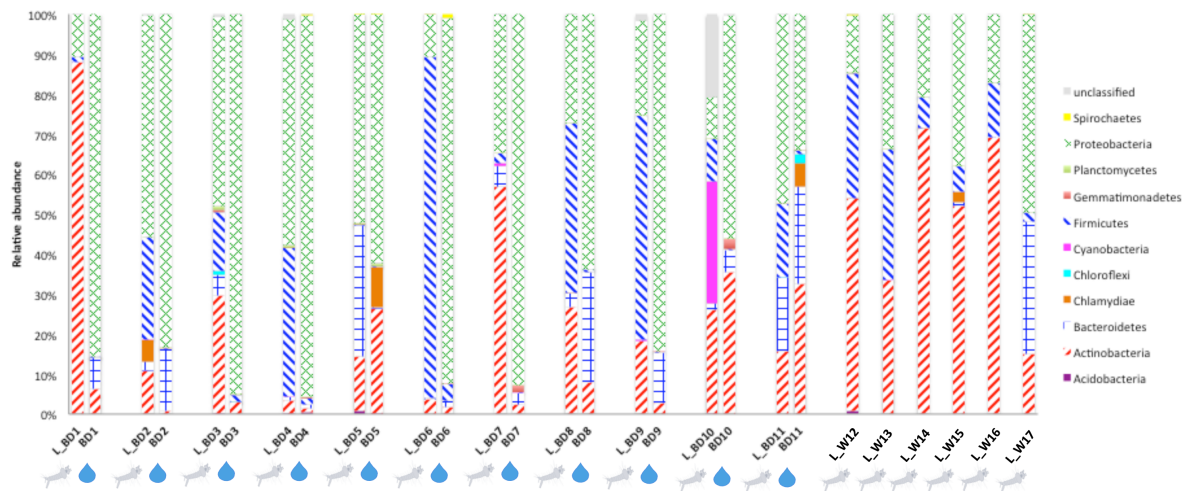
764



765

766 **Figure 6.** Correlation analysis between fungal communities associated with the breeding site  
 767 and larval mycobiota. OTU pairs for which the residual correlation was estimated to be  
 768 positive (respectively, negative) with 95% credibility level are shown in red (respectively,  
 769 green) colour. OTU\_19\_Nigrospora\_oryzae (92) is indicated in white colour as it is correlated  
 770 to no other OTU and it is present in water in relatively large abundance but only in a single  
 771 sample.

772



773

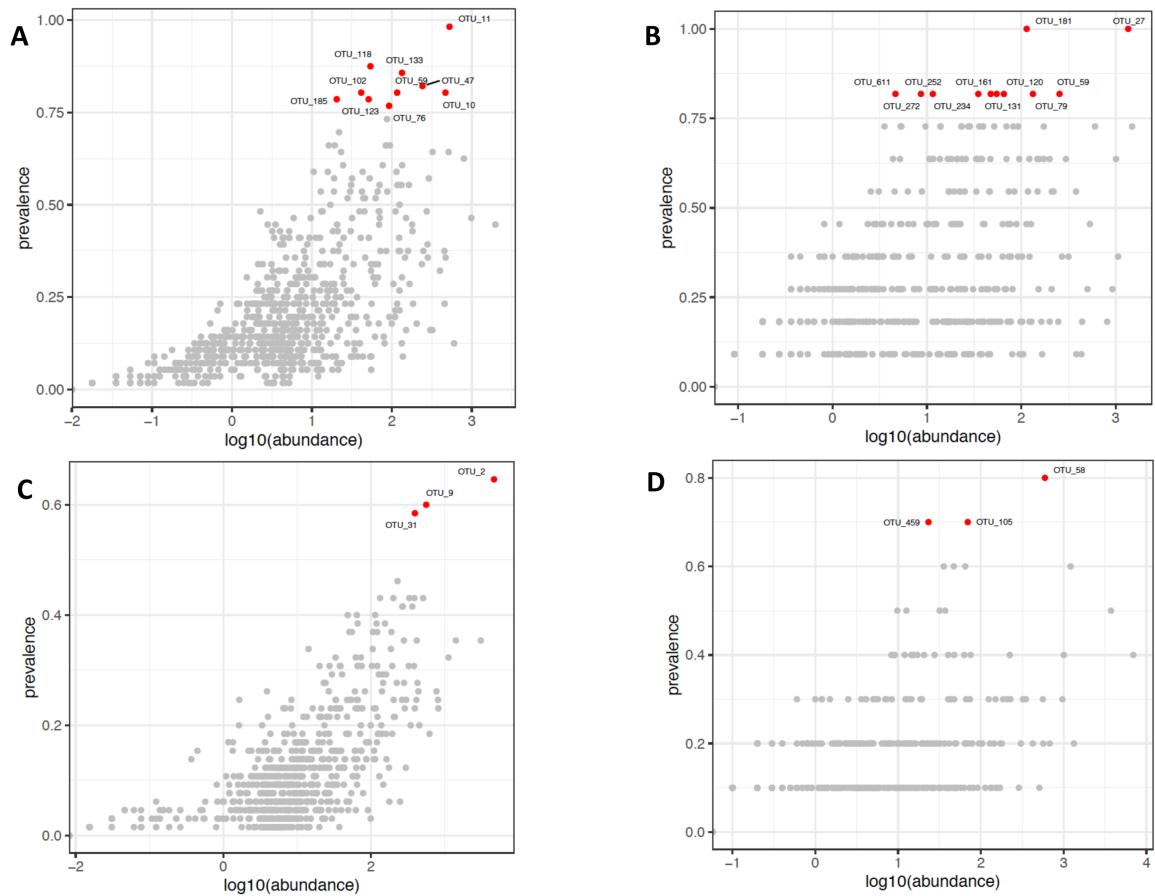
774 **Figure 7.** Relative abundance of the bacterial taxa at the phylum level. *Aedes aegypti* larvae

775 collected within the same breeding site are indicated by L\_BD and next to the water sample

776 they come from indicated by BD. L\_W indicates larvae belonging to the same breeding site

777 but for which the water sample from which they originated is absent.

778



779

780 **Figure 8.** Prevalence and relative abundance of operational taxonomic units in the different

781 modalities. (A) bacteria in larvae, (B) bacteria in water, (C) fungi in larvae and (D) fungi in

782 water. OTUs with the highest prevalence and abundance across the samples are highlighted in

783 red and labeled with their OTU ID (Panel A: Mycobacterium (OTU\_11), Microbacterium

784 (OTU\_118), Unclassified Microbacteriaceae (OTU\_133), Actinomycetospora (OTU\_102),

785 Uncultured Burkholderiaceae (OTU\_59), Clostridium sensu stricto (OTU\_47),

786 Curtobacterium (OTU\_185), Actinomycetospora (OTU\_123), Williamsia (OTU\_76),

787 Uncultured Peptostreptococcaceae (OTU\_10). Panel B: Uncultured Rhizobiales (OTU\_181),

788 Uncultured Rhizobiales incertae sedis (OTU\_27), Uncultured Beijerinckiaceae (OTU\_611),

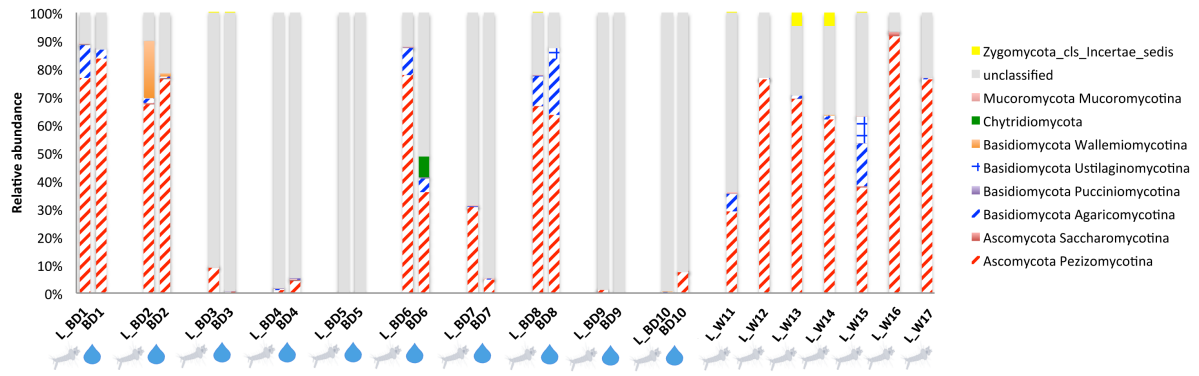
789 Methylobacterium (OTU\_252), Fodinicola (OTU\_161), Uncultured Rhizobiales (OTU\_120),

790 Uncultured Burkholderiaceae (OTU\_59), Uncultured Rhizobiales incertae sedis (OTU\_272),

791 Pelomonas (OTU\_234), Methylobacterium (OTU\_131), Uncultured Rhizobiaceae (OTU\_79).

792 Panel C: Unclassified OTU (OTU\_2), Trichosphaeriales incertae sedis (OTU\_9), Penicillium

793 citrinum (OTU\_31). Panel D: Uncultured Pleosporales (OTU\_58), Toxicocladosporium  
794 irritans (OTU\_459), uncultured Aureobasidium (OTU\_105). Prevalence is defined as the  
795 overall infection rate of a given OTU overall samples. It was calculated for water and larvae  
796 samples as follows: number of samples in which the OTU was detected / total number of  
797 samples. Abundance refers to proportion occupied by an OTU within a sample among either  
798 the bacterial or fungal community. It was calculated as follows: number of normalized reads  
799 assigned to the OTU in a given samples / total number of normalized reads.  
800



801

802 **Figure 9.** Relative abundance of the fungal taxa at the phylum and subphylum levels. *Aedes*

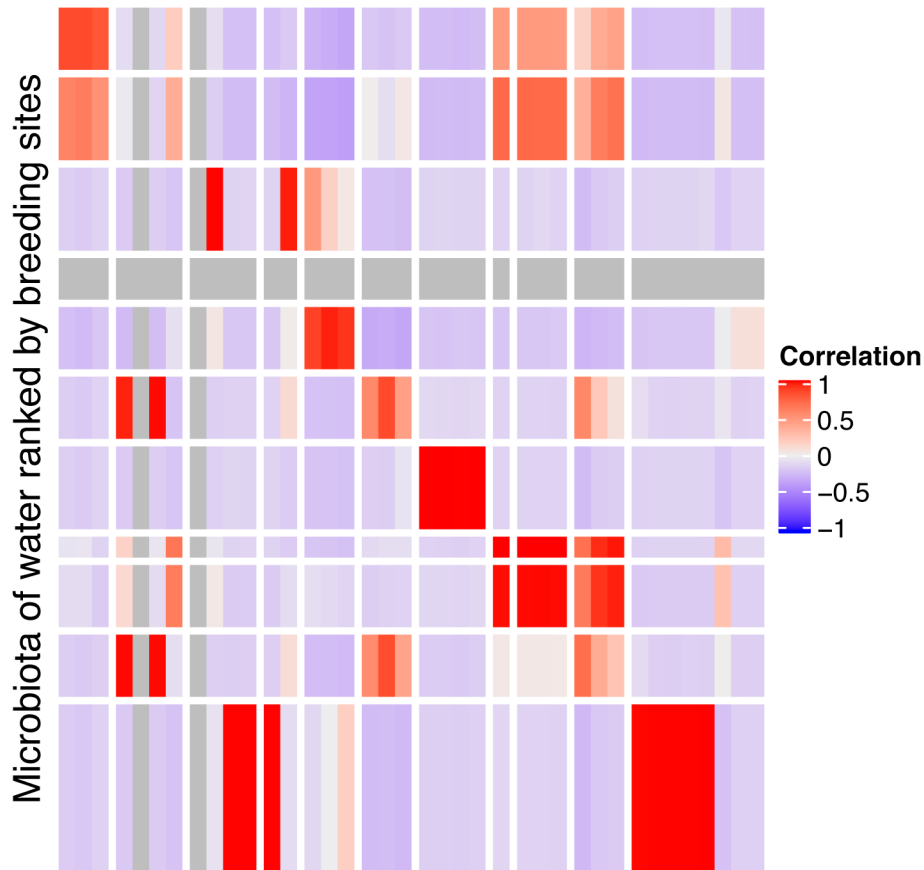
803 *aegypti* larvae collected within the same breeding site are indicated by L\_BD and next to the

804 water sample they come from indicated by BD. L\_W indicates larvae belonging to the same

805 breeding site but for which the water sample from which they originated is absent.

806

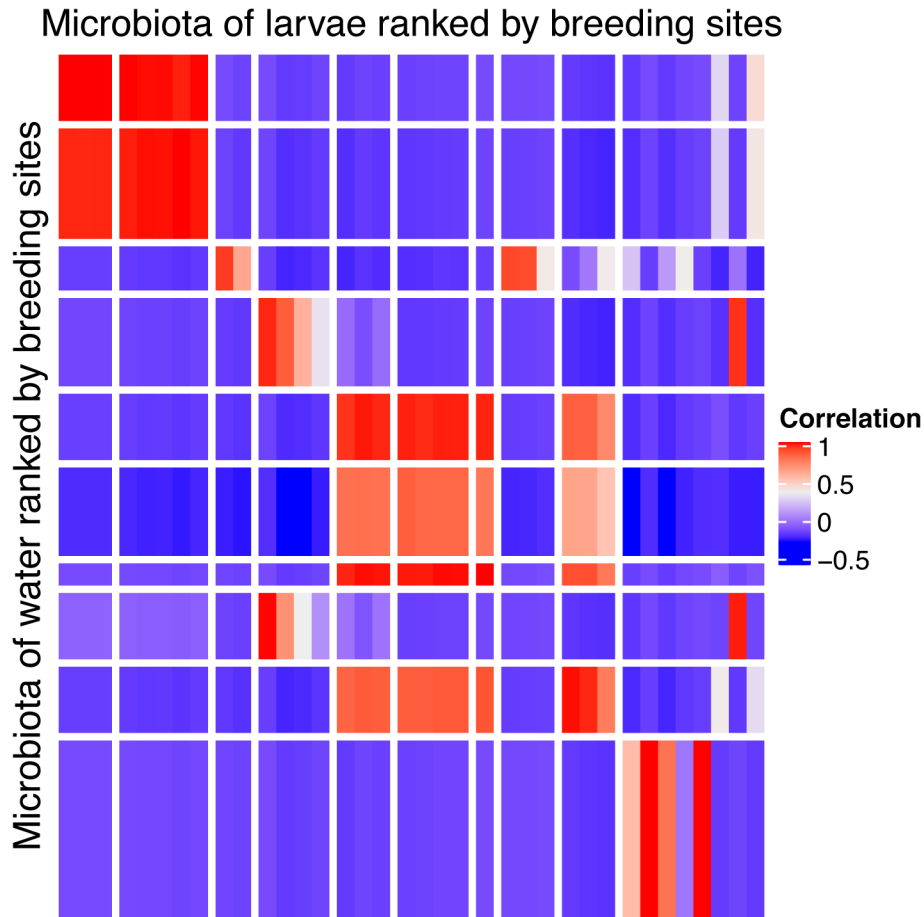
Microbiota of larvae ranked by breeding sites



808 **Figure S1.** Correlation analysis between bacterial communities associated with the aquatic  
809 habitat and larval bacterial microbiota, ranked by breeding sites. A white line separates each  
810 breeding site. Individuals for which the residual correlation was estimated to be positive  
811 (respectively, negative) with 95% credibility level are shown in red (respectively, blue)  
812 colour.

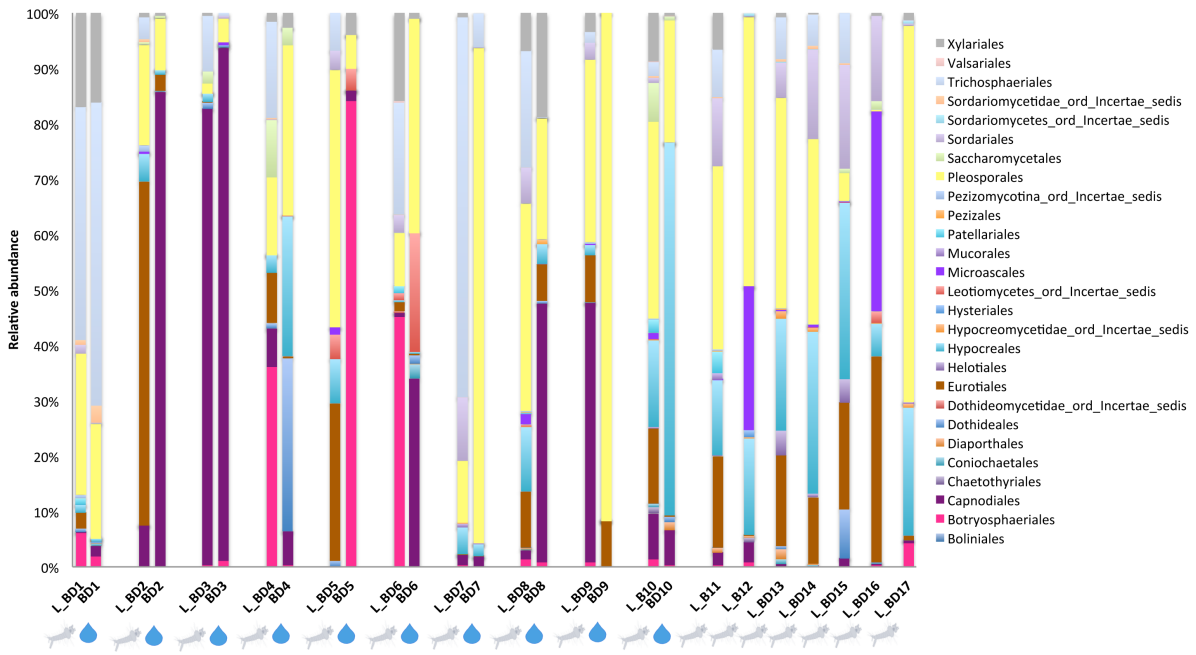
813





815 **Figure S2.** Correlation analysis between fungal communities associated with the aquatic  
 816 habitat and larval mycobiota, ranked by breeding sites. Each breeding site is separated by a  
 817 white line. Individuals for which the residual correlation was estimated to be positive  
 818 (respectively, negative) with 95% credibility level are shown in red (respectively, blue)  
 819 colour.  
 820

## ASCOMYCOTA



821

822 **Figure S3.** Relative abundance of the Ascomycota at the order level. *Aedes aegypti* larvae

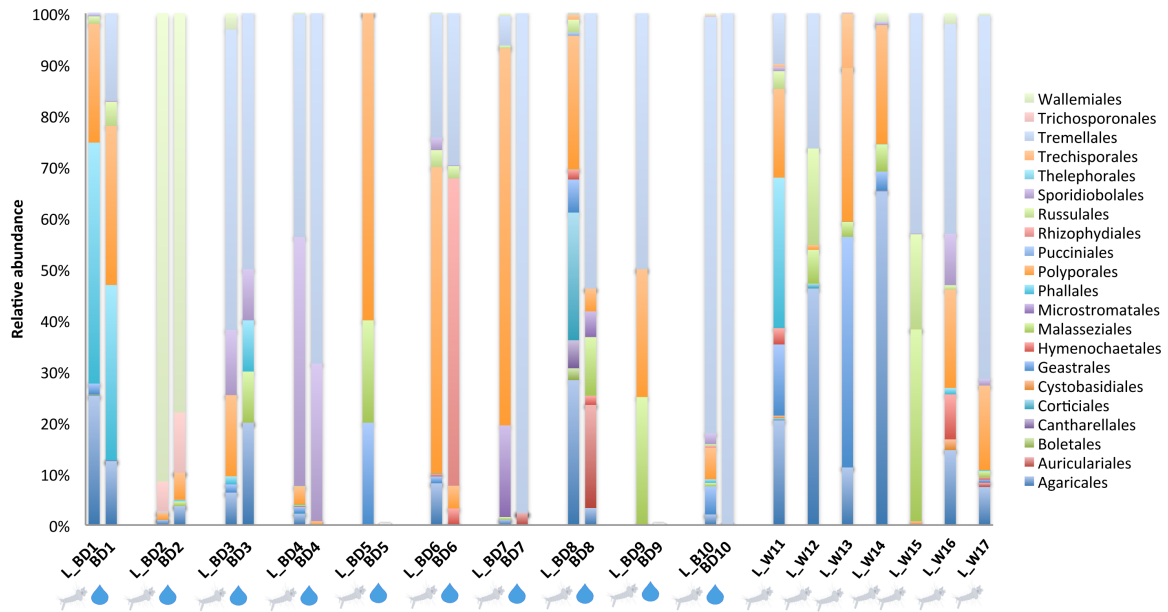
823 collected within the same breeding site are indicated by L\_BD and next to the water sample

824 they come from indicated by BD. L\_W indicates larvae belonging to the same breeding site

825 but for which the water sample from which they originated is absent.

826

## BASIDIOMYCOTA



827

828 **Figure S4.** Relative abundance of the Basidiomycota at the order level. *Aedes aegypti* larvae  
 829 collected within the same breeding site are indicated by L\_BD and next to the water sample  
 830 they come from indicated by BD. L\_W indicates larvae belonging to the same breeding site  
 831 but for which the water sample from which they originated is absent.

832 **Table S1.** List of samples analysed with related information. Third- and fourth-instar larvae  
833 as well as water were sampled in artificial breeding sites such as small flasks or containers.  
834 Samples were collected with a sterile plastic pipette into a sterile 50-ml falcon tube with  
835 filter-top lid. Water samples were agitated before sampling to mix detritus and the upper  
836 surface to a depth of 10cm was collected representing  $\approx$  50 ml per breeding site. In grey are  
837 indicated samples that were successfully sequenced and in white those for which sequencing  
838 failed.

839

840 **Table S2.** Number of bacterial OTUs and Shannon diversity index calculated for each sample  
841 (one sample = one larva or one water sample).

842

843 **Table S3.** Number of fungal OTUs and Shannon diversity index calculated for each sample  
844 (one sample = one larva or one water sample).

**Table S1. List of samples analysed with related information.** Third- and fourth-instar larvae as well as water were sampled in artificial breeding sites such as small flasks or containers. Samples were collected with a sterile plastic pipette into a sterile 50-ml falcon tube with filter-top lid. Water samples were agitated before sampling to mix detritus and the upper surface to a depth of 10cm was collected representing  $\approx$  50 ml per breeding site. In grey are indicated samples that were successfully sequenced and in white those for which sequencing failed.

Breeding dite (BD)		Date of sampling	Larvae		Water sequencing		Larva sequencing	
Type	Sample name		Number collected / BD	Sample name	16S	ITS	16S	ITS
Plastic flask	BD1	02/12/17	2	L1-BD1 L2-BD1				
Plastic flask	BD2	02/12/17	3	L1-BD2 L2-BD2 L3-BD2 L4-BD2				
Plastic bucket	BD3	03/12/17	3	L1-BD3 L2-BD3 L3-BD3				
Metal container	BD4	03/12/17	4	L1-BD4 L2-BD4 L3-BD4 L4-BD4				
Plastic flask	BD5	04/12/17	1	L1-BD5				
Stainless steel container	BD6	20/04/18	3	L1-BD6 L2-BD6 L3-BD6				
Plastic bucket	BD7	25/04/18	3	L1-BD7 L2-BD7 L3-BD7				

Plastic flask	BD8	29/04/18	8	L1-BD8 L2-BD8 L3-BD8 L4-BD8 L5-BD8 L6-BD8 L7-BD8 L8-BD8	
Plastic flask	BD9	25/04/18	3	L1-BD9 L2-BD9 L3-BD9	
Plastic flask	BD10	29/04/18	4	L1-BD10 L2-BD10 L3-BD10 L4-BD10 L5-BD10	
Plastic flask	BD11	02/05/18	5	L1-BD11 L2-BD11 L3-BD11 L4-BD11 L5-BD11	
Plastic flask	BD12	25/04/18	3	L1-BD12 L2-BD12 L3-BD12	
Metal container	BD13	28/04/18	2	L1-BD13 L2-BD13 L3-BD13 L4-BD13	
Metal container	BD14	03/05/18	3	L1-BD14	

				L2-BD14	
				L3-BD14	
Plastic cup	BD15	02/05/18	5	L1-BD15	
				L2-BD15	
				L3-BD15	
				L4-BD15	
				L5-BD15	
Plastic flask	BD16	21/04/18	2	L1-BD16	
				L2-BD16	
Plastic flask	BD17	01/05/18	2	L1-BD17	
				L2-BD17	

**Table S2. Number of bacterial OTUs and Shannon diversity index calculated for each sample (one sample = one larva or one water sample).**

<b>Sample type</b>	<b>Sample name</b>	<b>No. Of OTUs</b>	<b>Mean number of OTUs per larva</b>	<b>Shannon index</b>
Larva	L1-BD1	147	160±18	1.17
Larva	L2-BD1	173		1.18
Water	BD1	138		2.69
Larva	L1-BD2	146	169±21	2.54
Larva	L2-BD2	174		2.93
Larva	L3-BD2	187		3.32
Water	BD2	213		2.39
Larva	L1-BD3	273	275±18	3.50
Larva	L2-BD3	258		3.49
Larva	L3-BD3	293		3.84
Water	BD3	200		2.18
Larva	L1-BD4	252	218±64	3.50
Larva	L2-BD4	257		3.53
Larva	L3-BD4	122		1.05
Larva	L4-BD4	242		3.24
Water	BD4	266		2.56
Larva	L1-BD5	215	–	2.47
Water	BD5	205		3.04
Larva	L1-BD6	112	146±35	0.75
Larva	L2-BD6	181		1.09
Larva	L3-BD6	144		0.65
Water	BD6	160		3.22
Larva	L1-BD12	172	162±22	1.45
Larva	L2-BD12	178		2.68
Larva	L3-BD12	137		1.75
Larva	L1-BD7	192	240±43	3.42



Larva	L2-BD7	272		3.34
Larva	L3-BD7	207		3.15
<b>Water</b>	<b>BD7</b>	190		2.41
Larva	L1-BD9	278		2.93
Larva	L2-BD9	229	215±71	1.67
Larva	L3-BD9	138		1.72
<b>Water</b>	<b>BD9</b>	167		1.62
Larva	L1-BD10	174		3.07
Larva	L2-BD10	219	190±24	3.16
Larva	L3-BD10	168		3.06
Larva	L4-BD10	199		3.31
<b>Water</b>	<b>BD10</b>	223		3.20
Larva	L3-BD12	291	—	
Larva	L1-BD13	270	273±4	3.35
Larva	L2-BD13	276		2.83
Larva	L1-BD14	275		2.98
Larva	L2-BD14	280	281±7	1.17
Larva	L3-BD14	289		1.20
Larva	L1-BD15	263		1.62
Larva	L2-BD15	208		1.12
Larva	L3-BD15	296	226±85	0.92
Larva	L4-BD15	276		2.54
Larva	L5-BD15	86		2.94
Larva	L6-BD8	353		2.88
Larva	L7-BD8	272		2.63
Larva	L8-BD8	338	240±72	2.85
Larva	L1-BD8	159		1.36
Larva	L2-BD8	206		2.74
Larva	L3-BD8	199		3.16

Larva	L4-BD8	195		3.11
Larva	L5-BD8	197		3.05
<b>Water</b>	<b>BD8</b>	198		<b>3.43</b>
Larva	L1-BD16	294		3.55
Larva	L2-BD16	291	293±2	2.95
Larva	L1-BD11	189		2.49
Larva	L2-BD11	204		3.10
Larva	L3-BD11	230	239±46	3.25
Larva	L4-BD11	296		3.56
Larva	L5-BD11	276		2.96
<b>Water</b>	<b>BD11</b>	263		<b>3.26</b>
Larva	L1-BD17	284		2.48
Larva	L2-BD17	379	332±67	3.51

**Table S3. Number of fungal OTUs and Shannon diversity index calculated for each sample (one sample = one larva or one water sample).**

<b>Sample type</b>	<b>Sample name</b>	<b>No. Of OTUs</b>	<b>Mean number of OTUs per larva</b>	<b>Shannon index</b>
Larva	L1-BD1	312	268±62	4.07
Larva	L2-BD1	224		3.91
<b>Water</b>	<b>BD1</b>	202		3.26
larva	L1-BD2	56	91±24	2.81
Larva	L2-BD2	100		3.28
larva	L4-BD2	97		3.19
Larva	L3-BD2	109		3.09
<b>Water</b>	<b>BD2</b>	99		1.60
larva	L1-BD3	105	83±20	1.87
Larva	L2-BD3	77		1.53
Larva	L3-BD3	66		1.59
<b>Water</b>	<b>BD3</b>	47		1.42
Larva	L1-BD4	84	78±7	1.64
Larva	L2-BD4	79		1.52
Larva	L3-BD4	69		1.36
Larva	L4-BD4	81		1.32
<b>Water</b>	<b>BD4</b>	93		2.33
Larva	L1-BD5	44	–	0.15
<b>Water</b>	<b>BD5</b>	38		0.04
Larva	L1-BD6	141	141±24	3.03
Larva	L2-BD6	186		3.13
Larva	L3-BD6	151		3.25
<b>Water</b>	<b>BD6</b>	82		2.95
Larva	L1-BD12	67	58±13	1.88
Larva	L2-BD12	43		1.74
Larva	L3-BD12	65		2.08

larva	L1-BD7	54		1.74
Larva	L2-BD7	99	76±23	2.07
Larva	L3-BD7	75		1.66
<b>Water</b>	<b>BD7</b>	<b>81</b>		<b>1.40</b>
Larva	L1-BD9	70		1.01
Larva	L2-BD9	59	62±7	1.02
Larva	L3-BD9	56		1.15
<b>Water</b>	<b>BD9</b>	<b>39</b>		<b>0.23</b>
Larva	L1-BD10	72		1.34
Larva	L2-BD10	77		1.58
Larva	L3-BD10	81	76±6	1.47
Larva	L5-BD10	68		1.73
Larva	L4-BD10	81		1.45
<b>Water</b>	<b>BD10</b>	<b>85</b>		<b>1.42</b>
Larva	L3-BD13	97		2.98
Larva	L4-BD13	104	107±9	3.29
Larva	L1-BD13	111		3.34
Larva	L2-BD13	117		3.16
Larva	L1-BD14	106		3.20
Larva	L2-BD14	115	110±5	3.19
Larva	L3-BD14	110		3.37
Larva	L1-BD15	62		2.09
Larva	L2-BD15	71		2.19
Larva	L3-BD15	62	72±18	2.26
Larva	L4-BD15	60		2.36
Larva	L5-BD15	103		0.95
Larva	L6-BD8	137		3.31
Larva	L7-BD8	85	125±34	2.67
Larva	L8-BD8	146		3.11

Larva	L1-BD8	131		3.53
Larva	L2-BD8	76		3.19
Larva	L3-BD8	179		4.11
Larva	L4-BD8	138		3.72
Larva	L5-BD8	105		3.63
<b>Water</b>	<b>BD8</b>	72		2.90
Larva	L1-BD16	135	138±4	2.51
Larva	L2-BD16	140		2.35
Larva	L1-BD11	75		2.10
Larva	L2-BD11	72		2.11
Larva	L3-BD11	84	124±66	1.47
Larva	L4-BD11	211		4.18
Larva	L5-BD11	180		4.14
Larva	L1-BD17	105	139±48	3.30
Larva	L2-BD17	173		3.24