Title: Bacteria contribute to mosquito development via folate biosynthesis and energy storage

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Abstract

The mosquito microbiota impacts the physiology of its host and is essential for normal larval development, thereby influencing transmission of vector-borne pathogens. Germ-free mosquitoes generated with current methods show larval stunting and developmental deficits. Therefore, functional studies of the mosquito microbiota have so far mostly been limited to antibiotic treatments of emerging adults. In this study, we developed a novel approach to produce germ-free *Aedes aegypti* mosquitoes. It is based on reversible colonisation with bacteria genetically modified to allow complete decolonisation at the end of larval development. We show that, unlike germ-free mosquitoes previously produced using sterile diets, reversibly colonised mosquitoes show no developmental retardation and reach the same size as control adults. This uniquely allowed us to study the role of bacteria during larval development by inducing decolonisation at the beginning of the third larval instar. Based on a transcriptome analysis and diet supplementation experiments, we propose that bacteria support larval development by contributing to folate biosynthesis and by enhancing energy storage. Overall, this study provides a new tool to study the microbiota in insects and deepens our knowledge on the metabolic contribution of bacteria to mosquito development.

31 # Introduction

32

33 Vector-borne diseases are estimated to account for 17 % of all cases of infectious diseases
34 worldwide and cause over 700 000 deaths per year [1]. The mosquito *Aedes aegypti* is the main
35 vector for several arboviruses, and it is widely distributed across tropical and neotropical
36 regions. Due to the spread of insecticide resistance, the factors shaping mosquito fitness and
37 vector competence (*i.e.* its permissiveness to virus development) are under study in order to
38 identify alternative strategies for transmission blockade. Among these, the microbial
39 community colonising the mosquito is known to directly impact vector competence and other
40 life-history traits affecting transmission success, including mosquito development, lifespan,
41 mating choice, fecundity and fertility [2–7]. In particular, live bacteria are essential for normal
42 larval development, hindering possibilities for the production of germ-free controls for
43 microbiota studies. Therefore, most studies investigating the mosquito microbiota were based
44 on conventional rearing and use of antibiotics at adult emergence, which may have off-target
45 effects (notably on mitochondria) and does not fully sterilize mosquitoes. In the past, several
46 studies proposed rearing protocols of germ-free larvae, which could not be reproduced in recent
47 experiments [8–10]. Lately, mosquito larvae have been shown to develop in the absence of bacteria
48 when provided an enriched diet embedded in agar plugs rather than food in suspension, but
49 development time is tripled and resultant germ-free mosquitoes are smaller than their
50 conventional controls [10].
51 The exact contribution of the microbiota to larval development is still a conundrum. It has been
52 proposed that the bacteria growing in the mosquito gut consume oxygen and that the resulting
53 hypoxia is a developmental signal for moulting from one stage to the next [11]. However,
54 successful larval development in fully germ-free conditions challenged this model and
55 metabolic contribution of bacteria to larval development was alternatively hypothesised [10].

56 We developed a transient bacterial colonisation method to support larval development while
57 producing fully developed germ-free adults. Using this approach, we were able to support
58 development within a standard timeline and without affecting adult size, showing that these
59 germ-free individuals represent a valid tool for mosquito microbiota studies. We further used
60 transient colonisation during early larval development to characterise the role of bacteria in
61 larval development using a transcriptomic approach and diet complementation. Our data
62 indicate that bacteria contribute to folate biosynthesis and energy storage and that folic acid
63 supports larval development in the absence of a microbiota.

64

65 # Results

66

67 *Production of germ-free adult mosquitoes via transient bacterial colonisation*

68 After microbiological sterilisation of mosquito eggs, hatchlings provided with autoclaved
69 standard rearing diet are able to survive for weeks but remain first-instar larvae. Their
70 development can be rescued by bacterial colonisation, for instance with *Escherichia coli* [4,10].
71 Therefore, we hypothesised that transient bacterial colonisation would be an efficient way to
72 support larval development and produce germ-free adults. To test this, we used HA416, an *E.*
73 *coli* HS-derived strain that is auxotrophic for two non-standard amino-acids required for
74 peptidoglycan synthesis (*meso*-diaminopimelic acid, *m*-DAP, and D-alanine, D-Ala) and that
75 was previously used to transiently colonise the gut of germ-free mice [12]. We supplemented the
76 food of gnotobiotic larvae with *m*-DAP and D-Ala to support the proliferation of auxotrophic
77 *E. coli* during larval development only (Figure 1A). These supplements were toxic to first instar
78 larvae when provided at the concentrations previously used *in vitro*, but 4-fold lower
79 concentrations supported growth of auxotrophic *E. coli* (Supplementary Figure S1) without
80 causing toxicity in larvae. The auxotrophic *E. coli* strain rescued the development of axenic

81 larvae with the same efficiency as control wild-type *E. coli* (p = 0.29, Figure 1B). The
82 proportion of larvae reaching adulthood was even 18% higher among gnotobiotic larvae than
83 among untreated larvae, carrying their conventional microbiota ($F_{2,16}$ = 7.9, p < 0.001, Figure
84 1B).
85 To monitor colonisation efficiency, we quantified bacterial loads at different time points of
86 larval and pupal development in auxotrophic *E. coli*-carrying larvae, using wild-type *E. coli*
87 gnotobiotic larvae as controls (Figure 1C). After surface sterilisation, larval and pupal
88 homogenates were plated on LB supplemented with *m*-DAP and D-Ala. Comparable bacterial
89 amounts were found in third instar larvae harbouring each *E. coli* strain ($F_{1,8.9e6}$ = 0.14, p =
90 0.71). Bacterial counts gradually decreased in auxotrophic *E. coli* gnotobiotic hosts from the
91 fourth instar and were 1.3 and 8-fold lower than wild-type *E. coli*-carrying larvae at the first
92 and second day of the fourth instar, respectively (day 1: $F_{1,1.6e8}$ = 4.5, p = 0.033; day 2: $F_{1,3.4e8}$
93 = 44, p < 0.001). During the pupal stage, bacteria were undetected in most auxotroph-carrying
94 individuals (92.1 ± 7.7 %) while bacterial load also decreased over 100 fold in the controls, as
95 previously documented [13] ($F_{1,65}$ = 66, p < 0.001). The lower bacterial loads found in fourth-
96 instar larvae harbouring auxotrophic *E. coli* might be explained by the exhaustion of *m*-DAP
97 and D-Ala by auxotrophic bacteria and their resulting inability to grow. Since before
98 metamorphosis larvae stop to feed and empty their gut, the lower bacterial loads found in late
99 fourth instar larvae and in pupae might be further explained by the resulting intestinal
100 inaccessibility of *m*-DAP and D-Ala. No bacteria could be cultured from adults reared on
101 auxotrophic *E. coli* during the first three days following emergence, while bacteria were
102 culturable from 50 % of the wild-type *E. coli* gnotobiotic mosquitoes at the same time-points
103 (Figure 1D). The absence of culturable bacteria in some conventionally-reared mosquitoes
104 immediately after adult emergence has been previously observed in different mosquito species,
105 including *Ae. aegypti* [13]. To further quantify the loss of bacteria, we measured the bacterial

106 loads in adult mosquitoes originating from auxotroph-carrying gnotobiotic larvae on a larger
107 sample size. No culturable bacteria was isolated from 97.6 ± 2.1 % (34/34; 118/122; 311/321)
108 of 1-to-2-day old adults (Supplementary Figure S2). Bacterial DNA was still detectable via
109 qPCR in 22 % of 1-day old adult mosquitoes originating from auxotrophic *E. coli*-carrying
110 larvae as in 40 % of mosquitoes originating from wild-type *E. coli* gnotobiotic larvae (Fisher's
111 exact test, p=0.24, Supplementary Figure S3).
112 We investigated whether larvae harbouring auxotrophic *E. coli* presented any developmental
113 deficits. Firstly, we quantified the duration of each developmental stage to assess if the
114 colonising bacterial strains had an impact on development kinetics. The gnotobiotic treatments
115 slightly shortened the beginning of larval development, while total development time was
116 unaffected (Figure 1E, L1: $F_{2,550} = 18$, $p < 0.001$; L2: $F_{2,526} = 3.1$, $p = 0.044$; L3: $F_{2,503} = 6.9$,
117 $p = 0.001$; L4: $F_{2,475} = 2.9$, $p = 0.057$; P: $F_{2,467} = 1.0$, $p = 0.37$, see the figure for single
118 comparisons). Secondly, we evaluated whether auxotrophic *E. coli* supported the development
119 of both sexes. A 36% increase in the proportion of females was observed in wild-type *E. coli*-
120 carrying mosquitoes compared to conventionally reared individuals, but differences in sex ratio
121 between all three conditions were not statistically significant (Supplementary Figure S4, $F_2 =$
122 4.2, $p = 0.50$). Finally, we compared the larval body length and the wing length of
123 conventionally reared and gnotobiotic individuals to assess whether the different microbiota
124 compositions impacted the mosquito size, which is a readout of the insect nutritional and
125 metabolic status. At the fifth day after egg hatching, gnotobiotic fourth-instar larvae for both
126 *E. coli* strains reached the same length as conventionally-reared larvae (Figure 1F, $F_{2,481} = 2.5$,
127 $p = 0.083$) and wing size was comparable among the three rearing conditions in both males and
128 females (Figure 1G, males: $F_{2,199} = 2.0$, $p = 0.14$; females: $F_{2,176} = 1.7$, $p = 0.19$).
129
130 *Microbiota-dependent gene regulation during late larval development*

131 We tested whether reversible colonisation could also be arrested during larval development by
132 placing the auxotroph-harbouring larvae in a rearing medium lacking *m*-DAP and D-Ala
133 (Figure 2A). For that purpose, we rinsed third-instar larvae and placed them in sterile water
134 containing un-supplemented sterile fish food and monitored the bacterial loads in single larvae.
135 As soon as 2h after *m*-DAP and D-Ala depletion, we observed a 1000-fold reduction in
136 bacterial load with respect to non-transferred larvae, which became a 10^4-fold reduction after
137 5 h. Larvae were germ-free 12 h after amino-acid depletion (Supplementary Figure S5). We
138 processed wild-type *E. coli*-carrying larvae in the same way to test whether this reduction in
139 the microbiota load was specific to the bacterial auxotrophy or if it was only due to the transfer
140 of larvae to a new sterile rearing environment and the active expulsion of bacteria from the
141 larval gut. Two hours after the transfer, we observed a 500-fold reduced bacterial load,
142 suggesting that the change of medium has a substantial effect on the microbiota load ($F_{1,33}$ =
143 38, $p < 0.001$). However, the bacterial load of transferred larvae started to increase between 2
144 and 5h after the transfer and after 12 h, it was less than 10-fold different than in non-transferred
145 controls (Supplementary Figure S5, 5h: $F_{1,32} = 36$, $p < 0.001$; 12 h: $F_{1,3702} = 23$, $p < 0.001$; 20
146 h: $F_{1,2344} = 14$, $p < 0.001$). This indicates that the colonisation with auxotrophic bacteria can
147 efficiently be switched off during larval development. As larvae develop at a similar speed with
148 wild-type or auxotrophic bacteria, we reasoned that this switch would allow a well-controlled
149 investigation of the bacterial contribution to larval development. We decided to address this
150 question by transcriptomic analysis, focusing on third instar larvae because their size allowed
151 precise dissections and because this developmental stage is intermediate between two larval
152 stages, and therefore more representative of larval development than fourth instar larvae which
153 moult into pupae.
154 We compared the transcriptome between third instar larvae following clearance of auxotrophic
155 *E. coli* and individuals carrying wild-type *E. coli* (referred to as germ-free and colonised,

156 respectively). We synchronised larvae by selecting individuals starting their third instar within
157 a 5h timeframe, prior to transferring auxotroph-carrying larvae for *m*-DAP and D-Ala depletion
158 (Figure 2A). We sampled guts and whole larvae from germ-free and colonised conditions 12 h
159 and 20 h later, in order to detect early changes caused by the absence of bacteria prior to
160 potential developmental delay as well as later changes caused by bacterial loss throughout the
161 third instar. After extraction of total RNA, we confirmed the loss of bacteria in reversibly
162 colonised mosquitoes by qPCR (Supplementary Figure S6, gut 12 h: $F_{1,2} = 39$, $p = 0.023$; gut
163 20 h: $F_{1,3} = 81$, $p = 0.001$; whole larvae 12 h: $F_{1,4} = 45$, $p = 0.004$, whole larvae 20 h: $F_{1,2} = 68$,
164 $p = 0.015$). cDNA libraries were produced and sequenced on an Illumina NextSeq 500. A total
165 of 2735 transcripts were significantly differentially regulated (adjusted $p < 0.001$) at least in
166 one sample type at one time point. The sample distance heatmap showed that for each tissue
167 type and time-point, the three experimental replicates clustered together (Supplementary
168 Figure S7). The principal component analysis (PCA) on gut samples identified a first
169 component explaining 45 % of the variance and discriminating time-points and a second
170 component representing 26 % of the variance and separating samples by the presence/absence
171 of bacteria (Figure 2B). PCA on whole larvae data revealed a more important separation of
172 samples by time-point (77 % of the variance) and only 13 % of the variance explained by the
173 microbiota composition. The 12 h time point was early enough to detect early transcriptional
174 modifications in the gut before any body-scale transcriptional change, which was detectable 20
175 h post-amino-acid depletion (Figure 2B).
176 Among the differentially regulated genes, we selected the transcripts with a $\log_2$ fold value < -
177 1.5 or > 1.5 (*i.e.* > 4.5-fold down/up-regulation), obtaining a total of 573 up-regulated and 293
178 down-regulated genes in germ-free larvae compared to colonised larvae (Supplementary Table
179 S1). The Gene Ontology enrichment analysis of transcripts upregulated in germ-free larvae
180 included phosphatase/alkaline phosphatase/phosphoric ester hydrolase activity,

181 oxidoreductase activity, heme binding, iron ion binding, monooxygenase activity, cofactor
182 binding, and catalytic activity (Figure 2C and Supplementary Table S2). Five alkaline
183 phosphatases were found amongst the most highly upregulated genes (Figure 2D and
184 Supplementary Table S3). The Kyoto encyclopaedia of gene and genomes (KEGG) enrichment
185 analysis revealed that folate (Vitamin B9) biosynthesis and thiamine (Vitamin B1) metabolism
186 were upregulated in germ-free larvae in both gut and whole larva samples (Figure 2E). As
187 many genes of these pathways are missing in the genome of *Ae. aegypti*, this suggests that, as
188 reported in other insects, the mosquito microbiota participates in vitamin B biosynthesis.
189 Focusing on the genes downregulated in germ-free larvae, we detected 6 *hexamerin 2 beta*
190 genes as significantly downregulated, including 4 among the 10 most downregulated genes
191 (Figure 2D and Supplementary Table S4). Hexamerins are proteins reported to participate in
192 amino-acid storage during the end of larval development and metamorphosis. Interestingly, we
193 also identified several down-regulated genes encoding proteins involved in lipid storage and
194 transport, such as *vitellogenin*, a vitellogenin-related gene (*AAEL008598*) (Figure 2D and
195 Supplementary Table S4) and two *lipophorin* genes (*AAEL009955*, log2-fold -1.01 in larvae
196 20 h and *AAEL018219*, -0.8 in guts 20 h). Together, our transcriptomic analysis suggests that
197 the microbiota participates in vitamin B biosynthesis and in macromolecule storage during
198 larval development.
199 To determine whether hypoxia-induced signalling was affected in third-instar germ-free larvae,
200 we specifically checked the expression level of *hypoxia-induced factors (HIF)-alpha* and *-beta*
201 (*AAEL019499* and *AAEL010343*) and of *prolyl hydroxylase (PHD)-1* and *-2* genes
202 (*AAEL024908* and *AAEL028025*) in our samples, as HIF proteins were reported to be induced
203 in the presence of a microbiota in first instar larvae [14] and are targets of PHDs. Only *phd-2* was
204 slightly down-regulated at 20 h (gut: $\log_2$ fold = -1.4, *p*adj = 0.0015; whole larvae: $\log_2$ fold =
205 -0.6, *p*adj = 0.015). However, none of the *HIF* genes was differentially regulated in our

206 samples. We also measured the oxygen levels in larval guts 12 h post-transfer, which should
207 correspond to the lowest level of oxygen concentration in the presence of bacteria [11]. No
208 difference in oxygen levels was detected between colonised larvae (left in their medium or
209 transferred in new sterile medium) and germ-free larvae (Supplementary Figure S8, $F_{2,82}$ =
210 0.52, p = 0.60).
211
212 *Folic acid supports late stages of development*
213 The absence of a significant correlation between the loss of the microbiota and the hypoxia-
214 induced signalling, and the regulation of many genes involved in vitamin biosynthesis and
215 macromolecule storage after microbiota loss support the hypothesis that the microbiota
216 contributes metabolically to mosquito development. To further investigate this theory, we
217 tested whether, as already known for first-instar larvae, late larval development was affected
218 by the loss of bacteria. When third-instar larvae turned germ-free, they all moulted into the
219 fourth instar (99.4 ± 0.6 %, mean ± SEM), but only 20.2 ± 6.6 % moulted to pupa and only
220 17.9 ± 6.1 % fully developed into adults (Figure 3A, L4: $F_{2,302}$ = 0.7, p = 0.48; pupae: $F_{2,339}$ =
221 33.9, p < 0.001; adults: $F_{2,339}$ = 36.4, p < 0.001). The fourth instar of germ-free larvae was
222 twice longer (Figure 3B, $F_{2,116}$ = 54.4, p <0.001), while third instar and metamorphosis
223 durations were not affected (L3: $F_{2,325}$ = 2.3, p = 0.10; metamorphosis $F_{2,104}$ = 0.74, p = 0.48).
224 These observations suggest that bacteria are essential throughout all larval development, while
225 some individuals make it to the adult stage, hypothetically using energy stored during their
226 early development.
227 A 60% reduction in development success was also observed among wild-type *E. coli*-carrying
228 larvae that were transferred into sterile water (Figure 3A), indicating that the presence of a
229 sufficient microbiota load throughout larval development is critical. The duration of the fourth
230 instar was not significantly affected by this transient decrease in bacterial load (Figure 3B).

231 Based on our transcriptomic results, we investigated whether the addition of folic acid to the
232 rearing water of late germ-free larvae would support further larval development and
233 metamorphosis. We observed that development success to adulthood was over 4-fold higher
234 when providing folic acid upon *m*-DAP and D-Ala depletion, regardless folic acid
235 concentration, while development time was unaffected (Figure 3C, L4: $F_{3,377} = 0.8$, $p = 0.48$;
236 pupae: $F_{3,375} = 21.2$, $p < 0.001$; adults: $F_{3,375} = 25.0$, $p < 0.001$; Figure 3D, L3: $F_{3,376} = 0.67$, p
237 $= 0.57$; L4: $F_{3,162} = 1.37$, $p = 0.25$; metamorphosis: $F_{3,152} = 1.12$, $p = 0.34$).
238 Considering that folic acid partly rescued larval development, we wondered whether this
239 supplementation also had an effect on some genes detected in the RNASeq analysis as most
240 differentially regulated, focusing on amino acid and lipid storage and folate biosynthesis. Folic
241 acid supplementation for 20 h from decolonisation did not rescue the expression of *hexamerin*
242 genes (Figure 3E, WT: $t_2 = 1.28$, $p = 0.33$, WT-transf: $t_1 = -3.42$, $p = 0.18$, GF: $t_2 = 1.09$, $p =$
243 0.39; Supplementary Figure S9) or of genes implicated in lipid storage (Figure 3F, WT: $t_2 =$
244 2.05, $p = 0.18$, WT-transf: $t_1 = 0.98$, $p = 0.51$, GF: $t_2 = 1.46$, $p = 0.28$; Supplementary Figure
245 S9). The regulation of genes involved in folate biosynthesis was not directly related to folic-
246 acid supplementation (Figure 3G, WT: $t_2 = -5.25$, $p = 0.03$, WT-transf: $t_1 = 25.29$, $p = 0.02$,
247 GF: $t_2 = 2.41$, $p = 0.14$; Supplementary Figure S9).
248 We then explored the consequences of the second transcriptomic signal observed in germ-free
249 larvae, the downregulation of genes involved in amino-acid and lipid storage. To this aim, we
250 quantified by fluorescence the lipid levels in the gut and in the pelt of germ-free larvae and in
251 larvae carrying wild-type *E. coli*, transferred or not transferred to sterile rearing water. We
252 detected a 60 % higher fluorescent signal in the gut of germ-free larvae compared to larvae
253 carrying a microbiota (Figure 4A, B, $F_{2,124} = 7.7$, $p < 0.001$), while the amount of lipids in pelts,
254 which are composed for the majority of the cuticle and the fat body, was 20% lower in larvae
255 that lost their microbiota (Figure 4C, $F_{2,97} = 18$, $p < 0.001$). The lower lipid levels detected in

256 the pelt of germ-free larvae correlated with, and possibly led to, a slower growth of these larvae,
257 as reflected by the lower DNA signal and the smaller larval size (Figure 4D, $F_{2,97} = 16$, $p <$
258 0.001; Figure 4E, $F_{2,125} = 18$, $p < 0.001$).
259

260 # Discussion

261

262 The mosquito microbiota impacts host physiology at the larval and adult stage via previously
263 largely uncharacterised mechanisms. Our study led to the development of a novel approach to
264 produce fully developed germ-free adult mosquitoes and sheds light on the metabolic
265 contribution of the microbiota during larval development.
266 The production of a relevant negative control has long been a technical issue for the study of
267 the microbiota in adult mosquitoes, which was either solved by allowing normal development
268 and treating emerging mosquitoes with antibiotics or by producing stunted germ-free
269 mosquitoes [7,10]. However, antibiotic treatments are known to reduce bacterial load and modify
270 microbiota composition rather than produce germ-free mosquitoes [15], and a role of toxic effects
271 of antibiotics on the host cannot be ruled out. Moreover, several pieces of evidence underline
272 the requirement for standardised conditions during development due to carry over effects of
273 the larval environment from larvae to adults. For instance, larval microbiota composition and
274 larval diet affect host lifespan and vector competence, while crowding and starvation affect
275 metabolic profiling in the adult fat body [16–18]. Here we provide a tool which allows the
276 development of germ-free and colonised adults under comparable conditions, allowing further
277 functional studies of the bacterial microbiota in adults. We found that development success
278 was slightly higher in gnotobiotic than conventionally-reared mosquitoes (Figure 1B). This
279 may be due to the presence of bacteria with a low larvicidal activity in our colony, which could

280 also explain the slight variation in sex ratio (Supplementary Figure S3) as females develop
281 slightly slower than males and therefore may have a prolonged contact with such pathogens.
282 Alternatively, some individual larvae may not carry enough bacteria for successful larval
283 development, which correlates with development success deficits after the transfer of wild-type
284 *E. coli* harbouring third-instar larvae (Figure 3A). We were intrigued that as much as 99.8 %
285 of the bacteria were lost within 2 h after transferring larvae to sterile water, and that bacterial
286 load close to recovered in the next 10 h. This may be due to quantification of cuticular bacteria,
287 if the quick surface sterilisation has limited efficacy, or to an extremely fast shedding of gut
288 bacteria during development.
289 Our transcriptomic data points to gene upregulation in the folate biosynthesis pathway during
290 larval development in the absence of microbiota. The genome of *Ae. aegypti* lacks several
291 genes of this biosynthesis pathway, while the *E. coli* genome codes a complete biosynthetic
292 pathway except for the *gamma-glutamyl-hydrolase* [19], which we found up-regulated in germ-
293 free larvae. This suggests that the mosquito and its microbiota cooperate towards the
294 production of this vitamin. Accordingly, folate biosynthesis also appeared among the regulated
295 pathways in adult *Anopheles* mosquitoes deprived of their microbiota [20]. Vitamins B have
296 already been reported as a key contribution of the microbiota to development in other insects
297 [21,22] and are essential nutrients for mice lacking a microbiota, together with Vitamin K [23,24]. In
298 mosquitoes, older studies already identified folic acid as one of the essential nutrients for the
299 rearing of *Ae. aegypti* larvae in axenic conditions and pointed out its importance in late larval
300 development [9,25]. However, the actual role of the mosquito microbiota on folate biosynthesis
301 was still unclear, as a recent attempt failed to support development of axenic larvae using the
302 vitamin B-supplemented artificial diet proposed by Lang in 1972 [9,10]. Additionally, a
303 transcriptome study investigating the role of the microbiota on the mosquito early larval
304 development did not identify folate biosynthesis as a microbiota-regulated pathway [26]. Our

305 transcriptomic and diet supplementation data now provide strong evidence that bacteria and
306 mosquito cooperate for folate biosynthesis and that folic acid is essential for larval
307 development.
308 In our system, only a small proportion of larvae completed their development to adulthood
309 after losing their microbiota. These germ-free larvae had a prolonged fourth instar, while their
310 metamorphosis had a conventional timing. These data highlight that the transition between
311 larvae and pupae is a development checkpoint while pupal development cannot be modified.
312 The metamorphic onset is stimulated by a peak of ecdysone which is controlled by the size and
313 metabolic status of the fourth instar larva. Folic acid supplementation increased the proportion
314 of larvae passing this checkpoint and developing to adults, without restoring normal expression
315 of genes involved in amino acid and lipid storage in the conditions tested. We hypothesize that
316 this higher proportion of individuals reaching the metamorphosis requirements is achieved by
317 a combination between a prolonged fourth instar and a yet uncharacterised improvement in
318 energy intake during the fourth instar.
319 Besides folate metabolism, our transcriptomic analysis showed that several lipid and amino-
320 acid storage genes are expressed in a microbiota-dependent manner. This corroborates
321 published data on the bean bug *Riptortus pedestris*, where *hexamerin* and *vitellogenin* are
322 induced by a *Burkholderia* gut symbiont [27], but the mechanism associating the microbiota to
323 these storage proteins has not yet been elucidated. We observed a lipid accumulation in the gut
324 and a lipid deficit in the fat body of germ-free larvae (Figure 4A, B), which was previously
325 observed in axenic first instar *Ae. aegypti* larvae [14] and correlates with three signals of our
326 transcriptome data, specifically dietary lipid absorption, transport and accumulation in fat
327 tissues. In insects, diet-derived triacylglycerols (TAG) are hydrolysed by TAG lipase via a two-
328 step reaction in diacylglycerols (DAG), then monoacylglycerols, and free fatty acids (FFA) in
329 the midgut lumen. FFA are then absorbed by the intestinal epithelium via an uncharacterized

330 mechanism and stored in lipid droplets in enterocytes. FFA are then used as precursors for the
331 synthesis of DAG and TAG that are loaded onto Lipophorin which transports them via the
332 haemolymph to the fat body (reviewed in [28,29]). Firstly, we observed that *TAG lipase* is one of
333 the most up-regulated genes in all germ-free samples (Figure 2D). We hypothesize that in the
334 absence of a microbiota, mosquito larvae are not able to reconstruct DAG or TAG from FFA
335 in enterocytes and thus fail to load lipids onto Lipophorin. Secondly, two *lipophorins*, encoding
336 lipid transporters are downregulated in germ-free larvae. Thirdly, lipid storage may also be
337 decreased at the level of the fat body, due to the downregulation of *Vitellogenin* and
338 vitellogenin-related gene (*AAEL008598*) and to the up-regulation of *Angiopoietin-related*
339 *protein 2* (Supplementary Table S3). Mouse *Angiopoietin-like protein 4* is an inhibitor of TAG
340 deposition in adipocytes [28,29] that is repressed by the microbiota, a mechanism that is conserved
341 in zebrafish [30].
342 The most down-regulated gene family in the absence of a microbiota is Hexamerins, which are
343 insect-specific proteins encompassing six subunits. They are amino acid reserves stored in the
344 fat body that are consumed during non-feeding stages and vitellogenesis [31]. They might also
345 play a role in cuticle maturation and transport of riboflavin (Vitamin B2) and hormones.
346 Hexamerins accumulate during the end of larval development and represent as much as 60 %
347 of hemolymph proteins just before pupation [30]. In the mosquito two sub-classes of Hexamerins
348 exist, namely Hexamerins-1 and -2, which are orthologues to *Drosophila* Larval Serum
349 Proteins-1 (CG2559, CG4178, CG6821) and -2 (CG6806), respectively, and are enriched in
350 different amino acids. Repression of *hexamerins* might reflect a lower amino acid availability
351 caused by a reduced peptidase activity or amino acid transport, two functions found to be down-
352 regulated in the absence of bacteria in first instar axenic larvae [26]. However, in our system,
353 peptidases were present in both up- and down-regulated genes (Supplementary Table S2). This
354 might reflect a reduced ability of larvae to digest diet-derived proteins in the absence of bacteria

355 and, in parallel, an increased consumption of the proteins stored while still carrying a
356 microbiota.
357 In conclusion, our study provides a new tool for the rigorous functional analysis microbiota in
358 mosquitoes that may be also applicable to produce adults in other insect species requiring gut
359 bacteria for normal development. It allowed us to build a model for the metabolic contribution
360 of the microbiota to larval development. We suggest that bacteria are required for folate
361 biosynthesis, which supports the later stages of development, and for energy storage in the
362 form of proteins and lipids, which promotes larval growth.
363
364

365 Materials and Methods

366

367 *Bacteria and mosquitoes*

368 *E. coli* HS (wild-type) and *E. coli* HA 416 (auxotroph; [12]) were grown in lysogeny broth (LB).
369 *E. coli* HA 416 cultures were supplemented with 50 µg/mL kanamycin, 50 µg/mL *meso*-
370 diaminopimelic acid (*m*-DAP) and 200 µg/mL D-alanine (D-Ala). For all experiments, *E. coli*
371 cultures were inoculated from single fresh colonies in liquid LB with appropriate
372 supplementation and incubated at 30 °C, shaking at 200 rpm for 16 h. Growth curves of
373 auxotrophic *E. coli* with optimal amino-acid concentrations (50 µg/mL *m*-DAP and 200 µg/mL
374 D-Ala) and concentrations compatible with first-instar larvae (12.5 µg/mL *m*-DAP and 50
375 µg/mL D-Ala) were obtained by inoculating 2 mL of LB supplemented with the two amino-
376 acid concentrations with the same diluted bacterial culture in a 24-well plate. The optical
377 density at 600 nm (OD_{600}) was monitored every 15 minutes using a FLUOstar plate reader
378 (BMG Labtech). The experiment was performed two times.

379 The *Ae. aegypti* New Orleans colony was maintained under standard insectary conditions at
380 28-30 °C on a 12:12 h light/dark cycle. Gnotobiotic mosquitoes were maintained in a climatic
381 chamber at 80 % relative humidity on a 12:12 h light/dark 30 °C/25 °C cycle.
382
383 *Generation of germ-free (axenic) larvae*
384 Inside a microbiological safety cabinet, eggs were scraped off the paper they were laid on and
385 transferred onto a disposable vacuum filtration system. Eggs were surface sterilised via
386 incubation in 70 % ethanol for 5 min, then in 1 % bleach for 5 min and finally in 70 % ethanol
387 for 5 min. Bleach solution was extemporaneously prepared from a tablet. After each incubation
388 step, a vacuum was applied to the filtration unit for fast replacement of the sterilising liquid.
389 After rinsing three times with sterile water, the eggs were resuspended in ~20 mL of sterile
390 water and transferred to a sterile 25-mL cell-culture flask with a vented cap. Approximately
391 20-30 eggs were inoculated in 3 mL of liquid LB and incubated for 48 h at 30 °C shaking at
392 200 rpm to confirm sterility. Flasks were kept standing in a climatic chamber at 80 % relative
393 humidity on a 12:12 h light/dark 30 °C/25 °C cycle for eggs to hatch overnight.
394
395 *Generation of gnotobiotic larvae and axenic adults via reversible colonisation*
396 Depending on the experiment type, two rearing methods were used: for experiments aiming to
397 analyse adult mosquitoes, a batch rearing in cell-culture flasks (10-15 larvae per flask) was
398 used; for experiments requiring the follow-up of single larvae, larvae were placed in single
399 wells of sterile 24 well-plates [32].
400 A 16 h culture of each *E. coli* strain was pelleted and cells were resuspended in 5 times the
401 initial culture volume of sterile deionised water to reach a concentration of 2-5*10^8 CFU/mL.
402 For the generation of larvae gnotobiotic for the auxotrophic *E. coli*, *m*-DAP (12.5 µg/mL) and
403 D-Ala (50 µg/mL) were also added to the bacterial suspension. For batch rearing, 20 mL of

404 bacterial suspension and 750 µL of autoclaved suspension of TetraMin Baby fish food (50
405 mg/mL in water) were added to each flask. For rearing of individualised larvae, 2 mL of
406 bacteria and 50 µL of fish food suspension were added to each well. Larvae were kept in the
407 climatic chamber until pupation.
408 Pupae were rinsed once in sterile water and then transferred to 15 mL tubes placed in
409 autoclaved polypropylene cups (Eco2 NV) for emergence. A smaller tube containing a rod of
410 sterile cotton dipped into a 10 % sterile sucrose solution was placed in each cup. Tubes
411 containing pupae and sugar were held in place using specifically designed 3D printed
412 components (designs available upon request). See Supplementary Figure S10 for detailed
413 information on the preparation of sterile cups.
414
415 *Duration of development*
416 All measurements for the investigation of the developmental dynamics were performed on
417 wild-type and auxotrophic *E. coli*-carrying gnotobiotic individuals and from conventionally-
418 reared individuals obtained from non-sterilised eggs and reared in non-sterile conditions (using
419 tap water as conventionally used for our mosquito colony). Three independent mosquito
420 batches were examined for each type of analysis.
421 Gnotobiotic or conventionally-reared larvae were placed in single wells of 24-well plates to
422 monitor the duration of the different developmental stages in single individuals and to
423 determine the developmental success of each rearing condition, measured as the percentage of
424 larvae reaching the adult stage. Plates were inspected three times per day and the moment at
425 which larvae moulted was noted. The sex of the adult mosquito or the time of death were also
426 recorded for each individual. The experiment was stopped after 15 days, generally 5 days after
427 the last emergence. Individuals stacked at the larval stage were marked as non-developed. For
428 each replicate, at least 48 individuals per condition were analysed.

429 *Reversible colonisation dynamics*

430 To validate reversible colonisation, adult mosquitoes emerged from auxotrophic *E. coli*-
431 gnotobiotic larvae were collected 1-3 days after emergence, surface sterilised for 3-5 min in 70
432 % ethanol, rinsed three times in sterile PBS and homogenised individually in 100 µL of sterile
433 LB. To quantify the number of bacterial CFUs present in each individual, serial dilutions of
434 the homogenates were plated on LB agar plates supplemented with 50 µg/mL *m*-DAP and 200
435 µg/mL D-Ala. Supplementary Figure S3 show data from three independent replicates based on
436 34, 123 and 321 individuals. To investigate colonisation dynamics of the auxotrophic *E. coli*
437 strain, the bacterial loads in third and fourth instar larvae and pupae were investigated and
438 compared to those of individuals carrying the wild-type strain. Larvae and pupae were surface
439 sterilised for 15-30 s in 70 % ethanol, rinsed in sterile water and homogenised individually in
440 100 µL of sterile LB. Serial dilutions of the homogenates were plated on LB agar plates
441 supplemented with 50 µg/mL *m*-DAP and 200 µg/mL D-Ala and the number of bacterial cells
442 present in each individual was determined. Three replicates of at least 12 individuals per
443 replicate were analysed at each time point.

444 To detect the presence of bacterial DNA on adult mosquitoes, DNA was extracted from single
445 1-day old whole mosquitoes produced with the two *E. coli* strains. Mosquitoes were placed
446 individually in 2 mL screw-cap tubes containing 0.5 mm glass beads and homogenised using
447 a bead beater (Precellys Evolution, Bertin) for 2x1 min at 9,000 rpm in 190 µL of Lysis Buffer
448 (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X, 20 mg/mL lysozyme). Two blank samples
449 were extracted in parallel and used as negative controls. After 2 h incubation at 37 °C,
450 Proteinase K (1 mg/mL final concentration) was added and samples were incubated 4 h at 55
451 °C. DNA was purified using the ZymoBIOMICS DNA 96 MagBead kit (Zymo Research) and
452 a Kingfisher Duo Apparatus. The presence of bacterial DNA was detected and quantified via
453 amplification of the *16S* rRNA bacterial gene and the ribosomal mosquito gene *S7* via qPCR

454 using the SYBR qPCR Premix Ex Taq (Takara) in a Light Cycler 480 (Roche). Primer
455 sequences are listed in Supplementary Table S5. Mosquitoes were considered germ-free when
456 their *16S* Ct were higher than those of negative controls. Three replicates of 9 individuals per
457 replicate were analysed.
458
459 *Larval and wing length measurements*
460 For larval length measurements, larvae were collected at the fifth day from hatching. Larvae
461 were fixed in PBS with 4 % paraformaldehyde for 20 min and placed on a microscope slide.
462 For each larva, the distance between the anterior border of the head and the posterior border of
463 the last abdominal segment excluding the siphon [32] was measured using a dissection
464 microscope equipped with a camera. Four independent mosquito batches were examined and,
465 for each replicate, at least 28 individuals per condition were analysed.
466 For wing length measurements, adult mosquitoes were collected 2 days post emergence and
467 kept at -20 °C until analysis. The measurements were conducted on the left wing of both male
468 and female mosquitoes. Wings from several individuals were dissected and placed on a
469 microscope slide. The distance between the alular notch and the radius 3 vein was measured
470 for each wing using a dissection microscope equipped with a camera [32]. Four independent
471 mosquito batches were examined and, for each replicate, 8 to 20 individuals per condition were
472 analysed.
473
474 *Experimental setup and sample collection for transcriptomics on colonised vs germ-free larvae*
475 Gnotobiotic larvae for both bacterial strains were obtained as previously described, reared
476 individually in 24-well plates and kept in a climatic chamber at 80 % RH with 12:12 h
477 light/dark 30 °C/25 °C cycle. Larvae gnotobiotic for the auxotrophic strain were supplemented
478 with *m*-DAP (12.5 µg/mL) and D-Ala (50 µg/mL). To ensure synchronicity, larvae moulting

479 to third instar between 48 h and T_0=53 h after addition of bacteria were selected. At T_0, larvae
480 carrying the auxotrophic *E. coli* were washed in sterile water and individually transferred to
481 new 24-well plates containing 2 mL of sterile water and 50 µL of sterile fish food. Twelve
482 hours later (T_{12}), 120 larvae per condition were collected for sampling and another 120 larvae
483 reversibly colonised by the auxotrophic *E. coli* were transferred to new 24-well plates
484 containing 2 mL of sterile water and 50 µL of sterile fish food to further reduce bacterial
485 colonisation. Another 8 h later (T_{20}), 120 larvae per condition were collected for sampling.
486 At both T_{12} and T_{20}, for each condition, 60 larval guts (dissected on ice) and 60 whole larvae
487 were snap frozen in prechilled 2 mL screw-cap tubes containing 0.5 mm glass beads and kept
488 in a cooling block below -40 °C. A gut sample included gastric caeca, anterior and posterior
489 midgut, while hindgut and Malpighian tubules were excluded. Practically, T_0, T_{12} and T_{20} of
490 each condition was 1 hour apart to match our dissection speed (60 individuals/hour) and the
491 order of processing was inverted between replicates. Three replicates were performed using
492 independent mosquito batches.
493
494 *RNA extraction, library preparation, RNA sequencing and data analysis*
495 After collection, 1 mL of TRIzol (Thermo Fisher Scientific) was added to all samples. Samples
496 were homogenised using a bead beater (Precellys Evolution, Bertin) for 2x1 min at 9,000 rpm
497 and stored at -80 °C until RNA extraction. Total RNA was extracted using TRIzol according
498 to the manufacturer's instructions. After RNA extraction, samples were treated for 30 min at
499 37 °C with 10 U of DNase I (Thermo Fisher Scientific) and purified using the MagJET RNA
500 Kit (Thermo Fisher Scientific) in a KingFisher Duo Prime system (Thermo Fisher Scientific).
501 Samples were mixed with RNA stable (Biomatrica), vacuum dried using a SpeedVac and
502 shipped for library preparation and sequencing.

503 RNA quality evaluation, library preparation and sequencing were performed by Biofidal
504 (Vaulx-en-Velin, France - http://www.biofidal-lab.com). RNA integrity was evaluated on a
505 BioAnalyzer (Agilent Technologies) using an RNA 6000 Pico RNA chip. Single-end libraries
506 (75 bp) with poly(A) selection were constructed using the Universal Plus mRNA-Seq Library
507 Preparation with NuQuant kit (Nugen Tecan Genomics) from 1 µg of total RNA. After quality
508 check, libraries were pooled in equimolar concentrations and sequenced on a NextSeq 500
509 high-output flow cell (Illumina). The resulting raw reads were de-multiplexed and adaptor
510 sequences were trimmed by the sequencing facility. FASTQ sequences were trimmed for their
511 quality using Trimmomatic [33] (minimum quality score: 15, minimal length: 40) and their
512 quality was evaluated using FASTQC [34]. After quality trimming, an average of 15 million reads
513 (min 8.4 million, max 25 million) were obtained per sample. Reads were mapped on the *Ae.*
514 *aegypti* genome (assembly AeaegL5, geneset AeaegL5.2) using HISAT2 [35] with a mean
515 alignment rate of 94 %. Read counts were obtained with FeatureCounts [36] and differential
516 expression analysis was conducted in R using the DESeq2 [37] package. Only the genes with an
517 adjusted *p*-value < 0.001 and a $\log_2$ fold change < -1.5 or > 1.5 were subjected to the Gene
518 Ontology analysis with g:Profiler [38].
519 To quantify bacterial loss on samples subjected to RNA sequencing, RNA was retro-
520 transcribed using the PrimeScript RT-PCR Kit (Takara) and analysed via qPCR using the
521 SYBR qPCR Premix Ex Taq (Takara) in a Ligth Cycler 480 (Roche). Bacterial load was
522 quantified via amplification of the *16S* rRNA bacterial gene and normalised to the ribosomal
523 mosquito gene *S17*. Primer sequences are listed in Supplementary Table S5.
524
525 *Analysis of bacterial loss dynamics*
526 Third-instar synchronous gnotobiotic larvae were obtained as previously described. At T_0,
527 larvae carrying the wild-type or the auxotrophic *E. coli* were washed in sterile water and

528 individually transferred to new 24-well plates containing 2 mL of sterile water and 50 µL of
529 sterile fish food. Non-transferred larvae were reared in parallel for 2h. After 2h, 5h, 12 h and
530 20 h from the transfer, at least 6 larvae per condition were surface sterilized for 15-30 s in 70
531 % ethanol, rinsed in sterile water and homogenised individually in 100 µL of sterile LB. Serial
532 dilutions of the homogenates were plated on LB agar plates supplemented with 50 µg/mL *m*-
533 DAP and 200 µg/mL D-Ala and the number of bacterial cells present in each individual was
534 determined. Two replicates were analysed at each time point.
535
536 *Analysis of development and folic acid supplementation*
537 Third-instar larvae reared with wild-type *E. coli* and transferred to new rearing medium and
538 germ-free larvae were obtained as described above. Non-transferred larvae were reared in
539 parallel. To investigate the effect of folate on development success, 0.25, 0.5 or 1.25 mg/mL
540 folic acid were added to germ-free larvae. Plates were inspected daily and the developmental
541 stage of each individual was noted. At least three replicates were performed using independent
542 mosquito batches. With some egg batches we observed that all larvae completed their
543 development despite their rearing condition. We discarded those data and we considered valid
544 and experiment when wild-type *E. coli* gnotobiotic larvae transferred to new rearing medium
545 had a 40-60 % developmental success. For each replicate, 24 to 48 larvae were analysed.
546
547 *Expression analysis of genes involved on folate biosynthesis or on energy storage after folic*
548 *acid supplementation*
549 Third-instar colonised and germ-free larvae were obtained as described above. At the time of
550 transfer (T_0) 1.25 mg/mL folic acid was added to half of the larvae. At T_{20}, 18 to 24 larvae per
551 condition were sampled and immediately stored at -80 °C. Three replicates were performed
552 using independent mosquito batches, except for larvae reared with wild-type *E. coli* and

553 transferred to new rearing medium which were sampled from two replicates. RNA was
554 extracted as previously described, retro-transcribed using the PrimeScript RT-PCR Kit
555 (Takara) and analysed via qPCR using the SYBR qPCR Premix Ex Taq (Takara) in a 7300
556 Real-Time PCR System (Applied Biosystems).
557
558 *Gut hypoxia measurements*
559 Third-instar colonised and germ-free larvae were obtained as described above. At least 9 larvae
560 per condition were collected at the 12 h time-point, washed in sterile water and incubated at
561 dark for 30 min in sterile PBS supplemented with 5 µM Image-iT Red Hypoxia Reagent
562 (Thermo Fisher Scientific) and fish food. In parallel, two larvae per condition were processed
563 without the dye as a negative control. Larvae were frozen 3 min at -80 °C and placed in a single
564 slide. Images were acquired with an EVOS FL Auto system (Thermo Fisher Scientific)
565 equipped with a Texas Red filter using a 10X objective. The multiple images were stitched
566 automatically using the EVOS FL Auto Software to reconstitute a single image including all
567 samples. Fluorescence intensities and area surfaces of single guts were measured using FIJI [39].
568 Three replicates were performed using independent mosquito batches.
569
570 *Lipid content and length measurements*
571 Third-instar colonised and germ-free larvae were obtained as described above. Larvae were
572 sampled 44 h after the transfer, when wild-type *E. coli* carrying larvae were at the second day
573 of the fourth instar. Guts and pelts (composed by the cuticle and the fat body) were dissected,
574 placed in PBS with 4 % paraformaldehyde and fixed overnight at 4 °C.
575 After three 5 min washes in PBS, guts were stained in the dark for 20 min with DAPI (Thermo
576 Fisher Scientific, final concentration 0.1 µg/mL) and for 20 min with BODIPY 505/515
577 (Thermo Fisher Scientific, final concentration 1 µM), rinsed three times in PBS for 5 min and

578 imaged. Imaging and analysis were performed as described above, using DAPI and GFP filters.
579 A different protocol was used to quantify lipids in the pelts to avoid the loss of fat body parts
580 during staining and rinsing steps. After fixation, pelts were washed three times in PBS for 5
581 min and placed in individual tubes with 75 µL of DAPI (0.1 µg/mL) and 75 µL of BODIPY
582 505/515 (1 µM). Pelts were homogenized, incubated at the dark for 20 min and transferred to
583 a 96-well plate. Fluorescence intensities were measured using a FLUOstar plate reader
584 equipped with DAPI and GFP filters, using a solution of the two dyes as blank. At least 12
585 larvae per condition were analysed in three independent replicates.
586 Larval lengths were measured as previously described on larvae obtained with the same
587 protocol and collected at the 44 h time-point. Three independent replicates were performed
588 using 6 to 24 individuals per condition.
589 *Statistical analyses*
590 Statistical analyses were performed with generalized linear mixed models (GLMM) using the
591 lme4 package in R (version 3.6.0) and setting the rearing condition (bacterium) as fixed effects
592 and the replicate as a random effect. For categorical data (development success, sex ratio) an
593 ANOVA was performed on a logistic regression (glmer). For quantitative data (CFU counts,
594 duration of development, larval and wing length measurements, hypoxia, lipid and DNA
595 quantifications) an ANOVA was performed on a linear regression (lmer). For qPCR results, an
596 ANOVA with Turkey HSD test or a Welch Two Sample t-test were performed. Prevalence
597 data on qPCR were analysed with a Fisher's exact test. Variations are calculated as the
598 difference between treatments normalised to the control.
599
600
601

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703
704

705 # Figure legends

706

707 **Figure 1. Reversible colonisation allows production of germ-free mosquitoes without any**
708 **delay or deficit in development.** (**a**) Schematic representation of the experimental set up. Eggs
709 are surface sterilized to give rise to axenic larvae. A bacterial culture of the *E. coli* wild-type
710 (gnotobiotic control) or auxotrophic strain is added to axenic larvae to obtain gnotobiotic
711 larvae. To support the growth of auxotrophic bacteria, *m*-DAP and D-Ala are provided
712 throughout the whole larval developmental stage. After transferring pupae carrying
713 auxotrophic *E. coli* to a new medium lacking *m*-DAP and D-Ala, germ-free mosquitoes are
714 obtained. (**b**) Proportion of larvae developing to adulthood (striped bars) and of larvae dead or
715 stalled in the development (full bars) after conventional rearing (CR, black), rearing with wild-
716 type *E. coli* (WT, orange) and auxotrophic *E. coli* (AUX, blue). CR larvae developed in tap
717 water on autoclaved fish food. (**c**) Colony Forming Units (CFU) quantification of bacterial
718 loads (upper chart) and prevalence (lower chart) in larvae at third instar (L3), early fourth instar
719 (L4-1), late fourth instar (L4-2), and in pupae (P). (**d**) CFU quantification of bacterial loads
720 (upper chart) and prevalence (lower chart) in 1-3-day old mosquitoes. (**e**) Duration of the larval
721 instars and of the pupal stage, quantified from the addition of bacteria to first instar larvae (L1)
722 to the beginning of the indicated stages. (**f**) Larval length measured on the fifth day after
723 hatching. (**g**) Wing length of two-day old adult mosquitoes. In **b**-**g**, data show mean ± SEM of
724 three independent replicates where 48 (**b**, **e**), 12 (**c**, **d**), 28 (**f**) and 8-20 (**g**) individuals were
725 analysed per condition. NS, Non-significant; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. In **e**,
726 different letters represent significantly different data for each larval instar.

727

728 **Figure 2. Transcriptomic analysis in germ-free and colonised larvae.** (**a**) Schematic
729 representation of the experimental set up. To produce third-instar germ-free larvae, larvae
730 gnotobiotic for auxotrophic *E. coli* were transferred to a rearing medium lacking m-DAP and
731 d-Ala. Gut (n = 60) and whole larvae (n = 60) were sampled for RNA extraction 12 h and 20 h
732 after transfer, using wild-type *E. coli* gnotobiotic larvae as a control. Three independent
733 replicates were performed. (**b**) Principal Component Analysis (PCA) of guts and whole larvae
734 transcriptomic data of wild-type *E. coli* carrying (WT, orange) and germ-free larvae (GF,
735 white). (**c**) Venn diagram of Gene Ontology terms enriched for genes up-regulated in germ-
736 free conditions. (**d**) Log_2-fold values and function of the ten highest up-regulated (red) and
737 down-regulated (blue) genes in germ-free larvae, ordered by decreasing (up-regulated) or
738 increasing (down-regulated) mean value in the four sample types. Different colour codes
739 indicate genes with a shared function (orange: phosphatase activity; green: hydrolytic reaction;
740 yellow: chitin binding; purple: transferase activity; light blue: amino-acid storage; dark blue:
741 lipid transport/storage). (**e**) Representation of the mosquito gene products that participate in the
742 folate biosynthesis pathway and that were up-regulated in germ-free larvae. Enzymes
743 responsible for reactions between 7,8-Dihydroneopterin and 7,8-Dihydrofolate are not encoded
744 by the genome of *Ae. aegypti*. The up-regulation colour-code is the same as in panel **d** and
745 reflects the mean of log_2 fold in the four sample types.
746
747 **Figure 3. Late larval development is folic acid dependent.** (**a**) Proportion of individuals
748 reaching the fourth instar (L4), the pupal stage and adulthood when continuously colonised
749 (WT, orange), reared on wild-type *E. coli* and transferred in new rearing medium (WT
750 transferred, light orange) and after becoming germ-free (GF, white). (**b**) Duration of the third
751 instar (L3), fourth instar (L4) and pupal stage in the experiments shown in **a**. (**c**) Proportion of
752 larvae reaching the fourth instar (L4), the pupal stage and adulthood in germ-free conditions

753 with or without folic acid (Fol) supplementation. (**d**) Duration of the third instar (L3), fourth
754 instar (L4) and pupal stage in the experiments shown in **c**. (**e-g**) Effect of folic acid
755 supplementation (1.25 mg/mL) for 20 h on the expression of *hexamerin AAEL008045* (**e**),
756 *vitellogenin*-related gene *AAEL08598* (**f**) and *alkaline phosphatase AAEL000931* (**g**) in larvae
757 reared continuously with wild-type *E. coli* (WT, orange), transferred in new rearing medium
758 after being reared with wild-type *E. coli* (WT transf, light orange) and after becoming germ-
759 free (GF, white). Data show the mean ± SEM of three independent replicates where at least 48
760 (**a**, **b**), 24 (**c**, **d**) or 18 (**e**, **f**, **g**) individuals were analysed per condition, except for expression
761 data on WT transf larvae in **e**, **f**, **g**, which derive from two replicates. NS, Non-significant; *, p
762 < 0.05, **, $p < 0.01$, ***, $p < 0.001$.
763
764 **Figure 4. Consequences of the absence of a microbiota on lipid storage and larval growth.**
765 (**a**) Exemplifying image of three guts belonging to fourth-instar larvae gnotobiotic for *E. coli*
766 wild-type before (WT) and after the transferring to new rearing medium (WT transf) and germ-
767 free (GF) stained with BODIPY 505/515. (**b**, **c**) Quantification of BODIPY 505/515
768 fluorescence intensity in the gut (**b**, normalized to the gut area) and pelt (**c**, AU – Arbitrary
769 Units) of fourth instar larvae in the same three conditions, including samples shown in **a**. (**d**)
770 Quantification of DAPI fluorescence intensity in the pelt of fourth instar larvae in the three
771 gnotobiotic conditions. (**e**) Larval length measured at the second day of fourth instar in larvae.
772 Data represent the mean ± SEM of three independent replicates where 6-24 individuals were
773 analysed per condition. NS, Non-significant; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.
774

Figure 1

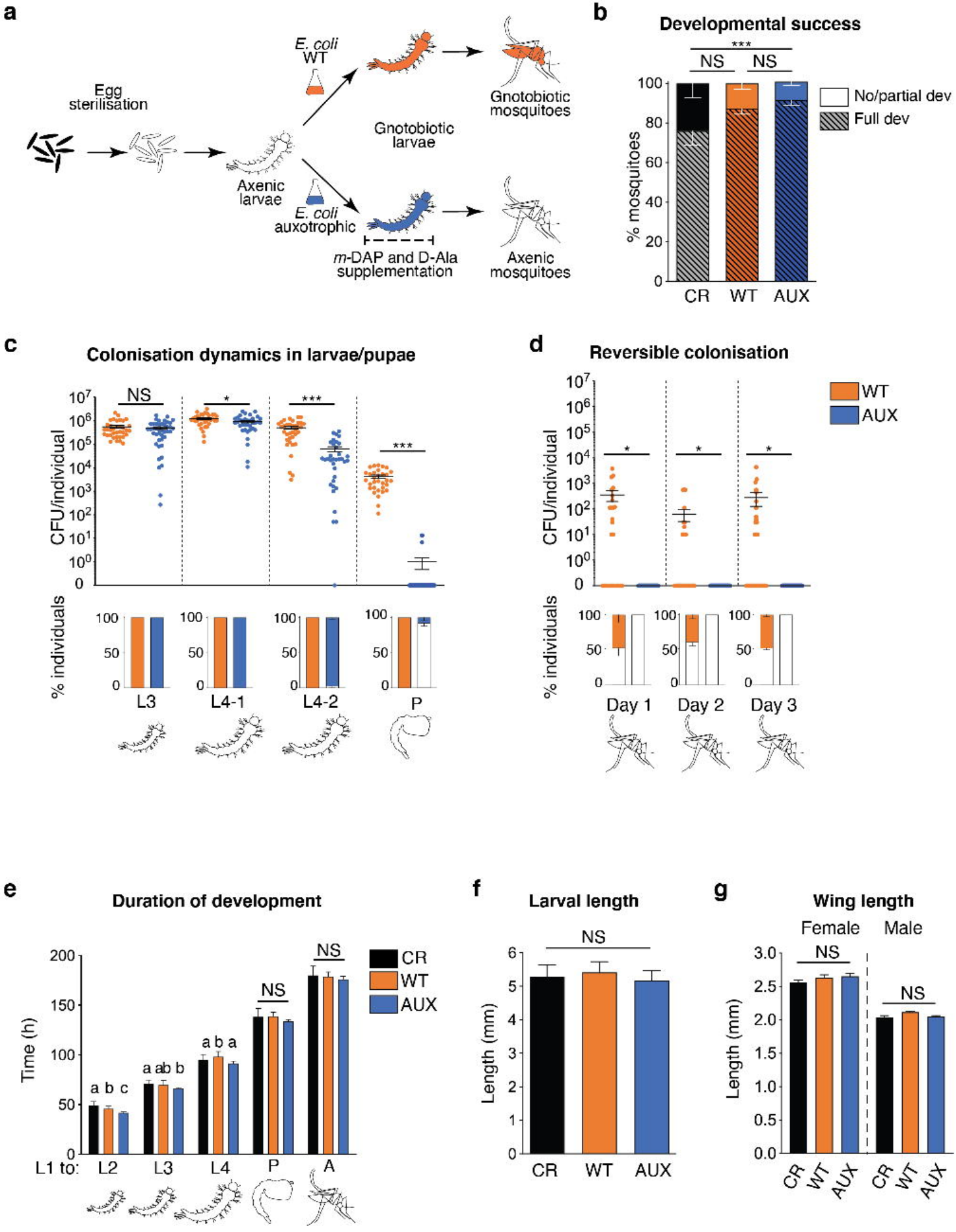
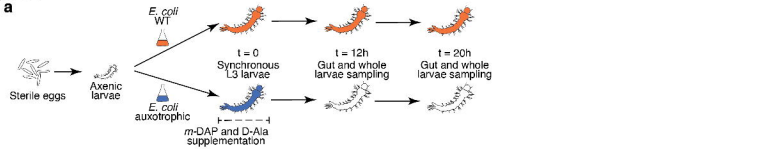
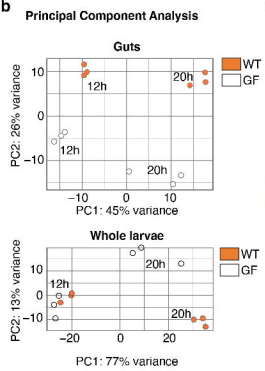
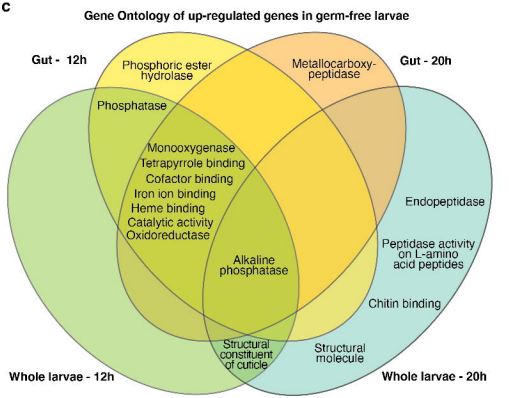

a
Egg sterilisation
Axenic larvae
E. coli WT
Gnotobiotic larvae
Gnotobiotic mosquitoes
E. coli auxotrophic
m-DAP and D-Ala supplementation
Axenic mosquitoes
b
Developmental success
***
NS
NS
% mosquitoes
No/partial dev
Full dev
CR
WT
AUX
c
Colonisation dynamics in larvae/pupae
CFU/individual
% individuals
NS
*
***
***
L3
L4-1
L4-2
P
d
Reversible colonisation
CFU/individual
% individuals
WT
AUX
Day 1
Day 2
Day 3
e
Duration of development
Time (h)
CR
WT
AUX
NS
NS
L1 to:
L2
L3
L4
P
A
f
Larval length
Length (mm)
NS
CR
WT
AUX
g
Wing length
Female
Male
Length (mm)
NS
NS
CR
WT
AUX
CR
WT
AUX

Figure 2







d

AAEL	Description					Function	Gut 12h	Gut 20h	Wl 12h	Wl 20h
000931	Alkaline phosphatase					Phosphatase activity	9.2	7.2	5.5	5.6
003317	Alkaline phosphatase					Phosphatase activity	7.1	6.1	7.6	6.0
003297	Alkaline phosphatase					Phosphatase activity	5.8	3.2	5.4	3.2
002555	Sodium/solute symporter					Transmembrane transport	5.7	4.5	3.6	3.1
002138	Triacylglycerol lipase					Hydrolytic reaction	4.4	3.9	4.0	4.0
003289	Alkaline phosphatase					Phosphatase activity	5.2	3.0	4.7	3.1
000323	Cysteine-rich venom protein					Trypsine inhibitor activity	4.0	3.9	3.7	3.6
029062	Unknown (Chitin binding)					Chitin binding	5.0	3.0	4.5	2.7
024870	Nose resistant to fluoxetine protein 6-like					Transferase activity	3.3	3.1	3.8	3.1
003905	Alkaline phosphatase					Phosphatase activity	4.5	2.5	3.1	3.1

AAEL	Description					Function	Gut 12h	Gut 20h	Wl 12h	Wl 20h
008045	Hexamerin 2 beta					Amino acid storage	0.0	-6.7	-5.3	-7.0
011169	Hexamerin 2 beta					Amino acid storage	-3.6	-4.2	-2.5	-4.2
011520	Sucrose transport protein					Sucrose transport	-4.5	-3.8	-3.1	-2.9
008817	Hexamerin 2 beta					Amino acid storage	0.0	-4.0	-3.1	-6.0
018240	Unknown (Fuseless region)					Pre-synaptic calcium channel regulation	-3.3	-3.4	-3.3	-3.0
008313	Unknown						0.0	-3.1	-4.5	-4.5
013757	Hexamerin 2 beta					Amino acid storage	0.0	-5.4	-2.2	-4.5
011661	Unknown (Cilia structure/activity)					Cilia structure/activity	-3.9	-4.1	0.0	-3.2
023132	Unknown (Lipase activity)					Hydrolytic reaction	-2.7	-4.0	0.0	-4.5
008598	Unknown (Vitellogenin domain)					Lipid transport/storage	0.0	-2.7	-2.0	-4.7

Log2Fold GF/WT: +9, 0, -9

e

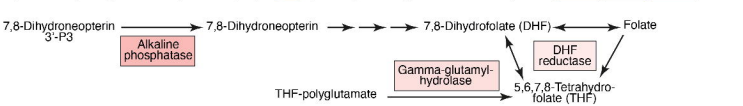


Figure 3

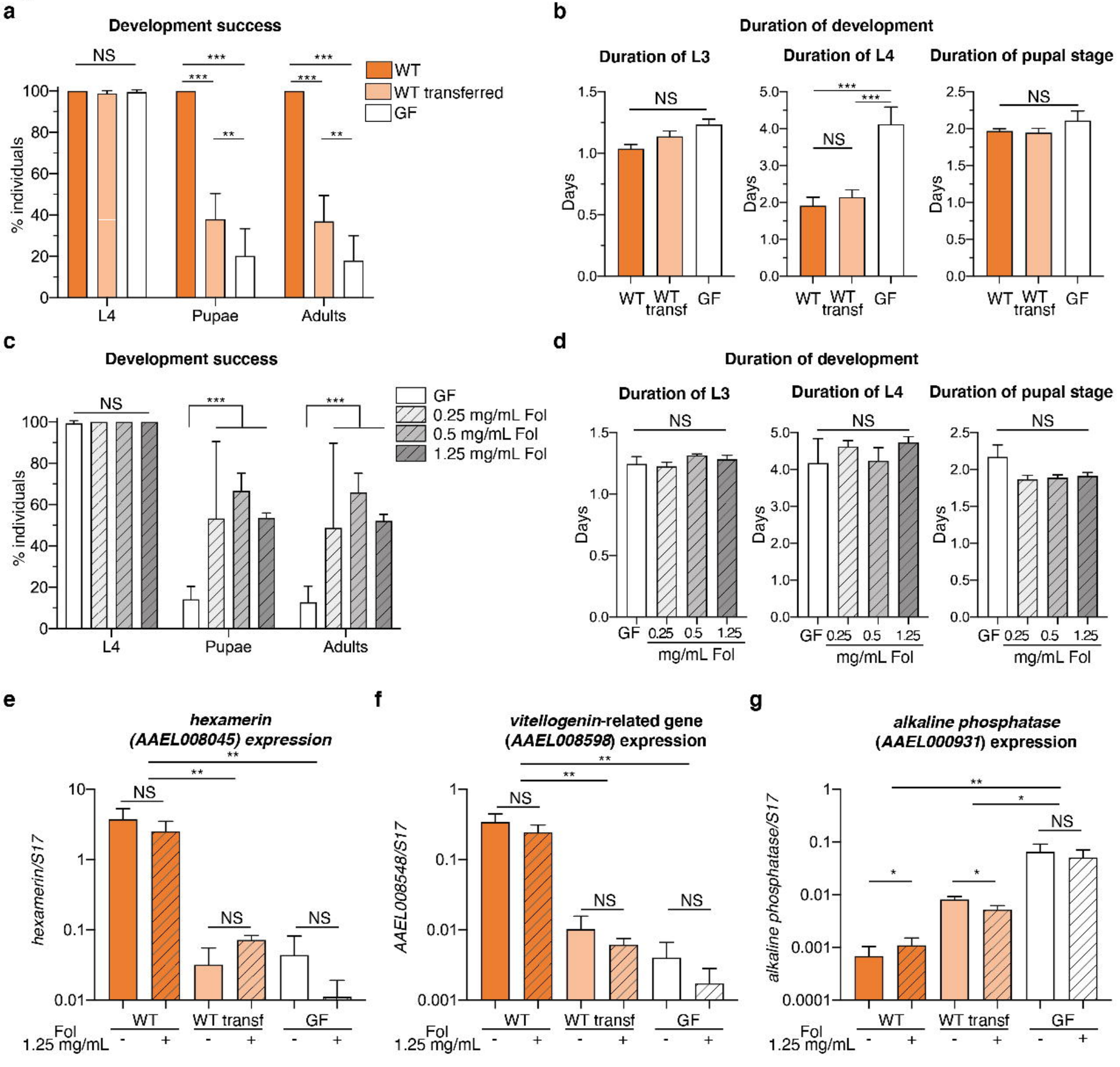
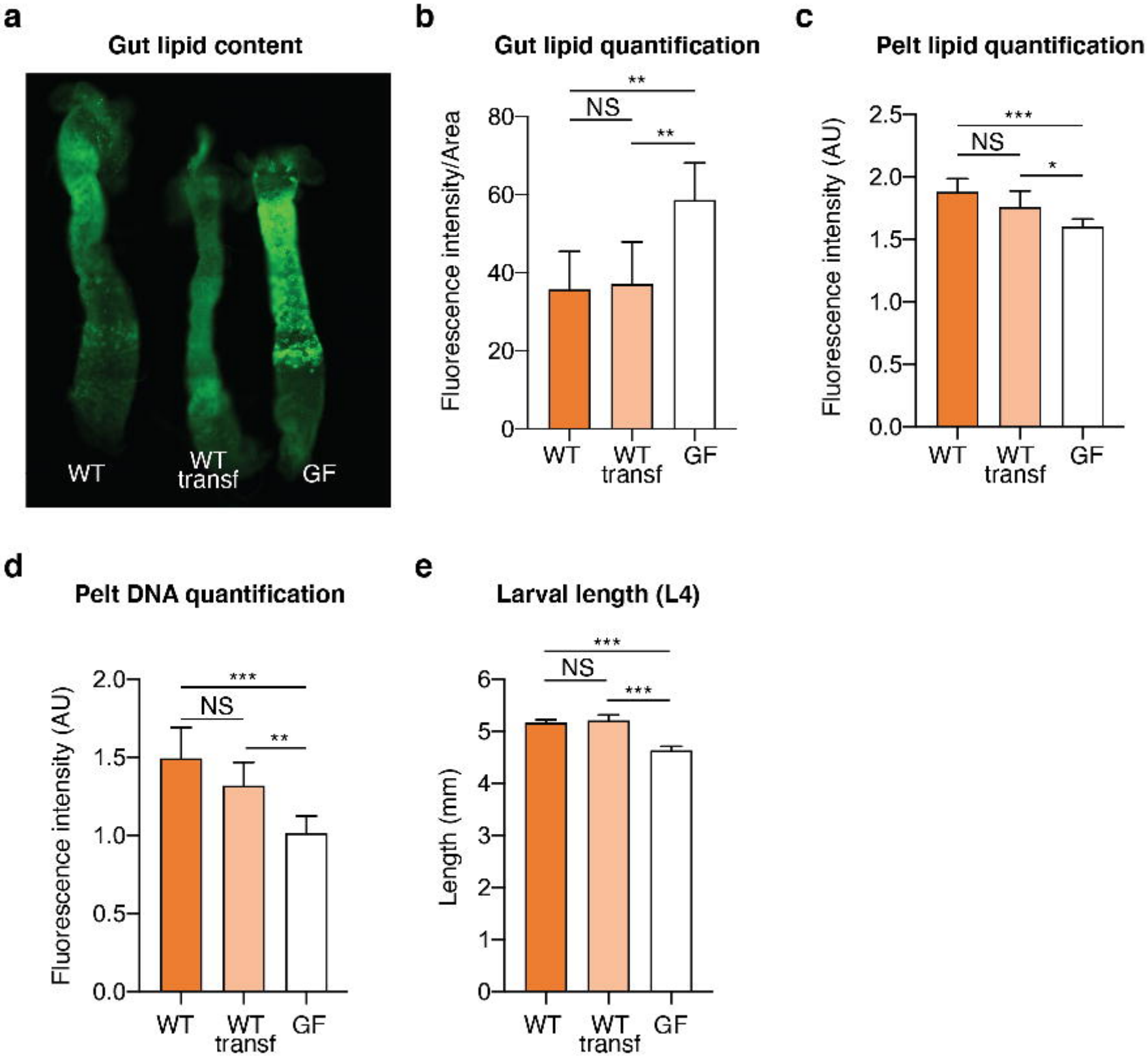

a
Development success
NS
***
***
***
***
**
**
WT
WT transferred
GF
% individuals
L4
Pupae
Adults
b
Duration of development
Duration of L3
Duration of L4
Duration of pupal stage
Days
NS
***
***
NS
WT
WT transf
GF
c
Development success
GF
0.25 mg/mL Fol
0.5 mg/mL Fol
1.25 mg/mL Fol
d
Duration of development
Duration of L3
Duration of L4
Duration of pupal stage
mg/mL Fol
e
hexamerin (AAEL008045) expression
hexamerin/S17
f
vitellogenin-related gene (AAEL008598) expression
AAEL008548/S17
g
alkaline phosphatase (AAEL000931) expression
alkaline phosphatase/S17
Fol
1.25 mg/mL

Figure 4


a
Gut lipid content
WT
WT transf
GF
b
Gut lipid quantification
Fluorescence intensity/Area
80
60
40
20
0
NS
**
**
WT
WT transf
GF
c
Pelt lipid quantification
Fluorescence intensity (AU)
2.5
2.0
1.5
1.0
0.5
0.0
NS
***
*
WT
WT transf
GF
d
Pelt DNA quantification
Fluorescence intensity (AU)
2.0
1.5
1.0
0.5
0.0
NS
***
**
WT
WT transf
GF
e
Larval length (L4)
Length (mm)
6
5
4
3
2
1
0
NS
***
***
WT
WT transf
GF