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1 Extracellular microbes are required for mosquito development
2 even in the presence of *Wolbachia*

3

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10 **Abstract (214 words)**

11 *Wolbachia* is an endosymbiotic bacterium infecting a wide array of invertebrates that gained
12 attention for its potential to curb the transmission of vector-borne diseases. Its capacity to colonize
13 arthropod populations is generally driven by vertical transmission and reproductive manipulation.
14 In some insect species, *Wolbachia* additionally became an essential nutritional symbiont,
15 providing vitamins to its host. As mosquito larvae require microbe-derived vitamins for
16 development, we studied whether such a support of *Wolbachia* would exist in mosquitoes but be
17 masked by the presence of other microbes. We chose *Culex quinquefasciatus* species to address
18 this question, as it is highly colonized with *Wolbachia*. We developed a method to produce *Culex*
19 *quinquefasciatus* devoid of extracellular microbiota and demonstrated that *Wolbachia* alone is
20 insufficient to support larval development. Using transient colonization with *Escherichia coli*, we
21 managed to produce adult *Culex quinquefasciatus* harboring *Wolbachia* only. When curbing
22 *Wolbachia* infection of these *E. coli*-colonized larvae via tetracycline treatment, we obtained a

23 higher larval development. Together, our data indicate that *Wolbachia* does not support
24 development but rather acts here as a metabolic burden, and that *E. coli* is sufficient for
25 development success even in a species that grows in “dirty” water. This opens the way towards
26 gnotobiology studies in *Culex quinquefasciatus* and highlights the complex relationships between
27 *Wolbachia* and its mosquito host.

28 **Author summary (175 words)**

29 *Wolbachia* is a bacterium infecting many invertebrates that gained attention for its potential to
30 curb the transmission of vector-borne diseases, such as dengue. In some insect species, *Wolbachia*
31 provides vitamins to its host. As mosquito larvae require microbe-derived vitamins for
32 development, we studied whether such a support of *Wolbachia* would exist in mosquitoes but be
33 masked by the presence of other microbes. We chose *Culex quinquefasciatus* species to address
34 this question, as it is highly colonized with *Wolbachia*. We developed a method to produce *Culex*
35 *quinquefasciatus* devoid of extracellular microbiota and demonstrated that *Wolbachia* alone is
36 insufficient to support larval development. We transiently colonized these larvae with *Escherichia*
37 *coli* to rescue larval development and to produce adults harboring *Wolbachia* only. When curbing
38 *Wolbachia* infection of these *E. coli*-colonized larvae via tetracycline treatment, we obtained a
39 higher larval development. Together, our data indicate that *Wolbachia* does not support
40 development but rather acts here as a metabolic burden, and that *E. coli* is sufficient for
41 development success even in a mosquito species that grows in “dirty” water.

42

43 **Introduction**

44 Mosquito-borne diseases are a worldwide health threat. New vector control tools that are safe for
45 people and the environment are needed. One efficient approach uses the release of *Aedes*
46 mosquitoes infected with the endosymbiotic bacterium *Wolbachia* to suppress mosquito
47 populations, ultimately reducing the incidence of mosquito-borne diseases (1). This approach has
48 allowed significant progress in reducing dengue incidence in high burden settings worldwide (2,3).

49 *Wolbachia* is a genus of intracellular alphaproteobacteria that infect many invertebrate species. It
50 has attracted particular interest due to its diverse effects on hosts. In arthropods, *Wolbachia* is
51 present in approximately 65 % of terrestrial species (4); and half of aquatic species (5). While its
52 interaction with nematodes is clearly mutualistic, contributing to worm nutrition, its role in
53 arthropods is generally parasitic, as it manipulates host reproduction for its own benefit (6–8).

54 Within arthropods, it is highly concentrated in female germlines, enabling vertical transmission
55 while simultaneously acquiring resources for survival and growth (8).

56 Among the reproductive manipulation phenomena, cytoplasmic incompatibility (CI) is most
57 common. In CI, infected males mating with uninfected females produce embryonic death, while
58 they can fertilize infected females, resulting in viable offspring. This increases the relative
59 competitiveness of infected females within the population, promoting *Wolbachia* transmission (9).

60 When two mosquito populations harbor different *Wolbachia* strains, bidirectional CI can occur.

61 *Wolbachia* also induces other phenotypic changes, often increasing the population's proportion of
62 females, which further enhances its spread. These changes include: a) induction of
63 parthenogenesis, leading to female offspring; b) male killing, where *Wolbachia* infection in mated
64 females selectively eliminates developing male embryos by manipulating the sex-determination

65 system; and c) feminization, where males develop female characteristics due to hormone
66 manipulation (10).

67 Recently, two hemipteran species have been found to maintain an unexpected nutritional link with
68 *Wolbachia*. In bedbugs, *wCle* (*Wolbachia* of *Cimex lectularius*) promotes nymph development,
69 lifespan and fecundity by producing riboflavin and biotin (8,11,12). In two planthoppers species,
70 *Wolbachia*-cured insects are sterile, but their fecundity levels are rescued by experimental
71 reinfection with *wLug* (*Wolbachia* of *Nilaparvata lugens*) and *wStri* (*Wolbachia* of *Laodelphax*
72 *striatellus*), respectively (13).

73 *Wolbachia* has been found naturally in some dipterans, such as mosquitoes and flies (14–16). This
74 includes the common domestic mosquitoes *Culex quinquefasciatus* and *Culex pipiens* where
75 *Wolbachia* is highly prevalent (17), in contrast to other mosquito species, notably *Aedes aegypti*
76 and *Anopheles* species, where *Wolbachia* has rarely been detected (18–20).

77 *Culex* mosquitoes are vectors of multiple medically significant arboviruses, such as West Nile
78 virus, Japanese encephalitis virus, Usutu virus, Saint Louis encephalitis virus, Western and Eastern
79 equine encephalitis viruses (21–24). In addition to viruses, *Cx* mosquitoes can also transmit
80 nematodes responsible for lymphatic filariasis and protists that cause avian malaria (25,26).

81 Another known role for *Wolbachia* in dipterans is increased resistance to viral replication in flies
82 (27) as well as in transinfected mosquitoes (28,29). In *Culex* mosquitoes, several studies have
83 shown that, except for bidirectional cytoplasmic incompatibility, the impact of *Wolbachia* on life
84 history traits is low or moderate. *Wolbachia*-cured mosquitoes exhibit an increased and a delayed
85 egg production, a minor decrease in lifespan, and a slight decrease in resistance to mosquitocidal
86 bacterial strains (30,31). *Wolbachia* in *Culex* mosquitoes exhibits a complex relationship with
87 insecticide resistance, potentially exploiting hosts with resistance genes by increasing bacterial

88 density, while also differentially affecting susceptibility to various insecticides, as seen with
89 increased deltamethrin sensitivity but neutral effects on DDT resistance (32,33).

90 In insects, the extracellular and intracellular microbiota contribute to essential microelements
91 beyond the host genome's metabolic capacities (34). Mosquitoes, with their life cycle comprising
92 immature aquatic instars and one aerial adult stage, can potentially host a wide spectrum of
93 bacteria. The microbiota plays various roles including larval nutritional support and direct impacts
94 on adult fitness, measured as lifespan and reproduction (35–41). Despite the significant impact of
95 microbiota on mosquitoes, there is a lack of studies on the role of maternally inherited *Wolbachia*
96 in the fitness of *Culex* mosquitoes in the absence of their microbes. It is unclear if *Wolbachia*'s
97 effects might be partially buffered by the microbiota. Our laboratory recently developed a method
98 to produce germ-free *Aedes aegypti* (39). We adapted this approach to *Cx quinquefasciatus* to
99 analyze *Wolbachia*'s role in larval development without other microbiota members.

100 **Results**

101 *Development of an egg sterilization protocol for Culex quinquefasciatus*

102 Mosquito production in sterile conditions requires protocol optimization, taking into account the
103 specificities of mosquito species. While gnotobiotic *Ae. aegypti* (i.e. carrying a defined microbiota
104 composition) have now been reared in several laboratories, we needed to define conditions to rear
105 *Culex* mosquitoes in microbiologically-controlled conditions. To this aim, we used as a starting
106 point a transient colonization method that was previously developed for *Aedes aegypti* in our
107 laboratory, allowing to support larval development via monocolonization with a bacterium that is
108 lost at the time of metamorphosis (39). This consists of a three-step procedure. First, eggs are
109 surface-sterilized, producing germ-free larvae. Second, newly hatched germ-free larvae are reared

110 in the presence of a mutant bacterium, *E. coli* HA416, which is auxotrophic for two amino-acids
111 essential for peptidoglycan biosynthesis. As long as larvae are provided with a special sterile diet
112 supplemented with these amino acids, *E. coli* HA416 (AUX) grows and supports larval
113 development. Third, pupae are transferred to a rearing environment devoid of the specific amino-
114 acids, allowing the emergence of germ-free adults. We decided to adapt this approach for field-
115 collected *Cx quinquefasciatus* egg rafts to produce mosquitoes with no culturable microbiota.

116 *Ae. aegypti* eggs are laid individually and survive for weeks after drying; their hatching is
117 stimulated when they are covered with water, notably due to the lack of oxygen. In contrast, *Cx*
118 *quinquefasciatus* eggs are laid as rafts containing up to several dozens of eggs (Figure 1A), which
119 float on the water surface. These eggs remain wet and close to the water surface throughout their
120 uninterrupted development, limiting laboratory flexibility. When using the *Ae. aegypti* egg-
121 sterilization protocol to surface-sterilize *Cx quinquefasciatus* eggs (Figure 1A), we observed that
122 the egg rafts disaggregated into individual eggs or small clusters, sinking to the bottom of the flask.
123 Almost no larvae hatched in these conditions. We hypothesized that either the eggs needed to
124 remain attached throughout development or the raft's location at the surface of the water was
125 crucial for successful development, possibly due to oxygenation. To investigate the importance of
126 the egg raft, we performed a delicate wash, minimizing raft disintegration while constantly
127 submerging it in sterilizing solutions (sodium hypochlorite and ethanol). Alternatively, egg rafts
128 were thoroughly washed to separate eggs, while a control group remained non-sterile. To maintain
129 egg proximity to the surface, we incubated eggs in a minimal water layer, 1-2 mm deep (3-5 mL
130 in a 25 cm² flask laid horizontally). The next day, only 6.6% of preserved sterilized rafts hatched,
131 compared to 71 % of individualized sterile eggs (Figure 1B-E). Non-sterile eggs exhibited the
132 highest hatching rate (91%).

133 The presence of *Wolbachia* in sterile larvae was confirmed by qPCR, and egg sterility was verified
134 after incubation in liquid Luria Bertani medium (LB) or on LB agar plates. We thus refer to these
135 individuals as germ-free^{Wol+}, indicating they are germ-free except for the presence of the
136 *Wolbachia* endosymbiont. To assess whether *Wolbachia* alone could support larval development,
137 we monitored the development and lifespan of larvae provided sterile conventional food for 10
138 days. Larvae remained in their first instar, and supplementing the diet with a cocktail of B vitamins
139 that are essential for *Cx quinquefasciatus* larval development (42) or with medium for bacterial
140 culture did not improve development (Figure 1F). However, these larvae were still able to develop,
141 as provision of live bacteria efficiently rescued 77% of individuals to adulthood. Approximately
142 80% of sterile larvae survived until day 5, but survival sharply declined in the following days,
143 resulting in around 20% of surviving individuals on day 10 (Figure 1G). We confirmed by qPCR
144 that *Wolbachia* colonization was unaffected by the absence of other bacteria. *Wolbachia* levels
145 remained stable in first instar larvae even after 72 hours, independently of the presence of *E. coli*
146 (Figure 1H).

147 Together, these data indicate that *Wolbachia* alone is insufficient to support larval development in
148 the absence of other bacteria. Furthermore, although *Cx quinquefasciatus* typically thrives in a
149 microbe-rich environment compared to other mosquito larvae, the sole presence of *E. coli* is
150 sufficient to complement its requirements for larval development.

151 *Production of germ-free^{Wol+} Culex quinquefasciatus adults*

152 Having successfully produced germ-free^{Wol+} larvae, we investigated whether the transient
153 colonization approach could yield germ-free^{Wol+} adults. To accomplish this, we added the
154 auxotrophic bacterial culture, using an *E. coli* wild-type as a positive control (Figure 2A).

155 We monitored development daily and found no significant differences in development success
156 rates or timing between larvae monocolonized with *E. coli* wild-type and those on AUX. Larval
157 development success to pupa was slightly higher on AUX (Figure 2B-E). Their sex ratio and adult
158 wing size, used as a readout of the adult size, were also similar (Figure 2F-G). Reanalyzing
159 developmental rates data with those from subsequent experiments, we found that the overall
160 development rate to adulthood in AUX condition increased and the development timing to pupa
161 significantly decreased (pupae: $p < 0.0001$, adults: $p = 0.18$, GLMM with lsmeans), although masked
162 here by replicate variation. Pupae transfer occurred under sterile conditions, and colony forming
163 unit (CFU) tests confirmed that 10-day old gnotobiotic mosquitoes were colonized with *E. coli*
164 while germ-free^{Wol⁺} mosquitoes were not (Figure 2H).

165 *Impact of Wolbachia on development success of germ-free Culex quinquefasciatus mosquitoes*

166 *Wolbachia* alone is insufficient to support larval development. However, previously published
167 *Culex*-derived *Wolbachia* genomes indicate the bacterium can synthesize several B vitamins,
168 including riboflavin, thiamin and pyridoxin, but not folate or biotin (7). Additionally, *Wolbachia*
169 possesses complete purine and pyrimidine biosynthesis pathways. Given that bacteria-derived
170 riboflavin and folate are essential larval development metabolites, and *Wolbachia* provides purine
171 and pyrimidine to fly larvae (39,41,43,44), we wondered if *Wolbachia* could aid larval
172 development under suboptimal *E. coli* conditions. Alternatively, we hypothesized that *Wolbachia*
173 might burden larval development by consuming some nutrients. Thus, we tested the impact of
174 *Wolbachia* load reduction on monocolonized larvae development success.

175 The most widely used method to clear *Wolbachia* in various insects is tetracycline antibiotic, which
176 is also the first-choice treatment for *Culex* mosquitoes (33,45). Our AUX bacterial strain, used for
177 transient colonization, also possesses a tetracycline-resistance cassette in addition to the

178 kanamycin resistance cassette employed in our standardized protocol (46). We thus decided to test
179 the impact of a tetracycline treatment on larvae colonized with AUX to assess larval development
180 success (47). We treated larvae with three tetracycline doses (5µg/mL, 25 µg/mL, or 50µg/mL,
181 see Methods) and confirmed that all concentrations effectively reduce *Wolbachia* load in larvae
182 and adults (Figure 3A-B). Development outcomes were highly concentration-dependent. Treating
183 with 5µg/mL tetracycline significantly increased development success to adulthood, indicating
184 that *Wolbachia* is a burden rather than a nutritional benefit during larval development (Figure 3F-
185 3G). This observation is consistent with the fact that WT-colonized larvae had a lower *Wolbachia*
186 load and a higher development success than their AUX-colonized counterparts (Figure 3A, 3F,
187 3G). Increasing tetracycline concentrations progressively decreased development success,
188 suggesting direct antibiotic toxicity to larvae (Figure 3F, 3G). The tetracycline solution at the
189 highest concentration (50 µg/mL) underwent a noticeable color change from light yellow to
190 brownish after 5-7 days, indicating antibiotic degradation (48). This transformation can potentially
191 harm larvae, as tetracycline and its degradation products have been shown to have toxic effects on
192 various organisms, including algae, at concentrations above 5 mg/mL (49).

193 Development of AUX-colonized larvae and pupae was delayed compared to WT-colonized ones;
194 this was partly rescued in 5 µg/mL tetracycline treated group (Figure 3H-3I). Similarly, AUX-
195 colonized larvae led to a slightly lower proportion of females than WT-colonized ones and 5
196 µg/mL tetracycline-treated ones. As females require more nutrients for development than males,
197 this points to a lower nutritional status in AUX conditions. CFU analysis confirmed that WT-
198 colonized mosquitoes indeed contained live *E. coli*, while the AUX and AUX+TET did not (Figure
199 3E), which matches the loss of bacteria after metamorphosis while not during larval stages (Figure
200 3C-D).

201 Discussion

202 In this study, we established a protocol to produce mosquitoes harboring solely *Wolbachia* and no
203 other microbiota. We demonstrated that *Wolbachia* alone is insufficient to support larval
204 development. Conversely, our data indicate that this endosymbiont negatively impacts mosquito
205 larval development.

206 *Culex* mosquitoes are generally known to grow in “dirty” water, i.e. stagnant bodies of water that
207 are turbid (1,50,51), whereas *Aedes* mosquitoes typically breed in cleaner water or with a moderate
208 amount of organic matter present (52). For instance, we generally set *Aedes* oviposition traps with
209 clear water, while we add chicken manure to attract *Culex*. Hence, one may expect that *Cx*
210 *quinquefasciatus* larvae require more bacteria, and potentially a higher diversity of
211 microorganisms, to grow compared to *Aedes aegypti*. However, we observed that *E. coli* alone was
212 able to rescue larval development of *Cx quinquefasciatus*, as already found for *Ae. aegypti*.
213 Nonetheless, we achieved a slightly lower success rate (~75 %) than what we typically observe
214 with *Ae. aegypti* (~85%), particularly when rearing mosquitoes on AUX *E. coli* (~60%), which
215 tends to be present at lower loads at the end of larval development (39). This suggests that there
216 might still be a difference in the requirement for microbial metabolites between the two mosquito
217 species, which may be quantitative rather than qualitative. Moreover, we observed that *E. coli* is
218 highly efficiently transmitted through metamorphosis in *Cx quinquefasciatus*, as 100% adults
219 reared on WT-*E coli* were positive to the bacterium, compared to only 50% in *Ae. aegypti* (Figure
220 2H and (39)).

221 The eggs of *Culex* mosquitoes are laid as a raft, allowing them to remain on the water surface.
222 Consequently, their upper side is dry and in direct contact with open air. Our rigorous sterilization
223 protocol caused the eggs to sink into the hatching water, and a low hatching rate was observed

224 only when the water layer was very thin and the eggs were individualized. We interpret these
225 results as indicating a high oxygen requirement for *Culex* egg development (53–55).

226 The presence of *Wolbachia* in a high proportion of terrestrial and aquatic insects is due to its ability
227 to manipulate reproduction through cytoplasmic incompatibility, its stability within the insect, and
228 its efficient vertical transmission. Our data corroborate this stability in *Cx quinquefasciatus*, as the
229 antibiotic treatment did not allow complete clearance of *Wolbachia*. In line with these
230 observations, protocols for antibiotic treatments to clear *Wolbachia* generally involve treatment on
231 several generations (45). Beyond its efficient colonization through manipulation of mosquito
232 reproduction, *Wolbachia*'s ability to colonize populations can be enhanced by positively impacting
233 mosquito fitness. Symbionts combining parasitism and mutualism to enhance their population
234 colonization are often termed “Jekyll and Hyde” symbionts (56), in reference to the *Dr Jekyll and*
235 *Mr Hyde* novel, whose main character recurrently shifts between being a criminal and a gentleman.
236 *Wolbachia* has notably been shown to provide biotin and riboflavin to bedbugs (8,12) and
237 planthoppers (13). It has recently been reported as a nutritional symbiont in *Drosophila*, shifting
238 to an energy-save profile during growth and development, and increasing resistance to stress (43).

239 As microbiota-derived B vitamins, especially riboflavin, are crucial for mosquito development
240 (41), we hypothesized that *Wolbachia* might also support larval development in *Culex sp.*, which
241 are heavily colonized by *Wolbachia*. The genome of *Culex*-associated *Wolbachia*, wPip, lacks the
242 biotin operon but is predicted to possess a complete riboflavin biosynthesis pathway (13).
243 However, our data indicate that *Wolbachia* clearance benefits mosquito development, suggesting
244 that *Wolbachia* is detrimental to larval development under our experimental conditions. We
245 hypothesize that *Wolbachia* competes with its host for resources. Our findings align with a
246 previous study showing a non-significant trend towards lower development success of *Wolbachia*-

247 colonized compared to *Wolbachia*-cured *Aedes albopictus* larvae at a medium larval density of 1
248 larva/mL (57).

249 These data indicate that *Wolbachia* is a metabolic parasite during larval development, in addition
250 to its previously characterized role as a reproductive parasite. Nevertheless, it may still be
251 considered a Jekyll and Hyde symbiont, as it can benefit its host inducing resistance to viruses in
252 flies and mosquitoes (58). This protective activity is suggested to enhance *Wolbachia*'s success.
253 Indeed, *Wolbachia*-mediated antiviral protection has been shown to prolong survival in various
254 host-virus combinations, thereby positively impacting host fitness (59). Furthermore, *Wolbachia*
255 has been found to protect *Drosophila* against viral infections at 25°C but not at 18°C, correlating
256 with higher *Wolbachia* colonization in tropical compared to temperate regions (59).

257 Our data further indicates a concentration-dependent toxicity of tetracycline in mosquito larvae.
258 Tetracycline inhibits bacterial protein synthesis via interacting with the 30S ribosomal subunit,
259 inhibiting the binding of aminoacyl-tRNAs (60). While ribosomes of eukaryotes, including in
260 insects, are composed of 60S and 40S subunits and are hence not sensitive to tetracyclines,
261 mitochondrial ribosomes may be targeted by this drug (61). So far, several negative impacts of
262 tetracycline on insects have been reported, yet some may be linked to the loss of the microbiota.
263 A tetracycline treatment to clear *Culex pipiens* of *Wolbachia* using 10-20% solutions resulted in a
264 development success as low as 10-15% (62), yet this strong impact may rather be due to the loss
265 of other microbes, which are essential for mosquito larval development. Some other studies on
266 *Wolbachia* clearance in *Culex* larvae did not report survival rates or indicated no effect on larval
267 development, which may be linked to the presence of tetracycline-resistant bacteria (30,45,47,63).
268 In ticks, tetracycline has been found to reduce the reproductive fitness by downregulating
269 vitellogenesis and embryogenesis, although this effect is likely linked to microbiota depletion (64).

270 Our results obtained in otherwise germ-free conditions, indicate a direct toxic impact of
271 tetracycline on mosquitoes. They are consistent with a previous report of long-lasting impact of
272 tetracycline on mitochondrial metabolism (65).

273 Beyond arthropods, tetracycline has been documented to cause DNA damage, metabolic
274 alterations, and oxidative stress in earthworms (66). In zebrafish embryos, exposure to this
275 antibiotic also leads to developmental delay, reactive oxygen species (ROS) production, and cell
276 death (67). In common freshwater green algae, the active form (tetracycline) and most common
277 degraded forms (anhydrotetracycline and epitetracycline) inhibited the growth of *Chlamydomonas*
278 *reinhardtii*, with tetracycline exhibiting the highest toxicity (49). We observed a brown staining
279 after a few days in our larval breeding water, indicating that it becomes oxidized over time. It
280 remains unclear whether the toxicity observed in our larvae can be attributed to tetracycline or to
281 its oxidized product.

282 **Conclusions**

283 While *Wolbachia* studies related to the impact in fitness in mosquitoes are sometimes hindered by
284 the fact that antibiotic treatments affect the insect microbiota, we managed to set up a method to
285 investigate the role of *Wolbachia* in *Culex* mosquitoes in the absence of other microbiota members.
286 We observed that *Wolbachia* is not able to support larval development on its own. This is
287 consistent with the absence of the biotin operon reported in *Wolbachia pipiens*, contrary to strains
288 found in bedbugs and planthoppers. Our results indicate that *Wolbachia* has a parasitic impact on
289 larval development, hence point that reproductive parasitism in itself is sufficient to support the
290 strong colonization capacity of *Wolbachia* in *Cx quinquefasciatus* populations.

291 **Materials and Methods**

292 *Culex quinquefasciatus* mosquitoes

293 Up to three breeding sites were placed inside the campus of the Institut Pasteur de la Guyane in
294 Cayenne, French Guiana (GPS coordinates: 4.943100, -52.325350) for collecting egg rafts. They
295 consisted in containers containing tap water, soil and chicken manure, as this organic matter-
296 enriched water attracts wild gravid *Cx quinquefasciatus* females to lay eggs. Each breeding site
297 was checked daily, covered with a net on weekends and changed every 14 days to avoid potential
298 escaping mosquitoes from uncollected larvae. Mosquito species were confirmed using a taxonomy
299 key at the beginning of the project (68,69). As this species is by far the commonest species in
300 urban areas in French Guiana (69), further checks were not performed in subsequent experiments,
301 but no variations in the shape of egg rafts, larvae or adults were noticed during stereoscopic
302 observations.

303 *Sterilization protocol and bacterial inoculum*

304 Egg rafts were recovered from the breeding site bucket and transported to the lab, a pre-wash with
305 tap water was made to remove organic matter from the liquid. Under the biosafety cabinet rafts
306 were sterilized following lab protocol (39). Sterile eggs were kept in sterile conditions and placed
307 in a 25 cm² culture flask with a minimal water layer (~3-5 mL depending on the number of eggs)
308 in horizontal position, a sterility control test in a petri dish with LB agar was tested each experiment
309 and kept until 48 h to verify sterility.

310 After egg rafts sterilization protocol, 24 h post-hatched first instar larvae were individually placed
311 into 24-wells plates with 1.5 mL of *E. coli* HA416 supplemented with D-Alanine (50 µg/mL) and
312 acid meso-diaminopimelic (12.5 µg/mL), with one drop (0.05-0.1 mL) of sterile 5 % (w/v)

313 TetraMin baby™ fish food solution. Plates were maintained in a climatic chamber at 28 °C with a
314 light-dark cycle 12 h/12 h with 80 % relative humidity.

315 For a control rearing, *E. coli* HS (wild-type, WT) was used in the same culture conditions as the
316 mutant bacteria.

317 Sterile larvae survival, pupation and development success tests, were recorded daily during 14
318 days. Hatching rates were counted under microscope at 24 h after sterilization.

319 *Antibiotic treatment during transient colonization rearing*

320 *E. coli* HA416 contains a tetracycline resistance cassette, a parallel treatment with the antibiotic to
321 remove *Wolbachia* was viable without hindering bacterial culture added to rear axenic mosquitoes.

322 An initial dose of 5, 25 and 50 µg/mL was added to each larva, and then at day 3 and 5, 25 and 50
323 µg/mL were added again to sustain a level on the antibiotic concentration, also at day 2 and 4
324 bacterial culture was added as well in “AUX” treatment (33,45).

325 *Wolbachia detection*

326 For detection of *Wolbachia*, individual female and male mosquitoes were placed in screwcaps
327 tubes with crystal beads and frozen at -80 °C until use. Samples were processed for DNA extraction
328 using the MagBio HighPrep Blood and Tissue DNA kit. Tissue lysis was performed with a
329 Precellys Evolution bead beater homogenizer (Berting Technologies) following manufacturer’s
330 conditions.

331 Protein digestion was completed with Proteinase K incubation overnight at 55 °C. Automatic
332 DNA extraction was accomplished with the kit in a KingFisher Duo Prime system (Thermo
333 Scientific). DNA was eluted in nuclease-free water and saved at -80 °C until use. Genomic DNA
334 was diluted to 25 ng/µL using a Nanodrop spectrophotometer (Thermo Scientific) for qPCR test.

335 Quantitative PCR test was performed in a Roche 480 Lightcycler with a 2x SYBR-green master
336 mix using standard protocol instructions (Takara). Total *Wolbachia* density was analyzed by
337 relative quantification of the *Wolbachia surface protein* against the mosquito *homothorax* gene.
338 *Wolbachia* primers, *wsp* (Fwd) 5' - ATCTTTTATAGCTGGTGGTGGT - 3', *wsp* (Rev) 5' -
339 AAAGTCCCTCAACATCAACCC -3'; housekeeping *hth* (Fwd) 5'-
340 TGGTCCTATATTGGCGAGCTA - 3', *hth* (Rev) 5'-TCGTTTTTGCAAGAAGGTCA - 3' (Ant
341 et al., 2020).

342 *Statistical analysis*

343 Egg rafts and individual first instar larvae were photographed with a stereomicroscope.
344 Numerical observation data was collected and organized in excel sheets, statistical analysis was
345 performed in R studio 2023.06.2+561 under R 4.3.1. Survival curve was made using survival and
346 survminer packages. Multicomparisons were performed using generalized mixing models (GLMM)
347 with the packages lsmeans (70), and glmer (71).
348 Bar and scatter plots were made using GraphPad Prism 10.3.1 for Mac.

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549

550 **Figure legends**

551

552 **Figure 1. *Culex quinquefasciatus* germ-free^{Wol+} larvae hatch but do not develop without**
553 **extracellular bacteria.**

554 **A.** Egg rafts collected from a local breeding site at time=0 h, just after collection. **B-D.** Aspect of
555 the eggs 24 h after collection and treatment, whether they were not sterilized (B), sterilized with a
556 harsh washing procedure to disrupt the rafts into individualized eggs (C) or following a delicate

557 washing procedure, so as to preserve rafts (D). E. Hatching rate of the eggs imaged in B-D. NS vs
558 SI $p < 0.0001$, SI vs SR $p < 0.0001$, NS vs SR $p < 0.0001$. GLMM fit by maximum likelihood,
559 binomial distribution, pairwise comparisons with Bonferroni adjustment. F. Development success
560 of larvae on sterile diets compared to larvae monocolonized with *E. coli*. B_vit vs LB $p = 1.0$, B vit
561 vs Water $p = 1.0$, B vit vs *E. coli* WT $p < 0.0001$, LB vs Water $p = 1.0$, LB vs *E. coli* WT $p < 0.0001$,
562 Water vs *E. coli* WT $p < 0.0001$. Type III ANOVA with Satterthwaite's method and pairwise
563 comparisons with Bonferroni adjustment. Dev: developed to adults by day 14; Non dev: stalled in
564 development or dead. G. Survival curve of sterile larvae provided with autoclaved Tetramin baby
565 fish food. H. qPCR quantification of the load of *Wolbachia* in germ-free^{Wol+} and *E. coli* colonized
566 larvae. No difference in water vs *E. coli* $p = 0.77$. Type III ANOVA with Satterthwaite's method
567 and pairwise comparisons with Tukey's adjustment. NS – Non-sterile, SI – Sterile individuals,
568 SR – Sterile rafts, B vit – B vitamin cocktail defined in (42), LB – lysogeny broth. In 1A-1D,
569 representative images from 6 independent replicates. 1E, 1F, 1H; average \pm SEM from 6, 3 and 2
570 independent replicates, respectively; 1G: average of 4 independent replicates.

571

572 **Figure 2. Transient colonization allows to produce germ-free^{Wol+} adults.** A. Experimental
573 design for rearing germ-free^{Wol+} or monocolonized^{Wol+} larvae via transient colonization. B.
574 Percentage of pupation success between groups with wild-type and auxotrophic bacteria. AUX vs
575 WT $p < 0.0001$. GLMM fit by maximum likelihood with binomial distribution and pairwise
576 comparison. C. Percentage of developmental success. AUX vs WT $p = 0.1432$. GLMM fit by
577 maximum likelihood with binomial distribution and pairwise comparison. D-E. Duration of larval
578 development to pupae (D, AUX vs WT $p < 0.0001$) and metamorphosis (E, AUX vs WT $p = 0.1767$)
579 in days. Type III ANOVA with Satterthwaite's method and pairwise comparisons. F. Sex ratio in

580 emerged adults' post-treatment, AUX vs WT $p=0.1591$. GLMM fit by maximum likelihood with
581 binomial distribution and pairwise comparison. **G.** Wing size. AUX vs WT $p=1.0$ Type III
582 ANOVA with Satterthwaite's method and pairwise comparison. **H.** Colony forming units per
583 mosquito. AUX vs WT $p<0.0001$. Type III ANOVA with Satterthwaite's method and pairwise
584 comparison. WT - Reared with wild-type *E. coli*. AUX – Reared with auxotrophic *E. coli*. In 2B-
585 F: average \pm SEM of 6 independent replicates. 2G-H: average \pm SEM of 3 independent replicates.

586

587

588 **Figure 3. *Wolbachia* negatively impacts development success.** **A.** *Wolbachia* load in larvae
589 pools during larval development. AUX vs AUX+TET25 $p<0.0001$, AUX vs AUX+TET5
590 $p=0.0002$, AUX vs WT $p=0.0433$, AUX+TET25 vs AUX+TET5 $p=1.0$, AUX+TET25 vs WT
591 $p=0.0944$, AUX+TET5 vs WT $p=0.1790$. LMM fit by REML and pairwise comparisons with
592 Bonferroni adjustment. **B.** *Wolbachia* load in adult mosquitoes. AUX vs AUX+TET25 $p<0.0001$,
593 AUX vs AUX+TET5 $p<0.0001$, AUX vs AUX+TET50 $p=0.0111$, AUX vs WT $p=0.03653$,
594 AUX+TET25 vs AUX+TET5 $p=1.0$, AUX+TET25 vs AUX+TET50 $p=1.0$, AUX+TET25 vs WT
595 $p=0.0047$, AUX+TET5 vs WT $p=0.0221$, AUX+TET50 vs WT $p=0.6282$. LMM fit by REML and
596 pairwise comparisons with Bonferroni adjustment. **C.** Bacterial decolonization in larvae. AUX vs
597 AUX+TET25 $p=1.0$, AUX vs AUX+TET5 $p=1.0$, AUX vs WT $p=1.0$, AUX+TET25 vs
598 AUX+TET5 $p=1.0$, AUX+TET25 vs WT $p=1.0$, AUX+TET5 vs WT $p=1.0$. LMM fit by REML
599 and pairwise comparisons with Bonferroni adjustment. **D.** Bacterial decolonization in mosquitoes.
600 AUX vs AUX+TET25 $p=1.0$, AUX vs AUX+TET5 $p=1.0$, AUX vs WT $p=0.0012$, AUX+TET25
601 vs AUX+TET5 $p=1.0$, AUX+TET25 vs WT $p=0.0012$, AUX+TET5 vs WT $p=0.0012$. LMM fit
602 by REML and pairwise comparisons with Bonferroni adjustment. **E.** Bacterial decolonization

603 measured with CFU test. AUX vs AUX+TET5 $p=1.0$, AUX vs WT $p<0.0001$, AUX+TET5 vs WT
604 $p<0.0001$. Type III ANOVA with Satterthwaite's method and pairwise comparison with
605 Bonferroni adjustment. **F.** Pupation ratio success in treated larvae with different tetracycline doses.
606 AUX vs AUX+TET25 $p=0.0007$, AUX vs AUX+TET5 $p<0.0001$, AUX vs AUX+TET50
607 $p=0.5103$, AUX vs WT $p<0.0001$, AUX+TET25 vs AUX+TET5 $p<0.0001$, AUX+TET25 vs
608 AUX+TET50 $p=1.0$, AUX+TET25 vs WT $p<0.0001$, AUX+TET5 vs AUX+TET50 $p<0.0001$,
609 AUX+TET5 vs WT $p=1.0$, AUX+TET50 vs WT $p<0.0001$. GLMM fit by maximum likelihood
610 and pairwise comparison with Bonferroni adjustment. **G.** Adult metamorphosis success in treated
611 groups with different tetracycline doses. AUX vs AUX+TET25 $p=0.0006$, AUX vs AUX+TET5
612 $p<0.0001$, AUX vs AUX+TET50 $p<0.0001$, AUX vs WT $p<0.0001$, AUX+TET25 vs AUX+TET5
613 $p<0.0001$, AUX+TET25 vs AUX+TET50 $p=1.0$, AUX+TET25 vs WT $p<0.0001$, AUX+TET5 vs
614 AUX+TET50 $p<0.0001$, AUX+TET5 vs WT $p=1.0$, AUX+TET50 vs WT $p<0.0001$. GLMM fit
615 by maximum likelihood and pairwise comparison with Bonferroni adjustment. **H.** Duration of
616 larval development until pupation. AUX vs AUX+TET25 $p=0.0003$, AUX vs AUX+TET5
617 $p<0.0001$, AUX vs WT $p<0.0001$, AUX+TET25 vs AUX+TET5 $p<0.0001$, AUX+TET25 vs WT
618 $p<0.0001$, AUX+TET5 vs WT $p<0.0001$. Type III ANOVA with Satterthwaite's method and
619 pairwise comparison with Bonferroni adjustment. **I.** Duration of metamorphosis from pupae to
620 adults. AUX vs AUX+TET25 $p=0.0003$, AUX vs AUX+TET5 $p<0.0001$, AUX vs WT $p<0.0001$,
621 AUX+TET25 vs AUX+TET5 $p<0.0001$, AUX+TET25 vs WT $p<0.0001$, AUX+TET5 vs WT
622 $p<0.0001$. Type III ANOVA with Satterthwaite's method and pairwise comparison with
623 Bonferroni adjustment. **J.** Sex ratio in treated groups. AUX vs AUX+TET25 $p=1.0$, AUX vs
624 AUX+TET5 $p=1.0$, AUX vs WT $p=0.0278$, AUX+TET25 vs AUX+TET5 $p=0.6009$,
625 AUX+TET25 vs WT $p=0.0630$, AUX+TET5 vs WT $p=1.0$. GLMM fit by maximum likelihood

626 and pairwise comparison with Bonferroni adjustment. Average \pm SEM of 3 (3A, 3E), 4 (3B, 3C)
627 and 2 (3D), independent replicates. In 3F-3J, average \pm SEM of 9 independent replicates (12
628 replicates), treated groups independent replicates were 4 in AUX+TET5 and AUX+TET25
629 conditions, and 3 for AUX+TET50 (F-J).

630

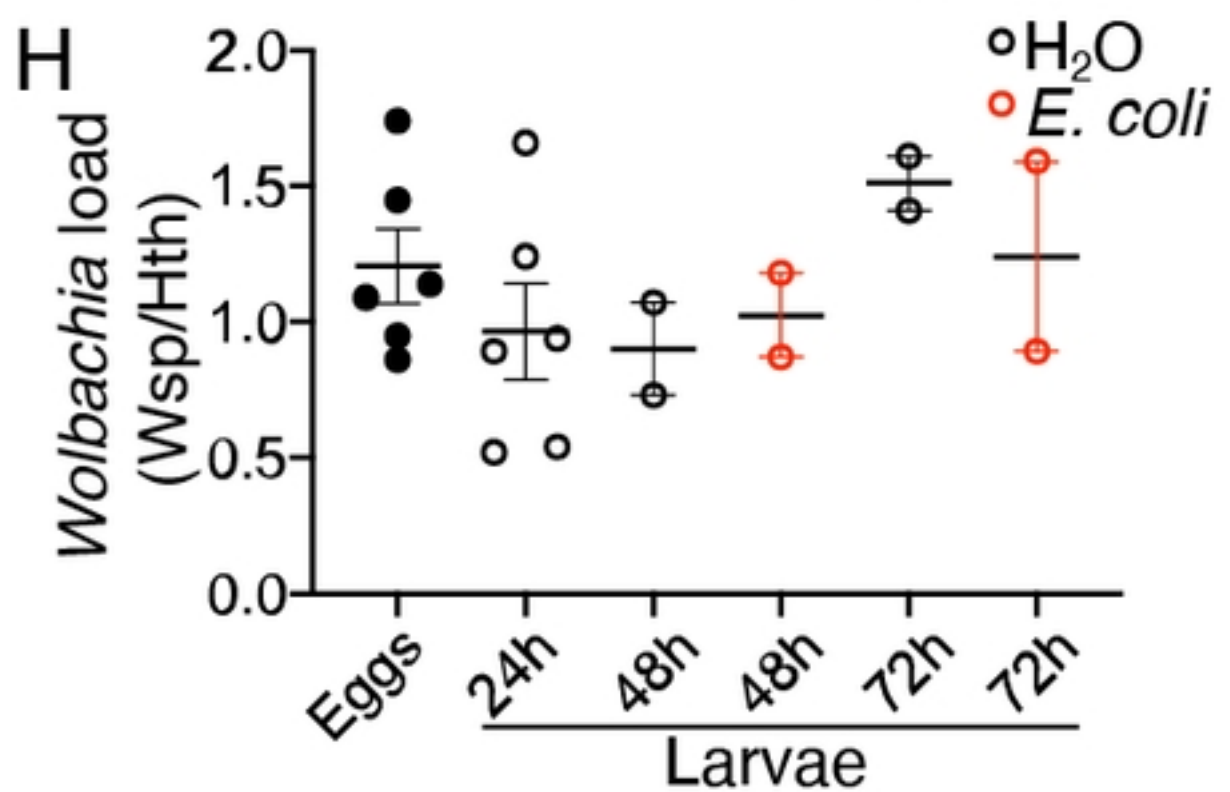
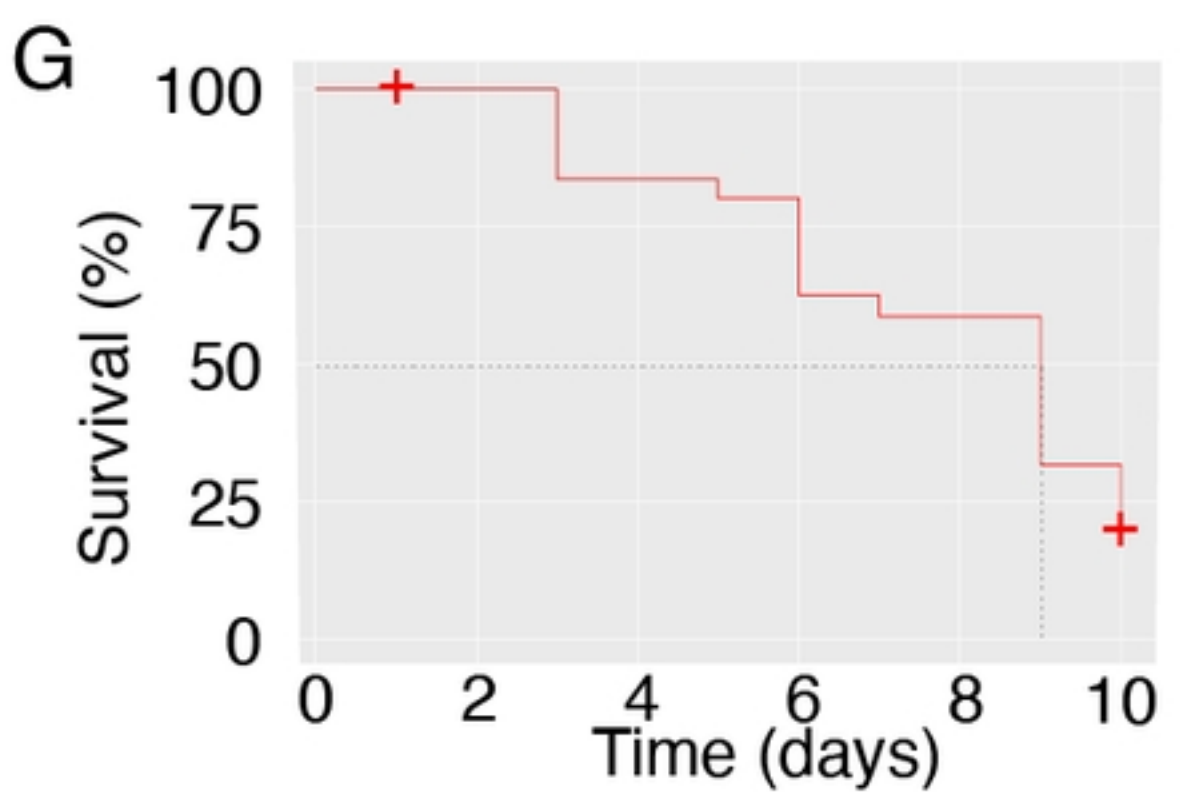
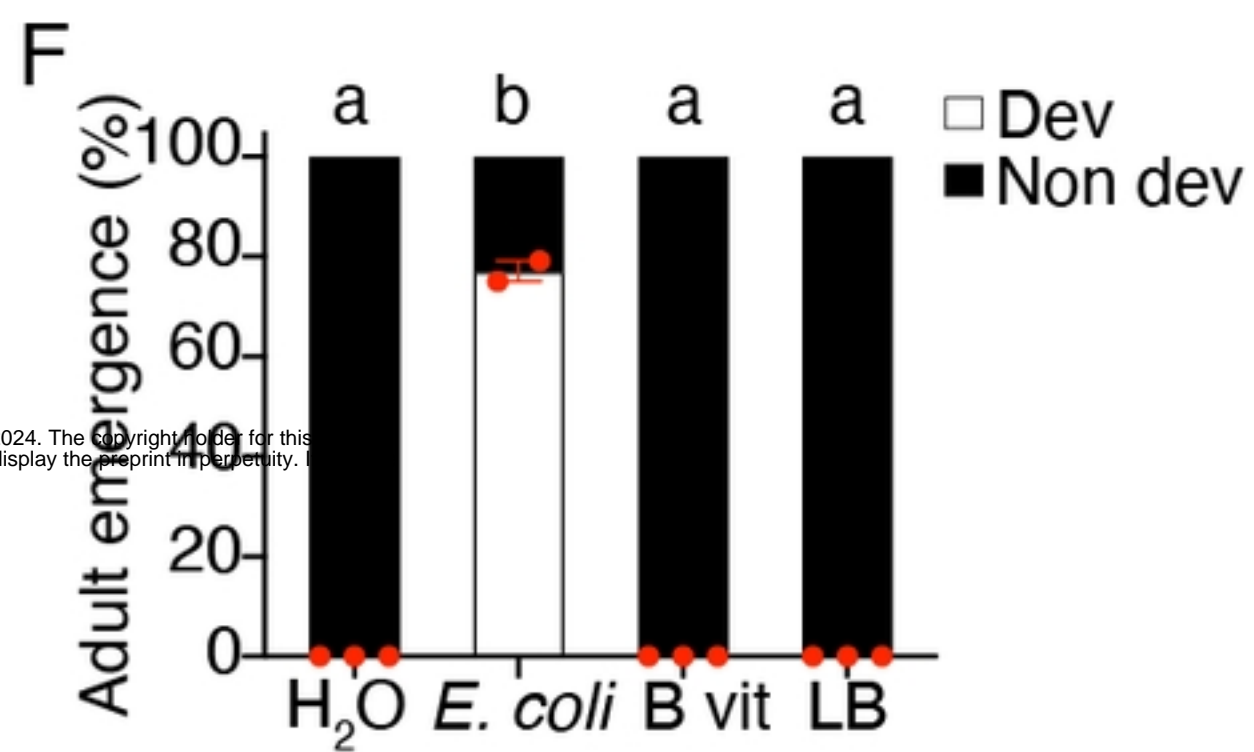
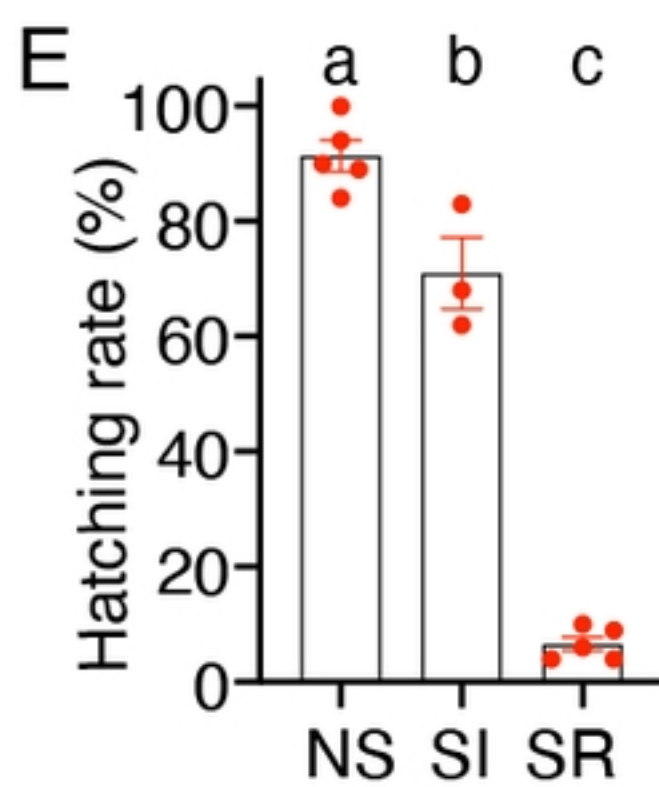
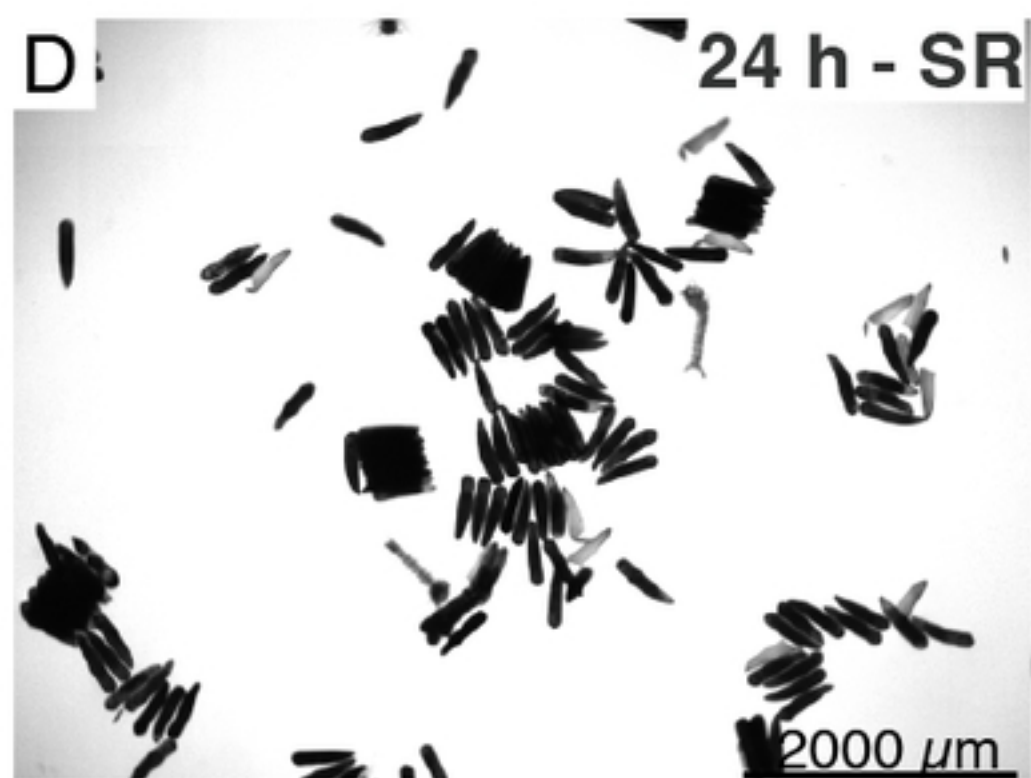
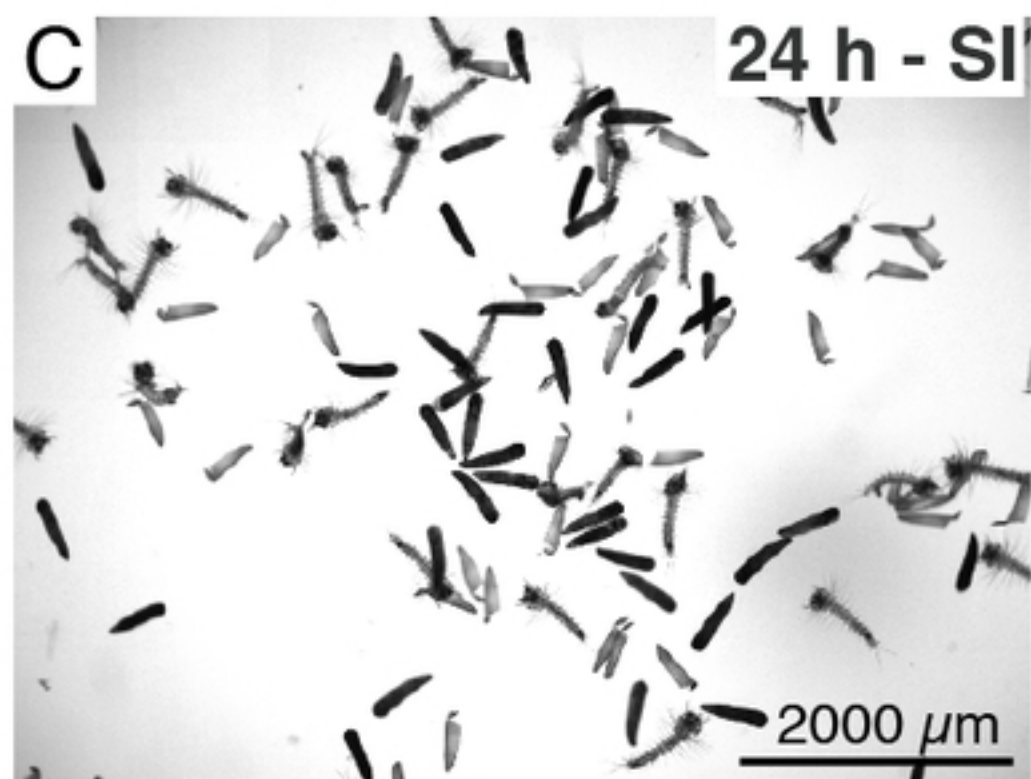
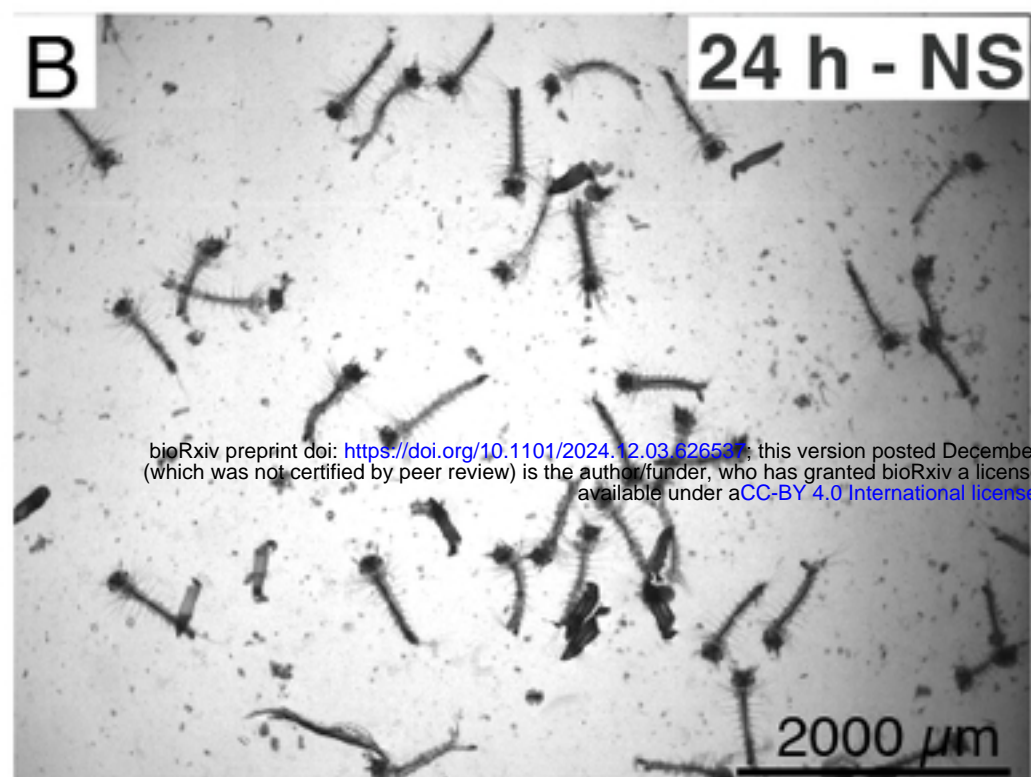
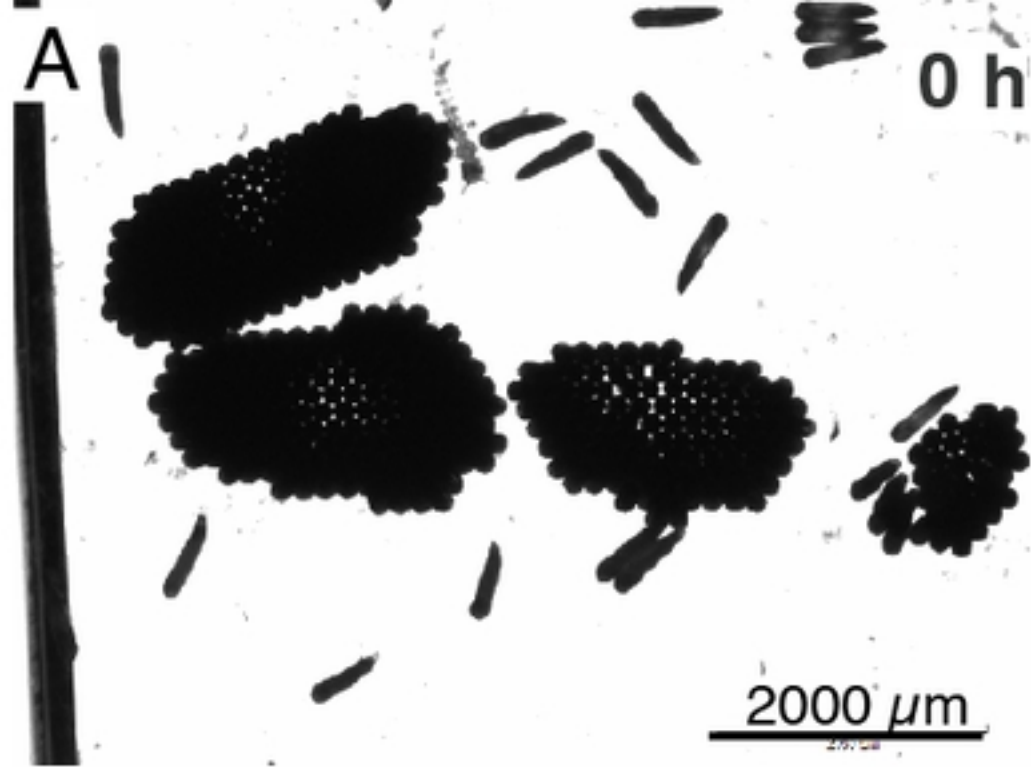


Figure 1

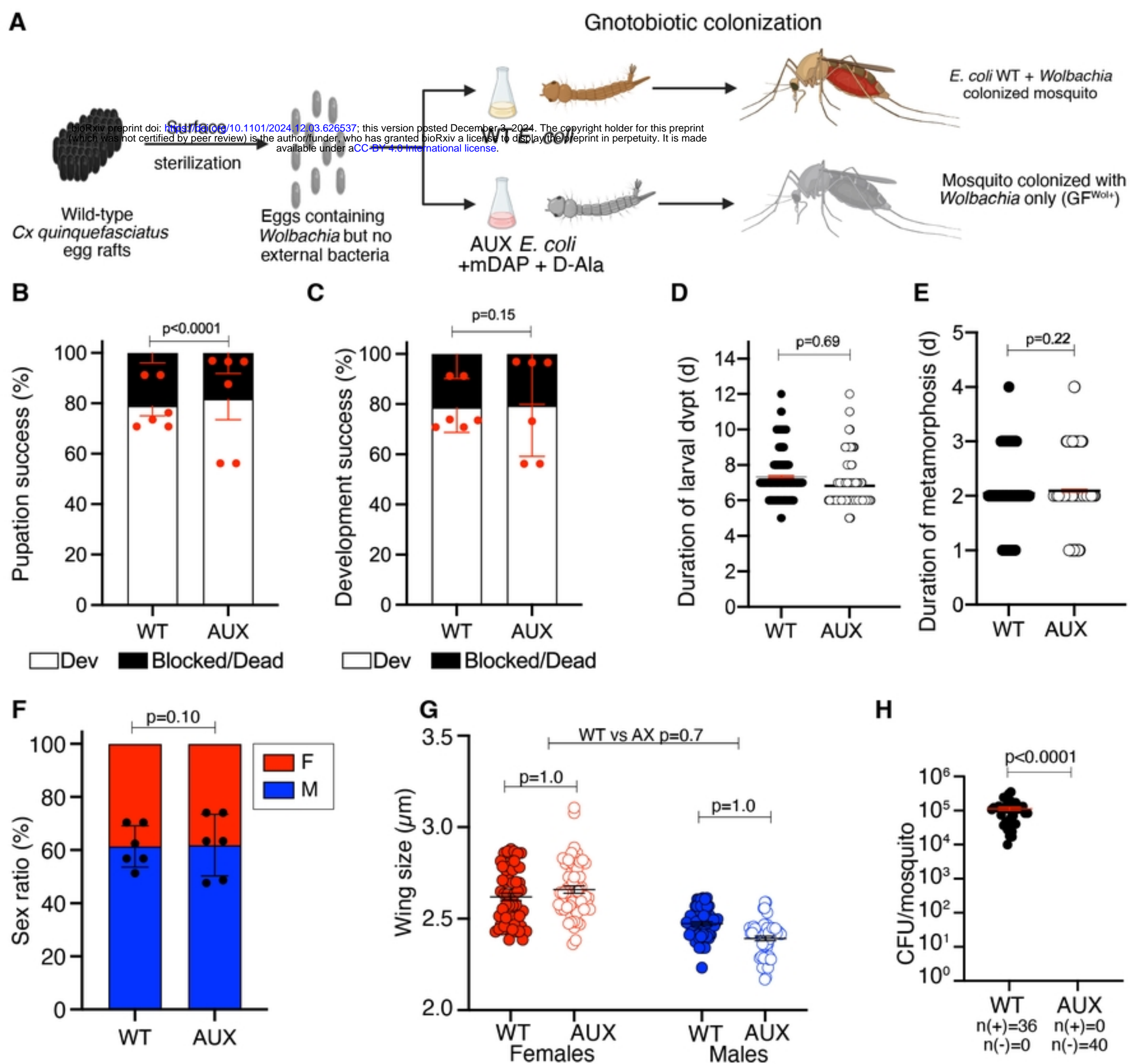


Figure 2

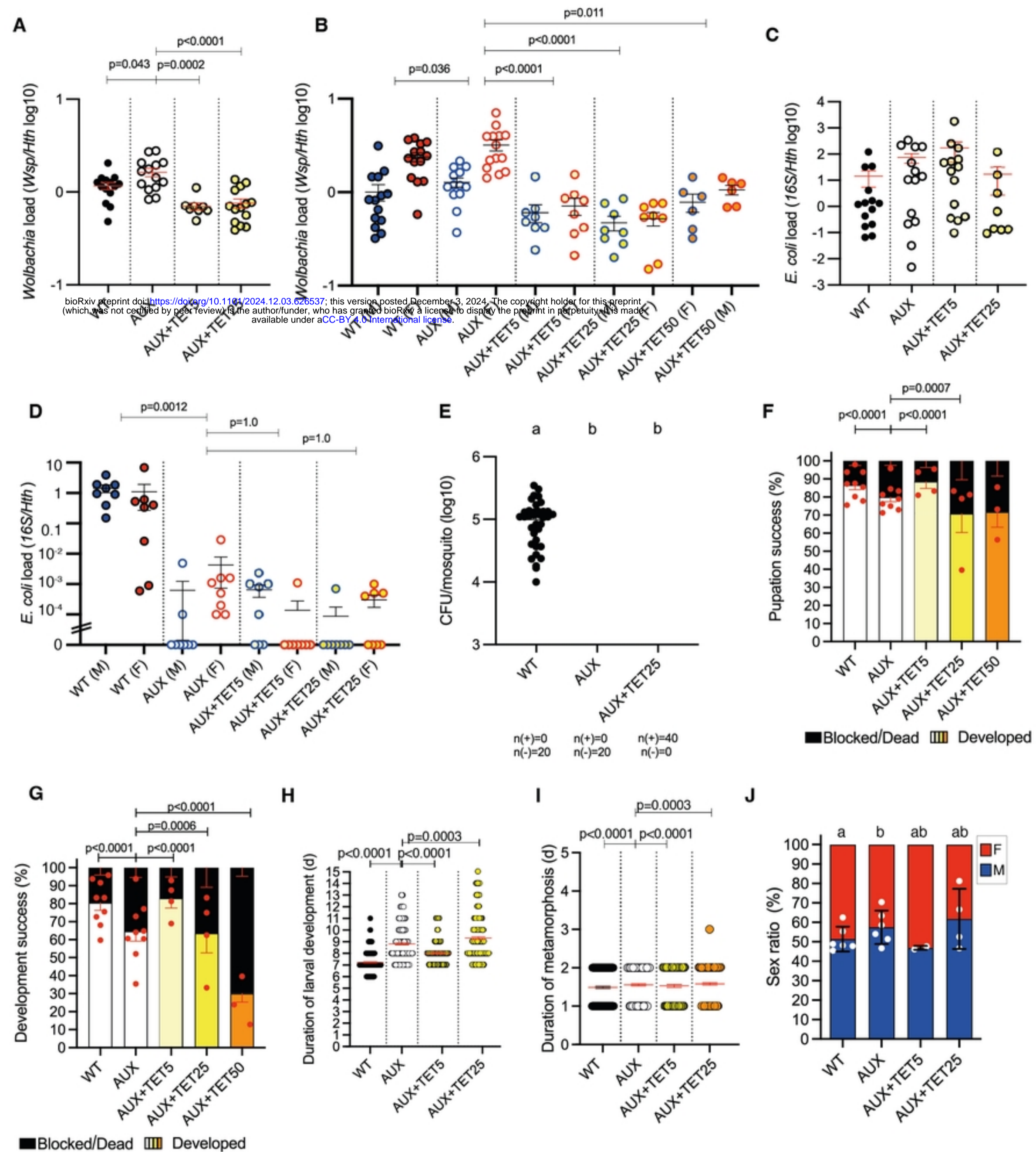


Figure 3