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5 **Axenic *Aedes aegypti* develop without live bacteria, but exhibit delayed development and**  
6 **reduced oviposition.**

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22 **Abstract**

23           The mosquito gut microbiome plays an important role in mosquito development and fitness,  
24 providing a promising avenue for novel mosquito control strategies. Here we present a method for rearing  
25 axenic (bacteria free) *Aedes aegypti* mosquitoes, which will greatly facilitate mechanistic studies  
26 documenting the structure and function of the microbiome. Through feeding sterilized larvae agar plugs  
27 containing attenuated *Escherichia coli*, mosquito development was observed in the absence of living  
28 bacteria. Axenic larvae were capable of full development into adults, which laid eggs that were  
29 subsequently hatched. However, axenic mosquitoes exhibited delayed development time and reduced egg  
30 clutch size in comparison to bacterially colonized mosquitoes. These findings suggest that mosquito  
31 development is not dependent on live bacteria, but their phenotype is modulated by the presence of  
32 microorganisms. This axenic system offers a new tool in which the mosquito microbiome can be  
33 systematically manipulated for a deeper understanding of microbiome host interactions.

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35

## 36 **Introduction**

37           It is increasingly clear that most, if not all, multicellular organisms live in association  
38 with a complex assemblage of microorganisms (i.e. microbiome) composed of bacteria, viruses,  
39 fungi, and archaea. While these communities can be found in every habitable organ, for most  
40 complex organisms the vast majority of microbes reside in the digestive tract. Because of the  
41 biomass and complexity of the indigenous gut microbiome, as well as its close association with  
42 the host, it is frequently considered an additional major organ<sup>1,2</sup>. Consequently, the influence of  
43 microbiota on host biology has garnered considerable attention<sup>3,4</sup>. These studies have revealed a  
44 link between the microbiome and a wide array of disease states in mammals, including obesity<sup>5</sup>,  
45 diabetes<sup>6</sup>, and autism<sup>7,8</sup>. Furthermore, the microbiome has been implicated in playing a  
46 significant role in the development and function of the immune system and autoimmune  
47 disorders<sup>9,10</sup>.

48           Invertebrates also harbor a diverse microbiome<sup>11,12</sup> that has been linked to a number of  
49 phenotypic outcomes, such as host-mating preference<sup>13</sup> and embryonic development<sup>14</sup>. It is clear  
50 from these studies that the microbiome can have profound effects on host physiology and health.  
51 Mosquitoes are important disease vectors for a number of human pathogens that include  
52 arboviruses, protozoa, and nematodes that pose a significant public health threat. Due to the lack  
53 of an effective vaccine for many of these pathogens and an increase in insecticide resistance in  
54 mosquitoes, the development and implementation of novel mosquito control strategies will be  
55 necessary to curtail their public health impact. The mosquito microbiome is emerging as a  
56 potential tool in this effort<sup>15</sup>. A number of descriptive studies, primarily focused on the bacterial  
57 components of the microbiome, have determined that it is relatively simple, typically composed  
58 of 10-70 bacterial strains, the majority of which are members of the phylum Proteobacteria,

59 specifically the family *Enterobacteriaceae*<sup>16,17</sup>. Based on the similarity in composition between  
60 the microbiome of mosquito larvae and the water they inhabit, it has been proposed that  
61 mosquitoes largely acquire their gut microbiota from the aquatic environment<sup>18</sup>. Further evidence  
62 suggests that at least some of the larval microbiome is transstadially transmitted to the adult after  
63 pupation<sup>19</sup>. While these studies demonstrate that environmental microorganisms readily colonize  
64 mosquitoes and these associations can be stable over the entire lifespan, the role these microbes  
65 play in mosquito development and biology is less clear.

66       Most mosquito-borne pathogens must infect or pass through the mosquito midgut prior to  
67 being transmitted. Not surprisingly, the complex interplay between pathogens, the mosquito  
68 midgut, and its associated microbiome have garnered considerable attention. For instance, it is  
69 known that bacterial load and/or microbiome community composition can significantly affect  
70 *Anopheles spp.* mosquito susceptibility to *Plasmodium* infection<sup>20,21</sup> and *Aedes aegypti*  
71 susceptibility to dengue virus is influenced by the intestinal microflora<sup>22,23</sup>. Microbiota have  
72 differing effects on vector competence in mosquitoes, with particular isolates either positively or  
73 negatively influencing mosquito infection rates depending on the species or bacterial strain<sup>22,24–</sup>  
74 <sup>26</sup>. Taken together, these observations demonstrate that the composition and structure of the  
75 microbiome can affect the ability of mosquitoes to acquire and transmit disease. Yet, because no  
76 current method exists to systematically manipulate the microbiome these studies are by  
77 definition correlational in nature. Furthermore, it is difficult to determine the impact of the  
78 microbiota on mosquito-pathogen interactions because many of these studies have relied upon  
79 antibiotic “clearance” of the bacterial communities. Recent reports show that the mosquito  
80 microbiome often contains antibiotic resistant bacteria<sup>27</sup>, antibiotics do not fully clear the gut  
81 microbiota, but rather cause a dysbiosis<sup>28</sup>, and extended use of antibiotics can cause toxicity and

82 mitochondrial dysfunction<sup>29</sup>. Consequently, these models cannot be truly considered “bacteria  
83 free” and do not address possible interactive effects between bacterial reduction and antibiotic  
84 exposure on mosquito biology and vector competence.

85 Systematic manipulation of the mosquito microbiome would be greatly facilitated by the  
86 existence of a microbiome-free, or axenic, mosquito<sup>30</sup>. Furthermore, the development of an  
87 axenic model could act as a blank template on which a microbiome of known composition could  
88 be imprinted, also known as a *gnotobiotic* organism<sup>31,32</sup>. Axenic rearing techniques have already  
89 been developed for a number of model organisms, including *Drosophila melanogaster*,  
90 *Caenorhabditis elegans*, and mice<sup>30</sup>. Early attempts to rear axenic mosquitoes reportedly  
91 obtained adults free from bacteria using a growth media of essential vitamins and nutrients<sup>33</sup>.  
92 However, these studies lacked the modern molecular based techniques that can detect  
93 microorganisms that are recalcitrant to laboratory cultivation; as is frequently cited, the majority  
94 of microorganisms in nature are unculturable<sup>34</sup>. In this regard, there is some uncertainty as to  
95 whether these mosquitoes were truly axenic. In fact, a series of recent studies have reported that  
96 mosquitoes require a live bacterial symbiont for development<sup>27,35,36</sup>. Yet the studies describing  
97 the necessity of live bacteria generally ignored the role of microflora in supplying essential  
98 nutrients to the host. Thus, there is somewhat of a contradiction in the literature; either  
99 mosquitoes are unique from *Drosophila* and *C. elegans* in requiring a live bacterial symbiont for  
100 development, or nutritional conditions sufficient to rear axenic mosquitoes have yet to be  
101 documented.

102 In this study, we tested a variety of reported methods and developed novel practices for  
103 the rearing of *Aedes aegypti* mosquitoes free of living bacteria. A means to rear axenic  
104 mosquitoes was achieved by hatching larvae from surface sterilized eggs fed on agar plugs

105 containing sonicated and heat-inactivated bacteria. Biometric comparisons revealed that  
106 developmental times differed between axenic mosquitoes and gnotobiotic mosquitoes colonized  
107 by *Escherichia coli*. Axenic mosquitoes also had a significant reduction in egg clutch size  
108 compared to their microbiome-colonized cohorts. The data presented here represents a  
109 methodological advancement in the field of mosquito microbiome research and provides a much-  
110 needed tool to elucidate the role of microbiota in mosquito physiology and pathogen  
111 susceptibility. Furthermore, our results challenge our current understanding of the interaction  
112 between the microbiome and mosquito development.

113

## 114 **Results**

### 115 *Testing mosquito diets to support axenic mosquito growth*

116 In order to define the nutritional requirements needed to support mosquito development,  
117 we tested multiple mosquito diets. Mosquito diets free of living bacteria generally resulted in  
118 widespread mortality or stalled larval development (Table 1). In contrast, the majority of  
119 gnotobiotic larvae (colonized by *E. coli* strain K12) survived to adulthood. Yet, supplementing  
120 the mosquito diet with heat killed or sonicated *E. coli* cells in the medium failed to rescue  
121 development (Table 1). Furthermore, the highest concentrations of heat-killed or sonicated *E.*  
122 *coli* cells resulted in larval mortality. A mixture of amino acids and vitamins commonly used for  
123 cell culture were also assessed for their ability to support larval growth. Larvae failed to develop  
124 at low concentrations of the added nutrients, whereas the mixture was lethal to the larvae at high  
125 concentrations (Table 1). To ascertain if the ability to rescue larval development was unique to  
126 bacteria, we inoculated the standard larval diet with 100  $\mu$ l of an active baker's yeast culture and

127 found that this too rescued development. However, solutions of 1% or 10% sterilized yeast  
128 extract did not rescue development, with 10% yeast extract causing larval mortality. These data  
129 suggest that both live bacteria and fungi are both capable of rescuing larval development. Yet a  
130 common observation across the different diets was that in high concentrations many of the  
131 supplements were toxic to the larvae (Table 1), suggesting mosquito larvae are sensitive to high  
132 concentrations of particular compounds in their environment.

133 A notable observation from the above experiments was that while gnotobiotic mosquitoes  
134 colonized by *E. coli* were generally capable of development, *E. coli* itself did not grow well in  
135 the larval media. Thus, we hypothesized that the majority of the *E. coli* were maintained inside  
136 the mosquito midgut rather than in the external environment. This observation, coupled with  
137 prior knowledge that axenic *Drosophila* larvae are reared on a solid-state cornmeal agar, which  
138 allows for the direct consumption of a highly nutrient-rich diet<sup>37</sup> led us to develop a mosquito  
139 diet with a high concentration of bacterial components embedded in an agar plug. This was the  
140 only diet able to support the development of mosquitoes in the absence of live bacteria. Sterility  
141 of the resulting larvae and adult mosquitoes was confirmed by both culture-dependent and  
142 independent methods (PCR of 16S rRNA genes, see methods) (Figure 1).

143

#### 144 *Development of axenic, gnotobiotic, and CR mosquitoes*

145 Having generated axenic mosquitoes and confirmed their sterility, we assessed if there  
146 were developmental effects associated with the axenic state by comparing axenic mosquitoes  
147 with gnotobiotic and conventionally reared mosquitoes (CR). The gnotobiotic group consisted of  
148 sterilized larvae colonized by *E. coli* and the CR group was sterilized larvae colonized by

149 bacteria from a conventionally reared mosquito colony (see methods below). Out of the three  
150 conditions, the axenic group exhibited the lowest rates of larval mortality (average:  
151  $1.85\% \pm 1.85\%$ ) within the fourteen-day experimental period, while the CR group exhibited the  
152 highest rates of mortality (average:  $61.1\% \pm 11.1\%$ ). Axenic mosquitoes also exhibited a  
153 significant delay in time to pupation when compared to their gnotobiotic counterparts ( $p < 0.0001$ ;  
154 Figure 2). On average, axenic larvae pupated approximately three days after the gnotobiotic  
155 larvae. There was no significant difference in pupation time between the axenic and CR groups.

156         The relatively rapid development of the gnotobiotic group is notable because *E. coli* K-12  
157 is a laboratory-adapted bacterium that carries mutations in lipopolysaccharide and amino acid  
158 synthesis, making it a poor colonizer of most hosts<sup>38</sup>. *E. coli* K-12 is therefore unlikely to be a  
159 normal member of the mosquito microbiome. Yet, we observed faster larval growth in the  
160 presence of a mono-culture of *E. coli* than in the normal microflora of laboratory-reared *Ae.*  
161 *aegypti* (Figure 2). This raises interesting questions regarding the interactions between the  
162 diversity and composition of the host's microbiome and the host's phenotype. However, the high  
163 mortality and longer development times of the CR group in comparison to the gnotobiotic group  
164 is likely attributable to the nature of our experimental setup. Larvae were reared individually in  
165 six-well plates, providing an ideal environment for growth of the native mosquito microbiota,  
166 which in turn caused widespread mortality in larvae in the CR group. Presumably, competition  
167 between the mosquitoes and bacteria for resources, or the production of inhibitory compounds by  
168 the bacteria were responsible for larval mortality. Additionally, the axenic group was the only  
169 group that did not exhibit any mortality during the pupal stage, which further supports that  
170 mortality may be related to the bacterial load in the system. Because the rates of mortality were



171 unusually high and did not reflect larval survivorship under normal mosquito rearing conditions,  
172 the CR group was excluded from the phenotypic analyses described below.

173

#### 174 *Biometric assessment of axenic and gnotobiotic mosquitoes*

175 To determine if our axenic rearing conditions altered mosquito phenotypic traits, we  
176 examined adult sex ratio, wing length, and survivorship. The axenic group contained a greater  
177 proportion of males than the gnotobiotic group, although the difference was not significant  
178 ( $p=0.23$ , Figure 3a). These results were somewhat surprising considering that a previous study  
179 demonstrated that *Culex molestus* mosquitoes reared on low concentration larval diets had higher  
180 proportions of males to females compared to those reared on a high concentration larval diet<sup>39</sup>.  
181 Interestingly, they also observed larger males and females, as measured by wing length, in the  
182 high versus low concentration larval diets, whereas we observed no significant differences in  
183 wing length (mm) among the males and females in the axenic and gnotobiotic groups (unpaired  
184 t-test, males:  $t(61) = 0.668$ ,  $p=0.506$ , females:  $t(37)=0.114$ ,  $p=0.99$ ; Figure 3b). Finally, there was  
185 no observable effect on adult survivorship after 14 days (data not shown).

186

#### 187 *Egg clutch size of axenic mosquitoes*

188 Conventionally reared females obtained from our laboratory-maintained colony had  
189 larger egg clutch sizes ( $74.29 \pm 3.26$ ,  $n=21$ ) than their axenic counterparts ( $60.75 \pm 3.34$ ,  $n=16$ ;  
190 unpaired t-test,  $t(35)=2.848$ ,  $p=0.007$ ; Figure 4), a reduction of approximately 18% fewer eggs.  
191 The reduced clutch sizes did not affect the viability of the eggs as larvae laid by axenic females  
192 readily hatched. Sterility of the subsequent generation was confirmed by culture-dependent and

193 independent means. These data indicate that under the proper conditions mosquitoes could be  
194 reared axenically over multiple generations, leading to the potential establishment of axenic  
195 mosquito colonies.

196

#### 197 *Characterizing bacterial components that rescue mosquito development*

198 The development of agar plugs containing liver:yeast extract and infused with attenuated  
199 *E. coli* was based on the hypothesis that large populations of actively growing bacteria were  
200 present in the mosquito midgut, and these populations of bacteria were producing the required  
201 nutrients to rescue larval development. Yet, the cellular components that rescued development  
202 were unknown. The initial agar plugs used in this study contained a mixture of sonicated, filter  
203 sterilized, and autoclaved *E. coli* cells derived from a mid-log phase culture. These agar plugs  
204 rescued larval development, and are the basis of the results presented above.

205 To better define the requisite bacterial components in the agar plugs needed for  
206 development, several cell fractions were tested for their ability to support larval development.  
207 Both sonicated and filter sterilized *E.coli* cells and autoclaved cells alone rescued larval  
208 development (Table 1), indicating that the cellular components supporting development were not  
209 heat labile. The initial agar plugs were made from mid-log phase cells based on the theory that  
210 the nutrients required for development were obtained from metabolically active and vigorously  
211 growing cells. However, agar plugs derived from stationary phase *E. coli* were similarly  
212 successful in rescuing development. Agar plugs consisting of the bacterial components but  
213 without the liver:yeast extract base were also tested. In this case, the agar plugs failed to rescue  
214 development (Table 1). Finally, agar plugs containing the liver:yeast extract base but no

215 attenuated *E. coli* did not support larval development, indicating the effect of the agar plugs was  
216 not due to the effects of a solid diet or nutrition provided by the agar.

217 Taken together, the above observations indicate that the components of the bacterial cells  
218 being utilized by the mosquitoes are heat stable and likely include some of the larger cellular  
219 components like the cell membrane, these components are present throughout the bacterial cell  
220 cycle, and, while required for larval development, they are not sufficient on their own to support  
221 full larval growth. Identifying the specific molecules supplied by the bacteria to fuel larval  
222 development will be a critical step in designing a fully synthetic defined medium for the rearing  
223 of axenic mosquitoes.

224

## 225 **Discussion**

226 Contrary to previous findings<sup>27,35,36</sup>, we demonstrate that it is possible to rear axenic  
227 mosquitoes under laboratory conditions. Furthermore, the colonization of axenic larvae to  
228 generate gnotobiotic mosquitoes demonstrates an ability to manipulate the microbiome,  
229 specifically to imprint axenic mosquitoes with bacterial communities of a known composition.  
230 This transitions the microbiome to an experimental variable that can be utilized to gain a better  
231 mechanistic understanding of the interaction between the microbiome and the phenotype of the  
232 host.

233 Here we show that bacterial components, when provided in high concentrations in a  
234 semi-solid form rather than freely in the mosquitoes' aquatic environment, can rescue larval  
235 development. This suggests that the primary association between mosquitoes and their gut  
236 microbiota is nutritional rather than symbiotic. Additionally, this relationship is not unique to

237 bacteria, as baker's yeast can also rescue development (Table 1). Finally, a companion paper to  
238 this study demonstrates that axenic larvae can be rescued through feeding on a diet consisting of  
239 cell-cultured live mosquito and *Drosophila* cells (Conor McMeniman, personal communication).  
240 In sum, these observations suggest that mosquitoes are unable to produce essential nutrients on  
241 their own, and that in nature these nutrients are supplied by the microbiome. A study of the  
242 transcriptional differences between axenic and colonized larvae may support this hypothesis.  
243 Axenic larvae (unable to develop due to a lack of a microbiome) displayed significant down-  
244 regulation of peptidase genes and an upregulation of amino acid transporters in comparison to  
245 their microbially colonized cohorts<sup>40</sup>. This suggests that protein and amino acid metabolism is  
246 significantly altered in axenic larvae. Yet when we supplemented the larval diet with an amino  
247 acid and vitamin mixture, low concentrations were insufficient to rescue larval development and  
248 high concentrations were lethal (Table 1). Thus, a diet with the appropriate concentration of  
249 amino acids and proteins appears to be critical for larval development, but we have so far been  
250 unable to identify the necessary components or concentration for a fully synthetic larval diet.

251 Previously, bacterial-mediated hypoxia was identified as a potential mechanism to  
252 explain the apparent requirement of live bacteria for mosquito development<sup>36</sup>. This report was  
253 based on the observation that *E. coli* mutants in cytochrome *bd* oxidase could not rescue larval  
254 development<sup>36</sup>. This oxidase has a high affinity for oxygen, and allows facultative anaerobes to  
255 maintain aerobic respiration under low oxygen conditions<sup>41</sup>. In this regard, the role of this  
256 enzyme complex is generally in response to anaerobic conditions, rather than a cause of them.  
257 Thus, the inability of mutants in this complex to rescue larval development may be due to an  
258 inability of cytochrome *bd* mutants to survive anoxic conditions. Furthermore, cytochrome *bd*  
259 oxidase complexes can protect bacteria from agents synthesized by the host immune system,

260 such as reactive oxygen species and nitric oxides<sup>42,43</sup>. Therefore, cytochrome *bd* oxidase mutants  
261 may be compromised in their ability to colonize host organisms<sup>44</sup>. Finally, our data show that  
262 disrupting *E. coli* cells, either through sonication or autoclaving and feeding them to mosquitoes,  
263 can rescue larval development, suggesting that larval development can occur without bacterially-  
264 mediated anoxia. Collectively, these observations support the hypothesis that the ability of the  
265 microbiome to rescue larval development is based on a nutritional relationship rather than an  
266 active interaction between the bacteria and the host.

267         The importance of the microbiome in the functional biology and physiology of  
268 vertebrates and invertebrates has been well documented<sup>1,2</sup>. It is unsurprising, therefore, that  
269 axenic mosquitoes exhibit phenotypic differences when compared to gnotobiotic or CR  
270 mosquitoes. Axenic mosquitoes demonstrated a delay in development when compared to the  
271 gnotobiotic group. A similar delay in development has been observed in other axenic organisms,  
272 including *Caenorhabditis elegans* and *Drosophila*<sup>45,46</sup>. In the case of *C. elegans*, an increase in  
273 development time is usually coupled with an increase in longevity, with axenic organisms living  
274 longer than organisms reared on a non-axenic culture<sup>45,47</sup>. This pattern is not observed in axenic  
275 *Drosophila*, the longevity of which appears to be diet-specific<sup>48,49</sup>. This suggests that the removal  
276 of the microbiome has differing effects on host biology and demonstrates why expanding the  
277 number of axenic models will help to uncover the common and diverging effects of the  
278 microbiome on host physiology. It is interesting to note that no significant difference in wing  
279 length was observed between axenic and gnotobiotic mosquitoes, suggesting that axenic  
280 mosquitoes are able to compensate for an increase in developmental time and reach full body  
281 size once a certain threshold in development has been surpassed.

282 Axenic mosquitoes also displayed a significant decrease in fecundity. A study on *Aedes*  
283 *aegypti* mosquitoes that were colonized by single bacterial strains showed that differences in  
284 fecundity of gnotobiotic females can be strain-specific<sup>50</sup>. Our results suggest that an absence of  
285 bacteria during development can detrimentally affect female egg clutch size. It is important,  
286 however, to note that mosquito egg clutch size has also been linked to nutrition<sup>51,52</sup>, which could  
287 explain the observed differences between the axenic and colonized groups. Bacterial turnover  
288 may supply the colonized mosquitoes with a steady source of nutrients, which is not available to  
289 the axenic group. Additionally, the microbiome is associated with an increased ability to extract  
290 energy from food<sup>53</sup>. Therefore, we would expect that this would lead to a better nutritional status  
291 for gnotobiotic and CR mosquitoes, possibly explaining reduced egg clutch size for the axenic  
292 mosquitoes.

293 In summary, this study presents a method to rear axenic *Aedes aegypti* mosquitoes from  
294 eggs to adults and into the subsequent generation in the complete absence of a microbiome. We  
295 show that axenic mosquitoes develop normally, but with a delay in the time of development.  
296 Axenic mosquitoes show decreased mortality and smaller egg clutch sizes in comparison to their  
297 bacteria colonized cohorts. As mosquitoes are a major global health concern, interventions that  
298 could decrease mosquito fecundity are a common objective for mosquito management. The data  
299 presented here suggest that the microbiome may be a potential target for future control strategies.  
300 Using bacteria as a tool in mosquito control, a method referred to as paratransgenesis<sup>15,56</sup>, has  
301 already been pursued. However, these studies have thus far been hampered by a lack of effective  
302 tools to manipulate the microbiome. The methods presented in this study add mosquitoes to the  
303 collection of organisms for which an axenic state can be maintained, underpinning our ability to  
304 treat the microbiome as a controlled experimental variable in organismal studies.

## 305 **Methods**

### 306 *Preparation of Mosquito Rearing Substrates*

307 Multiple diets and supplements were tested for their ability to support the development of  
308 axenic mosquitoes. The first group of treatments was based on the standard diet for the colony  
309 raised mosquitoes, which consisted of a 0.1% solution of 3 parts liver extract (Difco, desiccated,  
310 powdered beef liver) and 2 parts yeast extract (Fisher Scientific, granulated yeast extract). The  
311 standard diet was also supplemented with the following: 5 ml Luria Broth (LB), 1 ml or 5 ml of  
312 an overnight culture of sonicated *E. coli* cells (throughout the manuscript *E. coli* refers to the  
313 wild-type strain K-12<sup>54</sup>), 1 ml or 5 ml autoclaved *E. coli* cells (overnight culture), 0.2% or 2%  
314 (w/v) yeast extract, 100 µl of an overnight culture of live baker's yeast (*Saccharomyces*  
315 *cerevisiae*), and a 1x and 0.5x solution of an amino acid (Gibco MEM Amino Acids 50x stock)  
316 and vitamin (Gibco Vitamin Solution 100x stock) solution mixture. Two diets included a media  
317 base not consisting of the standard diet. These were: 0.1% sterile fish food (TetraminTropical  
318 Flakes) and a synthetic larval growth media previously described<sup>33</sup>.

319 The final diet tested was a mixture of sonicated and heat-killed *E. coli* embedded in agar  
320 plugs. A starter culture of *E. coli* was grown overnight at 37°C and used to inoculate two flasks  
321 of 500 ml of LB broth. To harvest mid log-phase cells, the inoculated flasks were placed on a  
322 shaker and incubated at 37°C for approximately 5 hours to an OD of ~0.8. The cells were then  
323 centrifuged at 1100 rpm for 10 minutes. After discarding the supernatant, the bacterial pellet was  
324 re-suspended in 20 ml of PBS and sonicated at 65% amplitude using a Fisher Scientific Model  
325 120 Dismembrator for a total of 3 minutes. The sonicated cells were then centrifuged at 4000  
326 rpm for 10 minutes. The supernatant was filter sterilized using a 0.2 µm PES membrane filter,  
327 and the pellet was re-suspended in 10 ml of sterile water and subsequently autoclaved at 121°C

328 for 30 minutes. The resultant filtrate and autoclaved pellet were then combined with 30 ml of a  
329 1.5% agar solution containing 3.3% 3:2 liver:yeast extract. The agar mixture was poured into  
330 standard Petri dishes and stored at 4°C.

331 For the plugs that contained only sonicated and filter sterilized *E. coli*, the above  
332 standard protocol for culturing and sonicating the bacteria was followed; however, 10 ml of  
333 sterile water was substituted for the autoclaved bacterial pellet. Similarly, for plugs that  
334 contained only mid-log phase autoclaved *E. coli*, the standard protocol was followed with the  
335 following exceptions: after re-suspension in 20 ml of PBS, the pellet was autoclaved at 121°C  
336 for 30 minutes and was then combined with 40 ml of a 1.5% agar solution containing 3.3% 3:2  
337 liver:yeast. For plugs containing stationary phase *E. coli*, two 500 ml flasks of LB were  
338 inoculated with *E. coli* and allowed to grow on a shaker at 37°C overnight. The culture was then  
339 centrifuged, re-suspended, autoclaved, and combined with agar and liver:yeast extract as detailed  
340 above. A full procedure documenting the making of agar plugs is attached as a supplemental file  
341 to this manuscript.

342

### 343 *Egg Sterilization*

344 *Aedes aegypti* eggs were acquired from a colony of laboratory-reared mosquitoes  
345 (Orlando strain) maintained in environmental chambers at 28°C with a 16:8 light:dark  
346 photoperiod. Sterilization of the *Aedes aegypti* eggs was carried out as previously described.<sup>35</sup>  
347 Briefly, a small segment of egg-covered filter paper was washed for five minutes in 70% ethanol,  
348 followed by a five-minute wash in a 3% bleach and 0.2% ROCCAL-D (Pfizer) solution, and an  
349 additional five minute wash in 70% ethanol. The sterilized eggs were then rinsed three times in



350 autoclaved distilled water and placed in Petri dishes filled with phosphate buffered saline (PBS).  
351 Eggs were hatched in a vacuum oven (Precision Scientific Model 29) at 25Hz for 15 minutes at  
352 room temperature. A schematic diagram showing the sterilization procedure is shown in Figure  
353 5.

#### 354 *Bacterial colonization of sterile larvae*

355 The sterile hatched eggs were split into three different treatment groups. Axenic larvae  
356 were incubated at 28°C in the presence of 75 µg/ml carbenicillin and 12.5 µg/ml tetracycline for  
357 4 hours. Gnotobiotic larvae were exposed to a 1 ml aliquot of an overnight culture of *E. coli* for 4  
358 hours at 28°C. Finally, the conventionally reared (“CR”) group were inoculated with a 1 ml  
359 aliquot obtained from pans of water used to rear larvae from a laboratory maintained *Ae. aegypti*  
360 colony. In this manner, each group went through the sterilization procedure, ensuring that any  
361 observed differences in mosquito development were not due to effects from surface sterilization  
362 of the eggs.

363

#### 364 *Rearing of larvae*

365 For each tested condition, six individual larvae were transferred from the Petri dishes to  
366 individual wells of a 6-well plate. Each well of the plate contained 5 ml of the rearing substrate,  
367 or, in the case of the agar plugs, a 0.4 g plug in a 5 ml solution of sterile saline. Development,  
368 time to pupation, and mortality were recorded each day for 14 days after hatching for a total of  
369 three replicate plates (i.e. 18 individuals).

370

371 *Confirming the Sterility of Axenic Mosquitoes*

372 A subset of the adults that were reared under axenic conditions was tested for the  
373 presence of live bacterial cell or bacterial genomic DNA via culturing and 16S rRNA gene PCR.  
374 Individual mosquitoes were transferred to a round bottom tube containing a steel BB and 150  $\mu$ l  
375 of sterile PBS and homogenized for 30 seconds at 30 1/s using a Mixer Mill. 50  $\mu$ l of each  
376 homogenate was inoculated into a 14 ml culture flask containing 2 ml of LB broth and incubated  
377 48 hours at 28°C. Negative results were confirmed by an absence of bacterial growth.

378 For the remainder of the homogenate, total DNA was extracted using a PowerSoil DNA  
379 Isolation kit (MoBio Laboratories, Inc.). DNA extractions were PCR amplified using standard  
380 16S rRNA gene primers (27F and 1492R<sup>55</sup>), using the following protocol: initial denaturation at  
381 95°C for three minutes, followed by 30 cycles consisting of 95°C for 45 seconds, annealing at  
382 55°C for 45 seconds, and extension at 72°C for 1 minute 45 seconds, with a final extension at  
383 72°C for 10 minutes. PCR results were verified by agarose gel electrophoresis. Although readily  
384 detected in colonized mosquitoes, bacterial DNA amplification was not detected by gel  
385 electrophoresis in axenic larvae and adult mosquitoes (Figure 1), despite *E. coli* DNA likely  
386 being present in the initial agar plugs. These data were collected from L4 larvae, thus incubation  
387 at 28°C, coupled with the presence of live mosquito larvae, presumably broke down any *E. coli*  
388 DNA present in the agar plugs. PCR of initially sterilized larvae and the growth media  
389 components were also consistently negative by bacterial 16S rRNA gene PCR.

390 To ensure that the axenic larvae were truly bacteria free we employed a three step  
391 verification of sterility: 1) In each experiment, a “sterile” control group of larvae was maintained.  
392 This group was processed in parallel to the other treatment groups and fed on the same batch of  
393 liver:yeast extract. Any larval development in this group past the L1 stage was taken as an

394 indication of contamination and the experiment was discarded; 2) In each experiment, a subset of  
395 axenic larvae and adult mosquitoes were tested for contamination by culturing. A positive test  
396 for bacterial presence in any experiment indicated contamination and the experiment was  
397 discarded; 3) A subset of axenic larvae (L4 growth stage) and newly emerged adult mosquitoes  
398 were tested for bacterial DNA through PCR of bacterial 16S rRNA genes. A positive test for  
399 bacterial presence in any experiment indicated contamination and the experiment was discarded.

400

#### 401 *Mass Rearing and Blood Feeding Axenic Mosquitoes*

402 After eggs were sterilized and hatched, larvae were incubated for four hours at 28°C in  
403 PBS supplemented with 12.5 µg/ml tetracycline and 75 µg/ml carbenicillin. Approximately 150  
404 larvae were placed in a sterile 500 ml polypropylene Nalgene jar containing 125 ml of distilled  
405 water and 4 grams of the *E. coli* agar food. After pupation, larvae were allowed to emerge into a  
406 UV-sterilized mosquito emergence chamber. Adult mosquitoes were fed filter sterilized 10%  
407 sucrose, and female axenic mosquitoes were blood fed sterile defibrinated sheep blood using a  
408 Hemotek membrane feeder in a biosafety cabinet. Hemotek feeders were autoclaved and UV-  
409 sterilized parafilm was used for the feeds. Similarly, all glassware and forceps used to sort  
410 mosquitoes were autoclaved. CR mosquitoes were acquired from the laboratory colony and  
411 blood fed using a circulating bath membrane feeder.

412

#### 413 *Biometric Assessment of Axenic Mosquitoes*

414 Using the method of mass rearing described above, an equal number of sterile and non-  
415 sterile larvae were placed in separate polypropylene jars, allowed to emerge, and were blood fed

416 using a Hemotek membrane feeder. Axenic larvae were fed 10 ml of the *E. coli* agar food and the  
417 CR mosquitoes were fed a 1% 3:2 liver: yeast extract solution. After blood feeding, axenic  
418 (n=16) and CR (n=21) females were individually placed in autoclaved 50 ml tubes containing  
419 sterilized egg-laying filter paper and water. After oviposition, the filter papers were removed  
420 from the tubes and the eggs counted.

421 To assess differences in wing length between the three groups axenic, gnotobiotic, and  
422 CR larvae were reared in 6 well plates as per the development assay. After emergence, males and  
423 females were knocked down on ice and wings were removed using forceps. Wings were then  
424 visualized using a Zeiss Axioplan 2 universal microscope and wing length was measured using  
425 Axiovision (v.4.8.1) software.

426

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434

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554

555

556 **Figure Legends**

557

558 **Figure 1. PCR detection of bacterial DNA.** Total DNA was extracted from axenic and conventionally  
559 reared (CR) mosquitoes and employed as a template for amplification of bacterial 16S rRNA genes.  
560 Lanes 1-8 show no visible PCR products from four of each axenic L4 larvae and adult mosquitoes,  
561 whereas amplification products were identified in the CR mosquitoes (lanes 9-10). A positive control (+)  
562 containing amplified *E. coli* K-12 DNA and a non-template control (NTC) are also included on the gel.  
563 Amplification consisted of 30 cycles (see methods).

564

565 **Figure 2: Delayed development in axenic larvae.** Time to pupation is shown for axenic (A), gnotobiotic  
566 (G), and conventionally reared (CR) larvae. Points represent time to pupation for individual larvae. The  
567 red bars signify mean time to pupation for the three replicates and the standard error.

568

569 **Figure 3: Biometric assessment of adult mosquitoes.** (a) Male:female ratio of adults for axenic (A) and  
570 gnotobiotic (G) mosquitoes. Using Fisher's exact test, no significant difference in sex ratio was identified  
571 ( $p > 0.05$ ) between the two groups. B) Wing length of adults for axenic and gnotobiotic mosquitoes. Points  
572 represent individual mosquitoes. Mean wing length and standard error of all individuals are signified by  
573 the black bars. No significant difference in wing length was observed between the two groups (unpaired t-  
574 test;  $p > 0.05$ ).

575

576 **Figure 4: Decreased egg clutch size in axenic mosquitoes.** Mean egg clutch size and distribution is  
577 shown for axenic (A) and conventionally reared (CR) mosquitoes. Points represent clutch size for  
578 individual females. Mean clutch size and standard error of all individuals are signified by the black bars.

579 Clutch size in CR mosquitoes was significantly greater than in axenic mosquitoes (unpaired t-test;  
580  $p=0.007$ ).

581

582 **Figure 5. Schematic diagram of egg sterilization.** *Ae. aegypti* eggs were collected from colony reared  
583 mosquitoes and surface sterilized as depicted. (a) Eggs were serially washed for 5 minutes in each  
584 solution. (b) Surface sterilized eggs were transferred to a Petri dish containing sterile PBS and antibiotics  
585 for axenic mosquitoes, *E. coli* cells for gnotobiotic mosquitoes, or water collected from colony rearing  
586 pans for CR mosquitoes. (c) After hatching the eggs in a vacuum oven and four hours of incubation,  
587 individual larvae were transferred from the Petri dishes to individual wells of a six well plate for  
588 development assays.

589

**Table 1.** Effect of sterile diets on larval development

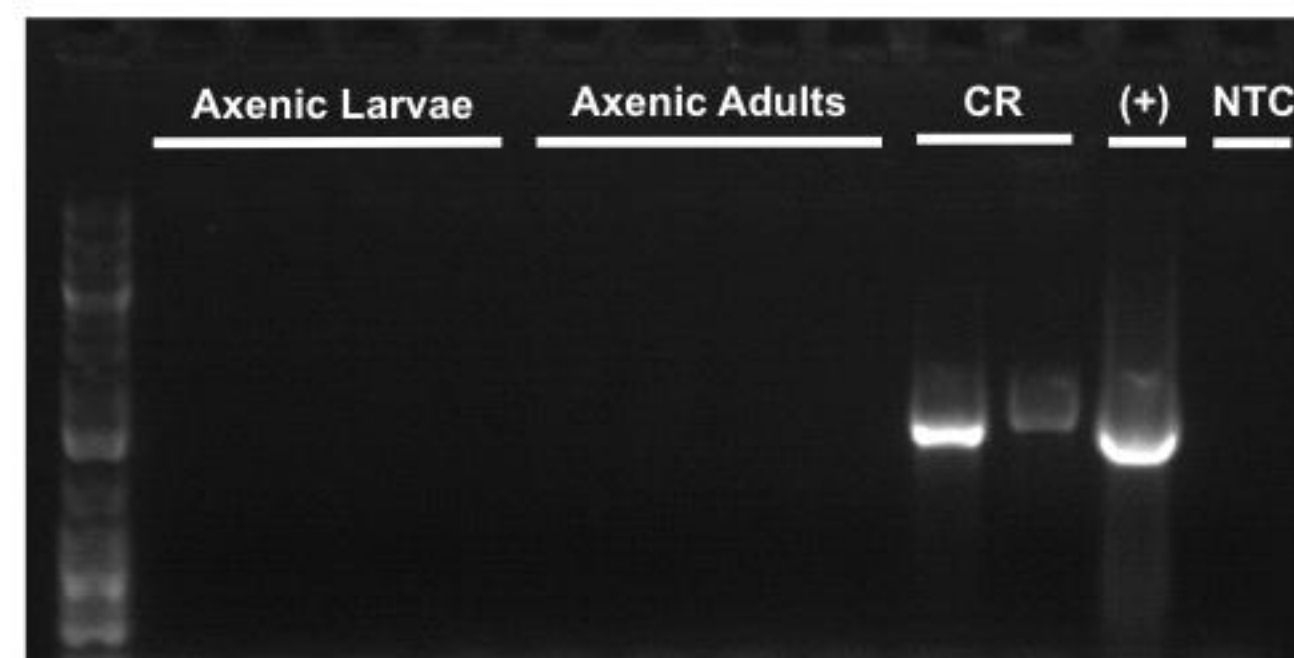
Diet	Axenic	Gnotobiotic <sup>1</sup>
<i>Base media</i>		
Liver:yeast extract (LY)	Stalled <sup>2</sup>	<b>Pupae</b> <sup>4</sup>
Luria broth	Stalled	Dead
Larval growth medium	Dead	<b>Pupae</b>
Fish food	Stalled	<b>Pupae</b>
<i>Supplemented media (LY base)</i>		
1 ml <i>E. coli</i> sonicate	Stalled	NA <sup>3</sup>
5 ml <i>E. coli</i> sonicate	Dead	NA
1 ml <i>E. coli</i> autoclaved	Stalled	NA
5 ml <i>E. coli</i> autoclaved	Dead	NA
1 ml 1% yeast extract	Stalled	<b>Pupae</b>
1 ml 10% yeast extract	Dead	Dead
Amino acid vitamin mix (0.5x)	Stalled	NA
Amino acid vitamin mix (1x)	Dead	NA
Active yeast culture	<b>Pupae</b>	<b>Pupae</b>
<i>Agar plugs (LY base)</i>		
Autoclaved and sonicated <i>E. coli</i>	<b>Pupae</b>	<b>Pupae</b>
Sonicated <i>E. coli</i>	<b>Pupae</b>	<b>Pupae</b>
Autoclaved <i>E. coli</i>	<b>Pupae</b>	<b>Pupae</b>
Stationary phase autoclaved <i>E. coli</i>	<b>Pupae</b>	<b>Pupae</b>
Autoclaved <i>E. coli</i> no LY base	Stalled	NA
LY base no <i>E. coli</i>	Stalled	NA

<sup>1</sup>Gnotobiotic larvae were colonized by *E. coli*.

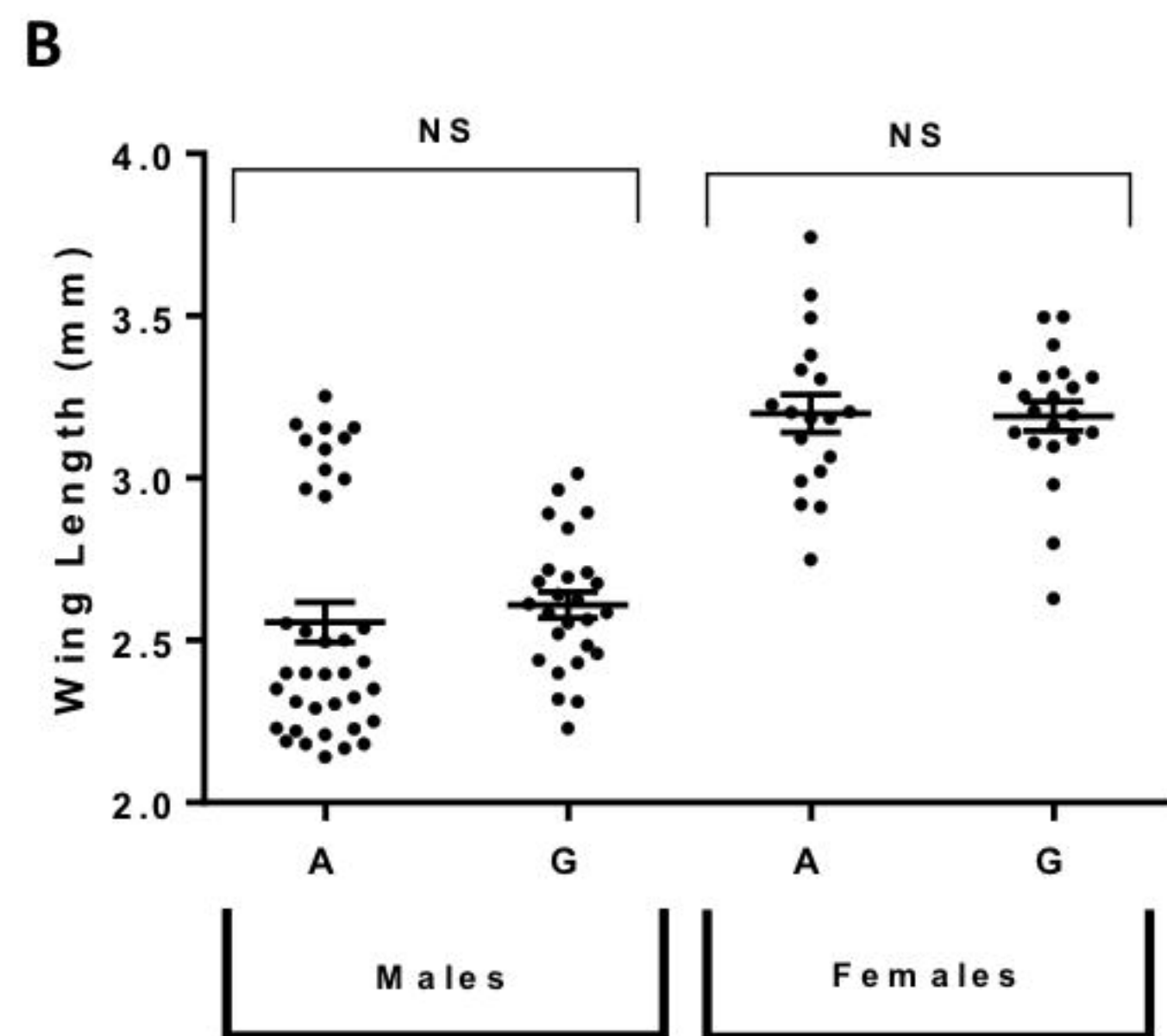
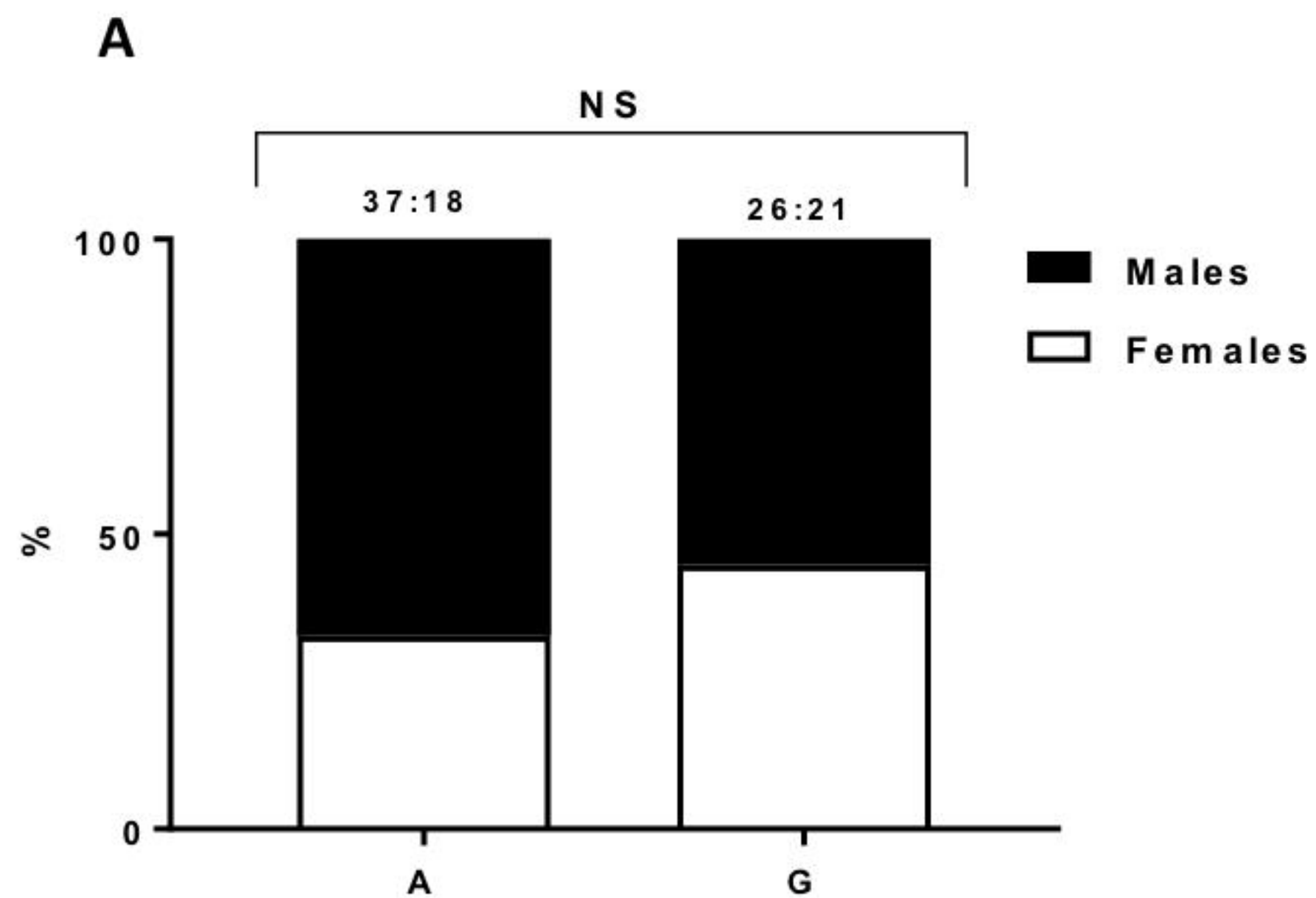
<sup>2</sup>Larva did not develop beyond the initial L1 stage.

<sup>3</sup>Not tested.

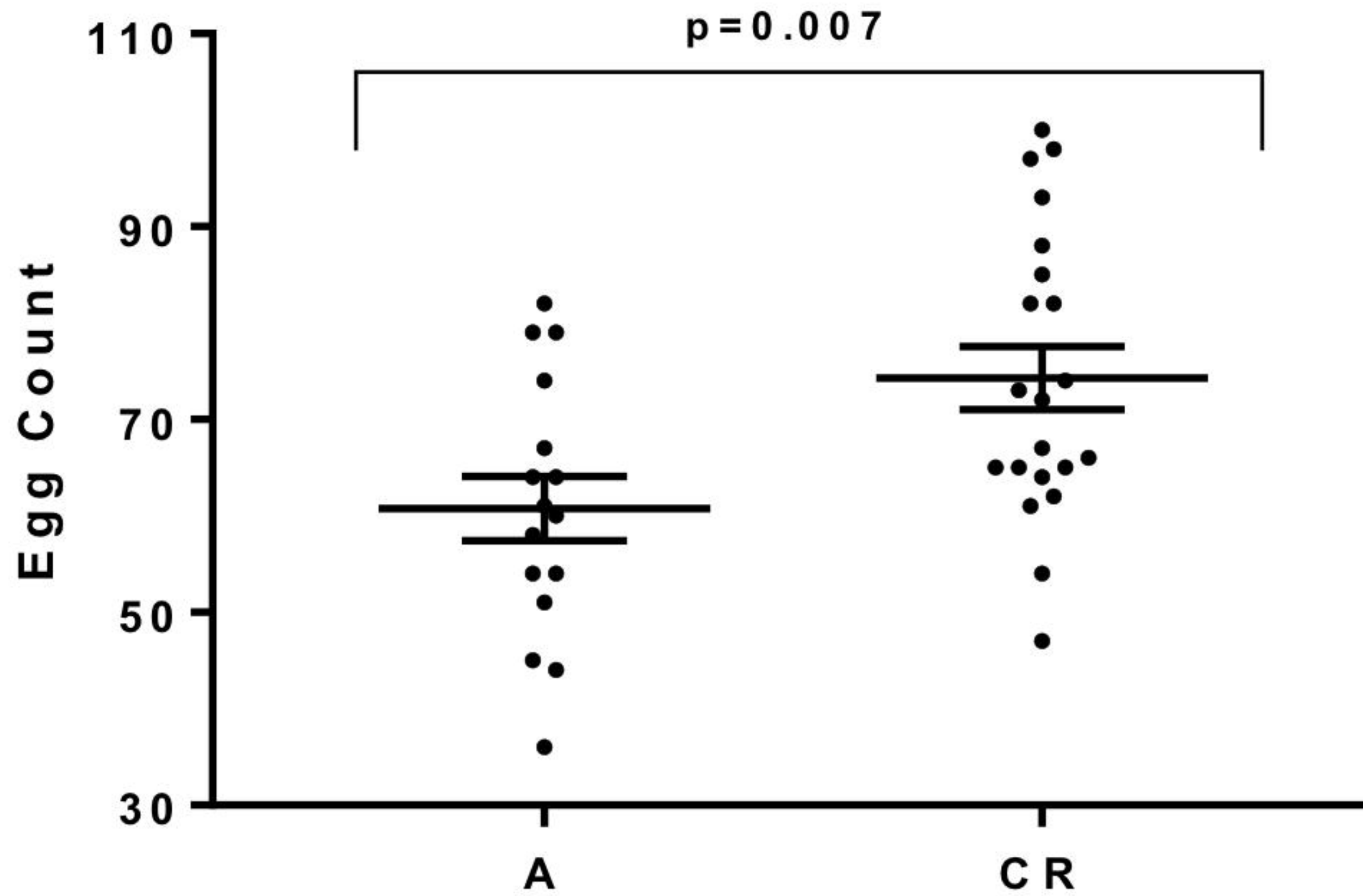
<sup>4</sup>Indicates full development of larvae to pupae.



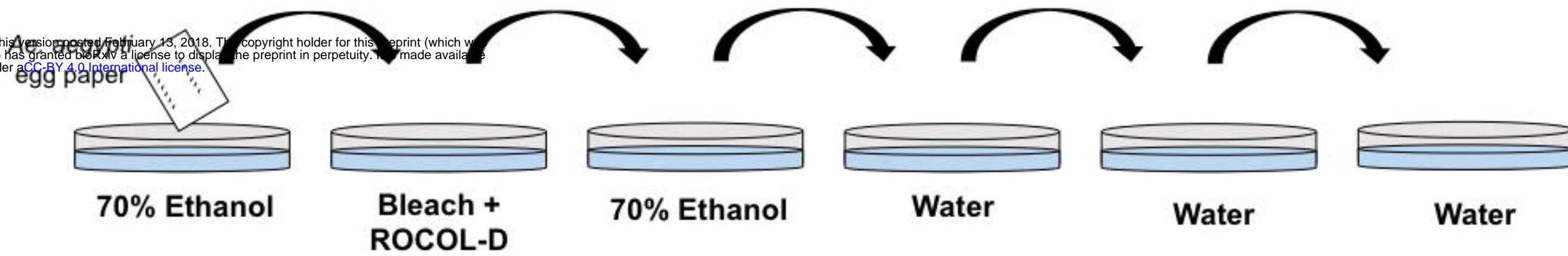




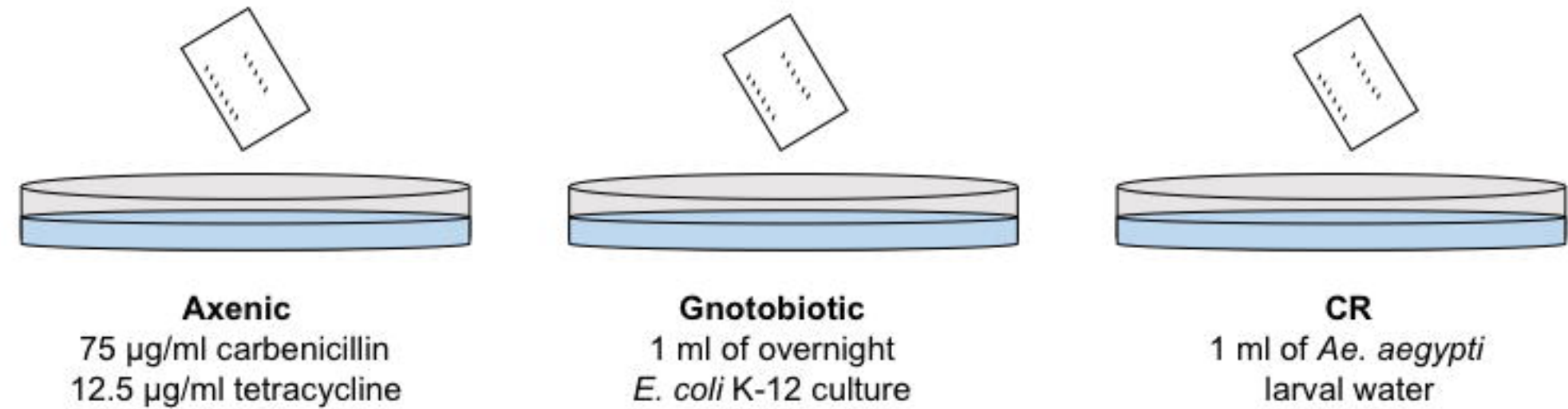




**a.**

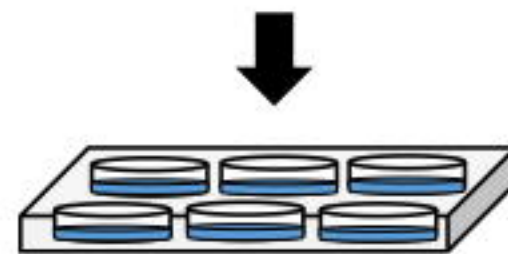


**b.**



**c.**

Hatch eggs in a vacuum oven  
Incubate at 28°C for 4 hours



Individual larvae are placed in 6-well plates and incubated at 28°C