1   # *Bacillus thuringiensis* bioinsecticides induce developmental defects

2   ## in non-target *Drosophila melanogaster* larvae

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9   Running Head: *Bt* impacts on *Drosophila* midgut

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14   **Summary statement**

15   Bioinsecticides based on *Bacillus thuringiensis kurstaki* spores are applied to specifically kill

16   pest lepidopteran larvae. Unexpectedly they display unintended effects on the intestinal

17   homeostasis of *Drosophila* midgut, resulting in developmental delay and growth defects.

18   **Abstract**

19   Bioinsecticides made from the bacterium *Bacillus thuringiensis* (*Bt*) are the best-selling

20   bioinsecticide worldwide. Among *Bt* bioinsecticides, those based on the strain *Bt var. kurstaki*

21   (*Btk*) are widely used in farming to specifically control pest lepidopteran larvae. Although

22   there is much evidence of the lack of acute lethality of *Btk* products for non-target animals,

23   only scarce data are available on their potential non-lethal developmental adverse effects.

24   Using doses that could be reached in the field upon sprayings, we have shown that *Btk*

25   products impair growth and developmental time of the non-target dipteran *Drosophila*

26   *melanogaster*. These effects are mediated by the synergy between *Btk* bacteria and *Btk*

27   insecticidal toxins, which induces a significant apoptosis of larval enterocytes, resulting in a

28   decreased intestinal capacity to digest proteins. The harmful effects can be mitigated by a

29   protein-rich diet or by adding the probiotic bacterium *Lactobacillus plantarum* into the food.

30   Finally, we showed that the larval midgut maintain its integrity upon *Btk* aggression thanks to

31   both the flattening of surviving enterocytes and the generation of new immature cells arising

32   from the adult midgut precursor cells.

33

34   **Keywords**
35   Bioinsecticides, *Bacillus thuringiensis*, *Drosophila melanogaster*, Developmental defects,
36   Gut homeostasis, *Lactobacillus plantarum.*
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39 **INTRODUCTION**

40 The use of conventional synthetic chemical pesticides is controversial mainly because of their

41 harmful effects on human health and ecosystems. Public opinion along with governmental

42 policies encourage to reduce their use (Casida and Bryant, 2017; Frisvold, 2018). However,

43 due to the world's population expansion, the use of pesticides will still be necessary to

44 produce enough food to feed the 9.5 billion humans expected in 2050

45 (https://population.un.org/wpp/). An emerging solution to reduce the use of conventional

46 pesticides is their replacement with biopesticides.

47 Among bioinsecticides, *Bacillus thuringiensis* (*Bt*) products are increasingly sprayed

48 in both organic farming and conventional agriculture (Fiuza et al., 2017). *Bt* products are the

49 second most used insecticides in the world with 32 000 tons sold in 2017 (Casida and Bryant,

50 2017). *Bt* is a Gram-positive spore-forming bacterium belonging to the *Bacillus cereus* group

51 (Vilas-Boas et al., 2007). It was first identified and characterized for its specific

52 entomopathogenic properties due to the presence of specific Cry toxins, which are produced

53 in a crystalline form during the sporulation of bacteria (Rabinovitch et al., 2017). Seventy-

54 eight different strains of *Bt* are currently inventoried (http://www.bgsc.org/), producing a total

55 of more than 300 distinct Cry toxins

56 (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) having a spectrum of toxicity

57 ranging from nematodes to human tumor cells (Adang et al., 2014; Frankenhuyzen, 2017).

58 However, only four strains are used commercially as bioinsecticides owing to the specific

59 acute toxicity of their Cry toxins to pest larvae: *Bt var. kurstaki (Btk)* and *Bt var. aizawai* to

60 kill lepidopteran larvae, *Bt var. israelensis* to kill mosquito larvae and *Bt var. morrisoni* to kill

61 coleopteran larvae (http://sitem.herts.ac.uk/aeru/bpdb/index.htm). Upon ingestion of

62 commercial formulations made of spores, crystals and additives, Cry protoxins are first

63 released from the crystal before being processed by gut proteases to give rise to the active Cry

64 toxins. Then, these Cry toxins bind to receptors at the surface of the intestinal cells, inducing

65   their death and ultimately the destruction of the gut epithelium. The spores germinate and,

66   thanks to the holes made by Cry toxins in the gut lining, vegetative bacteria invade the

67   internal cavity of the body, causing sepsis which kills the targeted pest in 2 or 3 days. The

68   specificity of the acute Cry toxicity relies on both the capacity of gut proteases to cleave and

69   activate Cry protoxins and on the presence of host receptors specifically recognized by Cry

70   toxins (Adang et al., 2014). Each variant of *Bt* produces between 1 and 6 different Cry toxins.

71   In general, all the Cry toxins contained in a *Bt* variant target a specific phylogenetic order

72   (*e.g.* lepidopteran, coleopteran…) (Palma et al., 2014). The most used *Bt* variant is *Btk,* which

73   produces 5 different Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab) (Caballero

74   et al., 2020). *Btk* is widely used in forestry and farming to fight lepidopteran larvae.

75   The increasing environmental dispersion of *Btk* bioinsecticides raises the question of

76   their long-term putative effects on both health and environment. Although there are many data

77   showing that the Cry toxins produced by *Btk* do not display harmful effects against non-target

78   organisms (Haller et al., 2017; Rubio-Infante and Moreno-Fierros, 2016), there are also

79   studies suggesting that Cry toxins and/or *Bt* products may have unintended impacts on non-

80   target animals. For example, the presence of Cry1Ab toxins produced by GM maize in water

81   streams surrounding maize fields delays the growth and increases the mortality of trichopteran

82   species whose larvae have an aquatic lifestyle (Rosi-Marshall et al., 2007; Tank et al., 2010).

83   More recently, Amichot and colleagues have shown that *Btk* bioinsecticides are acutely toxic

84   against *Trichogramma chilonis* females, a tiny egg endoparasitoid hymenopteran used as a

85   biocontrol agent in integrated pest management (Amichot et al., 2016). On vertebrates,

86   Grisolia and colleagues observed that the exposure of *Danio rerio* fry to different *Btk* Cry

87   toxins induced lethality and developmental delay (Grisolia et al., 2009).

88   Based on these data, we decided to use the model organism *Drosophila melanogaster*,

89   not targeted by *Btk*, to identify and characterize the putative impacts of *Btk* products on the

90   development. While the acute toxicity of the agricultural doses of *Btk* bioinsecticides

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91   efficiently kills lepidopteran larva, *Drosophila* larvae, being non-susceptible to these doses,

92   allow the study of non-lethal impacts. In addition, *D. melanogaster* has been used

93   successfully in many studies for deciphering the toxicology of insecticides and is a toolbox

94   widely used to identify disturbed cellular and molecular mechanisms (Scott and Buchon,

95   2019).

96   Here, using commercial *Btk* products at two realistic doses, a dose detected on

97   vegetables after one spraying and a dose only tenfold higher (but equivalent to the ten

98   cumulative treatments authorized by the European Union), we observed that *Btk*

99   bioinsecticides induce developmental delay and reduce growth of *D. melanogaster* larvae. We

100   further showed that *Btk* bioinsecticides trigger intestinal cell death and alter protein digestion.

101   Interestingly, we showed that a protein-rich diet or supplementing food with the probiotic

102   *Lactobacillus plantarum* (*L. plantarum*), known to enhance protein uptake (Erkosar et al.,

103   2015), mitigates the impacts of *Btk* bioinsecticide on growth and development. We also found

104   that *Btk* products do not interfere with the commensal flora of the larvae. Finally, we

105   unraveled new cellular mechanisms for maintaining intestinal integrity mounted by the larval

106   midgut epithelium in response to *Btk* aggression, particularly the flattening of enterocytes and

107   the production of new cells from the adult midgut precursors. Overall, our data show that

108   though not lethal for larvae of the non-target organism *Drosophila melanogaster*, agricultural

109   doses of *Btk* bioinsecticides damage gut epithelium and consequently impair larval growth

110   and developmental time.

111

112   **RESULTS**

113   ***Btk* bioinsecticide induces developmental delay and growth defect**

114   European and French Food Safety Agencies (Efsa and Anses, respectively) have estimated

115   that the amount of *Btk* spores on fruits and vegetables after treatment can reach 5×10^6 Colony

116   Forming Units (CFU)/g (ANSES Saisine n° 2013-SA-0039, 2013; EFSA BIOHAZ Panel,

117   2016). This quantification may be underestimated since up to 10 successive treatments can be

118   applied by farmers (ANSES Saisine n° 2013-SA-0039, 2013) (see also the European

119   directives SANCO/1541/08 – rev. 4 and SANCO/1543/08 – rev. 4). Therefore, we decided to

120   assess the impacts of the ingestion of two *Btk* bioinsecticides (i.e. Delfin and Dipel) on the

121   growth and development of *Drosophila* larvae at two field-realistic doses: $5x10^6$ CFU/g

122   (corresponding to the dose observed after one spraying) and $5x10^7$ CFU/g (a dose which

123   could be reached upon repeated treatments), named hereafter "1X" and "10X", respectively.

124   We first compared the impacts of Delfin with water (the diluent for commercial products) by

125   contaminating 2g of standard fly medium before depositing 20 eggs aged of 0-4h. We then

126   measured the timing of larval development by performing a pupation curve. In the control

127   experiment (H_2O), 90% of the larvae pupated at 169h after eggs laying (this threshold will

128   serve as a point of comparison throughout our study, Table 1 summarizes all the data at 10%,

129   50% and 90% of pupation entry). The 1X and 10X doses of Delfin postponed pupation by

130   10.5h (+6.2%) and 16h (+9.5%) respectively (Fig. 1A and Table 1). We then assessed the

131   impact of the second bioinsecticide Dipel. Because developmental delay was marked with

132   Delfin at the 10X dose, we used this same dose as a benchmark for Dipel. Strikingly, the

133   pupation delay was increased to reach 25h (+14.8%). Commercial products are made of

134   spores, toxin crystals and additives (see Materials And Methods). Dipel contains 46% of

135   "trade secret" components, while Delfin contains 15% of naphthalene sulfonic acid. To assess

136   the potential involvement of the additives present in Dipel in the augmented delay observed,

137   we plated the commercial product on LB agar, isolated a colony and prepared our own

138   mixture of spores and toxins (the *Btk* strain used in Dipel is named *ABTS-351*). When larvae

139   were fed with the 10X dose of the homemade preparation, we obtained developmental delays

140   similar to those with the Delfin treatments (Fig. 1A and Table 1), suggesting that the

141   unknown additives present in the commercial preparation of Dipel might contribute to the

142   increased delay we observed. Moreover, these data also suggested that the 15% of

143   naphthalene sulfonic acid presents in Delfin had no impact on the developmental timing.

144   Indeed feeding developing larvae with an amount of naphthalene sulfonic acid equivalent to

145   the 10X dose of Delfin had no effect on the developmental timing (Fig. 1B and Table 1).

146   Then, we wondered whether the developmental delay was due to the spores alone, the crystals

147   of toxins alone, or both. First, we fed the developing larvae with the 1X and 10X doses of

148   spores of a *Btk* strain devoid of Cry toxins (*Btk$^{\Delta Cry}$*). Whatever the dose, no developmental

149   delay was observed (Fig. 1B and Table 1). Then, we fed the developing larvae with an

150   equivalent 10X dose of toxin crystals, considering that crystals represent 30% of the dry

151   weight of bioinsecticides (Agaisse and Lereclus, 1995; Monro, 1961; Murty et al., 1994) (Fig.

152   S2). Again, we did not observe any developmental delay (Fig. 1B and Table 1). Therefore, we

153   assumed that the developmental delay we observed was mainly due to the mixture of spores

154   and crystals (Fig. 1A).

155   Since growth defects have a well-documented impact on the developmental timing

156   (Hietakangas and Cohen, 2009), we next measured the pupal volume, a known readout to

157   estimate growth defects (Delanoue et al., 2010). Interestingly, we observed a marked growth

158   defect when the larvae were fed with Delfin and Dipel, as well as a weaker one when the

159   larvae were fed with *ABTS- 351* (Fig. 1C). We did not detect any growth defect in all the

160   other conditions. Together, our data show that ingestion of food contaminated with a mixture

161   of *Btk* spores and crystals induces developmental delay and growth defect on the model

162   organism *D. melanogaster*, at doses that can be found in treated areas.

163   Since i) the composition of Delfin is clearly provided by the manufacturer compared

164   to Dipel (see Materials And Methods), ii) Delfin is composed of 85% of spores and toxin

165   crystals while only 54% for Dipel, and iii) the naphthalene sulfonic acid has no effect on the

166   developmental timing and growth, we decided to use only Delfin in the rest of the study.

167

168   ***Btk* bioinsecticide does not alter feeding behavior and food intake**

169   The *Btk* load in the midgut could explain the differences observed between $Btk^{\Delta Cry}$ and Delfin.

170   When we estimated the amount of *Btk* cells in the midgut of the L3 larvae (Fig. 2A), no

171   difference was observed for 1X or 10X doses of Delfin compared to the equivalent doses of

172    $Btk^{\Delta Cry}$. Therefore, the variation in the quantity of *Btk* bacterial cells in the midgut could not

173   explain the disturbed developmental timing and growth observed with Delfin.

174   Adult flies and larvae can sense the presence of harmful microbes, displaying an

175   aversion to the contaminated food (Babin et al., 2014; Keita et al., 2017; Stensmyr et al.,

176   2012; Surendran et al., 2017). Therefore, we tested whether the feeding behavior was affected

177   by the presence of Delfin. We added a blue dye in the food and counted the number of feeding

178   larvae containing blue food in their gut, either in L3 (Fig. 2B) or in L2 stage (Fig. 2C).

179   Although we observed a slight decrease in L2 larvae reared under Delfin 1X dose condition,

180   we did not detect any change in feeding behavior. We then quantified the food intake and,

181   once again, we did not detect any significant modification in the L3 larvae (Fig. 2D), unlike in

182   the L2 larvae, which were affected whatever the condition (excepted for the $Btk^{\Delta Cry}$ 10X

183   condition which was not significant) compared to the control (Fig. 2E). Therefore, although

184   food contaminated with *Btk* spores (with or without crystals of toxins) disturbed food intake

185   of L2 larvae but not of L3 larvae, these data do not explain the specific impact of Delfin on

186   the developmental time and growth of the larvae.

187

188   ***Btk* bioinsecticide impairs intestinal protein metabolism**

189   Proteins are a key nutrient to drive normal larval development and growth. Increasing protein

190   content in the diet accelerates the developmental time and/or stimulates growth, while an

191   increase in sugar content delays development and a change in lipid amounts has only a

192   marginal impact (Anagnostou et al., 2010; Colombani et al., 2003; Layalle et al., 2008; Reis,

193   2016; Rodrigues et al., 2015; Soultoukis and Partridge, 2016). Because we have previously

194   shown that the presence of opportunistic bacteria in the adult midgut impede the digestion of

195   proteins (Loudhaief et al., 2017), we wondered whether this could also be the case in larvae in

196   the presence of *Btk* bioinsecticide. While the 10X dose of $Btk^{\Delta Cry}$ had no consequence, we

197   observed a decreased capacity of the midgut of L3 larvae to digest proteins in a Delfin dose-

198   dependent manner (Fig. 3A).

199   We next wondered whether increasing the protein content of the diet could rescue the

200   developmental delay and the growth defects caused by the ingestion of Delfin. Our rearing

201   medium contains 2% yeast as a protein source (see Materials and Methods). We therefore

202   performed a pupation curve with larvae raised on a medium containing 10% yeast with the 1X

203   or 10X doses of Delfin (Fig. 3B). Compared to the control, no difference in the developmental

204   time was observed for the 1X dose of Delfin and only a slight delay for the 10X dose (Table

205   1). Noteworthy, the developmental time for all conditions on the protein-rich medium was

206   shortened compared to the control on our conventional medium (light green curve in Fig. 3B

207   and Table 1). Similarly, the pupal volume (Fig. 3C) was completely restored for the 1X dose

208   of Delfin and partially rescued for the 10X dose of Delfin (5.9% volume loss instead of 11.9%

209   in the poor-protein diet). Taken together, our data suggest that Delfin impairs protein

210   digestion, and thus gut functions, which can be rescued by adding extra dietary protein.

211

212   **The commensal bacterium *L. plantarum* helps to overcome *Btk* bioinsecticide impacts**

213   It has been recently shown that the commensal bacterium *L. plantarum* promotes the juvenile

214   systemic growth of *Drosophila* larvae and infant mice by stimulating digestion and absorption

215   of proteins, and protects the child against sepsis upon undernutrition conditions (Erkosar et

216   al., 2015; Panigrahi et al., 2017; Schwarzer et al., 2016; Storelli et al., 2011; Tefit and Leulier,

217   2017). Therefore, we wondered whether complementing our conventional medium with *L.*

218   *plantarum* would compensate for the impacts of the 10X dose of Delfin. First, we observed

219   that adding *L. plantarum* in the conventional medium shortened the developmental time of

220   control larvae (Fig. 4A, purple curve compared to green curve and Table 1). Interestingly, the

9

221   presence of *L. plantarum* fully counteracted Delfin-dependent developmental delay (Fig. 4,

222   pink curve compared to red curve and Table 1). We next observed that the presence of *L*

223   *plantarum* in the conventional medium reduced the pupal size of the control larvae by 4.4%

224   compared to the control without *L. plantarum* (Fig. 4B, purple bar compared to green bar) but

225   fully rescued the growth defects induced by the 10X dose of Delfin (Fig. 4B pink bar). Since

226   ingestion of *L. plantarum* and its establishment in the midgut (Loudhaief et al., 2017) might

227   compete for the presence of *Btk* and not act on protein intake as expected, we also controlled

228   the number of *Btk* CFUs in the absence or presence of *L. plantarum* in the midgut of L3

229   larvae and we found no difference (Fig. 4C). Taken together, our data demonstrated that

230   stimulation digestion and/or absorption of proteins by the commensal bacterium *L. plantarum*

231   helps to overcome the adverse effects of the *Btk* bioinsecticides on larval development.

232

233   **The impacts of *Btk* bioinsecticide do not rely on commensal bacteria disturbance**

234   *L. plantarum* is a widespread commensal bacterium of the *Drosophila* midgut (Broderick and

235   Lemaitre, 2012; Chandler et al., 2011; Fast et al., 2018). Because complementing the larval

236   diet with *L. plantarum* rescued the developmental delay and growth defects induced by *Btk*,

237   we wondered whether the indigenous commensal flora of larvae reared on our conventional

238   medium (i.e. poor in protein) was inhabited by *L. plantarum*. Only few *L. plantarum* and/or *L.*

239   *brevis* (belonging to the family of *Lactobacillaceae*) were present in the commensal flora of

240   our larvae (Fig. 4D). The dominant bacteria family we identified belonged to

241   *Acetobacteraceae* known to be common in flies feeding on sugary, acidic and alcoholic food

242   (Chandler et al., 2011; Téfit et al., 2018). This observation was in agreement with the

243   composition of our conventional rearing medium poor in protein but quite rich in simple sugar

244   (i.e. sucrose, see Materials And Methods) and it suggested that the *Btk* effects we observed

245   were independent of a putative deleterious impact on the indigenous *L. plantarum* of our

246   larvae.

247   However, we could not rule out an impact of the *Btk* bioinsecticide on other members

248 of the commensal flora. To test this hypothesis, we generated an axenic fly strain (see

249 Materials And Methods). Axenic larvae (Fig. 4D) were further fed on our conventional

250 medium treated or not with the 10X dose of Delfin. The developmental time of the control

251 axenic larvae was slightly shorter than those of the conventionally reared (CR) larvae (i.e.

252 non-axenic) (Fig. 4E and Table 1). Nevertheless, axenic larvae infected with Delfin 10X

253 displayed a 19h developmental delay compared to the control axenic larvae (+11.7%) (Fig. 4E

254 and Table 1), which was similar to the delay observed with *Btk*-treated CR larvae (see Fig. 1).

255 Then, while axenic larvae gave rise to pupae with a similar volume to that of CR larvae (Fig.

256 4F), Delfin 10X still induced a reduction of pupal volume in axenic larvae compared to the

257 axenic control (Fig. 4F) in a magnitude similar to what we observed for Delfin 10X with CR

258 larvae (see Fig. 1). Taken together our data strongly suggest that Delfin ingestion affects

259 developmental time and growth independently of the commensal bacteria.

260

261 ***Btk* bioinsecticide induces midgut perturbations**

262 Because we had previously shown that the ingestion of *Bt*k vegetative cells by adult

263 *Drosophila* reduced the accumulation of lipids in enterocytes (Loudhaief et al., 2017), we

264 wondered whether the commercial *Btk* product did the same in larvae. In unchallenged L3

265 larvae, lipid droplets strongly accumulate in enterocytes in the most anterior part of the

266 midgut and weakly in the posterior midgut (Fig. 5A) (Palm et al., 2012). Instead of lipid

267 droplets depletion, feeding the larvae with Delfin 10X provoked an accumulation of lipid

268 droplets in the posterior larval midgut (Fig. 5E). Furthermore, lipid droplets accumulated

269 apically, facing the lumen (Fig. 5F and F' compared to 5B and B'). Such an accumulation was

270 not observed when developing larvae were fed with $Btk^{\Delta Cry}$ spores (Fig. 5C, D and D').

271 Interestingly, it has been recently demonstrated that the pore-forming toxins hemolysin and

272 monalysin produced respectively by the enteropathogenic bacteria *Serratia marcescens* (*Sm*)

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273   and *Pseudomonas entomophila* (*Pe*) can induce an apical accumulation of lipid droplets in

274   adult enterocytes of *Drosophila*, honeybees and mice as well as in epithelial Caco-2 cells in

275   culture. This phenomenon precedes the expulsion of cytoplasm required to get rid of toxins

276   from the enterocytes (Lee et al., 2016). Since the Cry toxins produced by *Btk* are also pore-

277   forming toxins (Adang et al., 2014), we fed developing larvae with purified toxin crystals and

278   checked for the accumulation of lipid droplets. Unexpectedly, we did not observe any

279   accumulation of lipid droplets (Fig. 5G, H and H'). However, feeding our developing larvae

280   with the naphthalene sulfonic acid (the additive present in the commercial products, see

281   Supplemental Materials) provoked an accumulation of apical lipid droplets posterior to the

282   acidic domain (Fig. 5I, J and J'). To further assess whether such a lipid accumulation in the

283   midgut would impair lipid metabolism in the whole larva, we quantified the circulating lipids

284   (the diacylglycerides - DAG) in the hemolymph and the stored lipids (triacylglycerides -

285   TAG) (Tennessen et al., 2014) in mid-third instar larvae. We did not notice any significant

286   perturbation in lipid metabolism (Fig. 5K) though a downward trend was visible for

287   circulating DAG in larvae fed with Delfin 10X. Thereby, it is possible that such an apical

288   accumulation of lipid droplets serves for the trap and elimination of the naphthalene sulfonic

289   acid similarly to what happens for the elimination of the *Sm* and *Pe* pore-forming toxins (Lee

290   et al., 2016).

291   In adult *Drosophila*, pathogenic and opportunistic bacteria can induce enterocyte

292   apoptosis upon ingestion (Apidianakis et al., 2009; Loudhaief et al., 2017). Therefore, we

293   verified the appearance of dying cells in the midgut using the Caspase3::GFP sensor to

294   visualize apoptosis in living tissue (Schott et al., 2017) (see Materials And Methods). While

295   unchallenged and *Btk^{ΔCry}* fed larvae did not display any obvious apoptosis, the developing

296   larvae fed with Delfin harbored apoptotic enterocytes mainly in the posterior part of the

297   intestine (Fig. 5L-M and Fig. S3). Because in target lepidopteran larvae, Cry toxins make

298   holes in the gut lining, breaking down the gut barrier and allowing *Btk* cells to penetrate

299   inside the internal milieu (Adang et al., 2014), we checked whether the sealing of the gut

300   epithelium was maintained even in presence of widespread apoptosis. We fed our developing

301   larvae with the small fluorescent molecule Dextran-FITC that is unable to cross the midgut

302   epithelium (Fig. 5O) unless the intestinal barrier is broken, allowing the fluorescence to

303   spread throughout the larva like in the case of *Pe* infection (Fig. 5P). Interestingly, $Btk^{\Delta Cry}$

304   spores as well as Delfin did not induce the loss of gut barrier function (Fig. 5Q and R). Thus,

305   our data show that *Btk* bioinsecticide hampers intestinal lipid homeostasis and induces

306   enterocyte apoptosis without causing the rupture of the intestinal barrier. Such physiological

307   perturbations may be responsible for the developmental delay and the growth defect observed.

308

309   **Enterocyte flattening and incomplete differentiation of Adult Midgut Precursors help to**

310   **maintain intestinal integrity**

311   Intestinal physiology can also be impeded by a disturbance of the apico-basal polarity of the

312   gut epithelium. In the control L3 larval midgut, Armadillo (Arm, β-Catenin), a component of

313   the adherens junction, marks the basolateral compartment of the enterocytes and Disc Large

314   (Dlg), a component of the septate junction, marks the sub-apical compartment. Phalloidin that

315   binds to F-Actin reveals both the apical brush border (facing the lumen) and the basal visceral

316   muscles that surround the midgut lining (Fig. 6A and A'). This organization is similar to the

317   adult midgut (Chen et al., 2018; Loudhaief et al., 2017). Moreover, Arm also delineates the

318   islets of the adult midgut precursors (AMPs), which will give birth to the adult midgut during

319   metamorphosis (Fig. 6A) (Jiang and Edgar, 2009; Mathur et al., 2010; Micchelli et al., 2011).

320   When larvae were fed with the 10X dose of $Btk^{\Delta Cry}$ spores, we did not observe any major

321   perturbations of the architecture of late L3 midguts (Fig. 6B and B'). However, we observed

322   an increased number of AMPs per islet (Fig. 6D) correlated with an increase in AMP division

323   (Fig. 6E). When larvae were fed with Delfin 10X, we also observed an increased number of

324   AMPs by islet (Fig. 6D) and a huge increase in AMP proliferation (Fig. 6E) though at the

325   expense of the number of islets (Fig. 6F). Moreover, we also observed an increase in the

326   enterocyte surface (Fig. 6C and G). Interestingly, such an increase in enterocyte surface did

327   not rely on a higher ploidy (Fig. 6J) but rather on a change in cell shape, i.e. enterocyte

328   flattening (Fig. 6C' and H). Finally, we noticed the appearance of uncharacterized cells having

329   an "intermediate" size. These cells which were intercalated between large enterocytes and the

330   AMP islets, were neither mature polyploid enterocytes nor diploid AMPs or enteroendocrine

331   cells (Fig. 6I and I'). This observation was confirmed by analyzing the ploidy of the nuclei. In

332   the control larvae, 89% of the polyploid cells underwent 4 endocycles of replication (32C

333   nuclei, C corresponding to chromatin copy number) and 10% reached 5 cycles of

334   endoreplication (64C) (Fig. 6J). When larvae were raised on medium contaminated with

335    $Btk^{\Delta Cry}$ spores, 74% corresponded to 32C nuclei and 25% to 64C nuclei (Fig. 6J). When larvae

336   were fed with Delfin 10X, a new population of cells with 16C, 8C and 4C nuclei appeared,

337   representing 37% of the population of polyploid cells (Fig. 6J). While the adult midgut

338   quickly regenerates upon an oral infection thanks to the proliferation of adult intestinal stem

339   cells and the differentiation of their daughter cells (the enteroblasts) into new enterocytes

340   which repair the damages (Bonfini et al., 2016), such a mechanism has never been observed

341   in *Drosophila* larvae. Indeed, the number of enterocytes composing the L3 intestine is defined

342   during embryogenesis (Miguel-Aliaga et al., 2018) and the larval gut grows without cell

343   division but by increasing the ploidy and consequently the size of enterocytes. However, our

344   data suggested that upon Delfin ingestion, larval gut homeostasis is perturbed and the

345   apparition of undefined cells is promoted. In the larval intestine, AMPs are the only cells

346   capable of division (Jiang and Edgar, 2009; Mathur et al., 2010; Micchelli et al., 2011).

347   Therefore, we hypothesized that the unknown cells having a nucleus ranging from 4C to 16C

348   (Fig. 6J) and adjacent to the AMP islets (Fig. 6I) could arise from the differentiation of those

349   AMPs. To test our hypothesis, we used the lineage tracing genetic tools developed in

350   *Drosophila* named ReDDM (Antonello et al., 2015). The principle of this lineage tracing is

351   based on the expression of both a labile GFP and a stable RFP within the AMPs thanks to the

352   use of a promotor specifically expressed in AMPs and inducible at will (see Materials And

353   Methods). Accordingly, AMPs express both GFP and RFP, while the progenies, which are not

354   AMPs, only express RFP. In control larvae, GFP and RFP were only present in the islet cells

355   (Fig. 6K) (Jiang and Edgar, 2009; Mathur et al., 2010; Micchelli et al., 2011). When larvae

356   were fed with $Btk^{\Delta Cry}$ spores, GFP and RFP were also only expressed in the same AMP cells

357   (Fig. 6L). In contrast, when larvae were fed with Delfin 10X, cells harboring only RFP were

358   now detectable. Furthermore, the newborn cells had nuclei of intermediate size ranging from

359   4C to 16C (Fig. 6M). This result demonstrated that upon aggression by Btk bioinsecticides,

360   the larval midgut is able to produce new cells, most probably immature enterocytes arising

361   from AMPs. Altogether, our data suggest that the larval intestine maintains its integrity upon

362   aggression through two mechanisms, i) enterocyte flattening which increases the cell surface

363   without new cycles of endoreplication and ii) producing new immature enterocytes. Both

364   mechanisms may allow the intestine to maintain the intestinal barrier, avoiding the bacteria to

365   penetrate into the internal milieu and therefore preventing septicemia.

366

Discussion

368   The long-term effects of chronic exposure to low doses of potential noxious products cannot

369   be easily revealed by conventional epidemiological studies (which are long lasting and

370   expensive) or conventional (eco)toxicological approaches based on acute toxicity. The use of

371   amenable and cheap laboratory models becomes of prime importance to pinpoint discrete

372   chronic adverse effects. *D. melanogaster* fulfills all the required criteria to be such a model.

373   Indeed, *Drosophila* is easily handling, cheap to rear and the availability of genetic, cellular

374   and molecular tools allows to detect tenuous effects and to decipher the underlying

375   physiological mechanisms impaired (Scott and Buchon, 2019). Moreover, the main

376   physiological functions and genetic networks governing the *Drosophila* gut physiology are

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377   well conserved among the animal kingdom (Pasco et al., 2015), allowing to provide

378   informative outputs for both human health and wildlife.

379   *Btk* bioinsecticides have recently been shown to induce larval lethality and delay the

380   imago emergence of many different *Drosophila* species at doses ranging from 5×10^7 to 1×10^9

381   CFU of *Btk* spores/g of fly food medium (Babin et al., 2019; Cossentine et al., 2016). Larval

382   lethality is observed for the highest doses (Babin et al., 2019) and could be explained by the

383   cross-order toxicity of the Cry2A toxins (Cry2Aa and Cry2Ab are present in *Btk*

384   bioinsecticides) towards larvae of certain mosquito species (Frankenhuyzen, 2017) and

385   references herein). In this study, we investigated the physiological and cellular mechanisms

386   disturbed by *Btk* products which are responsible for developmental delay and growth defect.

387   To best mimic environmental conditions, we used doses potentially present in the field after

388   agricultural spraying. The lower dose we used corresponds to the amount deposited on the

389   vegetables after one treatment. The highest dose reflects the amount that may be encountered

390   after multiple treatments, even though data regarding *Bt* accumulation in the fields remain

391   scarce. Indeed, the half-life of the entomopathogenic Cry1 toxins being estimated at ten days

392   in the field (Hung et al., 2016), weekly sprays are recommended by the manufacturer and up

393   to 10 are authorized. Regarding the persistence of spores, it has been shown that 72 hours

394   after spraying of chards with *Btk* bioinsecticides, almost all of the *Btk* was still present at the

395   surface of the leaves (Bizzarri and Bishop, 2008). Ten days later, the amount of *Btk*

396   bioinsecticides corresponded to 50% of the initial dose sprayed on the leaves. The amount of

397   *Bt* found on the leaves approaches normal values only 28 days after treatment ($<10^1$ CFU/g)

398   (Raymond et al., 2010). Furthermore, once *Bt* has reached the soil, it persists more than a year

399   in the treated soil (Vettori et al., 2003). Our study highlights that such a persistence and

400   accumulation of *Btk* bioinsecticides may affect the development of non-target insects.

401   Nonetheless, the impacts are mainly due to the synergy between *Btk* spores and crystals since

402   either the spores ($Btk^{\Delta Cry}$) or the purified crystals alone do not promote detectable

403   developmental delay or growth defects in *Drosophila* larvae. These data are in agreement

404   with the study analyzing the safety of *Btk* purified Cry toxins on *Drosophila* larval

405   development (Haller et al., 2017).

406   Interestingly, the effects we have characterized here are reminiscent of those observed

407   on the target organisms *Helicoverpa armigera* and *H. zea*, *Spodoptera frugiperda* and

408   *Choristoneura fumiferana*. Indeed, when fed with sublethal doses of *Btk* spores, all these

409   target organisms display developmental delays and/or reduced pupal size (Bauce et al., 2006;

410   Polanczyk and Alves, 2005; Rabinovitch et al., 2017; Sedaratian et al., 2013). Moreover,

411   recent work has also shown that the potent pathogens *P. entomophila* and *P. aeruginosa* or

412   huge amounts of the opportunistic bacterium *Erwinia carotovora carotovora* induce

413   developmental delay and growth defects in *Drosophila* larvae (Houtz et al., 2019). These data

414   suggest that developmental delay and/or growth defects must be general consequences of

415   intestinal infections. The compromised gut digestive capacity of the infected larvae (whatever

416   the insect) is likely the main reason for such developmental defects. Indeed, we and others

417   have shown in the adult (Buchon et al., 2009; Chakrabarti et al., 2012; Erkosar et al., 2015;

418   Loudhaief et al., 2017; Wong et al., 2016) or in larva (this work) that allochthonous bacteria

419   induce a more or less widespread apoptosis of enterocytes leading to a decreased digestive

420   and/or absorptive capacities. The developmental delay and the growth defect likely reflect the

421   degree of damages suffered by the intestine. Houtz and colleagues observed a delay in

422   pupation of up to 4 days using strong pathogens (Houtz et al., 2019) while we observed here a

423   delay between 16 hours (Delfin) and 24 hours (Dipel) with *Btk* products. Therefore, while *Btk*

424   behaves as a strong pathogen on target organisms, ultimately leading to death (in fact the

425   expected effect), it has milder but significant impacts on the development of non-target insect.

426   Our work demonstrates that the composition of the diet is important to overcome *Btk*

427   intestinal infection. Increasing the protein content of the nutritive medium rescues both

428   growth defects and developmental delay. Our data are in agreement with previous

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429   observations in mammals and insects infected with various pathogenic bacteria or viruses

430   showing that protein intake is important to withstand attacks by pathogens, to support an

431   efficient immune defense and to promote growth, while intake of carbohydrates is deleterious

432   for all these functions (Bing et al., 2018; Lee et al., 2006; Reis, 2016; Rodrigues et al., 2015;

433   Shikano and Cory, 2014; Soultoukis and Partridge, 2016). Moreover, our data show that the

434   commensal bacterium *L. plantarum* also participates in the protection of larvae from *Btk*

435   intestinal aggression in accordance with previous works involving commensal bacteria in gut

436   protection against pathogens (Ubeda et al., 2017; Van Arnam et al., 2018). In *Drosophila*, *L.*

437   *plantarum* has been shown to protect adult flies from *Sm-* or *P. aeruginosa*-induced mortality

438   and this protection is specific since the commensal bacteria *Enterococcus faecalis* or *B.*

439   *subtilis* do not operate as such (Blum et al., 2013). Our work strongly suggests that the benefit

440   brought by *L plantarum* to the larvae of enduring *Btk* aggression passes through an enhanced

441   protein digestion and/or absorption by the gut in line with previous observations (Erkosar et

442   al., 2015). Finally, we did not observe a decrease in food intake suggesting that spores and

443   commercial products do not induce avoidance behavior. Together, our data support the model

444   that the impact of the *Btk* products on the development of the non-target organism *D.*

445   *melanogaster* (and probably many other arthropods) depends on both the nutritional

446   environment and the performance of its commensal flora. Consequently, spraying *Btk*

447   products when environmental conditions are stressful would amplify the adverse effects of

448   *Btk* products. Our work also sheds light on the pathogenic opportunism of *Btk* on a broad

449   range of organisms owing to the conservation of the digestive tract architecture and functions

450   (Pasco et al., 2015). For instance, the impacts of *Btk* on animals or humans suffering from

451   dietary deficiencies (very often associated with dysbiosis) may be more deleterious than on

452   individuals with a well-balanced diet. Of note, bacteria of the *B. cereus* group are well-known

453   opportunistic pathogens responsible for foodborne poisoning (EFSA BIOHAZ, 2016; Wells-

454   Bennik et al., 2016). Therefore, a more accurate monitoring of the presence of *Btk* in food and

455   environment is needed to avoid any setbacks, especially in the current context where organic

456   farming is gaining market share.

457   Finally, our investigations have unraveled two concomitant mechanisms allowing

458   *Drosophila* larvae to overcome an intestinal infection. It was thought so far that the

459   *Drosophila* larval gut was unable to regenerate due to the short period covering the larval

460   stages (about 5-6 days) and the inability to give birth to news cells to replenish the damaged

461   ones (Miguel-Aliaga et al., 2018). Only two choices were thus offered to the larvae: *Live or*

462   *let die*. However, we have uncovered a third choice for the infected larvae: *Die another day*.

463   First, we demonstrate that the adult midgut precursors (AMPs) can be diverted from their

464   original fate. Indeed, upon aggression of the larval intestinal epithelium, the AMPs can

465   engage toward a process of differentiation to give birth to enterocyte-like cells, which are

466   intercalated in between AMP islets and enterocytes. We can assume that such newborn cells

467   could plug the holes caused by the death of enterocytes, thus avoiding the entry of luminal

468   bacteria into the internal milieu. Unfortunately, newborn cells do not fully differentiate and

469   probably do not participate in digestive functions as efficiently as well-differentiated

470   enterocytes. The addition of these misdifferentiated cells to dying enterocytes can explain the

471   reduced protease activity of the intestine of infected larvae and the subsequent developmental

472   delay and growth defect. The huge increase in the proliferation of AMPs that we observed at

473   the end of L3 upon *Btk* spore ingestion could be explained by the necessity to replenish the

474   right number of AMPs before pupation to achieve a complete adult gut metamorphosis and

475   therefore produce a viable adult fly. These AMPs replenishment could also participate to the

476   developmental delay we observed. The work very recently published by Houtz and colleagues

477   reaches to similar conclusions (Houtz et al., 2019).

478   We have also uncovered a second unexpected mechanism involved in the maintenance

479   of gut integrity: the change in enterocyte shape. So far, it has been shown in many species

480   (insects, mammals or plants) that the loss of cells in response to stressors can be compensated

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481 by the rapid increase in the size of neighboring cells thanks to successive endocycles (Edgar

482 et al., 2014). Here, we evidence an increase in enterocyte area upon *Btk* spore ingestion but

483 concomitantly we have observed a thinning of the epithelium. Above all, we did not observe

484 an increase in the ploidy of the enterocyte nuclei. Interestingly, it has been shown that old

485 enterocytes in adult *Drosophila* midgut cannot re-enter the endocycle (Xiang et al., 2017).

486 Therefore, as for old adult enterocytes, L3 enterocytes (born during embryogenesis 7 days

487 earlier) are probably unable to re-enter a cycle of endoreplication to promote cell growth.

488 Hence, changing their shape through flattening allows to increase the area covered by one

489 enterocyte and to participate in maintaining epithelium sealing. Here again, the absence of

490 endoreplication likely participates to the reduced digestive capacities of the intestine and

491 therefore to the developmental delay and growth defects.

492 It has been proposed that lepidopteran larvae succumb to *Btk* spores and toxins

493 because they are unable to maintain their gut integrity. Conversely, one mean for a

494 lepidopteran larva to acquire resistance to *Btk* bioinsecticides may rely on its capacity to

495 maintain/regenerate its intestinal integrity (Castagnola and Jurat-Fuentes, 2016; Castro et al.,

496 2019; Dubovskiy et al., 2016). Non-target organisms are likely as such because they are able

497 to regenerate their gut lining faster than *Btk* bioinsecticides destroy it. However, increasing

498 the amount of *Btk* products provided to *Drosophila* larvae ultimately leads to their death

499 (Babin et al., 2019), suggesting that the harmfulness of *Btk* products relies on both the

500 amounts of *Btk* products ingested and the affinity of Cry toxins for more or less specific

501 receptors. Altogether, these data suggest that *Btk* spores used as bioinsecticides may be not as

502 safe as expected on non-target organisms. Moreover, the opportunistic virulence of *Btk* for

503 many organisms should not be overlooked, especially for those suffering from nutritional

504 stress, dysbiosis or with a compromised innate immunity. A situation that more and more

505 species will encounter with the climatic changes occurring worldwide. Many investigations

506 remain to be done to improve the use and efficiency of *Btk* products and to make their use as

507 safe as possible for both handlers and consumers (wildlife or humans).

508

509 **MATERIALS AND METHODS**

510 *Fly Stocks and genetics*

511 Canton S (WT) (Bloomington #64349); *w, dlg::GFP cc01936* (FlyTrap) was provided by A.

512 Spradling; *w; myo1A-Gal4* was a gift from N. Tapon; *w, UAS-GC3Ai^{G7S}* were kindly provided

513 by Magali Suzanne and described in (Schott et al., 2017). Apoptosis was monitored on F1

514 progeny *w/w; myo1A-Gal4/+; UAS-GC3Ai^{G7S}/+* L3 larvae. The ReDDM fly stock to trace

515 cell progenies in the midgut was kindly provided by Maria Dominguez (Antonello et al.,

516 2015). To perform lineage tracing, synchronized eggs (*w/+; esg-Gal4 UAS-GFP/+; tub-*

517 *Gal80^{ts} UAS-H2B::RFP/+*) were hatched on contaminated food (see below for the method)

518 and maintained for 24h at 25°C. Then, L1 larvae were transferred and maintained at 29°C to

519 alleviate the inhibitory effect of Gal80^{ts} on the Gal4 transcription factor. Intestine were

520 dissected and observed at mid L3 (between days 4 and 5).

521 *Bacterial strains*

522 *L. plantarum^{WJL}* that has been described in (Ryu et al., 2008) and sequenced in (Martino et al.,

523 2015) was kindly provided by Bernard Charroux (IDBM, Marseille, France) and by Renata

524 Matos (F. Leulier's Lab, IGFL, Lyon, France). *Pseudomonas entomophila* (Vodovar et al.,

525 2005) was kindly provided by Bruno Lemaitre's Lab (EPFL, Lausanne, Switzerland). *Pe* and

526 *L. plantarum^{WJL}* were grown as described in (Loudhaief et al., 2017). The *Btk$^{\Delta Cry}$* strain

527 (identified under the code 4D22) (Gonzalez et al., 1982) was obtained from the *Bacillus*

528 Genetic Stock Center (www.bgsc.org). Delfin and Dipel formulated bioinsecticides were

529 bought in an agricultural shop center. The strain *Btk* ABTS-351 was isolated from Dipel on

530 LB agar.

531 *Spore preparation*

532   *Btk* vegetative cells were let to grow and sporulate during two weeks at 30°C with shaking in

533   2L of PGSM medium. The culture was then heated 1h at 70°C to eliminate vegetative cells

534   and centrifuged for 15 min at 5000g. The pellet was first washed with 1 L of 0.15M NaCl and

535   twice with sterile water. The final pellet was suspended in 30 mL of sterile water, aliquoted

536   and lyophilized 24h-48h. The spore titration was determined by serial dilution of a known

537   weight of lyophilized spores and counting the number of "colony forming unit" (CFU) on LB

538   agar after incubation at 30°C for 18h.

539   ***Crystal purification***

540   Two grams of the commercial product were suspended in 60 mL of sterile water, dissolved

541   for 5h at 4°C with agitation and then sonicated 4 times 15s with a frequency set at 50%

542   (Fisherbrand™ Model 505 Sonic Dismembrator). Ten milliliters of the homogenate were

543   deposited on a step density gradient of 67/72/79% sucrose and centrifuged at 100 000g for

544   18h at 4°C. Crystals were collected on the interfaces between the different sucrose phases and

545   washed twice with sterile water. The final pellet was suspended in 12 mL of sterile water,

546   aliquoted and lyophilized 24h-48h.

547   ***Diet composition***

548   *Drosophila* were reared at 25°C on conventional medium (referred as the "poor-protein"

549   medium in this study): 8% cornmeal (AB Celnat), 2% yeast (Springaline® BA10/0–PW,

550   Amcan), 2.5% sucrose, 0.8% agar (ref 20768-361, VWR) and 0.6% methylparaben

551   (Tegosept,, ref 789063 Dutscher). The protein rich medium contains 10% yeast instead of 2%.

552   ***Intestinal CFU counting***

553   Colony forming unit counting were described in (Loudhaief et al., 2017).

554   ***Commensal flora estimation***

555   Ten flies or ten mid-third instar larvae were washed first with ethanol 70% for 30 seconds,

556   then washed with PBS and crushed in 500 µL of LB medium with a Tissue Lyser (Qiagen ref.

557   85600) at 50Hz for 5 minutes. The homogenate was plated on different selective media to

558 identify the common commensal bacteria. MRS agar (Sigma ref 69964 + 1 mL of Tween

559 20/L) at 37°C in anaerobic was used to identify *Lactobacillus plantarum* and *brevis*. BHI agar

560 (Sigma ref 70138) at 37°C allowed the identification of *Enterococcus faecalis*. LB agar

561 (Fisher ref BP1426-2) at 37°C allowed the identification of *Escherichia coli* and

562 *Staphylococcus aureus*. Mannitol medium (Bacto peptone 3g/L, Yeast extract 5g/L, D-

563 Mannitol 25g/L, agar 15g/L) at 30°C allowed the identification of *Commensalibacter intestini*

564 and *Acetobacter pomorum*.

Axenic flies

566 Adult Canton S flies were let to oviposit eggs for two days at 25°C and 12h/12h day/night

567 cycle on our conventional medium supplemented with a cocktail of four antibiotics:

568 ampicillin, kanamycin, tetracycline and erythromycin (50 µg/mL each). On the next

569 generation, newly hatched adults were immediately removed and transferred on fresh

570 conventional medium containing the four antibiotics. This operation was repeated until flies

571 with axenic gut were obtained. The presence of commensal flora was checked at each fly

572 generation (Fig. S1). Axenic flies were obtained after 6 generations.

Oral infection of larvae

574 Adult flies were let to oviposit eggs for 4h at 25°C on laying nests containing grape juice

575 medium (25% grape juice, 3% agar, 1.2% sucrose, 2% ethanol, 1% acetic acid). Twenty eggs

576 were collected and transferred in a *Drosophila* vial containing 2g of the desired medium

577 mixed with *Btk* spores, *Btk* crystals or the naphthalene additive. The conditions used for 2g of

578 medium and 20 eggs were: 10^7 CFU of *Btk* spores for the 1X dose; 10^8 CFU of *Btk* spores for

579 the 10X dose; 0.67mg of *Btk* purified crystals (equivalent to the 10X dose and considering

580 that crystals represents 15-30% of spore weight (Agaisse and Lereclus, 1995; Monro, 1961;

581 Murty et al., 1994)); 10^8 CFU of *L. plantarum* or *Pe*; 0.4mg of naphtalene-2-sulfonic acid

582 sodium salt (ref A13066, Alfa Aesar) (equivalent to the 10X dose). Vials were incubated at

583 25°C with a 12h/12h day/night cycle until further processing.

23

584   *Pupation curve*

585   Immobilized larvae forming white pupae were counted. The number of pupae was expressed

586   as the percentage of the final number of pupae. While 20 eggs were deposited in each tube, in

587   some conditions, especially with Dipel and Delfin, we never got 20 pupae probably due to

588   previously described larval lethality induced by the *Btk* products (Babin et al., 2019). Table 1

589   summarizes the developmental time at which 10%, 50% and 90% of the larvae were

590   immobilized for all the conditions we tested. For each condition described above, the

591   experiment was performed at least in three experimental blocks of triplicates.

592   *Pupal size*

593   On day 7, 8 and 9 after egg laying, pupae were gently removed from the vial, washed in water

594   and slightly dried with tissue paper. Pupae were placed on a microscope slide and pictures

595   were taken with a numeric microscope VHX-2000 (Keyence). Pupal length and width were

596   measured with Image J software and pupal volume was calculated using the following

597   formula for ellipsoid: $V = [(4/3) \times 3,14 \times (L/2) \times (l/2)^2]$ where V is the volume, L the length

598   and l the width of the pupa.

599   *Larval feeding behavior and Food intake*

600   Quantification of larval food intake was performed according to (Bjordal et al., 2014) with the

601   following modifications: we deposited a mix of 50% yeast/0.75% of the food blue dye

602   Erioglaucin (ref 861146, Sigma) in the center of a Petri dish lid (diameter: 35mm) to feed 20

603   mid-third larvae (previously infected or not) *ad libitum*. After 2h at 25°C, larvae were

604   collected and washed with water. The number of white larvae (indicating no feeding) and blue

605   larvae was counted. Ten blue larvae were transferred into a microtube containing 500 μL of

606   PBS, crushed at 50Hz for 2 minutes with a TissueLyser (Qiagen, ref 85600) and centrifuged

607   at 10000g for 5 minutes at 4°C. The amount of Erioglaucin in the supernatant was quantified

608   at 629 nm with a spectrophotometer (SpectraMax® Plus384, Molecular device).

609   *Midgut permeability*

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610   The same technique as described above was used except that the mix contained 50% yeast

611   and 1.25 mg/ml Dextran FITC (ref 46944, Sigma) in water. Twenty previously orally infected

612   mid-third instar larvae were placed in the mix. After 2h at 25°C, the larvae were collected,

613   washed with water and placed at least 30 minutes at 4°C. Pictures were taken with a

614   fluorescent stereoscopic AxioZoom V16 (Zeiss) microscope.

615   *Metabolic assay*

616   Intestinal protease activity assay was performed as described in (Nawrot-Esposito et al., 2017)

617   with 8 mid-third instar larval midguts per condition. Lipids were assayed as described in

618   (Tennessen et al., 2014). The tri- and diacylglycerol (TAG and DAG) were measured on 3µl

619   of hemolymph obtained from 5 days old larvae using the Triglyceride Reagent (ref T2449,

620   Sigma) and Free Glycerol Reagent (ref F6428, Sigma). The metabolite quantity was reported

621   per mg of protein or per µL of hemolymph. Protein assay was achieved with Biorad Protein

622   Assay Dye Reagent Concentrate (ref 5000006, Biorad). All experiments were performed at

623   least 3 times in triplicates.

624   *Lipids labelling*

625   Five days after infection (mid-third instar larvae), ten larval guts were dissected in PBS and

626   fixed 40 min in 4% formaldehyde in PBS. Guts were then washed twice in PBS and further

627   incubated for 30 min. either in 5µg/ml of BODIPY™ 493/503 (ref D3922, Invitrogen™

628   Molecular Probes™) or in a 0.06% Oil-red-O solution (ref 189400250, Acros Organics). Guts

629   were then washed twice with PBS. For BODIPY™ labelling guts were mounted in

630   Fluoroshield/DAPI (ref F6057, Sigma) and immediately observed under a fluorescent

631   microscope (Zeiss Axioplan Z1 with Apotome 2). For Oil-red-O labelling, guts were mounted

632   in 80% Glycerol/PBS and observed with a numeric microscope (VHX 2000, Keyence).

633   *Immunohistochemistry, image capture and processing*

634   Ten guts of late third instar larvae were dissected in PBS and fixed for 40 min. in 4%

635   formaldehyde in PBS. Guts were then washed twice with PBS-0.1% Triton X100, incubated

25

636   for 3h in Blotto (2.5% FBS, 0.1% Triton X-100, 0.02% sodium azide in PBS) and further

637   incubated with the primary antibody overnight at 4°C in Blotto. The following dilutions were

638   used: Mouse anti-Armadillo (DSHB #N2 7A1) at 1/50; Rabbit anti-Phospho-histone-3 (ref

639   9701S, Cell signaling) at 1/500. Guts were washed once with Blotto before incubation in the

640   secondary antibody and/or phalloidin (2h at room temperature in Blotto). The secondary

641   antibodies were the followings: AlexaFluor^{TM} 546 donkey anti-Rabbit IgG (ref A10040,

642   Invitrogen) used at 1:500; AlexaFluor^{TM} 647 goat anti-Mouse IgG (H+L) (ref A21235,

643   Invitrogen) used at 1/500; Alexa Fluor™ 555 Phalloidin (ref A34055, Invitrogen) used at

644   1/1000. Guts were finally washed several times in PBS, mounted in Fluoroshield/DAPI (ref

645   F6057, Sigma) and observed with a Zeiss Axioplan Z1 with Apotome 2 microscope. Images

646   were analyzed using ZEN (Zeiss) software or Photoshop. For cell death monitoring (in

647   *myo1A-Gal4 UAS- UAS-GC3Ai^{G7S} larvae*), guts were dissected in PBS, immediately mounted

648   in 80% Schneider medium/20% glycerol and pictures were rapidly taken with a fluorescent

649   stereoscopic microscope (AxioZoom V16, Zeiss). Image acquisition was performed at the

650   Microscopy platform of the Institut Sophia Agrobiotech (INRAE 1355-UCA-CNRS 7254-

651   Sophia Antipolis).

652   ***Polyploidy estimation***

653   Ploidy was calculated by measuring the area of the nuclei of late L3 larvae. Only round nuclei

654   were analyzed. The average nucleus area of diploid adult midgut precursor cells was

655   calculated. This average area (8µm²) was assigned to the 2C ploidy (C corresponding to

656   chromatin copy number). The theoretical average area of 4C, 8C, 16C, 32C and 64C nuclei

657   was calculated on the basis of the measured 2C average area. 109 nuclei for the control larvae,

658   184 nuclei for *Btk$^{\Delta Cry}$* 10X larvae and 306 nuclei for Dipel 10X larvae were counted and

659   binned into ploidy groups.

660   ***Statistics***

26

661   When "n" was equal or superior to 30, statistical analysis was performed using a parametric

662   T-test. An F-test was systematically done before applying the T-test to verify the homogeneity

663   of variances. When "n" was inferior to 30, we used the non-parametric pairwise comparisons

664   of the Wilcoxon-Mann-Whitney test. Significance symbols are the following: ***

665   ($P \leq 0.001$); ** ($P \leq 0.01$), * ($P \leq 0.05$).

666

685   **Author contributions**

686 **Marie-Paule Nawrot-Esposito:** Methodology, Validation, Formal analysis, Investigation,

687 Resources, Data Curation, Writing - Original Draft **Aurélie Babin:** Methodology, Validation,

688 Formal analysis, Investigation, Writing - Review & Editing **Matthieu Pasco:** Investigation

689 **Marylène Poirié:** Writing - Review & Editing, Funding acquisition **Jean-Luc Gatti:**

690 Validation, Writing - Review & Editing, Funding acquisition **Armel Gallet:**

691 Conceptualization, Validation, Formal analysis, Data Curation, Writing - Original Draft,

692 Writing - Review & Editing, Visualization, Supervision, Project administration, Funding

693 acquisition

694

References

696 **Adang, M. J., Crickmore, N. and Jurat-Fuentes, J. L.** (2014). Chapter Two – Diversity of
697 Bacillus thuringiensis Crystal Toxins and Mechanism of Action. *Advances in Insect*
698 *Physiology* **47**, 39–87.
699 **Agaisse, H. and Lereclus, D.** (1995). How does Bacillus thuringiensis produce so much
700 insecticidal crystal protein? *J Bacteriol.* **177**, 6027-32. doi: 10.1128/jb.177.21.6027-
701 6032.1995.
702 **Amichot, M., Curty, C., Benguettat-Magliano, O., Gallet, A. and Wajnberg, E.** (2016).
703 Side effects of Bacillus thuringiensis var. kurstaki on the hymenopterous parasitic wasp
704 Trichogramma chilonis. *Environ Sci Pollut Res Int* **23**, 3097-103. doi: 10.1007/s11356-015-
705 5830-7. Epub 2015 Nov 21.
706 **Anagnostou, C., Dorsch, M. and Rohlfs, M.** (2010). Influence of dietary yeasts on
707 Drosophila melanogaster life-history traits. *Entomologia Experimentalis et Applicata* **136**, 1–
708 11.
709 **ANSES Saisine n° 2013-SA-0039.** (2013). Risques en termes de sécurité sanitaire liés à
710 l'utilisation de souches de Bacillus thuringiensis (Bt) en tant que substances actives dans des
711 insecticides biologiques. Saisine n°2013-SA-0039,
712 https://www.anses.fr/fr/system/files/DPR2013sa0039.pdf.
713 **Antonello, Z. A., Reiff, T., Ballesta-Illan, E. and Dominguez, M.** (2015). Robust intestinal
714 homeostasis relies on cellular plasticity in enteroblasts mediated by miR-8-Escargot switch.
715 *EMBO* **34**, 2025-41. doi: 10.15252/embj.201591517. Epub 2015 Jun 15.
716 **Apidianakis, Y., Pitsouli, C., Perrimon, N. and Rahme, L.** (2009). Synergy between
717 bacterial infection and genetic predisposition in intestinal dysplasia. *Proc Natl Acad Sci U S A*
718 **106**, 20883-8. Epub 2009 Nov 23.
719 **Babin, A., Kolly, S., Schneider, F., Dolivo, V., Zini, M. and Kawecki, T. J.** (2014). Fruit
720 flies learn to avoid odours associated with virulent infection. *Biol Lett.* **10**, 20140048. doi:
721 10.1098/rsbl.2014.0048. Print 2014 Mar.
722 **Babin, A., Nawrot-Esposito, M.-P., Gallet, A., Gatti, J.-L. and Poirié, M.** (2019). Adverse
723 effects of *Bacillus thuringiensis* bioinsecticide on non-target *Drosophila* species. *bioRxiv*,
724 541847.
725 **Bauce, É., Carisey, N. and Dupont, A.** (2006). Carry over effects of the entomopathogen
726 Bacillus thuringiensis ssp. Kurstaki on Choristoneura fumiferana (Lepidoptera: Tortricidae)

727 progeny under various stressful environmental conditions. *Agricultural and Forest*
728 *Entomology* **8**, 63-76.
729 **Bing, X., Gerlach, J., Loeb, G. and Buchon, N.** (2018). Nutrient-Dependent Impact of
730 Microbes on Drosophila suzukii Development. *MBio.* **9(2).** mBio.02199-17. doi:
731 10.1128/mBio.02199-17.
732 **Bizzarri, M. F. and Bishop, A. H.** (2008). The ecology of Bacillus thuringiensis on the
733 Phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of Pieris
734 brassicae. *Microb Ecol* **56**, 133-9. Epub 2007 Nov 2.
735 **Bjordal, M., Arquier, N., Kniazeff, J., Pin, J. P. and Leopold, P.** (2014). Sensing of amino
736 acids in a dopaminergic circuitry promotes rejection of an incomplete diet in Drosophila. *Cell.*
737 **156**, 510-21. doi: 10.1016/j.cell.2013.12.024.
738 **Blum, J. E., Fischer, C. N., Miles, J. and Handelsman, J.** (2013). Frequent replenishment
739 sustains the beneficial microbiome of Drosophila melanogaster. *MBio.* **4**, e00860-13. doi:
740 10.1128/mBio.00860-13.
741 **Bonfini, A., Liu, X. and Buchon, N.** (2016). From pathogens to microbiota: How Drosophila
742 intestinal stem cells react to gut microbes. S0145-305X(16)30032-5. doi:
743 10.1016/j.dci.2016.02.008.
744 **Broderick, N. A. and Lemaitre, B.** (2012). Gut-associated microbes of Drosophila
745 melanogaster. *Gut Microbes.* **3**, 307-21. doi: 10.4161/gmic.19896. Epub 2012 May 10.
746 **Buchon, N., Broderick, N. A., Poidevin, M., Pradervand, S. and Lemaitre, B.** (2009).
747 Drosophila intestinal response to bacterial infection: activation of host defense and stem cell
748 proliferation. *Cell Host Microbe* **5**, 200-11. doi: 10.1016/j.chom.2009.01.003.
749 **Caballero, J., Jiménez-Moreno, N., Orera, I., Williams, T., Fernández, A. B., Villanueva,**
750 **M., Ferré, J., Caballero, P. and Ancín-Azpilicueta, C.** (2020). Unraveling the Composition
751 of Insecticidal Crystal Proteins in Bacillus thuringiensis: a Proteomics Approach. *Appl*
752 *Environ Microbiol.* **86**, e00476-20. doi: 10.1128/AEM.00476-20. Print 2020 Jun 2.
753 **Casida, J. E. and Bryant, R. J.** (2017). The ABCs of pesticide toxicology: amounts, biology,
754 and chemistry. *Toxicol Res (Camb).* **6**, 755-763. doi: 10.1039/c7tx00198c. eCollection 2017
755 Nov 1.
756 **Castagnola, A. and Jurat-Fuentes, J. L.** (2016). Intestinal regeneration as an insect
757 resistance mechanism to entomopathogenic bacteria. *Curr Opin Insect Sci.* **15:104-10.**,
758 10.1016/j.cois.2016.04.008. Epub 2016 Apr 20.
759 **Castro, B., Martinez, L. C., Barbosa, S. G., Serrao, J. E., Wilcken, C. F., Soares, M. A.,**
760 **da Silva, A. A., de Carvalho, A. G. and Zanuncio, J. C.** (2019). Toxicity and cytopathology
761 mediated by Bacillus thuringiensis in the midgut of Anticarsia gemmatalis (Lepidoptera:
762 Noctuidae). *Sci Rep* **9**, 6667.
763 **Chakrabarti, S., Liehl, P., Buchon, N. and Lemaitre, B.** (2012). Infection-induced host
764 translational blockage inhibits immune responses and epithelial renewal in the Drosophila gut.
765 *Cell Host Microbe* **12**, 60-70. doi: 10.1016/j.chom.2012.06.001.
766 **Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. and Kopp, A.** (2011). Bacterial
767 communities of diverse Drosophila species: ecological context of a host-microbe model
768 system. *PLoS Genet.* **7**, e1002272. doi: 10.1371/journal.pgen.1002272. Epub 2011 Sep 22.
769 **Chen, J., Sayadian, A. C., Lowe, N., Lovegrove, H. E. and St Johnston, D.** (2018). An
770 alternative mode of epithelial polarity in the Drosophila midgut. *PLoS Biol.* **16**, e3000041.
771 doi: 10.1371/journal.pbio.3000041. eCollection 2018 Oct.
772 **Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J. and Leopold, P.**
773 (2003). A nutrient sensor mechanism controls Drosophila growth. *Cell.* **114**, 739-49.
774 **Cossentine, J., Robertson, M. and Xu, D.** (2016). Biological Activity of Bacillus
775 thuringiensis in Drosophila suzukii (Diptera: Drosophilidae). *J Econ Entomol* **22**.
776 **Delanoue, R., Slaidina, M. and Leopold, P.** (2010). The steroid hormone ecdysone controls
777 systemic growth by repressing dMyc function in Drosophila fat cells. *Dev Cell.* **18**, 1012-21.
778 doi: 10.1016/j.devcel.2010.05.007.

779   **Dubovskiy, I. M., Grizanova, E. V., Whitten, M. M., Mukherjee, K., Greig, C., Alikina,**
780   **T., Kabilov, M., Vilcinskas, A., Glupov, V. V. and Butt, T. M.** (2016). Immuno-
781   physiological adaptations confer wax moth Galleria mellonella resistance to Bacillus
782   thuringiensis. *Virulence* **7**, 860-870. Epub 2016 Mar 30.

783   **Edgar, B. A., Zielke, N. and Gutierrez, C.** (2014). Endocycles: a recurrent evolutionary
784   innovation for post-mitotic cell growth. *Nat Rev Mol Cell Biol.* **15**, 197-210. doi:
785   10.1038/nrm3756.

786   **EFSA BIOHAZ.** (2016). Risks for public health related to the presence of Bacillus cereus
787   and other Bacillus spp. including Bacillus thuringiensis in foodstuffs. *EFSA Journal* **14**,
788   e04524.

789   **EFSA BIOHAZ Panel.** (2016). Scientific opinion on the risks for public health related to the
790   presence of Bacillus cereus and other Bacillus spp. including Bacillus thuringiensis in
791   foodstuffs. EFSA Journal 2016;14(7):4524, 93 pp. doi:10.2903/j.efsa.2016.4524.

792   **Erkosar, B., Storelli, G., Mitchell, M., Bozonnet, L., Bozonnet, N. and Leulier, F.** (2015).
793   Pathogen Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via
794   Inhibition of Protein Digestion. *Cell Host Microbe* **18**, 445-55. doi:
795   10.1016/j.chom.2015.09.001. Epub 2015 Oct 1.

796   **Fast, D., Duggal, A. and Foley, E.** (2018). Monoassociation with Lactobacillus plantarum
797   Disrupts Intestinal Homeostasis in Adult Drosophila melanogaster. *MBio.* **9(4).** mBio.01114-
798   18. doi: 10.1128/mBio.01114-18.

799   **Fiuza, L. M., Polanczyk, R. A. and Crickmore, N.** (2017). Bacillus thuringiensis and
800   Lysinibacillus sphaericus: Springer International Publishing.

801   **Frankenhuyzen, K.** (2017). Specificity and Cross-order Activity of Bacillus thuringiensis
802   Pesticidal Proteins. In *Bacillus thuringiensis and Lysinibacillus sphaericus*, (ed. L. M. Fiuza
803   R. A. Polanczyk and N. Crickmore), pp. 127-172: Springer International Publishing.

804   **Frisvold, G. B.** (2018). How low can you go? Estimating impacts of reduced pesticide use.
805   *Pest Manag Sci* **8**.

806   **Gonzalez, J. M., Jr., Brown, B. J. and Carlton, B. C.** (1982). Transfer of Bacillus
807   thuringiensis plasmids coding for delta-endotoxin among strains of B. thuringiensis and B.
808   cereus. *Proc Natl Acad Sci U S A* **79**, 6951-5.

809   **Grisolia, C. K., Oliveira, R., Domingues, I., Oliveira-Filho, E. C., Monerat, R. G. and**
810   **Soares, A. M.** (2009). Genotoxic evaluation of different delta-endotoxins from Bacillus
811   thuringiensis on zebrafish adults and development in early life stages. *Mutat Res* **672**, 119-23.

812   **Haller, S., Romeis, J. and Meissle, M.** (2017). Effects of purified or plant-produced Cry
813   proteins on Drosophila melanogaster (Diptera: Drosophilidae) larvae. *Sci Rep.* **7**, 11172. doi:
814   10.1038/s41598-017-10801-4.

815   **Hietakangas, V. and Cohen, S. M.** (2009). Regulation of tissue growth through nutrient
816   sensing. *Annu Rev Genet* **43**, 389-410.

817   **Houtz, P., Bonfini, A., Bing, X. and Buchon, N.** (2019). Recruitment of Adult Precursor
818   Cells Underlies Limited Repair of the Infected Larval Midgut in Drosophila. *Cell Host*
819   *Microbe* **28**, 30409-3.

820   **Hung, T. P., Truong, L. V., Binh, N. D., Frutos, R., Quiquampoix, H. and Staunton, S.**
821   (2016). Fate of insecticidal Bacillus thuringiensis Cry protein in soil: differences between
822   purified toxin and biopesticide formulation. *Pest Manag Sci.* **72**, 2247-2253. doi:
823   10.1002/ps.4262. Epub 2016 Mar 24.

824   **Jiang, H. and Edgar, B. A.** (2009). EGFR signaling regulates the proliferation of Drosophila
825   adult midgut progenitors. *Development.* **136**, 483-93. doi: 10.1242/dev.026955.

826   **Keita, S., Masuzzo, A., Royet, J. and Kurz, C. L.** (2017). Drosophila larvae food intake
827   cessation following exposure to Erwinia contaminated media requires odor perception, Trpa1
828   channel and evf virulence factor. *J Insect Physiol.* **99:25-32.**, 10.1016/j.jinsphys.2017.02.004.
829   Epub 2017 Feb 21.

830 **Layalle, S., Arquier, N. and Leopold, P.** (2008). The TOR pathway couples nutrition and
831 developmental timing in Drosophila. *Dev Cell* **15**, 568-77. doi: 10.1016/j.devcel.2008.08.003.
832 **Lee, K. P., Cory, J. S., Wilson, K., Raubenheimer, D. and Simpson, S. J.** (2006). Flexible
833 diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proc Biol Sci.* **273**,
834 823-9. doi: 10.1098/rspb.2005.3385.
835 **Lee, K. Z., Lestradet, M., Socha, C., Schirmeier, S., Schmitz, A., Spenle, C., Lefebvre,**
836 **O., Keime, C., Yamba, W. M., Bou Aoun, R. et al.** (2016). Enterocyte Purge and Rapid
837 Recovery Is a Resilience Reaction of the Gut Epithelium to Pore-Forming Toxin Attack. *Cell*
838 *Host Microbe.* **20**, 716-730. doi: 10.1016/j.chom.2016.10.010. Epub 2016 Nov 23.
839 **Loudhaief, R., Brun-Barale, A., Benguettat, O., Nawrot-Esposito, M. P., Pauron, D.,**
840 **Amichot, M. and Gallet, A.** (2017). Apoptosis restores cellular density by eliminating a
841 physiologically or genetically induced excess of enterocytes in the Drosophila midgut.
842 *Development* **144**, 808-819. doi: 10.1242/dev.142539.
843 **Martino, M. E., Bayjanov, J. R., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M.,**
844 **Siezen, R., van Hijum, S. A. and Leulier, F.** (2015). Resequencing of the Lactobacillus
845 plantarum Strain WJL Genome. *Genome Announc* **3(6).** e01382-15. doi:
846 10.1128/genomeA.01382-15.
847 **Mathur, D., Bost, A., Driver, I. and Ohlstein, B.** (2010). A transient niche regulates the
848 specification of Drosophila intestinal stem cells. *Science.* **327**, 210-3. doi:
849 10.1126/science.1181958.
850 **Micchelli, C. A., Sudmeier, L., Perrimon, N., Tang, S. and Beehler-Evans, R.** (2011).
851 Identification of adult midgut precursors in Drosophila. *Gene Expr Patterns.* **11**, 12-21. doi:
852 10.1016/j.gep.2010.08.005. Epub 2010 Sep 23.
853 **Miguel-Aliaga, I., Jasper, H. and Lemaitre, B.** (2018). Anatomy and Physiology of the
854 Digestive Tract of Drosophila melanogaster. *Genetics.* **210**, 357-396. doi:
855 10.1534/genetics.118.300224.
856 **Monro, R. E.** (1961). Protein turnover and the formation of protein inclusions during
857 sporulation of Bacillus thuringiensis. *Biochem J.* **81:225-32.**, 10.1042/bj0810225.
858 **Murty, M. G., Srinivas, G., Bora, R. S. and Sekar, V.** (1994). A simple method for
859 separation of the protein crystal from Bacillus thuringiensis using carboxymethyl cellulose
860 column chromatography. *Journal of Microbiological Methods* **19**, 103-110.
861 **Nawrot-Esposito, M., Loudhaie, f. R. and Gallet, A.** (2017). Protease activity assay in fly
862 intestines. *Bio-protocol* **7**, e2560.
863 **Palm, W., Sampaio, J. L., Brankatschk, M., Carvalho, M., Mahmoud, A., Shevchenko,**
864 **A. and Eaton, S.** (2012). Lipoproteins in Drosophila melanogaster--assembly, function, and
865 influence on tissue lipid composition. *PLoS Genet* **8**, e1002828. doi:
866 10.1371/journal.pgen.1002828. Epub 2012 Jul 26.
867 **Palma, L., Munoz, D., Berry, C., Murillo, J. and Caballero, P.** (2014). Bacillus
868 thuringiensis toxins: an overview of their biocidal activity. *Toxins (Basel).* **6**, 3296-325. doi:
869 10.3390/toxins6123296.
870 **Panigrahi, P., Parida, S., Nanda, N. C., Satpathy, R., Pradhan, L., Chandel, D. S.,**
871 **Baccaglini, L., Mohapatra, A., Mohapatra, S. S., Misra, P. R. et al.** (2017). A randomized
872 synbiotic trial to prevent sepsis among infants in rural India. *Nature.* **548**, 407-412. doi:
873 10.1038/nature23480. Epub 2017 Aug 16.
874 **Pasco, M. Y., Loudhaief, R. and Gallet, A.** (2015). The cellular homeostasis of the gut:
875 what the Drosophila model points out. *Histol Histopathol.* **Vol 30**, pp 277-292.
876 **Polanczyk, R. A. and Alves, S. B.** (2005). Biological parameters of Spodoptera frugiperda
877 (J.E. Smith) (Lepidoptera: Noctuidae) assayed with Bacillus thuringiensis berliner. *Scientia*
878 *Agricola* **62**, 464-468.
879 **Rabinovitch, L., Vivoni, A. M., Machado, V., Knaak, N., Berlitz, D. L., Polanczyk, R. A.**
880 **and Fiuza, L. M.** (2017). Bacillus thuringiensis Characterization: Morphology, Physiology,
881 Biochemistry, Pathotype, Cellular, and Molecular Aspects. In *Bacillus thuringiensis and*

882 *Lysinibacillus sphaericus*, (ed. L. M. Fiuza R. A. Polanczyk and N. Crickmore), pp. 1-18:
883 Springer International Publishing.

884 **Raymond, B., Wyres, K. L., Sheppard, S. K., Ellis, R. J. and Bonsall, M. B.** (2010).
885 Environmental factors determining the epidemiology and population genetic structure of the
886 Bacillus cereus group in the field. *PLoS* **6**, e1000905.

887 **Reis, T.** (2016). Effects of Synthetic Diets Enriched in Specific Nutrients on Drosophila
888 Development, Body Fat, and Lifespan. *PLoS One.* **11**, e0146758. doi:
889 10.1371/journal.pone.0146758. eCollection 2016.

890 **Rodrigues, M. A., Martins, N. E., Balance, L. F., Broom, L. N., Dias, A. J., Fernandes, A.**
891 **S., Rodrigues, F., Sucena, E. and Mirth, C. K.** (2015). Drosophila melanogaster larvae
892 make nutritional choices that minimize developmental time. *J Insect Physiol.* **81:69-80.**,
893 10.1016/j.jinsphys.2015.07.002. Epub 2015 Jul 4.

894 **Rosi-Marshall, E. J., Tank, J. L., Royer, T. V., Whiles, M. R., Evans-White, M.,**
895 **Chambers, C., Griffiths, N. A., Pokelsek, J. and Stephen, M. L.** (2007). Toxins in
896 transgenic crop byproducts may affect headwater stream ecosystems. *Proc Natl Acad Sci U S*
897 *A* **104**, 16204-8.

898 **Rubio-Infante, N. and Moreno-Fierros, L.** (2016). An overview of the safety and biological
899 effects of Bacillus thuringiensis Cry toxins in mammals. *J Appl Toxicol* **36**, 630-48. doi:
900 10.1002/jat.3252. Epub 2015 Nov 4.

901 **Ryu, J. H., Kim, S. H., Lee, H. Y., Bai, J. Y., Nam, Y. D., Bae, J. W., Lee, D. G., Shin, S.**
902 **C., Ha, E. M. and Lee, W. J.** (2008). Innate immune homeostasis by the homeobox gene
903 caudal and commensal-gut mutualism in Drosophila. *Science* **319**, 777-82. doi:
904 10.1126/science.1149357. Epub 2008 Jan 24.

905 **Schott, S., Ambrosini, A., Barbaste, A., Benassayag, C., Gracia, M., Proag, A., Rayer,**
906 **M., Monier, B. and Suzanne, M.** (2017). A fluorescent toolkit for spatiotemporal tracking of
907 apoptotic cells in living Drosophila tissues. *Development.* **144**, 3840-3846. doi:
908 10.1242/dev.149807. Epub 2017 Sep 4.

909 **Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova,**
910 **P., Martino, M. E., Balmand, S., Hudcovic, T., Heddi, A. et al.** (2016). Lactobacillus
911 plantarum strain maintains growth of infant mice during chronic undernutrition. *Science* **351**,
912 854-7. doi: 10.1126/science.aad8588.

913 **Scott, J. G. and Buchon, N.** (2019). Drosophila melanogaster as a powerful tool for studying
914 insect toxicology. *Pesticide Biochemistry and Physiology* **161**, 95-103.

915 **Sedaratian, A., Fathipour, Y., Talaei-Hassanloui, R. and Jurat-Fuentes, J. L.** (2013).
916 Fitness costs of sublethal exposure to Bacillus thuringiensis in Helicoverpa armigera: a
917 carryover study on offspring. *Journal of Applied Entomology* **137**, 540-549.

918 **Shikano, I. and Cory, J. S.** (2014). Dietary mechanism behind the costs associated with
919 resistance to Bacillus thuringiensis in the cabbage looper, Trichoplusia ni. *PLoS One* **9**,
920 e105864. doi: 10.1371/journal.pone.0105864. eCollection 2014.

921 **Soultoukis, G. A. and Partridge, L.** (2016). Dietary Protein, Metabolism, and Aging. *Annu*
922 *Rev Biochem* **85**, 5-34.

923 **Stensmyr, M. C., Dweck, H. K., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J.,**
924 **Grabe, V., Steck, K., Lavista-Llanos, S. et al.** (2012). A conserved dedicated olfactory
925 circuit for detecting harmful microbes in Drosophila. *Cell.* **151**, 1345-57. doi:
926 10.1016/j.cell.2012.09.046.

927 **Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J. and Leulier, F.** (2011).
928 Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal
929 signals through TOR-dependent nutrient sensing. *Cell Metab* **14**, 403-14. doi:
930 10.1016/j.cmet.2011.07.012.

931 **Surendran, S., Huckesfeld, S., Waschle, B. and Pankratz, M. J.** (2017). Pathogen-induced
932 food evasion behavior in Drosophila larvae. *J Exp Biol.* **220**, 1774-1780. doi:
933 10.1242/jeb.153395. Epub 2017 Mar 2.

934 **Tank, J. L., Rosi-Marshall, E. J., Royer, T. V., Whiles, M. R., Griffiths, N. A.,**
935 **Frauendorf, T. C. and Treering, D. J.** (2010). Occurrence of maize detritus and a transgenic
936 insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. *Proc*
937 *Natl Acad Sci U S A* **107**, 17645-50. Epub 2010 Sep 27.
938 **Téfit, M. A., Gillet, B., Joncour, P., Hughes, S. and Leulier, F.** (2018). Stable association
939 of a Drosophila-derived microbiota with its animal partner and the nutritional environment
940 throughout a fly population's life cycle. *Journal of Insect Physiology* **106**, 2-12.
941 **Tefit, M. A. and Leulier, F.** (2017). Lactobacillus plantarum favors the early emergence of
942 fit and fertile adult Drosophila upon chronic undernutrition. *J Exp Biol.* **220**, 900-907. doi:
943 10.1242/jeb.151522. Epub 2017 Jan 6.
944 **Tennessen, J. M., Barry, W. E., Cox, J. and Thummel, C. S.** (2014). Methods for studying
945 metabolism in Drosophila. *Methods* **68**, 105-15. doi: 10.1016/j.ymeth.2014.02.034. Epub
946 2014 Mar 12.
947 **Ubeda, C., Djukovic, A. and Isaac, S.** (2017). Roles of the intestinal microbiota in pathogen
948 protection. *Clin Transl Immunology.* **6**, e128. doi: 10.1038/cti.2017.2. eCollection 2017 Feb.
949 **Van Arnam, E. B., Currie, C. R. and Clardy, J.** (2018). Defense contracts: molecular
950 protection in insect-microbe symbioses. *Chem Soc Rev.* **47**, 1638-1651. doi:
951 10.1039/c7cs00340d.
952 **Vettori, C., Paffettib, D., Saxenac, D., Stotzkyc, G. and Giannini, R.** (2003). Persistence of
953 toxins and cells of Bacillus thuringiensis subsp kurstaki introduced in sprays to Sardinia soils.
954 *Soil biology & biochemistry* **35**, 1635-1642.
955 **Vilas-Boas, G. T., Peruca, A. P. and Arantes, O. M.** (2007). Biology and taxonomy of
956 Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis. *Can J Microbiol* **53**, 673-87.
957 **Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., Boccard, F.**
958 **and Lemaitre, B.** (2005). Drosophila host defense after oral infection by an
959 entomopathogenic Pseudomonas species. *Proc Natl Acad Sci U S A* **102**, 11414-9. Epub 2005
960 Aug 1.
961 **Wells-Bennik, M. H., Eijlander, R. T., den Besten, H. M., Berendsen, E. M., Warda, A.**
962 **K., Krawczyk, A. O., Nierop Groot, M. N., Xiao, Y., Zwietering, M. H., Kuipers, O. P. et**
963 **al.** (2016). Bacterial Spores in Food: Survival, Emergence, and Outgrowth. *Annu Rev Food*
964 *Sci Technol* **7:457-82.**, 10.1146/annurev-food-041715-033144.
965 **Wong, A. C., Vanhove, A. S. and Watnick, P. I.** (2016). The interplay between intestinal
966 bacteria and host metabolism in health and disease: lessons from Drosophila melanogaster.
967 *Dis Model Mech.* **9**, 271-81. doi: 10.1242/dmm.023408.
968 **Xiang, J., Bandura, J., Zhang, P., Jin, Y., Reuter, H. and Edgar, B. A.** (2017). EGFR-
969 dependent TOR-independent endocycles support Drosophila gut epithelial regeneration. *Nat*
970 *Commun.* **8:15125.**, 10.1038/ncomms15125.
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972

973 **Table 1**

974 Time in hours when 10%, 50% and 90% larvae enter the pupation stage (immobilized)

975 expressed in hours after egg laying. "n" corresponds to the number of larvae counted in each

976 condition. Note that we compiled the controls per year of experiments (encompassing years

977 from 2017 to 2019).

978

% of pupation	10%	50%	90%	n
Control 2017	140.5	156.0	169.0	183
Control 2018	142.0	155.0	168.5	211
Control 2019	142.5	155.5	171.0	203
Delfin 1X	143.0	159.5	179.5	262
Delfin 10X	149.5	165.5	185.0	214
Dipel 10X	151.5	170.0	194.0	152
ABTS-351 10X	147.5	165.0	189.0	159
$Btk^{\Delta Cry}$ 1X	141.5	157.0	170.5	263
$Btk^{\Delta Cry}$ 10X	141.5	157.5	170.5	204
Naphthalene	142.5	155.0	168.0	212
Crystals	142.0	156.5	169.5	143
Control protein-rich medium	117.0	128.5	137.5	212
Delfin 1X protein-rich medium	115.0	127.0	138.0	195
Delfin 10X protein-rich medium	117.0	130.5	141.0	207
L. plantarum	128.0	146.0	162.5	144
Delfin 10X + *L plantarum*	127.0	143.0	158.0	153
Control Axenic	134.0	151.5	162.5	171
Delfin 10X Axenic	142.0	159.0	182.0	172

34

979

980   **Figure legends**

981

982   **Figure 1: *Btk* bioinsecticides impacts on developmental delay and growth defects.**

983   (**A**) Pupation curve of larvae fed with commercial products (Delfin and Dipel) or with a

984   homemade preparation of spores of the *Btk* strain ABTS-351 (Dipel). In this figure and in all

985   the following figures, the cumulative percentage of pupation is shown over time. (**B**) Pupation

986   curve of larvae fed with a *Btk* strain devoid of Cry toxins (*Btk$^{\Delta Cry}$*) or with Cry toxin

987   containing crystals purified from Dipel or with Naphthalene sulfonic acid at a dose equivalent

988   to the amount found in Delfin 10X. In A and B, red dashed lines mark 90% of the population

989   reaching pupation. (**C**) Pupae volume in the different conditions listed on the abscissa. n=

990   number of individual counted. For the controls, n correspond to the total number of pupae

991   measured when one compiled all the internal controls. Errors bars represent the Standard

992   Deviation of the Mean (SEM). *=P<0.05; ***=P<0.001 compared with controls.

993

994   **Figure 2: *Btk* bioinsecticides and larval feeding behavior.**

995   (**A**) L3 midgut load of larvae fed with either spores of *Btk$^{\Delta Cry}$* or Delfin. (**B** and **C**) Percentage

996   of L3 (B) and L2 (C) larvae presenting blue dyed food in the intestine. (**D** and **E**) Amount of

997   dye ingested by L3 (D) and L2 (E) larvae. Green bars: control larvae (fed with water); light

998   blue bars: 1X dose of *Btk$^{\Delta Cry}$* spores; dark blue bars: 10X dose of *Btk$^{\Delta Cry}$* spores; yellow bars:

999   1X dose of Delfin; Red bars: 10X dose of Delfin. n= number of individuals analyzed. Errors

1000   bars represent the SEM. *=P<0.05, **=P<0.01 compared with controls.

1001

1002   **Figure 3: Influence of a protein rich-diet on the Delfin impacts**

35

1003   (**A**) Measurement of the protease activity of the intestine of L3 larvae raised on control

1004   medium contaminated with H2O (control) or with 10X doses of spores of $Btk^{\Delta Cry}$ or Delfin or

1005   1X dose of spores of Delfin. (**B**) Pupation curve of larvae raised on a protein rich medium

1006   (10% yeast instead of 2%) contaminated with 1X or 10X doses of Delfin. Note the shortening

1007   of the developmental time of the control larvae raised on the protein-rich medium (10% yeast)

1008   compared to larvae raised on the protein poor-medium (2% yeast). (**C**) Pupae volume derived

1009   from larvae raised on the protein-rich medium and in the different conditions listed on the

1010   abscissa.

1011

1012   **Figure 4: *Btk* bioinsecticides interaction with commensal flora**

1013   (**A**) Pupation curve of larvae reared on a medium complemented with 10^6 CFU of *L*

1014   *plantarum* and contaminated with Delfin 10X (pink) or not (purple) compared to control

1015   larvae (green) or Delfin 10X only (red). (**B**) Pupae volume derived from larvae raised on a

1016   medium complemented with 10^6 CFU of *L plantarum* and contaminated with Delfin 10X

1017   (pink) or not (purple) compared to control pupae reared on control medium (green) or

1018   medium contaminated with Delfin 10X only (red). (**C**) *Btk* load in the midgut of L3 larvae

1019   reared on a medium contaminated with Delfin 10X and complemented (pink) or not (red) with

1020 10^6 CFU of *L plantarum.* (**D**) Commensal bacterial load in the midgut of L3 larvae of our

1021   *Drosophila* Canton S strain reared on our conventional medium (left bar) or reared in axenic

1022   condition (right bar). The *Commensalibacter intestini* and *Acetobacter pomorum* grow on

1023   mannitol plate (orange), *L plantarum* and *L brevis* grow on MRS plate (grey), *Enterococcus*

1024   *faecalis* grows on BHI plate (green) and *Escherichia coli* and *Staphylococcus aureus* grow on

1025   LB plate (blue). (**E**) Pupation curve of axenic larvae raised on a medium contaminated with

1026   Delfin 10X (dark blue) or not (light blue, control Ax) compared to larvae conventionally

1027   reared (CR, green). (**F**) Pupae volume derived from conventionally reared larvae (green) or

1028   from axenic larvae raised on a sterile medium contaminated with Delfin 10X (dark blue) or

1029   not (light blue).

1030

1031   **Figure 5: *Btk* bioinsecticides impacts on gut integrity**

1032   L3 larvae raised on a control medium (**A-B'**) or a medium contaminated with $Btk^{\Delta Cry}$ 10X (**C-**

1033   **D'**), Delfin 10X (**E-F**), purified Crystals 10X (**G-H'**), naphthalene 10X (**I-J'**). (**A-J'**)

1034   Labelling of lipid droplets with ORO (red, A, C, E, G and I) or with Bodipy (green, B, B', D,

1035   D', F, F', H, H', J and J'). DAPI staining in blue marks the nuclei. Midguts are oriented

1036   anterior to the left. Red brackets delineate the acidic region of the midgut. (B',D', F', H' and J')

1037   are x/z section with the apical pole of the epithelium facing the lumen. (**K**) Stored (TAG,

1038   upper panel) and circulating (DAG, lower panel) lipid dosages in control larvae (green bars)

1039   and in larvae raised on Delfin 10X contaminated medium (red bars). (**L-N**)

1040   *myo1A>Casp::GFP* larvae raised on control a medium (L) or on a medium contaminated with

1041 $Btk^{\Delta Cry}$ 10X (M) or Delfin 10X (N). Arrows point enterocytes in which Caspase 3 is activated.

1042   (**O-P**) L3 larvae raised on a medium complemented with Dextran^{FITC} (O) or with Dextran^{FITC}

1043   and contaminated with *P. entomophila* (P), with $Btk^{\Delta Cry}$ 10X (Q) or Delfin 10X (R).
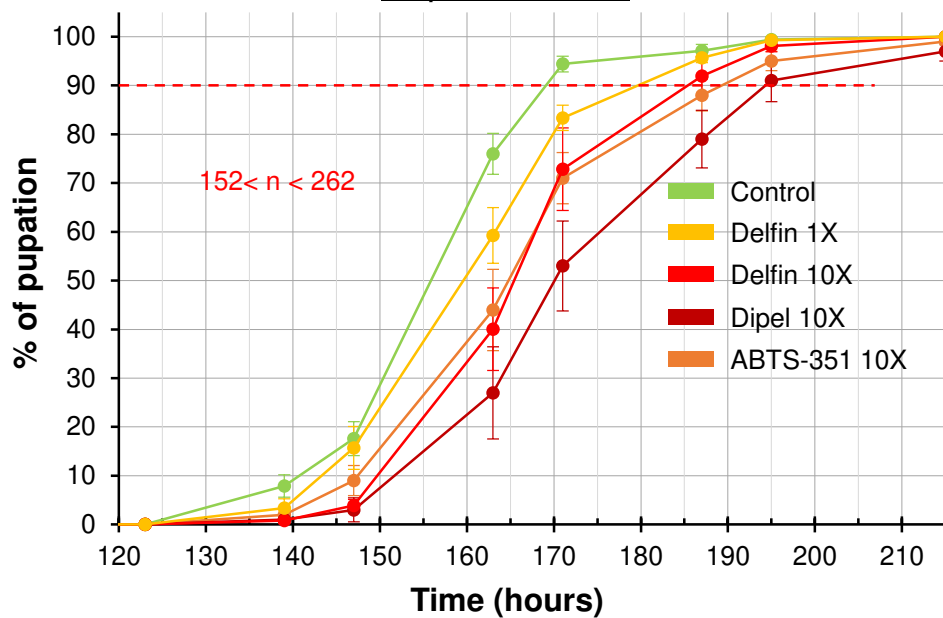
1044

1045   **Figure 6: Larval midgut regeneration upon aggression**

1046   (**A-C'**) Immunolabelling of *dlg::GFP* L3 midgut from larvae raised on control medium (A

1047   and A'), on medium contaminated with $Btk^{\Delta Cry}$ 10X (B and B') or Delfin 10X (C and C').

1048   Midguts were stained with the adherens junction marker Armadillo (Arm, purple) that

1049   strongly delineates the Adult Midgut Precursors (AMP) (A, B and C) and marks the

1050   basolateral compartment of the enterocytes (A', B' and C'). DAPI (blue) marks the nuclei and

1051   Phalloidin (red) labels the visceral muscle surrounding the intestine and the apical pole of the

1052   enterocytes facing the lumen (in A', B' and C'). Dlg::GFP (green) marks the sub apical

1053   compartment of the enterocytes. (**D-H**) L3 midgut from larvae raised on control medium

37

1054   (green bars), on medium contaminated with $Btk^{\Delta Cry}$ 10X (dark blue bars) or Delfin 10X (red

1055   bars). (**D**) Number of AMPs per islets, (**E**) number of dividing AMPs per intestine (mitotic

1056   AMPs are labeled by the Phospho-Histone 3 (PH3) immunostaining), (**F**) Number of AMP

1057   islets (an islet contains more than one diploid cell) per unit of surface, (**G**) enterocyte area and

1058   (**H**) epithelium thickness in the posterior midgut (3 independent measures were taken by

1059   image capture). (**I** and enlargement in **I'**) *dlg::GFP* L3 midgut from larvae raised on medium

1060   contaminated with Delfin 10X and stained for Arm (purple) and DAPI (blue). Arrows point

1061   the cells with intermediate size (smallest than enterocytes but biggest than AMPs). (**J**)

1062   Assessment of enterocyte ploidy in posterior L3 midgut from larvae raised on control

1063   medium, on medium contaminated with $Btk^{\Delta Cry}$ 10X or Delfin 10X (C corresponding to

1064   chromatin copy number). (**K-M**) Lineage tracing of AMP progeny in the *w; esg-Gal4, UAS-*

1065   *GFP/+; UAS-H2B::RFP, tub-Gal80^{ts}/+* (esg REDDM) L3 midgut from larvae raised on

1066   control medium (K), on medium contaminated with $Btk^{\Delta Cry}$ 10X (L) or Delfin 10X (M). DAPI

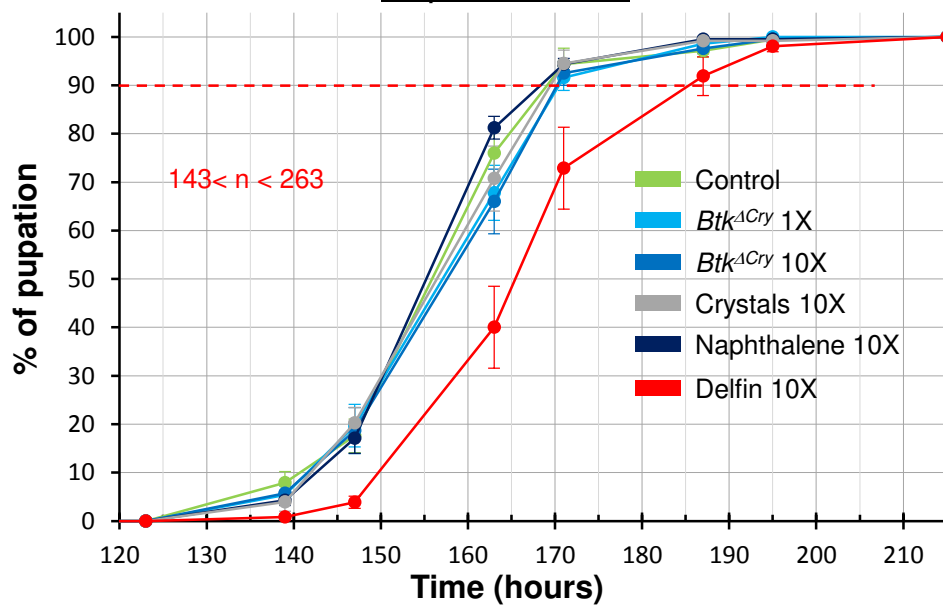1067   (blue) marks the nuclei. **=P<0.01, ***=P<0.001 compared with controls.

1068

Figure 1

Figure 2

A <u>Intestinal protease activity</u>

C <u>Pupae volume (protein-rich medium)</u>
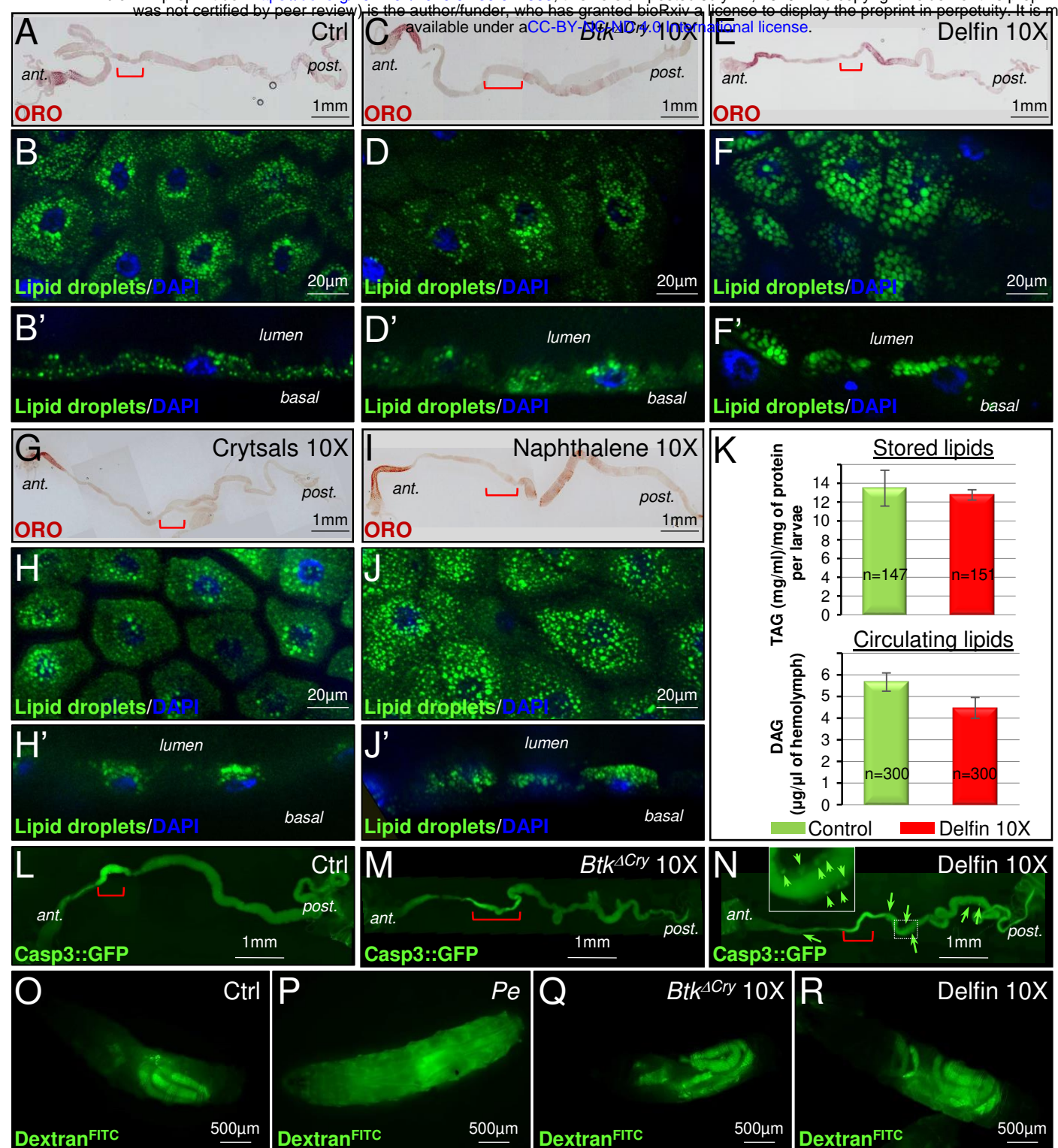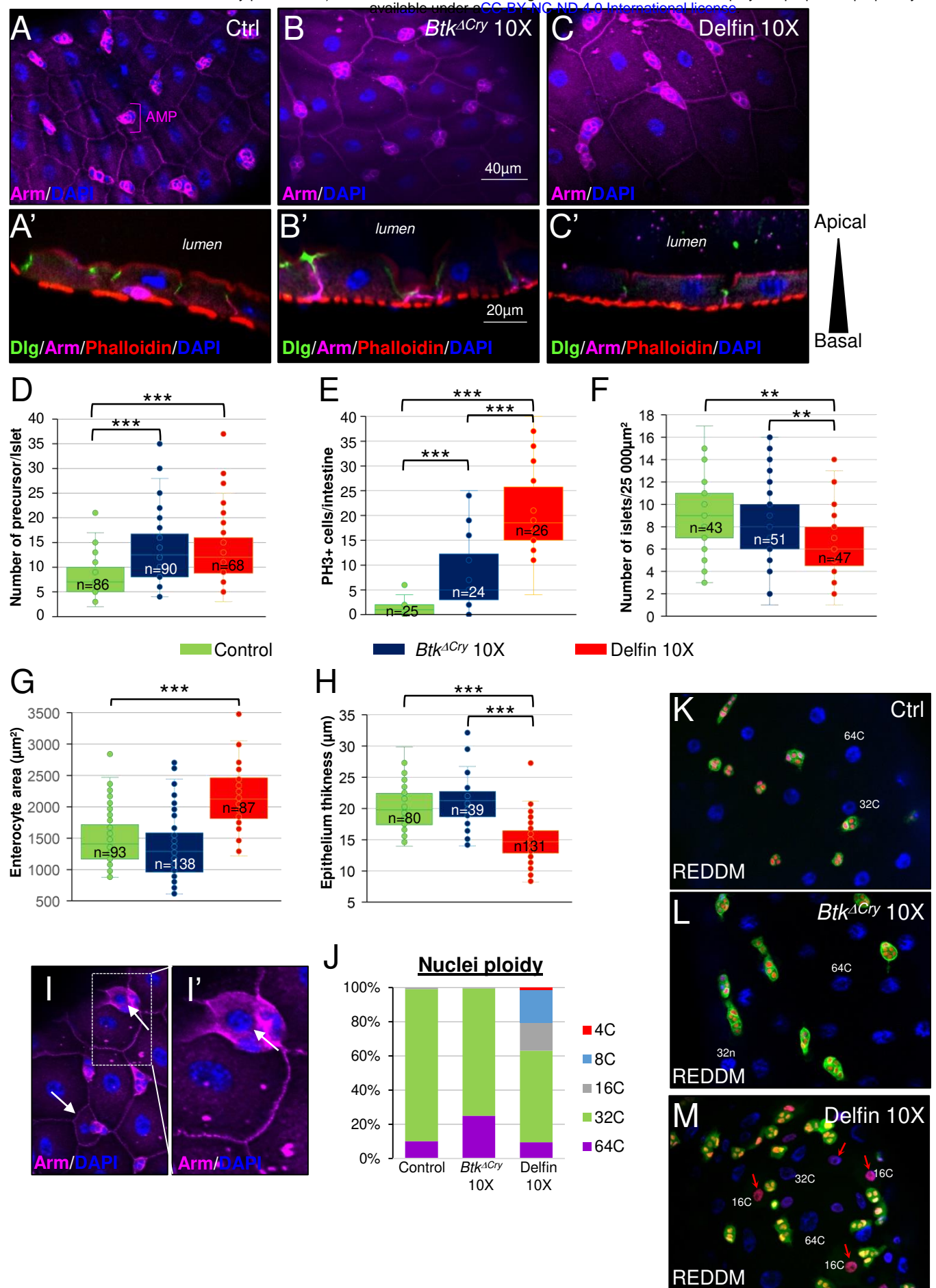

B <u>Pupation curve (protein-rich medium)</u>
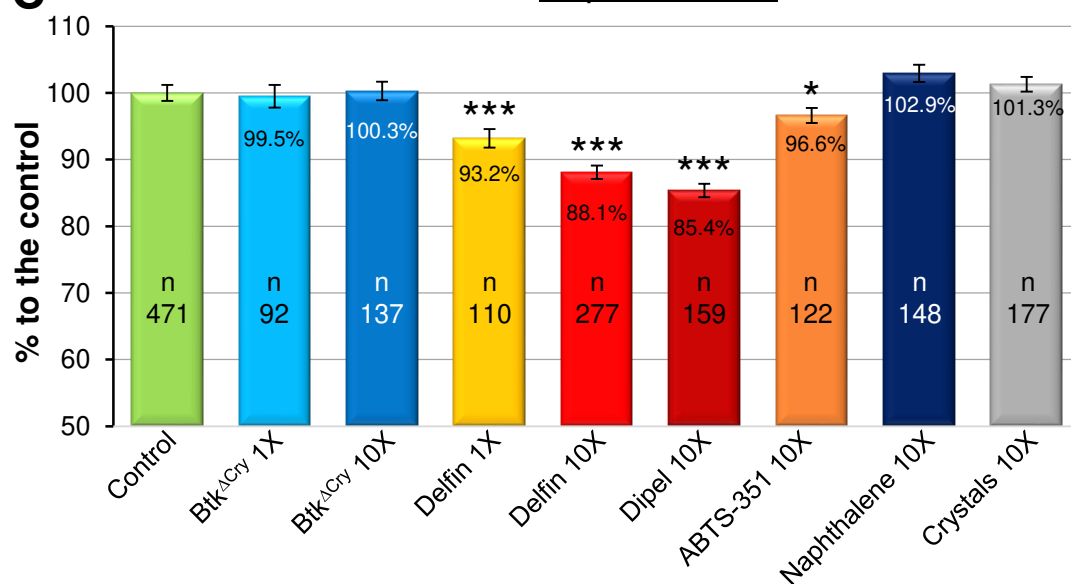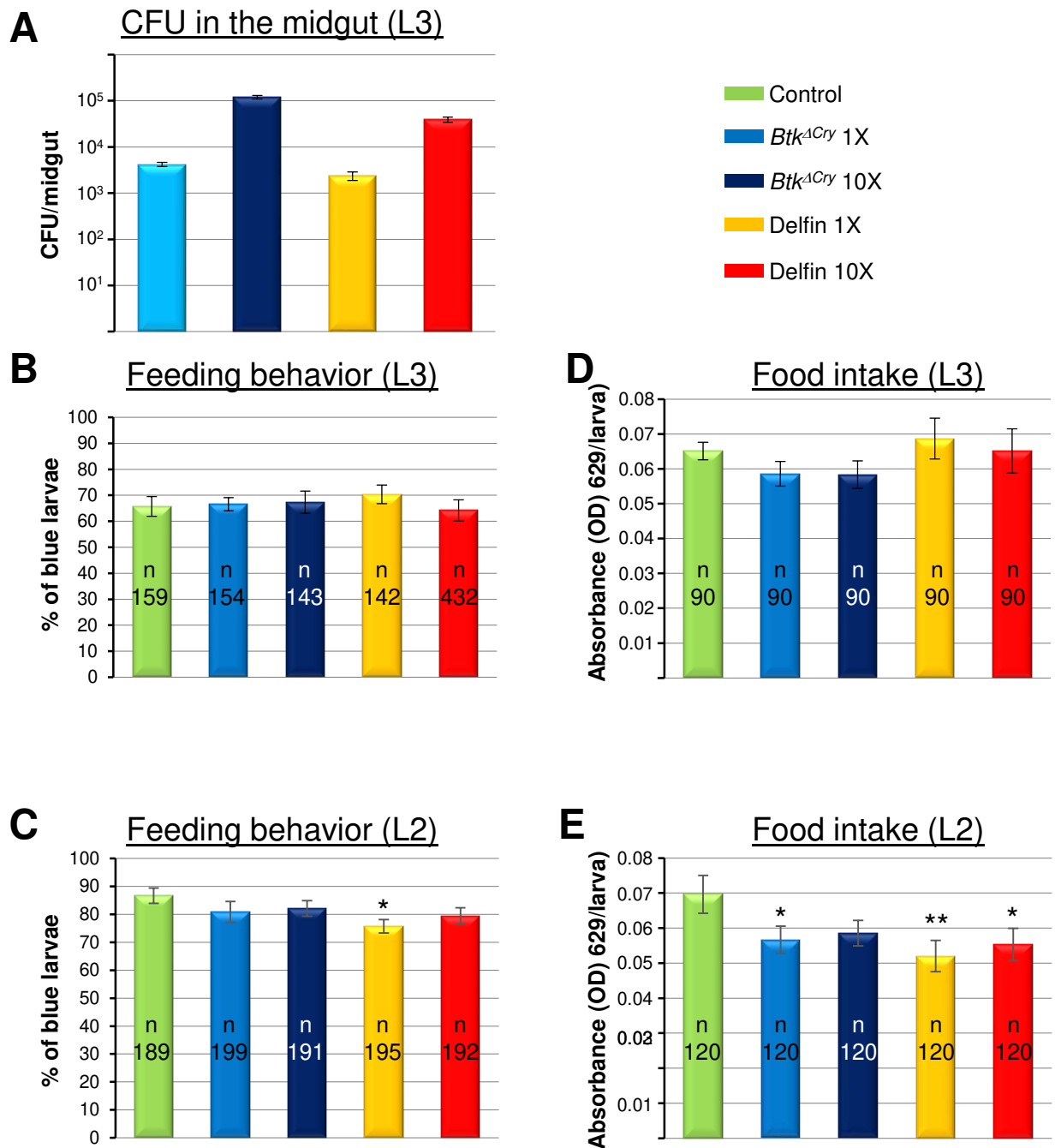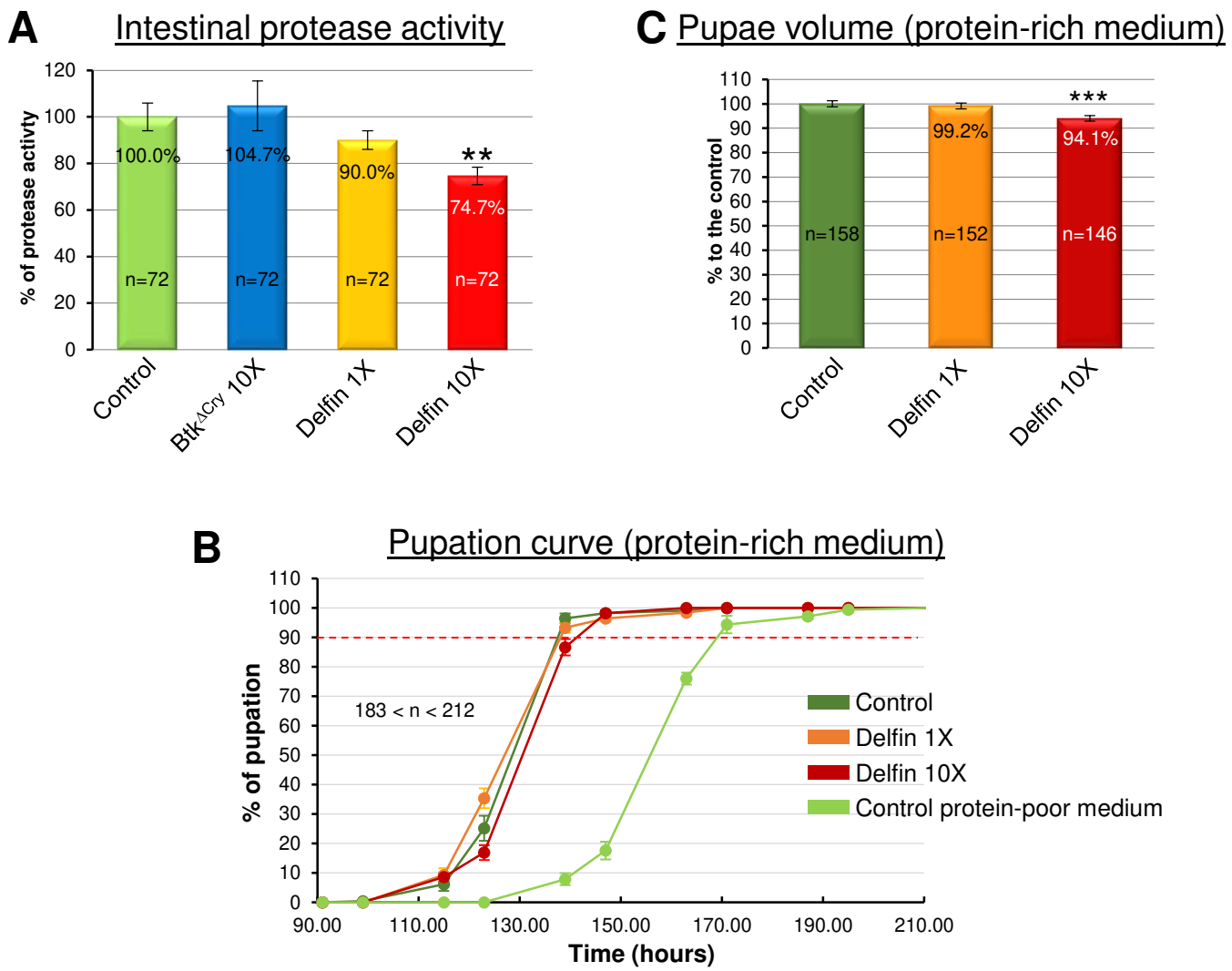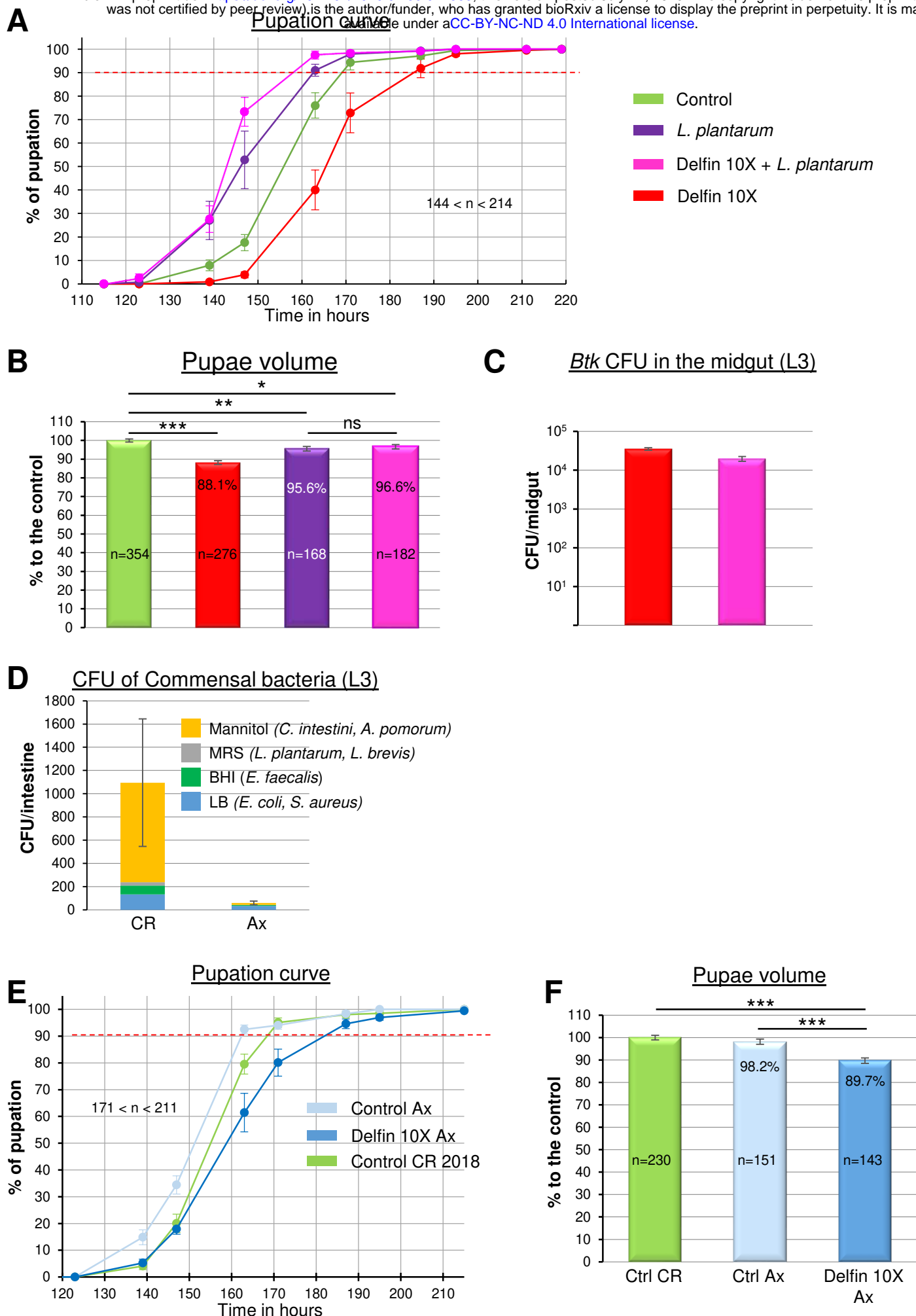
Figure 3

Figure 4

Figure 5

Figure 6