

1 **Adverse effects of *Bacillus thuringiensis* bioinsecticide on non-target *Drosophila* species**

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12 **Running title: Unintentional impact of *Bt* on *Drosophila* species**

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14 **Keywords:** *Drosophila*, *Bacillus thuringiensis*, biopesticide, toxicity, non-intentional effects,
15 development, fitness, longevity, offspring number, ecotoxicology

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17 **Word count (main text): 6 687**

18 **Figures:** 7 figures and 1 table

19 **Supplementary information**

20 **Abstract**

21 Biopesticides based on *Bacillus thuringiensis kurstaki* (*Btk*) and *israelensis* (*Bti*) spores and toxins
22 are widely used to control insect pests, increasing environmental risks to non-target biodiversity.
23 Here, we tested for potential effects of larval ingestion of *Bt* commercial formulations on
24 *Drosophila* species. Doses equivalent to those recommended for field application ($\leq 10^6$ CFU/g of
25 fly medium) had no effect whereas *Btk* doses 10 to 100-fold higher (10^7 - 10^8 CFU/g) altered the
26 development (decreased emergence due to larval mortality and increased development time), and
27 moderately influenced adult fitness-related traits. At the highest *Btk* and *Bti* dose (10^9 CFU/g), all
28 larvae died before pupation. The impact of *Btk* formulations resulted from the spores/cleaved toxins
29 synergy, but also additives. While recommended doses had no effect on non-target *Drosophila*
30 species, the accumulation of *Bt* bioinsecticides in the environment could have adverse side-effects
31 on the populations of these species and therefore their associated communities.

32 **Introduction**

33 The world's population is expected to reach more than 9 billion people by 2050 (United Nations,
34 2015), increasing the demand for agricultural resources in the future. Increasing agricultural
35 production requires improved management of pests, especially insects that cause more than 30% of
36 losses (Pimentel and Burgess, 2014). Nowadays, their management still largely relies on
37 conventional chemical insecticides. However, their use and efficiency have been considerably
38 reduced due to the emergence of pests' resistance, development of secondary pests, adverse side-
39 effects on non-target species (natural enemies of pests, pollinators) (Devine and Furlong, 2007;
40 Sanchis and Bourguet, 2008), and more generally the impacts on biodiversity and human health (e.g.
41 neurological disorders, functional impairment of reproduction, cancers) (WHO Report, 2007; Baldi
42 et al. 2013; Gilden et al. 2016; Rizzati et al. 2016). Developed as an alternative, biopesticides are
43 considered more specific and safer for the environment and human health. Today, they still
44 represent less than 5% of the pesticide market, the large majority being microbial insecticide
45 formulations based on viable spores and toxins of *Bacillus thuringiensis* (*Bt*) (over 400 registered
46 formulations) (Sanchis and Bourguet, 2008; Lacey et al. 2015).

47 *Bt* is a Gram-positive endospore-forming bacterium that synthesizes a wide range of toxins
48 with different chemical structures, modes of action and biological targets. The most abundant and
49 studied are Cry δ -endotoxins encoded by genes located on large plasmids and produced as
50 parasporal crystalline inclusions during the stationary growth phase (Crickmore 2017, Adang et al.
51 2014). *Bt* produces other insecticidal toxins, the Cyt (cytolytic δ -endotoxins) and Vip (secreted
52 Vegetative Insecticidal Proteins) that synergize their effects with Cry toxins, virulence factors such
53 as β -exotoxins (or thuringiensin), a secreted nucleotide toxic for almost all tested life forms thus
54 prohibited in commercial formulations (WHO Report, 1999), and anti-fungal factors (Bravo et al.
55 2017; Rabinovitch et al. 2017). *Bt* subspecies and strains can differ in their plasmid number and in
56 the synthesized toxins cocktail responsible for their biological activity, which was used to delineate
57 potential target insects (Palma et al. 2014). For instance, *Bt* subsp. *kurstaki* (*Btk*) produces the 5 Cry

58 toxins, Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (Ben-Dov et al. 1997; Adang et al. 2014),
59 while *Bt* subsp. *israelensis* (*Bti*) produces a combination of Cry4Aa, Cry4Ba, Cry10Aa, and
60 Cry11Aa (Ben-Dov et al. 1999; Berry et al. 2002), both strains being commercially used. The
61 different toxin cocktails produced by some *Bt* subspecies can also be harmful to non-insect
62 organisms such as nematodes and protozoa (Palma et al. 2014).

63 The formulations based on spores and toxin crystals of *Btk* and *Bti* are the most sprayed in
64 organic farming and natural areas (e.g. forests, swamps) to deal with larvae of Lepidopteran pests
65 and Dipteran larvae of mosquitoes and black flies, respectively. It is generally accepted that once
66 ingested by insect larvae, the toxin crystals are dissolved by the midgut alkaline pH, releasing ~130
67 kDa pro-toxins that are then processed by digestive proteases into smaller, soluble, active toxin
68 fragments of ~ 60-70 kDa (Bravo et al. 2007; Wei et al. 2016). Active toxins bind to specific
69 receptors of midgut epithelial cells, eliciting pores formation in the cell membrane, cell lysis and
70 gut epithelium disorganization (Schnepf et al. 1998; Bravo et al. 2011). This allows gut bacteria,
71 including *Bt*, to colonize the hemocoel, and leads to rapid septicaemia and death (Obata et al. 2009;
72 Caccia et al. 2016).

73 The increasing use of *Bt* has recently raised concern about its potential impact on non-target
74 species. Numerous impact studies of field application rates and acute intoxications have concluded
75 that *Bt* is safe or has a limited impact on non-target vertebrates and invertebrates, and associated
76 species communities (Glare and O'Callaghan, 2000). Yet, there is growing evidence of direct and
77 indirect cross-effects of *Bt* formulations and toxins across insect species and orders, or even across
78 phyla, suggesting that *Bt* targeting is only partly specific (van Frankenhuyzen 2017; Venter and
79 Bøhn, 2016). In addition, data showed that almost all of the *Btk* was still present on the leaves
80 surface 72 hours after spraying (Bizzarri and Bishop, 2008), its amount returning close to
81 environmental levels only 28 days after treatment (Raymond et al. 2010). Finally, *Bt* spores can
82 survive in the soil and different supports for months and even years after application (Hendriksen et
83 al. 2002; Duchet et al. 2014; Hung et al. 2016a, b; Enger et al. 2018). *Bt* formulations contain also

84 numerous compounds to protect spores and crystals and aggregate them into a wettable form,
85 surfactants to facilitate spraying and dispersion on plants, and phagostimulants (Couch, 2000; Brar
86 et al. 2006). Nevertheless, spores and toxins are somewhat sensitive to biotic and abiotic conditions
87 (e.g. UV, pH, rainfall), which requires frequent applications to achieve the required pest control
88 level (Brar et al. 2006). All this can lead to *Bt* accumulation in the environment, thus raising the
89 rarely addressed issue of potential side-effects of chronic exposure (*i.e.* continuous and increasing
90 exposure for an extended period) of non-target species to doses unexpectedly above the
91 recommended application rates.

92 Diptera are worldwide distributed insects, most of which are not targets for *Bt* and its toxins.
93 This is the case of the genus *Drosophila*, represented by ~ 1500 described species (Bächli, 1999-
94 2008), including the model organism *D. melanogaster*. In the field, most of these flies feed and
95 reproduce mainly on ripening or rotting fruits and are therefore present in areas treated with *Bt* such
96 as orchards, vineyards and gardening areas. Unable to disperse between food patches, early stages
97 of *Drosophila* larvae eat intensively and grow exponentially (Tennessee and Thumel, 2011), and
98 may thus ingest high doses of *Bt* bioinsecticides. Surprisingly, few studies have focused on
99 *Drosophila* species (Benz and Perron, 1967; Saadoun et al. 2001; Khyami-Horani 2002; Obeidat
100 2008; Obeidat et al. 2012; Cossentine et al. 2016; Biganski et al. 2017; Haller et al. 2017) and most
101 of them showed susceptibility of these species to *Btk*. However, definitive conclusions were
102 difficult to draw since most of these studies used mainly late 3rd instar larvae preparing for pupation,
103 *i.e.* when they feed much less than younger larvae, and the tested *Bt* preparations possibly contain
104 highly toxic β -exotoxins, especially in the case of field isolates.

105 Here, we have tested the dose-dependent chronic side-effects of different commercial
106 formulations of *Btk* (devoid of β -exotoxins) and, to a lesser extent of *Bti*, on the wild-type *D.*
107 *melanogaster* Canton S, with a focus on developmental traits (developmental time, emergence rate).
108 The spore-forming Gram-positive *Bacillus subtilis* and the *Btk* strain (4D22), devoid of Cry toxin
109 genes and thus of crystals, were used as non-pathogenic controls. We also analysed two fitness-

110 related traits of adult flies (male and female longevity, offspring number) after entire development
111 in presence of *Btk* formulation. To test for effects specific to the fly genetic background,
112 developmental traits upon exposure to *Btk* formulation were also measured on several other *D.*
113 *melanogaster* strains. Finally, we extended further these development experiments to several other
114 *Drosophila* species, including cosmopolitan species and the invasive *D. suzukii*, which are
115 frequently present in *Bt* treated areas, to explore the potential implications of chronic exposure to
116 *Btk* formulation in terms of competition and associated communities.

117

118 **Results**

119 ***Btk* formulations adversely impact the development of *D. melanogaster*.**

120 The wild-type Canton S strain of *D. melanogaster* was used to evaluate the dose-dependent effect
121 of the commercial *Btk* formulation Delfin[®] A on the emergence rate (ER, proportion of emerged
122 flies from the initial egg pool) and developmental time (DT, mean number of days from egg to adult
123 emergence). Eggs were transferred on a standard low-protein/high-sugar fly medium containing
124 Delfin[®] A at doses ranging from 5×10^5 CFU/g of medium (mean equivalent of the maximum
125 recommended doses for field application; see Methods and Supplementary information S1) to 10^9
126 CFU/g (~ 1,000 times the recommended dose). To check for specific effects of *Btk* formulations
127 and the respective role of *Btk* spores and Cry toxins, we tested the same dose range of the
128 commercial *Bti* formulation Vectobac[®] targeting mosquitoes that contains different Cry toxins
129 (Bravo et al. 2011), of the Cry-free strain *Btk* 4D22, and of the *Drosophila* non-pathogenic spore-
130 forming *Bacillus subtilis*.

131 Developmental traits (ER and DT) of exposed and non-exposed control flies were similar at
132 doses up to 10^7 CFU/g of Delfin[®] A (Fig. 1a-b; Table 1). At higher doses, both ER and DT were
133 affected in a dose-dependent manner: ER was reduced by 17% at 5×10^7 CFU/g (although not
134 statistically significant), up to 100% at 10^9 CFU/g, dose at which no individual reached the pupal
135 stage. The lethal dose 50 (LD50) was estimated between 5×10^7 and 10^8 CFU/g (Fig. 1a). DT was

136 increased of about 0.5 day at 5×10^7 CFU/g (+4% compared to controls), up to 1.5 days (+14%) at
137 10^8 CFU/g (Fig. 1b; Table 1). The sex-ratio at emergence (SR, proportion of males) was strongly
138 biased towards males at the highest dose at which complete development occurred (10^8 CFU/g),
139 with 58% more males compared to controls (Supplementary information S2). Because addition of
140 *Btk* formulation could modify parameters of the fly medium and thus contribute to these effects, we
141 checked the pH of the dose-responses medium. The presence or dose of the formulation had no
142 effect (Supplementary information S4).

143 We observed no change in ER using the same dose range of the *Btk* Cry-free strain 4D22 (Fig.
144 1a, 1e; Table 1) and the non-pathogenic *Bacillus subtilis* (Fig. 1a, Table 1). Addition of *Bti*
145 Vectobac[®] did not affect ER up to 10^8 CFU/g but reduced it by 89% at 10^9 CFU/g (~2,000 times the
146 highest recommended dose for field application; Fig. 1a; Table 1; Supplementary information S1).
147 DT varied with the dose of *Btk* 4D22, mainly due to differences between doses other than the
148 control. DT increased by ~1.5 days at the highest dose of Vectobac[®] (Fig. 1b; Table 1) and showed
149 a similar trend with *B. subtilis* ($p = 0.06$; Fig. 1b; Table 1). None of these three treatments
150 influenced dramatically the SR, the slight decrease in male proportion for most of the Vectobac[®]
151 doses being due to the higher average sex-ratio for the control dose compared to those for the two
152 other treatments (Supplementary information S2).

153 To test whether these effects are generic to *Btk* formulations, the fly development was
154 evaluated on two other formulations, Delfin[®] B (same brand) and Scutello DF (brand Dipel[®]), at the
155 critical doses 10^8 and 10^9 CFU/g. As Delfin[®] A, these formulations contain spores and Cry toxins
156 such as Cry-1A as pro-toxins of ~130 kDa, activated toxins of ~60-70 kDa, but also as smaller
157 fragments (Wei et al. 2016; Fig. 1e, red asterisks). ER remained unchanged at 10^8 CFU/g whereas
158 no individual reached pupation at 10^9 CFU/g on Delfin[®] B and very few individuals reached the
159 adult stage on Scutello DF[®], DT being increased by more than 2 days (Fig. 1c-d; Table 1). No
160 significant bias in SR was observed for either formulation (Supplementary information S2).

161

162 **Adverse effects of *Btk* formulation strongly impact the early development.**

163 Larval stages were tested for their susceptibility to *Btk* formulation in two independent and
164 complementary dose-response tests of survival on Delfin[®] A, at doses ranging from 10⁵ to 10⁹
165 CFU/g of high protein/sugar free medium (this medium is used to rear fly species which are
166 difficult to rear in the lab (see below) and is less limiting for the development of early larval stages).
167 We focused on the 1st and 2nd larval instars, during which growth is exponential (Tennessee and
168 Thummel 2011), so that larvae are most heavily exposed to the bioinsecticide. In the first test, the
169 cumulative survival was measured by counting late 1st and 2nd instar larvae alive which have been
170 exposed to Delfin[®] A from the egg stage. Larval survival was not influenced at 10⁷ CFU/g, whereas
171 it decreased for both larval instars above that dose to reach up to 37% mortality at 10⁹ CFU/g (Fig.
172 2a). Reduced survival tended to occur at a lower dose when cumulative survival was measured later
173 in the development, *i.e.* 10⁹ for late 1st instar larvae and 10⁸ CFU/g for 2nd instar larvae (Fig. 2a;
174 Table 1). For both instars, larvae surviving 10⁹ CFU/g were noticeably smaller and less active than
175 those surviving lower doses. In emergence assays with planned exposure from the egg to the adult
176 stage, none of these individuals reached the pupal stage (see results above). In the second test, larval
177 survival was measured after early 1st and 2nd instar larvae had been exposed for 24 hours to Delfin[®]
178 A. Survival of 1st instar larvae decreased by 36% on 10⁹ CFU/g whereas that of 2nd instar larvae did
179 not change (Fig. 2b, Table 1).

180

181 **Developmental exposure to *Btk* formulation does not strongly influence fitness-related traits**
182 **in adults.**

183 Long-term consequences on flies of exposure to *Btk* formulation throughout the development were
184 evaluated on two fitness-related traits, longevity and total offspring number. Traits were measured
185 on a *Btk*-free low-protein/high-sugar medium after individuals had completed their development on
186 the same fly medium but in presence of selected doses of Delfin[®] A: 5×10⁶ CFU/g, which had no
187 impact on development, and 5×10⁷ and 10⁸ CFU/g, which caused moderate and strong

188 developmental alterations, respectively (see Fig. 1a).

189 Adult longevity was analysed in two independent experiments on groups of 15 females and 15
190 males held together. Despite large variation between experiments (Table 1), the longevity of adults
191 reared on 5×10^6 CFU/g of Delfin[®] A was similar to that of non-exposed controls (Fig. 3). Males
192 and females which developed on the two higher doses showed a moderate longevity benefit, higher
193 in females for 10^8 CFU/g (Fig. 3a-b, d-e; Table 1). Males generally survived better than females
194 (Table 1) but their longevity benefit of developing on 10^8 CFU/g was only observed in the second
195 experiment (Fig. 3b, e).

196 The female offspring number - the sum of offspring produced by the 15 females of each fly
197 group during the longevity experiment - varied depending on both the experiment and the Delfin[®] A
198 dose (Table 1). In the 1st experiment, adults from larvae reared on 10^8 CFU/g had fewer offspring
199 compared to control adults and to adults developed on the other doses whereas the total offspring
200 number varied regardless of the *Btk* dose in the 2nd experiment (Fig. 3c, f, Table 1).

201

202 ***Btk*-formulation dose-dependent alterations of development are not specific to the *D.***
203 ***melanogaster* strain.**

204 Dose-dependent effects of *Btk* formulation on the development were tested on three additional *D.*
205 *melanogaster* strains: the wild-type Nasrallah (strain 1333), the wild-type Sefra population reared in
206 the laboratory for 4 years, and the double mutant YW1118. The emergence rates (ER) and
207 developmental times (DT) were measured on a high-protein/sugar-free medium (rearing medium of
208 these strains) mixed with Delfin[®] A doses ranging from 10^5 to 10^9 CFU/g. To allow the comparison
209 with previous results with Canton S flies on low-protein/high sugar fly medium, Canton S was also
210 reared and tested on the high-protein/sugar-free medium along with the other strains.

211 None of the fly strains was impacted at doses up to 10^7 CFU/g, whereas ER was strongly reduced
212 and DT was increased at higher doses for all the strains (Fig. 4a-b, Table 1), with no individual
213 reaching the pupal stage at 10^9 CFU/g (LD50 between 10^8 and 10^9 CFU/g). At 10^8 CFU/g, the

214 magnitude of effects on Canton S flies was lower than that observed on the low-protein/high-sugar
215 medium. At this dose, ER varied between strains, the largest reduction being observed for Sefra
216 (Table 1). We observed no dose-dependent bias in SR (Supplementary information S3).

217

218 ***Btk* formulation also affects other *Drosophila* species.**

219 The ER and DT were analysed for seven other *Drosophila* species from different phylogenetic
220 clades at doses of Delfin[®] A from 10⁵ to 10⁹ CFU/g of high-protein/sugar-free medium (rearing
221 medium of all the species). Tested species were *D. simulans* (*D. melanogaster* sister species), the
222 African *D. yakuba*, *D. subobscura*, *D. immigrans*, *D. hydei*, and the invasive *D. sukukii*, all
223 belonging to the *Drosophila* subgenus, and *D. busckii* from the *Dorsilopha* subgenus. For all the
224 species, doses up to 10⁶ CFU/g of Delfin[®] A had no effect on ER and DT whereas all individuals
225 failed to reach the pupal stage and no fly emerged at 10⁹ CFU/g (Fig. 5-6). Amplitudes of
226 development alterations at 10⁷ and 10⁸ CFU/g varied between species (Fig. 5-6; Table 1). All
227 species were affected at 10⁸ CFU/g as was *D. melanogaster* (see Fig. 4a for comparison). *D.*
228 *simulans* and *D. busckii* had unchanged ER, but DT was slightly increased for *D. simulans*
229 (although slightly reduced at 10⁷ CFU/g; similar results with a Japanese strain, data not shown) and
230 strongly increased for *D. busckii* (by 20%, *i.e.* ~ 4 days) (Fig. 5-6, Table 1). *D. yakuba* ER and DT
231 were similar to those of *D. melanogaster*, with an LD50 around 10⁸ CFU/g and a moderate DT
232 increase of ~ 1 day (Fig. 5-6, Table 1; similar results with a strain from Sweden, data not shown).
233 The ER of *D. hydei* and *D. subobscura* were very low at 10⁸ CFU/g (LD50 below this dose), with a
234 high DT (Fig. 5-6; Table 1), while *D. immigrans* did not survive. No *D. sukukii* individual emerged
235 at 10⁸ CFU/g and development was already moderately impacted at 10⁷ CFU/g (Fig. 5-6). No dose-
236 dependent bias in SR was detected for either species (Supplementary information S5).

237

238 **Development alterations may result from a synergy between formulation components.**

239 *Bt* spores and toxins can represent more than half the weight of commercial formulations, with up to

240 about 10% of insecticidal protein toxins within this fraction, mainly Cry pro-toxins and activated
241 toxins (see Fig. 1e) (Koch et al. 2015). The remaining weight consists of various compounds such
242 as residues of culture medium and various additives including surfactant, anti-foaming agents, etc.
243 (Glare and O'Callaghan, 2000; Brar et al. 2006). Indeed it has been shown for some products that
244 additives can be more harmful in some cases than the active ingredient (Bradberry et al. 2004), we
245 explored the role of small diffusible molecular weight components of Delfin[®] A in the alterations of
246 ER and DT of *D. melanogaster* Canton S. For that, we mixed a 10 kDa dialyzed suspension of
247 Delfin[®] A at 10⁷, 10⁸, and 10⁹ CFU/g with low-protein/high-sugar medium. ER and DT were
248 unaffected by the presence of the dialyzed suspension from the 10⁷ CFU/g dose, whereas no
249 individual reached the adult stage (no pupation) with the suspension from the 10⁹ CFU/g dose (Fig.
250 7a; Table 1). At 10⁸ CFU/g, ER was not modified but DT increased by ~ 1 day, only in one of the
251 two experiments, partially reproducing the changes observed without dialysis (Fig. 7a-b; see also
252 Fig. 1a-b, Table 1; 3 independent experiments for ER, 2 independent experiments for DT).

253 Cry1A profiles of dialyzed Delfin[®] A suspensions, like those of the non-dialyzed ones,
254 comprised 130-kDa pro-toxins and 60-70 kDa activated toxins, but also showed toxin degradation
255 as evidenced by additional smaller fragments of activated toxins (Fig. 7c). The respective roles of
256 *Btk* toxin fragments and spores in the alterations of *D. melanogaster* development were further
257 explored through experiments of dialysis such as those previously described followed by successive
258 centrifugations to eliminate most of the spores and toxin crystals. Despite variation between
259 experiments, ER was strongly affected only in one of the three experiments while DT was always
260 significantly increased when flies were reared in presence of centrifuged supernatants that
261 contained a limited range of Cry 1A toxin fragments (Supplementary information S6).

262

263 Discussion

264 The increasing use of bioinsecticides based on *Bacillus thuringiensis* (*Bt*) raised concern about
265 potential side-effects on non-target biodiversity because of their partial specific targeting (de Souza

266 Machado et al. 2017; van Frankenhuyzen 2017; Venter and Bohn 2016), persistence in the
267 environment (Duchet et al. 2014; Hung et al. 2016a, b), and requirement of repeated spraying to
268 reach the desired pest control level (Brar et al. 2006). Especially, side-effects of chronic exposure
269 on non-target biodiversity, including insects present on treated areas, remain under-evaluated. Here,
270 we tested the side-effects of ingestion of *Bt* formulations (mainly *Bt kurstaki* (*Btk*) but also *Bt*
271 *israelensis* (*Bti*)) throughout the entire development of several non-target species of *Drosophila*
272 flies which are naturally present in treated areas. While formulation doses up to those recommended
273 for field sprayings ($\leq 10^6$ CFU/g of medium) had no effect on *Drosophila* development, mortality
274 and/or developmental delay occurred from doses only 10 times and 50 times higher than the
275 maximum recommended dose of the main tested *Btk* formulation for *D. suzukii* (10^7 CFU/g) and the
276 *D. melanogaster* strains (5×10^7 CFU/g), respectively. Besides, all the tested species except *D.*
277 *simulans* were strongly affected at 10^8 CFU/g, and no (or extremely limited) fly development
278 occurred at the highest tested dose (10^9 CFU/g), equivalent to 1000 times the maximum
279 recommended dose but below common acute intoxication doses (WHO Report, 2007).
280 Recommended doses are those for each spraying on a homogeneous and dry zone without covering
281 areas. In the field, both repeated spraying of stabilized formulation and rainfall washouts can
282 increase *Bt* spores and toxins presence in both space and time. While the highest dose tested here
283 would hardly be reached in the field, the minimal doses at which flies development was impacted
284 may be readily obtained. Furthermore, the minimal quantity of *Bt* formulation inducing
285 developmental alterations may be even lower since a single *Drosophila* larva is unlikely to process
286 1g of medium given its size and feeding rate. Our data also evidence a window of susceptibility to
287 *Btk* during the larval development, ingestion during the 1st larval instar being responsible for a large
288 part of the observed detrimental effects on the development.

289 When testing for generic effects of *Bt* formulations, slightly different results were observed
290 with two other *Btk* formulations and a formulation of *Bti*: there was no effect on *D. melanogaster*
291 development at the doses up to 10^8 CFU/g but a strong detrimental effect at the highest dose tested,

292 10^9 CFU/g. All the *Btk* formulations, based on two different bacterial strains (see Methods), had
293 similar profiles of Cry1A protoxins and activated toxins, but they differed in their efficient spore
294 contents, formulation type, and likely additives, which may account for the observed variation in
295 the half-lethal dose. The *Bti* formulation, widely used against Dipteran Nematoceran insects (e.g.
296 mosquitoes, black flies; Becker 2000), impacted *D. melanogaster* development only at the highest
297 dose tested. These impacts of *Bt* formulations on *D. melanogaster* development are consistent with
298 growing evidence suggesting a partly specific targeting of *Bt* (van Frankenhuyzen 2013; Venter and
299 Bøhn, 2016). Until recently, it has generally been accepted that the mode of action of *Bt* after
300 ingestion by insects relies on key steps of specific binding of proteolyzed *Bt* toxins to receptors of
301 midgut epithelial cells, defining targets for each *Bt* subspecies (Bravo et al. 2007, 2011; Palma et al.
302 2014). Several primary and secondary types of toxin receptors, including cadherin-like proteins,
303 aminopeptidases, GPI-anchored alkaline phosphatases (Adang et al. 2014), and more recently the
304 ATP dependent binding cassette reporter C2 (Stevens et al. 2017), have been identified in
305 Lepidoptera and Dipteran mosquitoes. Focusing on the action of *Btk* targeting Lepidoptera, no
306 Lepidoptera cadherin-like Cry receptor orthologues were found in *Drosophila* (Stevens et al. 2017),
307 supporting the idea that these flies would not be affected by the spraying of *Btk* formulation. The
308 existence of other types of Cry receptors in *Drosophila* flies remains to be investigated. In addition,
309 the substantial amounts of active Cry1A toxin fragments in *Btk* formulations could compensate for
310 the possible lack of solubilization of protoxin crystals in the fly midgut and proteolytic activation of
311 toxins by fly gut proteases, both required for Cry activity in insect larvae (Bravo et al. 2007). Other
312 toxins synthesized by *Btk* and present in the formulations could also play a role in the observed
313 cross-order activity as some, such as Cry2A, have an insecticidal effect on both Lepidoptera and
314 Diptera (George and Crickmore 2012).

315 The lack of effect of ingestion of *Bacillus subtilis* or *Btk* Cry-free 4D22 on the development
316 of *D. melanogaster* excludes that developmental alterations result from severe disruption of
317 digestion and nutrient uptake/competition in the presence of high spore/bacteria loads in the larval

318 gut throughout development. It supports the idea of a synergistic action of *Btk* spores and Cry
319 toxins, consistent with the models of *Bt* action on insect larvae in which toxins first breach the gut
320 epithelium, allowing the gut content, including *Bt* spores, to colonize the hemocoel (Bravo et al.
321 2007; Obata et al. 2009; Bravo et al. 2011; Caccia et al. 2016). The partially reproduced mortality
322 rate and delayed development in dialysis experiments further indicate that low diffusible molecular
323 weight compounds in *Btk* formulations (e.g., culture media residues, salts, additives) may contribute
324 to these developmental alterations. This is supported by the lack of impact on *D. melanogaster*
325 development of the ingestion of laboratory-produced spores and Cry toxins of a *Btk* 4D1 strain (or
326 HD1, a reference strain used as a control strain, not used in the commercial formulations) used
327 without additives, even at the highest dose 10^9 CFU/g (additional information S7; Fig. S7a, b). The
328 *Btk* 4D1 culture contained few active Cry toxins and smaller toxin fragments, in contrast to
329 commercial *Btk* formulations (Fig. S7c), supporting the possible contribution of these toxin
330 fragments to the cross-order activity of *Btk* formulations on *Drosophila*.

331 As observed for *D. suzukii* exposed to laboratory-produced *Btk* cultures (Cossentine et al.
332 2016), mortality of *D. melanogaster* during development on *Btk* formulation already occurred early
333 in development. First and second instars larvae are probably highly exposed due to their high
334 feeding rate and their exponential growth (Santos et al. 1997). As the observed larval mortality was
335 only about 40% at the highest dose (10^9 CFU/g) (Figure 2), while none of the individuals reached
336 the pupal stage at this dose (Figure 4), the remaining mortality likely occurred during the third
337 larval stage, maybe due to delayed action of *Btk* spores and toxins. Interestingly, alterations of the
338 development (mortality and delayed emergence) mimicked those typically generated by nutritional
339 stress conditions in insect larvae (Nepoux et al. 2010; Vantaux et al. 2016). Accordingly, the
340 developmental alterations were partially rescued on a protein rich fly medium, probably through
341 compensatory protein intake, as in other arthropod species (Moret and Schmid-Hempel 2000;
342 Kutzer and Armitage 2016; Vantaux et al. 2016). In addition, the sex ratio of flies was strongly
343 biased towards males after development on the dose of *Btk* formulation affecting fly emergence (10^8

344 CFU/g) and under low protein conditions. This highlights the importance of nutritional conditions
345 in *Btk* impacts on development, with sex-specific differences in larval susceptibility to
346 environmental stressors, here the accumulation of *Btk* formulation, under protein restriction
347 conditions as previously reported in *D. melanogaster* (Andersen et al. 2010).

348 The development on sublethal doses of *Btk* formulation did not dramatically affect the
349 longevity of *D. melanogaster* adults and the offspring number throughout life. Developmental
350 exposure to *Btk* doses that slightly and strongly reduced the likelihood of reaching the adult stage
351 even gave males and females a dose-dependent longevity benefit, in addition to the male higher
352 longevity observed in mixed-sex populations (Khazaeli and Curtsinger, 2000), and slightly
353 increased the offspring number (although not significantly). Surviving the exposure to *Btk*
354 formulation throughout the development has likely selected for fitter individuals. This is similar to
355 the increased longevity of adult insects that have survived developmental nutritional stress (Rion
356 and Kawecki, 2007, Burger et al. 2010), or are resistant to environmental stressors (Khazaeli and
357 Curtsinger 2000).

358 The origin of *Drosophila* (species and population/strain) influenced the magnitude of the
359 impacts of the *Btk* formulation on the development. Within the *D. melanogaster* species, all strains
360 tested were susceptible to the *Btk* formulation with both mortality and delayed development at the
361 same dose, but with variation in the effect magnitude. This suggests potential population-specific
362 differences in susceptibility to *Btk* formulation accumulation in the environment, and hence
363 potential spatial and temporal heterogeneity of *Btk* spraying impacts for each *Drosophila* species.
364 At the fly community level, differences in susceptibility to *Btk* formulation, in terms of effect
365 magnitude and type of developmental alteration (mortality and/or developmental delay) occurred
366 between *Drosophila* species, regardless of their phylogenetic distances. In the *Drosophila*
367 subgenus, *D. simulans* was less susceptible than its sister species *D. melanogaster*, whereas the
368 African *D. yakuba* experienced similar impacts on the development as *D. melanogaster*. The three
369 species *D. immigrans*, *D. subobscura* and *D. hydei* were similarly more susceptible than *D.*

370 *melanogaster*, but with slight differences in effect magnitudes. The phylogenetically distant *D.*
371 *busckii* (*Dorsilopha* subgenus) was the least affected of all the species tested in terms of
372 developmental mortality, but its development was strongly delayed. The five species *D.*
373 *melanogaster*, *D. simulans*, *D. hydei*, *D. immigrans*, and *D. busckii* belong to the guild of
374 cosmopolitan domestic *Drosophila* species, *D. subobscura* is sub-cosmopolitan species, and *D.*
375 *busckii* is an opportunistic frugivorous species (Atkinson and Shorrocks 1977). All these species
376 coexist frequently and compete on the same discrete and ephemeral rotting fruit patches, with
377 seasonal variations in the composition of the fly community (Shorrocks 1991; Benado and Brncic
378 1994; Nunney 1996; Mitsui and Kimura 2000). Differences in species susceptibility to
379 accumulation of *Btk* formulation could modify larval competition conditions and lead to additional
380 local and temporal variations in *Drosophila* communities' composition. The potential side-effects
381 of *Bt* sprays on non-target *Drosophila* communities would be hardly predictable as they depend on
382 spatial patterns of *Bt* accumulation.

383 The *Btk* formulation clearly impacted the development of the invasive *D. suzukii*, as recently
384 reported by Cossentine et al. (2016), this species being the most susceptible here with effects
385 already detectable at 10 times the recommended spraying dose. Compared with the other seven
386 species that live on rotten fruits, *D. suzukii* poses a threat to fruit production because it feeds and
387 lays eggs on healthy ripening fruits (Walsh et al. 2011; Delbac et al. 2014; Poyet et al. 2014) and
388 hence colonizes orchards and vineyards earlier during the fruit season. The greater susceptibility of
389 *D. suzukii* to the accumulation of *Btk* formulation in the environment might mitigate the potential
390 ecological burden of its invasion for local communities of *Drosophila* frugivorous species in
391 orchards. Alternatively, as *D. suzukii* attacks on fruits can accelerate their decomposition by
392 microorganisms, its higher susceptibility to *Btk* could reduce the number of fruits made suitable for
393 other *Drosophila* species.

394 In conclusion, we show here that repeated spraying and accumulation of *Btk* formulation
395 can potentially impact non-target insect communities, and the magnitude of this impact could

396 depend on the formulation used and the insect species. Recent studies have reported similar adverse
397 side-effects due to repeated spraying of the *Bti* formulation, directly on non-target organisms (e.g.
398 Duguma et al. 2015), and indirectly on predators via food webs (e.g. Poulin et al. 2010). These
399 studies and the data presented here highlight that pest control with *Bt* bioinsecticides should be done
400 with caution in the field to avoid, or at least limit, potential negative impacts on non-target
401 biodiversity and species communities within ecosystems. At last, *D. melanogaster*, a model species
402 in many research fields, could also serve as a study model to assess the toxicity of *Bt* on non-target
403 species, and identify the mechanisms underlying these side-effects.

404

405 **Methods**

406 *Commercial formulations, Bacillus productions and Colony Forming Unit measurement*

407 The tested commercial brands of *Bacillus thuringiensis kurstaki* (*Btk*; serotype 3a, b, c; Zeigler,
408 1999) were Delfin[®] A and B (strain SA11; wettable granules, Valent BioSciences, AMM 9200482,
409 32,000 UI/mg) and Scutello DF (a Dipel[®] sub-brand; strain ABTS-351; wettable granules, Biobest[®],
410 AMM 2010513, 540g/kg). The commercial brand of *Bacillus thuringiensis israelensis* (*Bti*; strain
411 HD-14; serotype 14; Ziegler, 1999) was VectoBac[®] WG (wettable granules, Bayer, AMM 2020029,
412 3000 UTI/mg). For each formulation, the number of viable spores (expressed as Colony Forming
413 Units (CFU) per mg of granules) was estimated using serial dilutions of a suspension on LB agar
414 plates and counting of bacterial colonies after overnight incubation at 30°C. CFU estimations were
415 5×10^7 CFU/mg for *Btk* Delfin[®] A; 2.5×10^7 CFU/mg for *Btk* Delfin[®] B; 2.2×10^7 CFU/mg for *Btk*
416 Scutello DF; 6×10^7 CFU/mg for *Bti* VectoBac[®]. No change in CFU estimations occurred during the
417 time frame of the experiments. Manufacturer-recommended doses for Delfin[®] range from 0.15 to
418 1.5 kg/ha depending on the crop type. Based on our CFU estimations, this corresponds to
419 recommended doses of 7.5×10^4 to 7.5×10^5 CFU/cm² of Delfin[®] A, and 3.75×10^4 to 3.75×10^5
420 CFU/cm² of Delfin[®] B for each spraying in the field. For Scutello DF, recommended doses range
421 from 0.1 to 1 kg/ha, equivalent to 2.2×10^4 to 2.2×10^5 CFU/cm². Vectobac[®] WG is used at 0.125 to

422 1 kg/ha, equivalent to 7.5×10^4 to 6×10^5 CFU/cm².

423 The acrystillipherous (Cry toxin-free) *Btk* 4D22 strain (depleted for the toxin-encoding
424 plasmids; Gonzalez et al. 1982) obtained from the Bacillus Genetic Stock Center (<http://bgsc.org>;
425 Columbus USA), and a *Drosophila* non-pathogenic *Bacillus subtilis* (gift from Dr. E. Bremer,
426 University of Marburg, Germany; A. Brun-Barale, pers. comm.) were grown at 30°C in the
427 sporulation-specific medium PGSM (Bactopeptone[®] 7.5 g, KH₂PO₄ 3.4 g, K₂HPO₄ 4.35 g, glucose
428 7.5 g, PGSM salts 5 mL, CaCl₂ 0.25 M, distilled water qsp 1L, pH 7.2; PGSM salts: MgSO₄·7H₂O,
429 MnSO₄·H₂O, ZnSO₄·7H₂O, FeSO₄·7H₂O) for about 14 days for sporulation to occur. Following
430 elimination of vegetative cells (1h at 70 °C), spore pellets were collected after centrifugation (4,500
431 rpm, 20 min, 4 °C), washed with sterile water, and lyophilized. CFU numbers were counted for
432 each preparation as described above.

433

434 *Fly stocks*

435 The four tested strains of *Drosophila melanogaster* (phylogenetic subgroup: melanogaster) were the
436 standard wild-type Canton S (Bloomington Drosophila Centre) used as a reference strain, the wild-
437 type Nasrallah strain from Tunisia (strain 1333, Gif-sur-Yvette), the double mutant standard strain
438 YW1118 (white and yellow mutations; gift from Dr. B. Charroux, IBD, Marseille-Luminy), and a
439 recently field-collected strain (caught in Southern France in 2013) that we named “Sefra”. For
440 *Drosophila* species comparison, we included 6 species of the *Drosophila* subgenus, *D. simulans*
441 (strain 1132; phylogenetic subgroup: melanogaster), *D. yakuba* (strain 1880; phylogenetic subgroup:
442 melanogaster), *D. hydei* (phylogenetic subgroup: hydei) and *D. suzukii* (phylogenetic subgroup:
443 immigrans) (both kindly provided by Dr. R. Allemand, LBBE, University Lyon 1), *D. immigrans*
444 (phylogenetic subgroup: immigrans), *D. subobscura* (phylogenetic subgroup: obscura), and one
445 species of the *Dorsilopha* subgenus, *D. busckii* (all three species collected in South-East of France
446 in Spring 2015).

447 All strains and species were maintained at controlled densities (150-200 eggs/40 ml of fly

448 medium) under standard laboratory conditions (25°C or 20°C for recently collected species, 60 %
449 relative humidity, 12:12 light/dark cycle), on a high-protein/sugar-free fly medium (10 % cornmeal,
450 10 % yeast, 0 % sugar). The *D. melanogaster* Canton S strain was also reared on a standard low-
451 protein/sugar-free fly medium (8 % cornmeal, 2 % yeast, 2.5 % sugar) to test for the influence of
452 the medium composition on *Btk* exposure effects.

453

454 *Intoxication method and dose-response assay*

455 Commercial formulations and laboratory spore productions were suspended and diluted in buffer to
456 perform dose-response assays with doses from 10^5 to 10^9 CFU/g of fly medium. All doses were
457 prepared in 100 μ l and homogenized thoroughly with the fly medium (100 μ l/g). *Drosophila* eggs
458 and larvae were collected from stock vials at the suitable developmental stage and transferred
459 carefully to the intoxication vials and dishes, then maintained under standard laboratory conditions
460 until a) the emergence of adults, or, in the larvae survival tests, b) until a given developmental stage
461 was reached from the egg, and c) for 24h. Control groups of individuals were transferred on fly
462 medium homogenized with the same volume of buffer.

463

464 *Development-related traits and larval survival*

465 To evaluate emergence rates and developmental times upon intoxication throughout the entire
466 development, precise numbers of eggs from mass oviposition were transferred to intoxication vials
467 containing fly medium mixed with doses of *Bt* formulations or bacteria productions and let to
468 develop under standard laboratory conditions until the fly emergence. Eggs without chorion and
469 transparent eggs were discarded. The initial number of eggs was adjusted depending on the species
470 biology and the vial size: 20 eggs for 2 g of fly medium in small vials (\varnothing 3.3 cm, surface ~ 8.5 cm²,
471 0.24 g/cm²) for tests with *D. melanogaster* Canton S, 50 eggs for 6 g of fly medium for comparison
472 of *D. melanogaster* strains and *Drosophila* species in wider vials (\varnothing 4.6 cm, surface ~ 16 cm², 0.37
473 g/cm²) except for *D. hydei*, *D. suzukii* and *D. immigrans* for which 30 eggs were transferred on 6 g

474 of fly medium. Numbers and sex of emerging flies were recorded once a day until the day the pupae
475 of the next generation should form. From these data, the emergence rate (proportion of emerged
476 flies from the initial eggs; ER), the mean developmental time (mean number of days for completion
477 of development; DT), and the sex-ratio (proportion of male flies; SR) were calculated for each
478 intoxication vial.

479 For the larval survival tests, 20 eggs or larvae from a 4-hour mass oviposition at the indicated
480 developmental stage, were transferred to small dishes containing 1 g of fly medium (\varnothing 3 cm,
481 surface ~ 7 cm²) homogenized with increasing doses of Delfin[®] A. Surviving larvae were counted at
482 the indicated developmental stage, or after 24-hour intoxication, to calculate the proportion of
483 surviving larvae. For the test from the egg, eggs which did not hatch were not included in the
484 counting. As a control measurement, we measured the pH of the fly medium in the presence of the
485 dose range of *Bt* formulations (see Supplementary Information S4).

486

487 *Adult fitness-related traits*

488 For the longevity and offspring number tests, males and females emerged from several rearing vials
489 for each dose of Delfin[®] A were pooled when aged 2 days. Groups of 15 males and 15 females were
490 transferred into vials with fresh fly medium without formulation. Fly medium was renewed every 3-
491 4 days. After each fly transfer to fresh food, discarded maintenance vials were incubated under
492 standard laboratory conditions for the offspring to develop. Mortality and sex of dead flies were
493 recorded daily until the last fly died. Offspring numbers were counted from the first emergence
494 until pupae of the next generation appeared. The tests were repeated twice. Due to the variation in
495 the duration of the two longevity experiments, offspring numbers of each vial were summed to
496 obtain a total offspring number per dose of Delfin[®] A for each experiment.

497

498 *Dialysis and Cry toxin analysis*

499 A suspension of 2×10^{10} CFU of Delfin[®] A was dialyzed against PBS (KH₂PO₄ 1.06 mM,

500 $\text{Na}_2\text{HPO}_4(2\text{H}_2\text{O})$ 3mM, NaCl 154 mM, qsp distilled water, pH 7.2), at 250 rpm, 4°C overnight,
501 using an 8-10 kDa MW cut-off membrane (ZelluTrans, Roth®). The CFUs of the dialyzed
502 suspension and the effects on ER and DT were analysed as described above. The dialyzed
503 suspension was also subject to a 12.5 % SDS-PAGE and compared to the non-dialyzed suspension
504 after silver staining. The presence of Cry1A pro-toxins, activated toxins and toxin fragments was
505 probed by Western-blot using an in-house anti-Cry1A rabbit polyclonal antibody.

506

507 *Data analysis*

508 Data on development traits (emergence rate ER and developmental time DT), sex-ratio (SR),
509 survival of larval stages and offspring number were analysed with mixed effect models including
510 the dose of *Btk* formulation/spore production, the *D. melanogaster* strain, the *Drosophila* species or
511 the developmental stage as fixed effects, and replicate (plus the experiment when necessary) as
512 random effects (for ER data, data were analysed with bias-corrected models with replicate as fixed
513 effect to allow pairwise comparisons; similar results obtained with models including replicate as
514 random effect). ER, SR and survival of larval stages were analysed with generalized linear models,
515 with binomial distribution and logit link. DT and offspring number were analysed with linear
516 models. DT were transformed into developmental rates (1/developmental time) to fulfil the
517 assumptions of the analysis of variance (homoscedasticity and residuals normality). Adult longevity
518 data were analysed with proportional hazard Cox regression models including fly sex and dose of
519 *Btk* formulation as fixed effects, and replicates as a random effect. For all the data sets, the main
520 fixed effects and their interactions were tested with log-likelihood ratio tests. *Post hoc* pairwise
521 comparisons were made for pairs of *D. melanogaster* strains, formulation/spore treatments, and
522 between the control dose and the other doses. All the analyses were performed in R (R
523 Development Core Team, 2008) using the packages lme4 (Bates et al. 2015), brglm (Kosmidis
524 2017), multcomp (Horton et al. 2008), survival (Terry et al. 2000) and coxme (Terry and Therneau,
525 2015).

526

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- 733

734 **Acknowledgements**

735 We thank Xiao Han, Jingru Li and Abir Oueslati for help with preliminary experiments, L.
736 Kremmer, C. Rebuf and O. Magliano for providing and rearing flies and help in preparing fly
737 medium, A. Brun-Barale for the *Bacillus subtilis* spores, D. Pauron for preparation of Cry1A toxin,
738 Hugo Mathé-Hubert for advice on statistical analyses, and M. Amichot for helpful discussions. The
739 Cry1A antibody was produced in collaboration with the INRA-PFIE platform (Nouzilly, France).

740

741 **Financial Supports**

742 This work was supported by the French National Agency for Research (ANR-13-CESA-0003-001
743 ImBio), the European Union's Seventh Framework Program for research, technological
744 development and demonstration under grant agreement No. 613678 (DROPSA), the "Investments
745 for the Future" LABEX SIGNALIFE (ANR-11-LABX-0028), and the INRA Plant Health
746 Department.

747

748 **Author contributions**

749 AB, MP NE, AG, JLG and MP designed the experiments. AB performed the experiments with
750 contributions of MPNE. AB performed the statistical analyses. AB, JLG, and MP wrote the
751 manuscript with contributions from all the authors.

752

753 **Additional information**

754 **Supplementary information**

755 **Competing financial interest:** The authors declare no competing financial interests.

756

757

758 **Table 1.** Results of statistical analyses to assess the effect of the dose of formulation/spore
759 production and its interaction with the treatment, the larval instar, the experiment, the sex, the fly
760 strain and the fly species when appropriate. See figures for *post hoc* comparisons of the doses with
761 the control dose.
762

Source of variation/Data	χ^2 / Deviance	d.f.	P value
Development on <i>Btk</i> Delfin[®] A, <i>Btk</i> 4D22, <i>Bti</i> Vectobac[®], <i>Bacillus subtilis</i>			
<i>Emergence rate</i>			
Dose × Treatment	285.7	20	< 0.0001
Dose for each treatment:			
- Delfin [®] A	237.5	6	< 0.0001
- 4D22	7.0	7	0.40
- Vectobac [®]	165.8	5	< 0.0001
- <i>B. subtilis</i>	1.9	6	0.93
<i>Developmental time</i>			
Dose × Treatment	220.8	19	< 0.0001
Dose for each treatment:			
- Delfin [®] A	68.8	6	< 0.0001
- 4D22	16.08	7	0.024
- Vectobac [®]	37.5	6	< 0.0001
- <i>B. subtilis</i>	13.5	7	0.060
Development on <i>Btk</i> Delfin[®] B and Scutello DF (dose effect)			
<i>Emergence rate</i>			
- Delfin [®] B	151.2	2	< 0.0001
- Scutello DF	105.1	2	< 0.0001
<i>Developmental time</i>			
- Delfin [®] B	2.5	1	0.12
- Scutello DF	30.9	2	< 0.0001
Role of formulation components in the development alterations (dialysis)			
Dose effect			
<i>Emergence rate</i>	459.8	3	< 0.0001
<i>Developmental time</i>	13.7	2	0.0011
Survival of larval stages on Delfin[®] A			
<i>Cumulative survival</i>			
Dose × Larval instar	16.2	5	0.0063
Dose for each instar:			
- late 1 st instar	87.4	5	< 0.0001
- late 2 nd instar	25.7	5	0.0001
<i>24-hour survival</i>			
Dose × Larval instar	15.9	5	0.007
Dose for each instar:			
- late 1 st instar	55.9	5	< 0.0001
- late 2 nd instar	3.76	5	0.58
Adult fitness-related traits after development on Delfin[®] A			
<i>Longevity</i>			
Experiment	20.1	1	< 0.0001
- 1 st experiment:			
Dose	12.3	3	0.0065
Sex	35.0	1	< 0.0001
(e ^β coefficient males vs females ± se: 0.55 ± 0.16)			
Dose × Sex	20.4	3	0.00014
<i>Sexes analyzed separately</i>			
- females	12.0	3	0.0073
(e ^β coefficients vs control ± se: 5×10 ⁶ : 1.05 ± 0.17, 5×10 ⁷ : 0.71 ± 0.16, 10 ⁸ : 0.60 ± 0.21)			
- males	20.4	3	0.00014
(e ^β coefficients vs control ± se: 5×10 ⁶ : 0.80 ± 0.16, 5×10 ⁷ : 0.66 ± 0.16, 10 ⁸ : 1.53 ± 0.18)			

763

764

Source of variation/Data	χ^2 / Deviance	d.f.	P value
Adult fitness-related traits after development on Delfin[®] A			
<i>- 2nd experiment:</i>			
Dose	16.5	3	0.00090
Sex	31.5	1	< 0.0001
(e ^β coefficient males vs females ± se: 0.45 ± 0.22)			
Dose × Sex	0.69	3	0.88
<i>Sexes analyzed separately</i>			
- females	13.2	3	0.0043
(e ^β coefficients doses vs control ± se: 5×10 ⁶ : 0.92 ± 0.22, 5×10 ⁷ : 0.63 ± 0.21, 10 ⁸ : 0.51 ± 0.21)			
- males	7.01	3	0.072
(e ^β coefficients doses vs control ± se: 5×10 ⁶ : 1.02 ± 0.22, 5×10 ⁷ : 0.70 ± 0.22, 10 ⁸ : 0.64 ± 0.22)			
<i>Total numbers of offspring</i>			
Dose × Experiment	28.1	3	< 0.0001
<i>Dose for each experiment:</i>			
- 1 st experiment	26.3	3	< 0.0001
- 2 nd experiment	4.1	3	0.25
Development of other strains of <i>D. melanogaster</i> on Delfin[®] A (including Canton S)			
<i>Emergence rate</i>			
Dose × Fly strain	105.5	15	< 0.0001
<i>Dose for each fly strain:</i>			
- Canton S	588.6	5	< 0.0001
- Nasrallah	745.3	5	< 0.0001
- Sefra	900.7	5	< 0.0001
- YW1118	636.9	5	< 0.0001
<i>Developmental time</i>			
Dose × Fly strain	9.3	12	0.68
<i>Dose for each fly strain:</i>			
- Canton S	40.3	4	< 0.0001
- Nasrallah	18.0	4	0.0012
- Sefra	27.2	4	< 0.0001
- YW1118	28.9	4	< 0.0001
Development of other <i>Drosophila</i> species on Delfin[®] A			
<i>Emergence rate</i>			
Dose × Fly species	538.2	30	< 0.0001
<i>Dose for each species:</i>			
- <i>D. simulans</i>	461.0	5	< 0.0001
- <i>D. yakuba</i>	750.7	5	< 0.0001
- <i>D. hydei</i>	596.8	5	< 0.0001
- <i>D. immigrans</i>	726.3	5	< 0.0001
- <i>D. subobscura</i>	729.6	5	< 0.0001
- <i>D. sukukii</i>	725.0	5	< 0.0001
- <i>D. busckii</i>	586.0	5	< 0.0001
<i>Developmental time</i>			
Dose × Fly species	59.9	22	< 0.0001
<i>Dose for each species:</i>			
- <i>D. simulans</i>	25.9	4	< 0.0001
- <i>D. yakuba</i>	34.7	4	< 0.0001
- <i>D. hydei</i>	11.5	4	0.022
- <i>D. immigrans</i>	6.01	3	0.11
- <i>D. subobscura</i>	68.8	4	< 0.0001
- <i>D. sukukii</i>	11.7	3	0.0085
- <i>D. busckii</i>	58.8	4	< 0.0001

766 **Figure legends**

767 **Figure 1. Development of *D. melanogaster* Canton S flies on *Btk* and *Bti* commercial**
768 **formulations. (a)** Emergence rate (mean \pm sem) and **(b)** developmental time (mean \pm sem) of 20
769 initial eggs on increasing doses of *Btk* Delfin[®] A (red triangles), the Cry-free *Btk* 4D22 (open
770 lozenges), the mosquito-targeting *Bti* Vectobac[®] (green squares), and the non-pathogenic *Bacillus*
771 *subtilis* (light grey circles). For Vectobac[®] and *B. subtilis*, $N = 4-7$ per dose; for Delfin[®] A and *Btk*
772 4D22, $N = 9-12$ for the control, $N = 3$ for $5 \cdot 10^5$ and 10^9 , $N = 4-9$ for 10^6 , $N = 7-14$ from $5 \cdot 10^6$ to 10^8 .
773 **(c)** Emergence rate (mean \pm sem) and **(d)** developmental time (mean \pm sem) on increasing doses of
774 the two *Btk* formulations Delfin[®] B (dark red circles) and Scutello DF (orange squares). $N = 4$
775 replicates of 20 eggs per dose and formulation, except for controls and 10^8 CFU/g of Delfin[®] B (9-
776 10 replicates of 20 eggs). Results of *post hoc* comparisons of each dose to the control: $\cdot 0.05 < p < 0.1$;
777 $* 0.01 < p < 0.05$; $** 0.001 < p < 0.01$; $*** p < 0.001$. **(e)** Immunoblotting with an anti-Cry1A polyclonal
778 antibody on proteins from a suspension of laboratory-produced spores of Cry-free *Btk* 4D22, the
779 three *Btk* formulations Delfin[®] A, B, Scutello DF, and a suspension of laboratory-produced Cry1A
780 toxins. Red asterisks indicate the Cry protoxins (~ 130 kDa) and the activated fragments (~ 60 kDa
781 and ~ 70 kDa).

782

783 **Figure 2. Survival of *D. melanogaster* Canton S larval stages on increasing doses of *Btk***
784 **Delfin[®] A. (a)** Proportion of surviving larvae (mean \pm sem) upon *Btk* exposure from the egg to late
785 1st instar (open lozenges) and late 2nd instar (black triangles). **(b)** Proportion of surviving larvae
786 (mean \pm sem) upon 24-hour *Btk* exposure of early 1st instar larvae (open lozenges) and 2nd instar
787 larvae (black triangles). $N = 5-7$ replicates of 20 individuals per dose. Results of *post hoc*
788 comparisons of each dose with the control: $* 0.01 < p < 0.05$; $** 0.001 < p < 0.01$; $*** p < 0.001$.

789

790 **Figure 3. Fitness-related traits of adults (longevity and total offspring number) after**
791 **development on *Btk* Delfin[®] A. (a, d)** Female longevity (mean survival fraction over time \pm sem),

792 (b, e) Male longevity (mean \pm sem), and (c, f) total offspring number (mean \pm sem), measured on
793 individuals that developed without *Btk* (blue items) and on 5×10^6 CFU/g of *Btk* Delfin[®] A (green
794 items), 5×10^7 CFU/g (red items), and 10^8 CFU/g (dark red items). Data from 2 experiments (a-c,
795 experiment 1; d-f, experiment 2). For each trait, $N = 3$ -5 replicates of 15 males and 15 females per
796 dose in experiment 1, $N = 3$ replicates of 15 males and 15 females in experiment 2. Results of *post*
797 *hoc* comparisons of each dose with the control: * $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

798

799 **Figure 4. Development of four *D. melanogaster* strains on increasing doses of *Btk* Delfin[®] A.** (a)
800 Emergence rate (mean \pm sem), (b) Developmental time (mean \pm sem) of the strains Canton S (blue
801 lozenges), Nasrallah (yellow triangles), Sefra (green squares), and YW1118 (red circles). $N = 4$
802 groups of 50 eggs per dose and fly strain for each trait. Results of *post hoc* comparisons of each
803 dose to the control: $\cdot 0.05 < p < 0.1$; * $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

804

805 **Figure 5. Emergence rate of seven *Drosophila* species on increasing doses of *Btk* Delfin[®] A.**
806 Mean emergence rate (\pm sem). $N = 4$ replicates of 50 eggs per dose for *D. simulans*, *D. yakuba*, *D.*
807 *subobscura*, and *D. busckii*, $N = 4$ replicates of 30 eggs per dose for *D. hydei*, *D. suzukii*, and *D.*
808 *immigrans*. Results of *post hoc* comparisons of each dose with the control: $\cdot 0.05 < p < 0.1$; *
809 $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$.

810

811 **Figure 6. Developmental time of seven *Drosophila* species on increasing doses of *Btk* Delfin[®] A.**
812 Mean developmental time (\pm sem). $N = 4$ replicates of 50 eggs per dose for *D. simulans*, *D. yakuba*,
813 *D. subobscura*, and *D. busckii*, $N = 4$ replicates of 30 eggs per dose for *D. hydei*, *D. suzukii*, and *D.*
814 *immigrans*. Results of *post hoc* comparisons of each dose with the control: * $0.01 < p < 0.05$; **
815 $0.001 < p < 0.01$; *** $p < 0.001$.

816

817

818 **Figure 7. Evaluation of the role of small molecular weight components of *Btk* Delfin[®] A**
819 **(dialysis; membrane cut-off: 8-10 kDa) in the altered development of *D. melanogaster* Canton**
820 **S. (a) Emergence rate (mean ± sem) and (b) developmental time (mean ± sem) on increasing doses**
821 **of dialyzed Delfin[®] A. *N* = 3 experiments of 4 replicates with 20 eggs per dose for the emergence**
822 **rate, *N* = 2 experiments of 4 replicates per dose for the developmental time. Results of *post hoc***
823 **comparisons of each dose with the control: · 0.05 < *p* < 0.1; * 0.01 < *p* < 0.05; ** 0.001 < *p* < 0.01; *****
824 ***p* < 0.001. (c) Anti-Cry1A probed immunoblot of non-dialyzed (ND) and dialyzed (D) suspensions**
825 **showing the decrease in the amount of ~ 130 kDa protoxins and the increase in that of ~ 60/70 kDa**
826 **activated toxins after dialysis.**

Figure 1

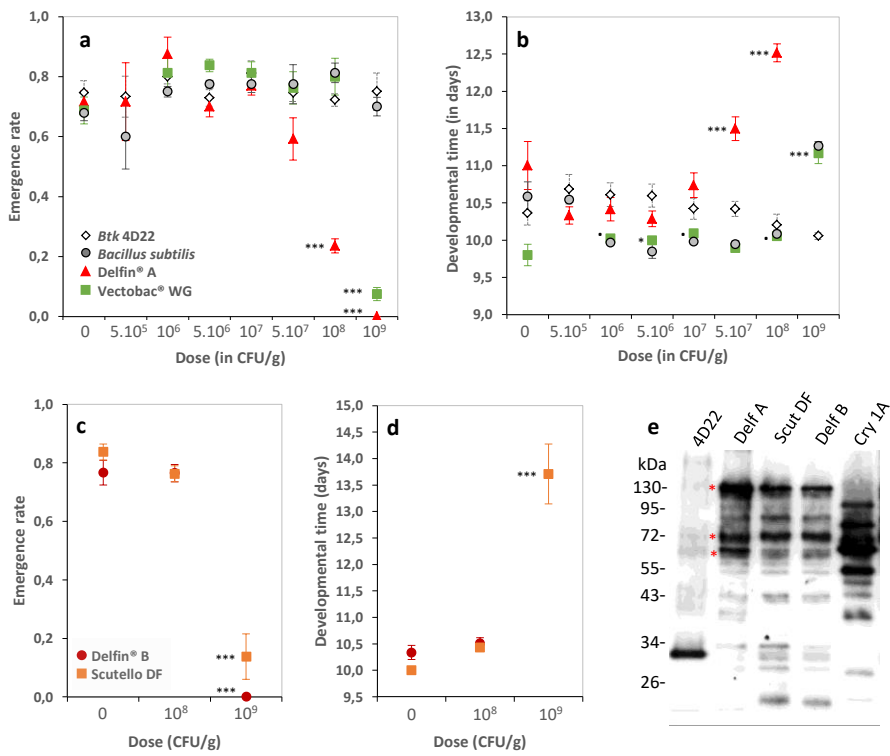


Figure 2

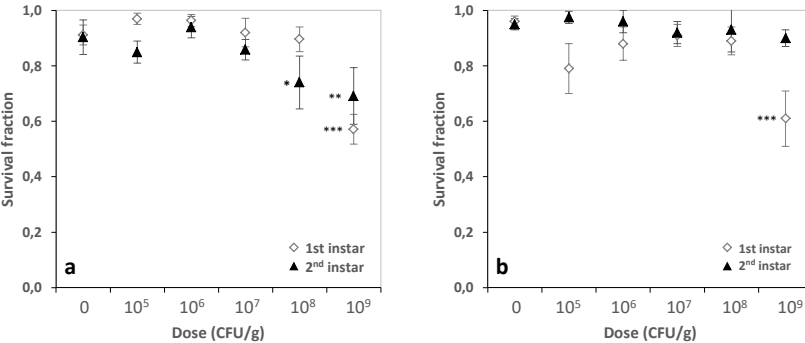


Figure 3

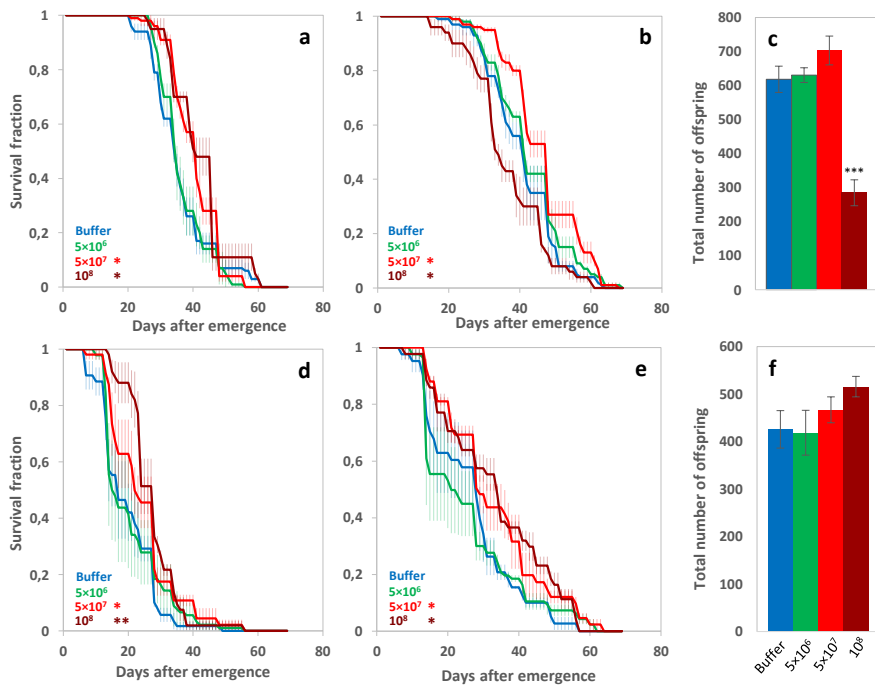


Figure 4

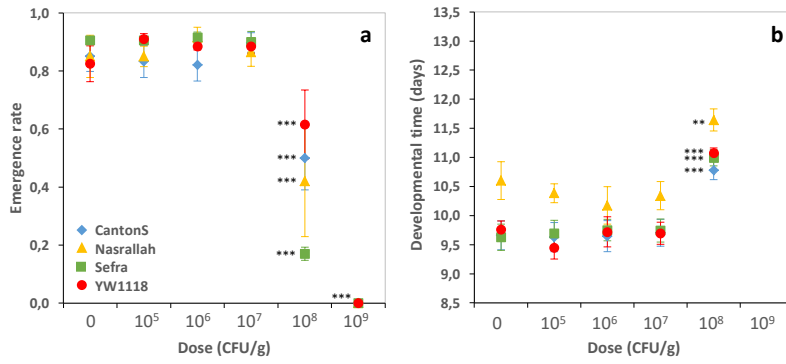


Figure 5

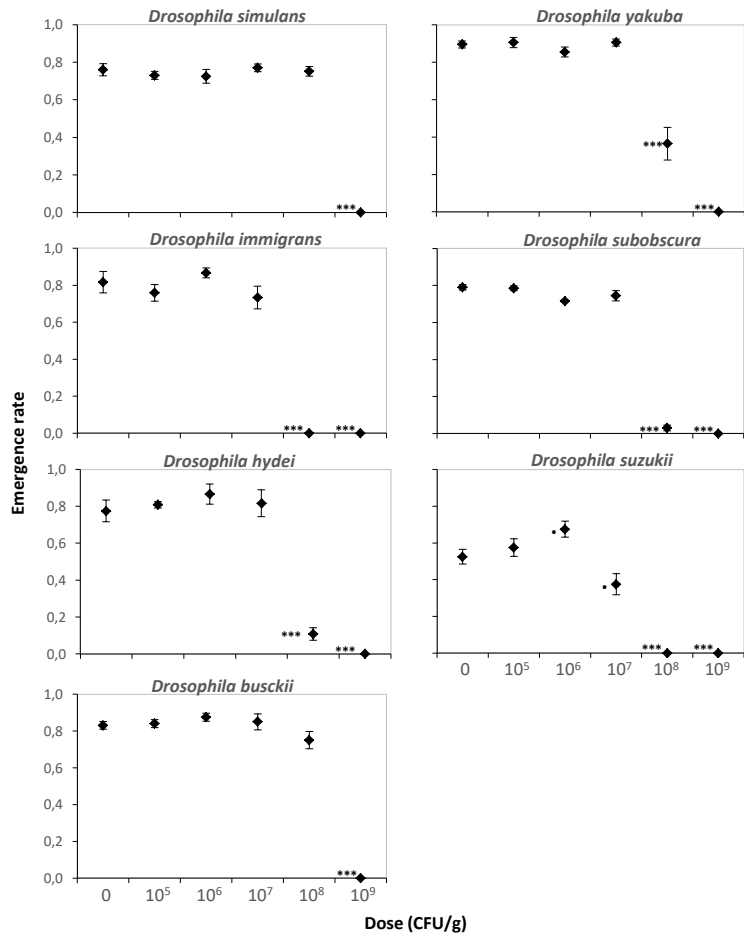


Figure 6

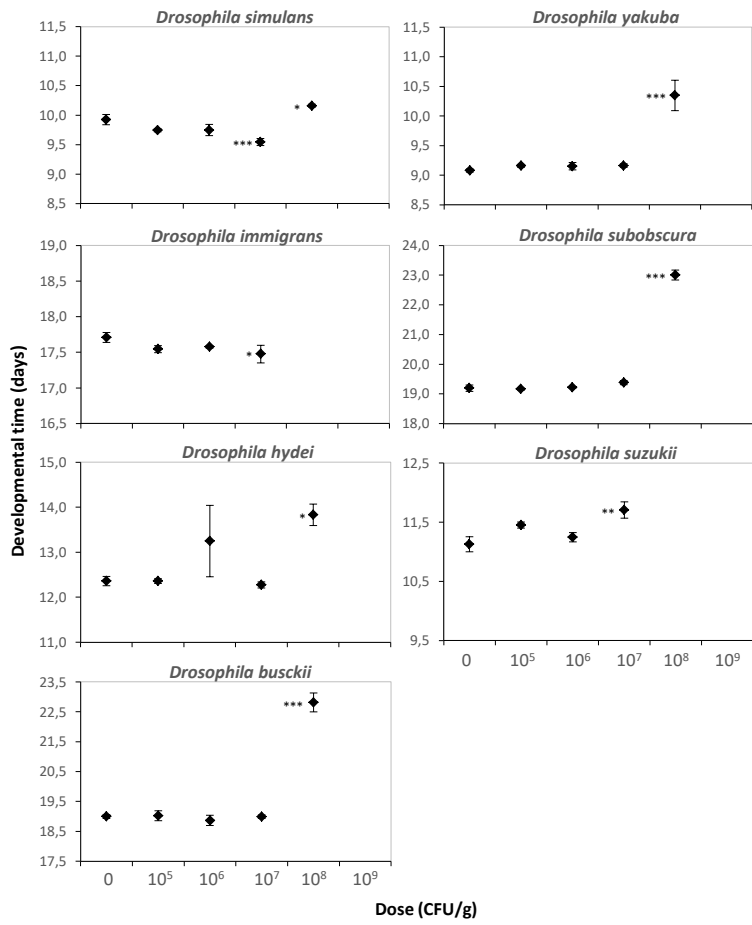


Figure 7

