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

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Results

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

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

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

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

Larval stages were tested for their susceptibility to *Btk* formulation in two independent and complementary dose-response tests of survival on Delfin® A, at doses ranging from 10^5 to 10^9 CFU/g of high protein/sugar free medium (this medium is used to rear fly species which are difficult to rear in the lab (see below) and is less limiting for the development of early larval stages). We focused on the 1st and 2nd larval instars, during which growth is exponential (Tennessen and Thummel 2011), so that larvae are most heavily exposed to the bioinsecticide. In the first test, the cumulative survival was measured by counting late 1st and 2nd instar larvae alive which have been exposed to Delfin® A from the egg stage. Larval survival was not influenced at 10^7 CFU/g, whereas it decreased for both larval instars above that dose to reach up to 37% mortality at 10^9 CFU/g (Fig. 2a). Reduced survival tended to occur at a lower dose when cumulative survival was measured later in the development, *i.e.* 10^9 for late 1st instar larvae and 10^8 CFU/g for 2nd instar larvae (Fig. 2a; Table 1). For both instars, larvae surviving 10^9 CFU/g were noticeably smaller and less active than those surviving lower doses. In emergence assays with planned exposure from the egg to the adult stage, none of these individuals reached the pupal stage (see results above). In the second test, larval survival was measured after early 1st and 2nd instar larvae had been exposed for 24 hours to Delfin® A. Survival of 1st instar larvae decreased by 36% on 10^9 CFU/g whereas that of 2nd instar larvae did not change (Fig. 2b, Table 1).

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

Long-term consequences on flies of exposure to *Btk* formulation throughout the development were evaluated on two fitness-related traits, longevity and total offspring number**.** Traits were measured on a *Btk*-free low-protein/high-sugar medium after individuals had completed their development on the same fly medium but in presence of selected doses of Delfin® A: 5×10^6 CFU/g, which had no impact on development, and 5×10^7 and 10^8 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

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

Btk formulation also affects other _Drosophila_ species.

The ER and DT were analysed for seven other *Drosophila* species from different phylogenetic clades at doses of Delfin® A from 10^5 to 10^9 CFU/g of high-protein/sugar-free medium (rearing medium of all the species). Tested species were *D. simulans* (*D. melanogaster* sister species), the African *D. yakuba*, *D. subobscura*, *D. immigrans, D. hydei*, and the invasive *D. suzukii*, all belonging to the *Drosophila* subgenus, and *D. busckii* from the *Dorsilopha* subgenus. For all the species, doses up to 10^6 CFU/g of Delfin® A had no effect on ER and DT whereas all individuals failed to reach the pupal stage and no fly emerged at 10^9 CFU/g (Fig. 5-6). Amplitudes of development alterations at 10^7 and 10^8 CFU/g varied between species (Fig. 5-6; Table 1). All species were affected at 10^8 CFU/g as was *D. melanogaster* (see Fig. 4a for comparison). *D. simulans* and *D. busckii* had unchanged ER, but DT was slightly increased for *D. simulans* (although slightly reduced at 10^7 CFU/g; similar results with a Japanese strain, data not shown) and strongly increased for *D. busckii* (by 20%, *i.e.* ~ 4 days) (Fig. 5-6, Table 1). *D. yakuba* ER and DT were similar to those of *D. melanogaster*, with an LD50 around 10^8 CFU/g and a moderate DT increase of ~ 1 day (Fig. 5-6, Table 1; similar results with a strain from Sweden, data not shown). The ER of *D. hydei* and *D. subobscura* were very low at 10^8 CFU/g (LD50 below this dose), with a high DT (Fig. 5-6; Table 1), while *D. immigrans* did not survive. No *D. suzukii* individual emerged at 10^8 CFU/g and development was already moderately impacted at 10^7 CFU/g (Fig. 5-6). No dose-dependent bias in SR was detected for either species (Supplementary information S5).

Development alterations may result from a synergy between formulation components.

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

about 10% of insecticidal protein toxins within this fraction, mainly Cry pro-toxins and activated toxins (see Fig. 1e) (Koch et al. 2015). The remaining weight consists of various compounds such as residues of culture medium and various additives including surfactant, anti-foaming agents, etc. (Glare and O'Callaghan, 2000; Brar et al. 2006). Indeed it has been shown for some products that additives can be more harmful in some cases than the active ingredient (Bradberry et al. 2004), we explored the role of small diffusible molecular weight components of Delfin® A in the alterations of ER and DT of *D. melanogaster* Canton S. For that, we mixed a 10 kDa dialyzed suspension of Delfin® A at 10^7, 10^8, and 10^9 CFU/g with low-protein/high-sugar medium. ER and DT were unaffected by the presence of the dialyzed suspension from the 10^7 CFU/g dose, whereas no individual reached the adult stage (no pupation) with the suspension from the 10^9 CFU/g dose (Fig. 7a; Table 1). At 10^8 CFU/g, ER was not modified but DT increased by ~ 1 day, only in one of the two experiments, partially reproducing the changes observed without dialysis (Fig. 7a-b; see also Fig. 1a-b, Table 1; 3 independent experiments for ER, 2 independent experiments for DT).

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

Discussion

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

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

When testing for generic effects of *Bt* formulations, slightly different results were observed with two other *Btk* formulations and a formulation of *Bti*: there was no effect on *D. melanogaster* 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

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

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

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

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

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

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

Methods

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

The tested commercial brands of *Bacillus thuringiensis kurstaki* (*Btk*; serotype 3a, b, c; Zeigler, 1999) were Delfin® A and B (strain SA11; wettable granules, Valent BioSciences, AMM 9200482, 32,000 UI/mg) and Scutello DF (a Dipel® sub-brand; strain ABTS-351; wettable granules, Biobest®, AMM 2010513, 540g/kg). The commercial brand of *Bacillus thuringiensis israelensis* (*Bti*; strain HD-14; serotype 14; Ziegler, 1999) was VectoBac® WG (wettable granules, Bayer, AMM 2020029, 3000 UTI/mg). For each formulation, the number of viable spores (expressed as Colony Forming Units (CFU) per mg of granules) was estimated using serial dilutions of a suspension on LB agar plates and counting of bacterial colonies after overnight incubation at 30°C. CFU estimations were 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* Scutello DF; 6×10^7 CFU/mg for *Bti* VectoBac®. No change in CFU estimations occurred during the time frame of the experiments. Manufacturer-recommended doses for Delfin® range from 0.15 to 1.5 kg/ha depending on the crop type. Based on our CFU estimations, this corresponds to recommended doses of 7.5×10^4 to 7.5×10^5 CFU/cm^2 of Delfin® A, and 3.75×10^4 to 3.75×10^5 CFU/cm^2 of Delfin® B for each spraying in the field. For Scutello DF, recommended doses range from 0.1 to 1 kg/ha, equivalent to 2.2×10^4 to 2.2×10^5 CFU/cm^2. Vectobac® WG is used at 0.125 to

1 kg/ha, equivalent to 7.5×10^4 to 6×10^5 CFU/cm^2.

The acrystillipherous (Cry toxin-free) *Btk* 4D22 strain (depleted for the toxin-encoding plasmids; Gonzalez et al. 1982) obtained from the Bacillus Genetic Stock Center (http://bgsc.org; Columbus USA), and a *Drosophila* non-pathogenic *Bacillus subtilis* (gift from Dr. E. Bremer, University of Marburg, Germany; A. Brun-Barale, pers. comm.) were grown at 30°C in the sporulation-specific medium PGSM (Bactopeptone® 7.5 g, KH_2PO_4 3.4 g, K_2HPO_4 4.35 g, glucose 7.5 g, PGSM salts 5 mL, CaCl2 0.25 M, distilled water qsp 1L, pH 7.2; PGSM salts: $MgSO_4.7H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$) for about 14 days for sporulation to occur. Following elimination of vegetative cells (1h at 70 °C), spore pellets were collected after centrifugation (4,500 rpm, 20 min, 4 °C), washed with sterile water, and lyophilized. CFU numbers were counted for each preparation as described above.

Fly stocks

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

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 µl and homogenized thoroughly with the fly medium (100µl/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 (Ø 3.3 cm, surface ~8.5 cm^2,
471 0.24 g/cm^2) 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 (Ø 4.6 cm, surface ~16 cm^2, 0.37
473 g/cm^2) except for *D. hydei*, *D. suzukii* and *D. immigrans* for which 30 eggs were transferred on 6 g

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

For the larval survival tests, 20 eggs or larvae from a 4-hour mass oviposition at the indicated developmental stage, were transferred to small dishes containing 1 g of fly medium (Ø 3 cm, surface ~7 cm^2) homogenized with increasing doses of Delfin® A. Surviving larvae were counted at the indicated developmental stage, or after 24-hour intoxication, to calculate the proportion of surviving larvae. For the test from the egg, eggs which did not hatch were not included in the counting. As a control measurement, we measured the pH of the fly medium in the presence of the dose range of *Bt* formulations (see Supplementary Information S4).

Adult fitness-related traits

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

Dialysis and Cry toxin analysis

A suspension of 2×10^{10} CFU of Delfin® A was dialyzed against PBS (KH_2PO_4 1.06 mM,

$Na_2HPO_4(2H_2O)$ 3mM, NaCl 154 mM, qsp distilled water, pH 7.2), at 250 rpm, 4°C overnight, using an 8-10 kDa MW cut-off membrane (ZelluTrans, Roth®). The CFUs of the dialyzed suspension and the effects on ER and DT were analysed as described above. The dialyzed suspension was also subject to a 12.5 % SDS-PAGE and compared to the non-dialyzed suspension after silver staining. The presence of Cry1A pro-toxins, activated toxins and toxin fragments was probed by Western-blot using an in-house anti-Cry1A rabbit polyclonal antibody.

Data analysis

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

526

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Author contributions

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

Additional information

Supplementary information

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

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 *Btk* Delfin® A, *Btk* 4D22, *Bti* Vectobac®, *Bacillus subtilis*			
Emergence rate			
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**
- *B. subtilis*	1.9	6	0.93
Developmental time			
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**
- *B. subtilis*	13.5	7	0.060
Development on *Btk* Delfin® B and Scutello DF (dose effect)			
Emergence rate			
- Delfin® B	151.2	2	**< 0.0001**
- Scutello DF	105.1	2	**< 0.0001**
Developmental time			
- 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			
Emergence rate	459.8	3	**< 0.0001**
Developmental time	13.7	2	**0.0011**
Survival of larval stages on Delfin® A			
Cumulative survival			
Dose × Larval instar	16.2	5	**0.0063**
Dose for each instar:			
- late 1st instar	87.4	5	**< 0.0001**
- late 2nd instar	25.7	5	**0.0001**
24-hour survival			
Dose × Larval instar	15.9	5	**0.007**
Dose for each instar:			
- late 1st instar	55.9	5	**< 0.0001**
- late 2nd instar	3.76	5	0.58
Adult fitness-related traits after development on Delfin® A			
Longevity			
Experiment	20.1	1	**< 0.0001**
- *1st 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**
Sexes analyzed separately			
- 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			
- *2nd experiment:*			
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
Sexes analyzed separately			
- 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)			
Total numbers of offspring			
Dose × Experiment	28.1	3	**< 0.0001**
Dose for each experiment:			
- 1st experiment	26.3	3	**< 0.0001**
- 2nd experiment	4.1	3	0.25
Development of other strains of *D. melanogaster* on Delfin® A (including Canton S)			
Emergence rate			
Dose × Fly strain	105.5	15	**< 0.0001**
Dose for each fly strain:			
- 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**
Developmental time			
Dose × Fly strain	9.3	12	0.68
Dose for each fly strain:			
- 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 *Drosophila* species on Delfin® A			
Emergence rate			
Dose × Fly species	538.2	30	**< 0.0001**
Dose for each species:			
- *D. simulans*	461.0	5	**< 0.0001**
- *D. yakuba*	750.7	5	**< 0.0001**
- *D. hydei*	596.8	5	**< 0.0001**
- *D. immigrans*	726.3	5	**< 0.0001**
- *D. subobscura*	729.6	5	**< 0.0001**
- *D. suzukii*	725.0	5	**< 0.0001**
- *D. busckii*	586.0	5	**< 0.0001**
Developmental time			
Dose × Fly species	59.9	22	**< 0.0001**
Dose for each species:			
- *D. simulans*	25.9	4	**< 0.0001**
- *D. yakuba*	34.7	4	**< 0.0001**
- *D. hydei*	11.5	4	**0.022**
- *D. immigrans*	6.01	3	0.11
- *D. subobscura*	68.8	4	**< 0.0001**
- *D. suzukii*	11.7	3	**0.0085**
- *D. busckii*	58.8	4	**< 0.0001**

765

766 **Figure legends**

767 **Figure 1. Development of *D. melanogaster* Canton S flies on *Btk* and *Bti* commercial**
768 **formulations.** (**a**) Emergence rate (mean ± sem) and (**b**) developmental time (mean ± 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 5.10^5 and 10^9, N = 4-9 for 10^6, N = 7-14 from 5.10^6 to 10^8.
773 (**c**) Emergence rate (mean ± sem) and (**d**) developmental time (mean ± 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: · $0.05<p<0.1$;
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 ± 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 ± 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 ± sem),

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

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

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

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

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

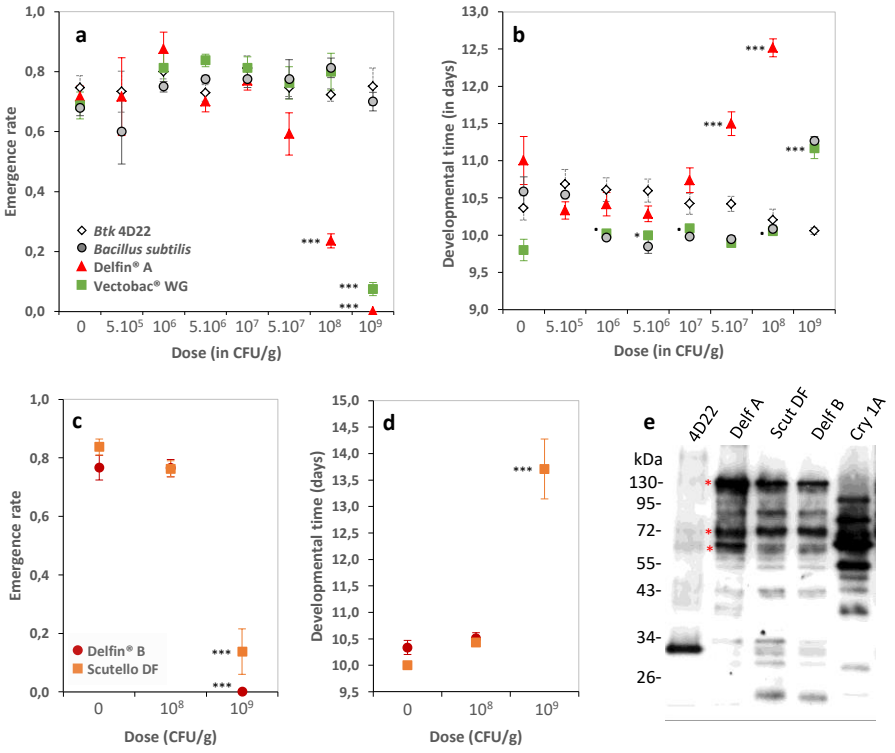

a
Emergence rate
Dose (in CFU/g)
Btk 4D22
Bacillus subtilis
Delfin® A
Vectobac® WG
b
Developmental time (in days)
Dose (in CFU/g)
c
Emergence rate
Dose (CFU/g)
Delfin® B
Scutello DF
d
Developmental time (days)
Dose (CFU/g)
e
4D22
Delf A
Scut DF
Delf B
Cry 1A
kDa
130-
95-
72-
55-
43-
34-
26-

Figure 2

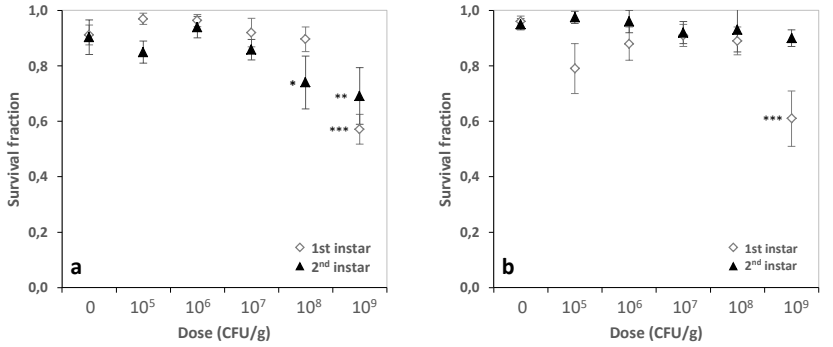

1,0
0,8
0,6
0,4
0,2
0,0
Survival fraction
a
1st instar
2nd instar
*
**
***
0
10^5
10^6
10^7
10^8
10^9
Dose (CFU/g)
b
1st instar
2nd instar
***
0
10^5
10^6
10^7
10^8
10^9
Dose (CFU/g)

Figure 3

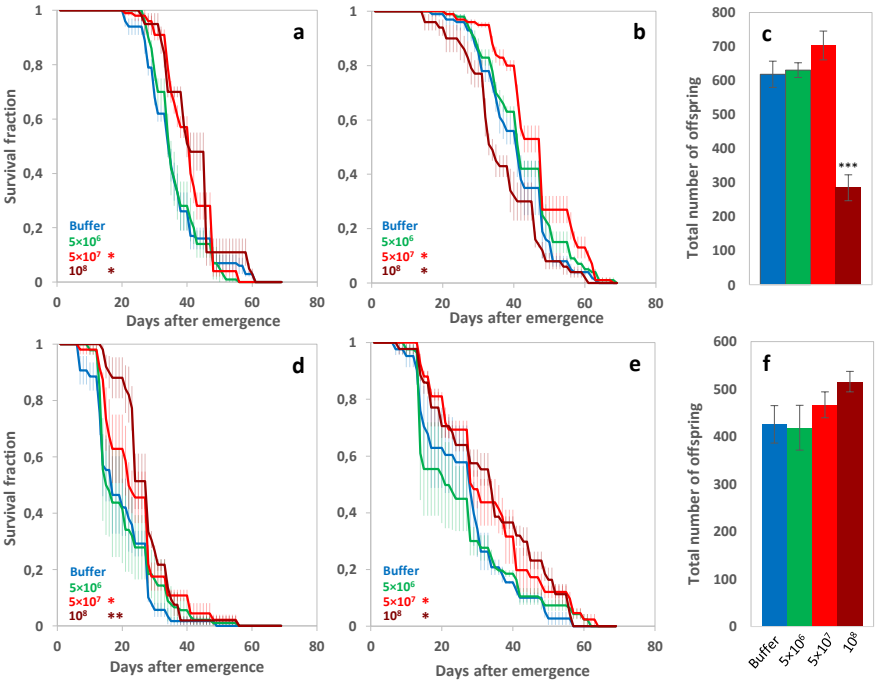

a
b
c
d
e
f
Survival fraction
Days after emergence
Total number of offspring
Buffer
5×10⁶
5×10⁷ *
10⁸ *
5×10⁷ *
10⁸ **
***
Buffer
5×10⁶
5×10⁷
10⁸

Figure 4

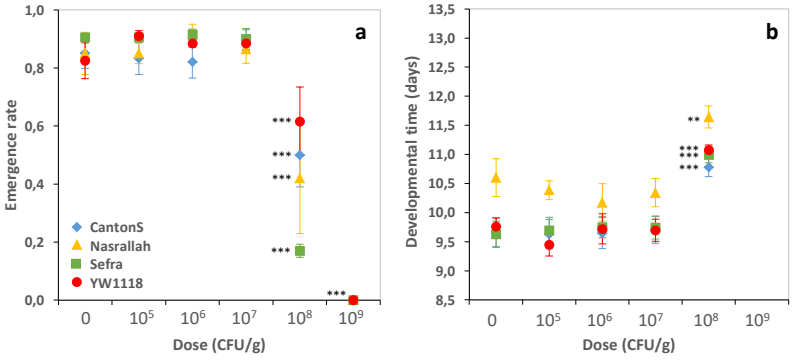

a
b
Emergence rate
Developmental time (days)
Dose (CFU/g)
Dose (CFU/g)
CantonS
Nasrallah
Sefra
YW1118
1,0
0,8
0,6
0,4
0,2
0,0
13,5
13,0
12,5
12,0
11,5
11,0
10,5
10,0
9,5
9,0
8,5
0
10^5
10^6
10^7
10^8
10^9
***
**

Figure 5

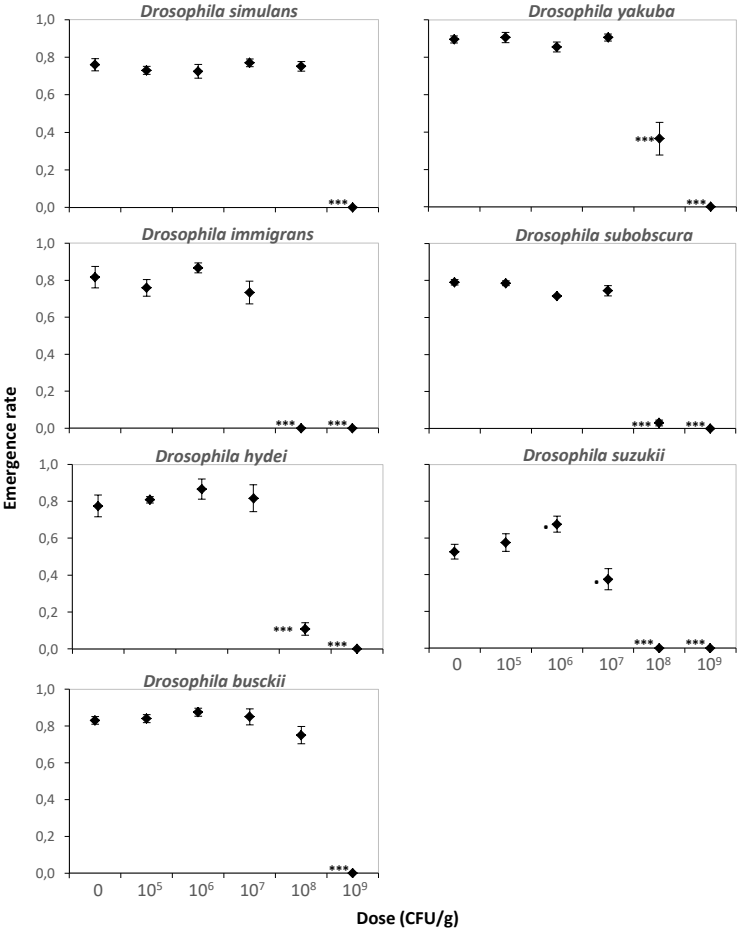

Drosophila simulans
Drosophila yakuba
Drosophila immigrans
Drosophila subobscura
Drosophila hydei
Drosophila suzukii
Drosophila busckii
Emergence rate
1,0
0,8
0,6
0,4
0,2
0,0
***
0
10^5
10^6
10^7
10^8
10^9
Dose (CFU/g)

Figure 6

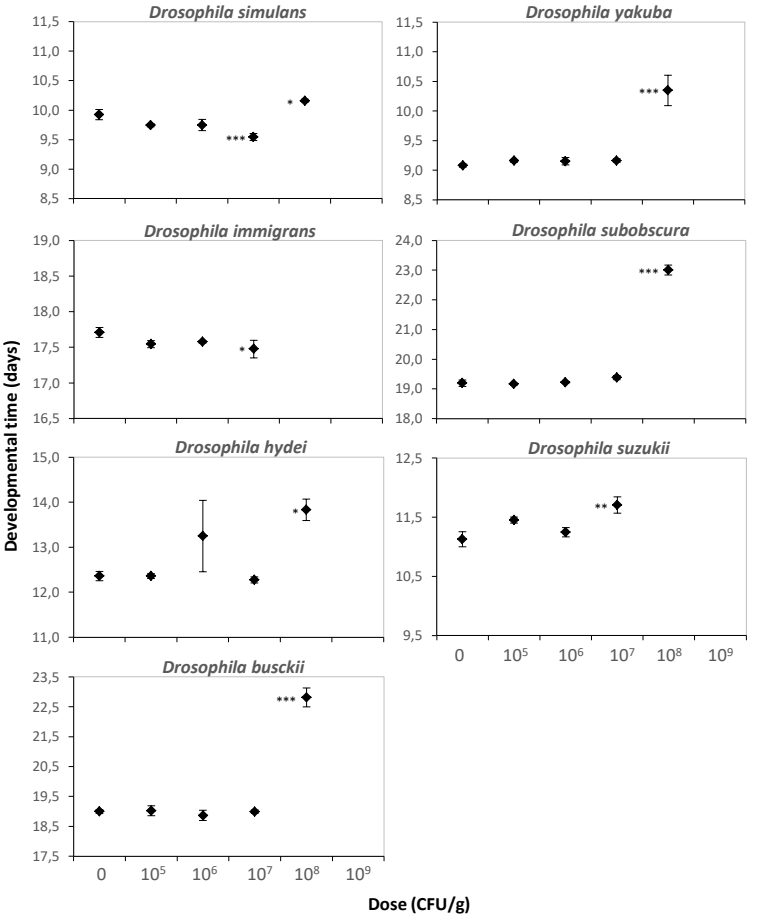
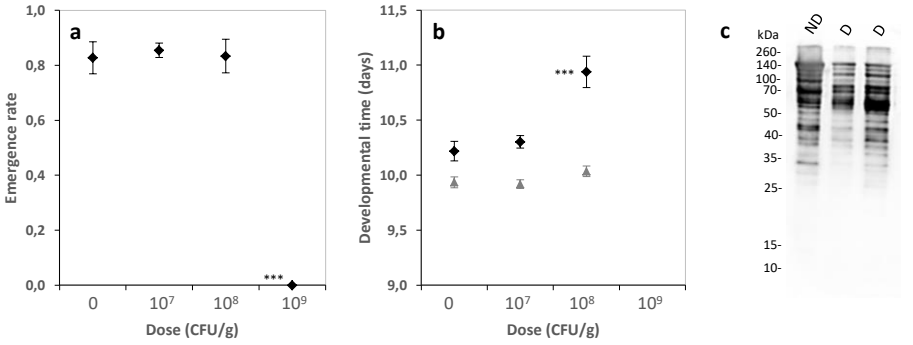

Drosophila simulans
Drosophila yakuba
Drosophila immigrans
Drosophila subobscura
Drosophila hydei
Drosophila suzukii
Drosophila busckii
Developmental time (days)
Dose (CFU/g)
0
10^5
10^6
10^7
10^8
10^9

Figure 7


a
Emergence rate
1,0
0,8
0,6
0,4
0,2
0,0
***
0
10^7
10^8
10^9
Dose (CFU/g)
b
Developmental time (days)
11,5
11,0
10,5
10,0
9,5
9,0
***
0
10^7
10^8
10^9
Dose (CFU/g)
c
ND
D
D
kDa
260-
140-
100-
70-
50-
40-
35-
25-
15-
10-