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1 ***Bacillus thuringiensis* bioinsecticide influences *Drosophila* oviposition**

2 **decision**

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16 **Abstract**

17 Behavioural avoidance has obvious benefits for animals facing environmental stressors such
18 as pathogen-contaminated foods. Most current bioinsecticides are based on the environmental
19 and opportunistic bacterium *Bacillus thuringiensis* (*Bt*) that kills targeted insect pests upon
20 ingestion. While food and oviposition avoidance of *Bt* bioinsecticide by targeted insect
21 species was reported, this remained to be addressed in non-target organisms, especially those
22 affected by chronic exposure to *Bt* bioinsecticide such as *Drosophila* species. Here, using a
23 two-choice oviposition test, we showed that female flies of three *Drosophila* species (four
24 strains of *D. melanogaster*, *D. busckii* and *D. suzukii*) avoided laying eggs in the presence of
25 *Bt* var. *kurstaki* bioinsecticide, with potential benefits for the offspring and female's fitness.
26 Avoidance occurred rapidly, regardless of the fraction of the bioinsecticide suspension (spores
27 and toxin crystals versus soluble toxins/components) and independently of the female
28 motivation for egg laying. Our results suggest that, in addition to recent findings of
29 developmental and physiological alterations upon chronic exposure of non-target *Drosophila*,
30 this bioinsecticide may have greater ecological implications in the field for the *Drosophila*
31 community and their associated natural enemies than previously thought.

32 1. Introduction

33 When exposed to environmental stressors, animals face two main options: dealing with the
34 stressor, which may ultimately lead to the evolution of special features, or physically avoiding
35 it. In interactions with opportunistic pathogens, broad-sense immunity includes components
36 for dealing with pathogens when interactions occur (physical barriers and cellular and
37 humoral effectors of the immune system) as well as a behavioural component to physically
38 avoid pathogens and reduce the infection risk.^[1-3] The immune response being costly (energy,
39 nutrients, and immunopathology resulting from damage to host tissues by effectors of its
40 innate immune response)^[2,4], obvious benefits come from physically avoid pathogens.

41 Behavioural avoidance of toxic compounds and microorganisms in a foraging context
42 is well documented. Both innate avoidance ('disgust') and learned avoidance based on
43 associative learning of hazardous food, are commonly expressed by vertebrates^[5] and
44 invertebrates, mainly insects.^[6-8] For instance, phytophagous insects avoid plants that
45 accumulate toxic alkaloids^[9] and the nematode *Caenorhabditis elegans* prefers feeding on
46 non-pathogenic bacteria rather than pathogenic ones^[10,11]. Exposed to opportunistic pathogens
47 through their diet of overripe fruits, *Drosophila melanogaster* females are able to learn to
48 adjust their preference for a food odour when that odour has previously been associated with a
49 gut infection by the virulent bacterium *Pseudomonas entomophila*,^[12] as do *C. elegans*
50 nematodes when exposed to pathogenic bacteria.^[13] *Drosophila melanogaster* males and
51 females also express strong innate aversive responses to bacterial lipopolysaccharides when
52 feeding and egg laying respectively, mediated by dTRPA1 cation channels of gustatory
53 neurons.^[14]

54 Naturally ubiquitous in the environment, *Bacillus thuringiensis* (*Bt*) is an opportunistic
55 Gram-positive bacterium, which synthesizes insecticidal toxins including Cry proteins as
56 crystals along with spores.^[15,16] The insecticidal action relies on the organisms' feeding

57 activity on *Bt*-contaminated food sources.^[17] In the context of the growing global food
58 demand and the need for safer and more specific insect pest control, these natural insecticidal
59 properties have led the development of *Bt*-based bioinsecticides (products made of viable *Bt*
60 spores and toxin crystals) or *Bt* transgenic crops to control insect pests in agriculture and
61 forestry (mainly Lepidoptera), and mosquitoes and black flies (Diptera).^[18,19] Many studies
62 concluded that *Bt* bioinsecticides and *Bt* crops are harmless or have limited impacts on the
63 non-target fauna.^[20,21] However, the partial targeting specificity of Cry toxins and the
64 potential for environmental accumulation of spores and toxins upon repeated treatments have
65 raised concern about potential side-effects on non-target organisms.^[16,22-25] In insects, recent
66 studies have reported deleterious effects of the Lepidoptera-targeting *Bt* var. *kurstaki* (*Btk*)
67 bioinsecticide on several species of non-target *Drosophila* flies likely present in *Btk*-treated
68 areas. Chronic exposure of fly larvae to subacute doses through the diet altered their growth,
69 development duration, survival, and complete development success.^[26-29] *Btk* bioinsecticide
70 also impacted the larval metabolism and midgut physiology, impairing protein digestion and
71 disturbing the gut epithelium organisation.^[28] One way for non-target insects that would
72 alleviate *Bt* bioinsecticide impacts is the expression of behavioural avoidance of *Bt*-treated
73 substrates. As *Bt* bioinsecticides act after ingestion, behavioural avoidance would be
74 advantageous upon food foraging, but also upon female oviposition with direct benefits for
75 the offspring and indirect benefits for the female's fitness.

76 So far, *Bt* behavioural avoidance has been investigated mainly in *Bt*-target
77 invertebrates: studies have reported no change in the oviposition behaviour of *Culex*
78 mosquitoes exposed to *Bt* var. *israelensis*^[30] or in the feeding behaviour of the Western corn
79 rootworm *Diabrotica virgifera virgifera*,^[31] and even an attractive effect of *Bt* maize on the
80 oviposition of the fall armyworm *Spodoptera frugiperda*.^[32] By contrast, behavioural
81 avoidance of *Bt* upon food foraging was reported in the nematode *Caenorhabditis elegans*^{[33-}

82 ^{36]} and in two Lepidopteran pests, the cotton bollworm *Helicoverpa armigera* and the cotton
83 leafworm *Spodoptera litura*.^[37] Females of *H. armigera* and of the diamondback moth
84 *Plutella xylostella* also avoid *Bt* when laying eggs in a choice situation.^[38,39] *Bt* avoidance was
85 also reported in insects' offspring: neonates of the European corn borer, *Ostrinia nubilalis*,
86 disperse more on *Bt* corn^[40] and avoid *Bt* when facing a choice with untreated diet,^[41] while
87 neonates of the tobacco budworm *Heliothis virescens* avoid diets containing Cry toxins or the
88 *Bt* bioinsecticide at doses that do not alter their development and survival.^[42]

89 By contrast, *Bt* behavioural avoidance has been scarcely addressed in non-target
90 invertebrates. Foraging activity and learning ability of *Apis mellifera ligustica* honey bees
91 remained unchanged on *Bt* corn,^[43] while collective nest building and prey attacks were
92 altered by cuticular *Bt* inoculation to the African social spider *Stegodyphus dumicola*.^[44]
93 Altered reproduction and survival were recorded in *Bombus terrestris* bumble bees exposed to
94 *Bt* depending on the *Bt* subspecies and the exposure route, but without altering the foraging
95 behaviour and colony performance.^[45] *Bt* bioinsecticides being increasingly applied in the
96 field, studies exploring the behavioural avoidance by non-target invertebrates are needed for
97 an accurate assessment of the potential bioinsecticide side-effects on non-target fauna.

98 Here, we explored the expression of behavioural avoidance toward the lepidopteran-
99 targeting *Bt* var. *kurstaki* (*Btk*) bioinsecticide by non-target *Drosophila* species that exhibit
100 developmental and physiological alterations in the chronic presence of bioinsecticide.^[27,28]
101 *Drosophila* larvae are particularly exposed to food-borne stressors as they intensively search
102 for food to fuel their exponential growth but have a low dispersal capacity. Bioinsecticide
103 avoidance by adult females when searching for oviposition sites would mitigate the
104 consequences on larval development. We focused on three *Drosophila* species with different
105 ecological features and varying developmental alterations elicited by chronic *Btk* exposure:
106 two cosmopolitan domestic species which frequently coexist on overripe fruits, *D.*

107 *melanogaster* (four strains) and the phylogenetically distant and opportunistic *D. busckii*,^[46-50]
108 and the invasive *D. suzukii* that feeds and lays eggs on ripe fruits and is a threat to
109 agriculture.^[51-54] We measured the females' oviposition preference in two-choice tests where
110 they were offered food with or without *Btk* bioinsecticide at a specific dose. The preference
111 dynamics during the choice test was recorded and the effect of different fractions of the *Btk*
112 bioinsecticide suspension (spores and toxin crystals, and soluble toxins/components) on the
113 fly preference was also assessed.

114

115 **2. Material and Methods**

116 *(a) Fly stocks*

117 Four *D. melanogaster* strains were tested: the wild-type Canton-S (Bloomington Drosophila
118 Center) used here as a reference strain, the wild-type "Nasrallah" from Tunisia (strain 1333,
119 Gif-sur-Yvette), a wild-type strain "Sefra" derived from flies collected in Southern France in
120 2013, and the yellow-white double mutant YW1118 (gift from Dr. B. Charroux, IBD,
121 Marseille-Luminy). Those strains and the two other *Drosophila* species tested, *D. busckii*
122 (derived from flies collected in South-East France in 2015) and *D. suzukii* (gift from Dr. R.
123 Allemand, LBBE, University of Lyon 1), were reared under controlled laboratory conditions
124 (150-200 eggs/40 ml fly medium; 25°C for *D. melanogaster* and 20°C for the two other fly
125 species; 60% relative humidity; 12:12 light/dark cycle) on a high-protein/sugar-free fly
126 medium (10% cornmeal, 10% yeast, 0% sugar). All the experiments were performed under
127 these laboratory conditions.

128

129 *(b) Bacillus thuringiensis bioinsecticide product*

130 Spores and Cry toxins of *Bt. var. kurstaki* strain SA-11 were from a commercial bioinsecticide
131 product (Delfin[®] wettable granules, Valent BioSciences, AMM 9200482, 32,000 UI/mg).

132 Viable spores were estimated at 5×10^7 CFU/mg product by counting Colony Forming Units
133 (CFUs) on LB agar, and this value remained stable during the timeframe of this study. For the
134 experiments, suspensions of *Btk* bioinsecticide were prepared in Ringer buffer (NaCl 7.5g/l,
135 NaHCO₃ 0.1g/l, KCl 0.2g/l, CaCl₂ 0.2g/l, in distilled water) to reach the desired CFUs in 100
136 μ l.

137

138 (c) Oviposition choice test

139 Two-to-five day-old mated females (20 *D. melanogaster*, 30 *D. suzukii*, 30 *D. busckii*) were
140 transferred to aerated plastic cages (\emptyset 10.5 cm, h 7.5 cm) containing two dishes (\emptyset 3 cm, \sim 7
141 cm², 1g of fly medium) diametrically opposed at the cage bottom. The test lasted 18 h for *D.*
142 *melanogaster* and 24 h for *D. suzukii* and *D. busckii* which lay fewer eggs. To avoid
143 confounding effects, cage orientation and location in the experimental chamber were
144 randomized.

145

146 (d) Oviposition in presence of *Btk* bioinsecticide

147 Flies were given the choice between a dish filled with fly medium mixed with a suspension of
148 *Btk* bioinsecticide in Ringer buffer at a given concentration, and a control dish filled with fly
149 medium mixed with the same volume of Ringer buffer (dose “0”). In control replicate cages,
150 females were offered the choice between two dishes filled with fly medium mixed with
151 Ringer buffer. Oviposition preference for *Btk* was calculated by dividing the number of eggs
152 laid on the *Btk* substrate divided by the sum of eggs on the two substrates of the cage.
153 Oviposition preference equal to 0.5 indicates no preference or avoidance of the bioinsecticide;
154 preference values above 0.5 indicate bioinsecticide appetitiveness, while values below 0.5
155 indicate bioinsecticide avoidance. Oviposition preference in control cages was the egg

156 proportion on one of the two Ringer substrates. For each cage, the fly motivation for egg
157 laying was assessed by summing the eggs laid on the two substrates.

158 Three *Btk* bioinsecticide doses previously described in ^[27] were used: 10^6 CFU/g fly
159 medium that has no effect on the *Drosophila* development and falls in the recommendation
160 range (equivalent to the field application of 1.4×10^5 CFU/cm²) and 10^8 CFU/g and 10^9 CFU/g
161 which strongly alters *Drosophila* larval development (equivalent to the application of 1.4×10^7
162 CFU/cm² and 1.4×10^8 CFU/cm², respectively). The dynamics of egg laying over the 18-h
163 choice test were explored with the *D. melanogaster* Canton-S strain by measuring the
164 oviposition preference at 2 h, 4 h, and 18 h (endpoint) of choice test. Oviposition preference
165 of *D. sukuzii* and *D. busckii* was measured with the choice over 24 h between a Ringer control
166 substrate and a substrate containing 10^9 CFU/g of *Btk* bioinsecticide.

167 To disentangle the effects on the oviposition preference of *Btk* spores, toxin crystals
168 and soluble toxins, from those of the commercial product additives, a 2×10^{10} CFU suspension
169 of the bioinsecticide product was dialyzed to remove low molecular weight compounds.^[27] A
170 fraction of the dialyzed suspension was centrifuged at 15,000 g, 15 min, 18°C to collect the
171 pellet containing mainly spores and toxin crystals, and the supernatant containing toxin
172 fragments and non-dialyzable compounds.^[27] The oviposition preference and motivation for
173 egg laying of *Drosophila melanogaster* Canton-S females was assessed during 18 h when
174 flies were offered the choice between a control Ringer substrate and a substrate containing the
175 non-dialyzed bioinsecticide, the dialyzed bioinsecticide, the centrifugation pellet (all adjusted
176 to 10^9 CFU/g), the supernatant, or the PBS buffer used for dialysis.

177

178 (e) Statistical analysis

179 Binomial data on oviposition preference were analysed with mixed-effects generalized linear
180 models that included, when appropriate, the *D. melanogaster* strain, the *Btk* treatment (Ringer

181 control, *Btk* bioinsecticide doses, dialysis and centrifugation fractions), the choice test
182 duration and their two-way interactions as fixed factors. The replicate cage was included as
183 random factor. Count data on egg-laying motivation were transformed into decimal logarithm
184 values and analysed with mixed-effect models including the same fixed and random effects as
185 described above (similar statistical results and biological conclusions were obtained with
186 untransformed data). Significance of fixed effects and interactions was tested by model
187 comparisons. Pairwise *post hoc* comparisons of each *Btk* dose with the no-*Btk* control and of
188 each fly strain with Canton-S were performed. The deviation of the oviposition preference
189 from a 50%-50% distribution of eggs on the two substrates was tested with t tests under the
190 H₀ hypothesis of a mean egg proportion of 0.5. The replicate number being relatively small,
191 Wilcoxon tests with the same H₀ hypothesis were performed and yielded similar biological
192 conclusions. Statistical analyses were performed in R^[55] using the packages lme4^[56] and
193 multcomp.^[57]

194

195 **3. Results**

196 *(a) Drosophila melanogaster expressed a rapid, dose-dependent oviposition avoidance of Btk*
197 *bioinsecticide*

198 The presence of *Btk* bioinsecticide impacted the oviposition preference of *D. melanogaster*
199 females over 18h compared to the controls without bioinsecticide, yet with varying
200 amplitudes between fly strains (Figure 1; Table S1.1). Canton-S females laid eggs evenly
201 when offered two control substrates, while they laid fewer eggs on *Btk* substrate when offered
202 a choice between substrates with and without *Btk* (Table S1.1; significance of *post hoc*
203 control-*Btk* dose pairwise comparisons in Figure 1). Their *Btk* avoidance increased with the
204 bioinsecticide dose, and deviated significantly from the “neutral” preference of 0.5 at the two
205 highest doses, 10⁸ and 10⁹ CFU/g (Table S1.1), dropping to 0.19 on average at 10⁹ CFU/g

206 (95% confidence interval: 0.07 – 0.30). The oviposition preference of Nasrallah females also
207 decreased with the increasing *Btk* dose (Figure 1; Table S1.1), dropping significantly below
208 0.5 only at 10^9 CFU/g with a smaller amplitude than that of Canton-S females (0.27 on
209 average, 95% CI: 0.12 – 0.41; Table S1.1). Similarly, the average preference of Sefra females
210 was 0.29 at this dose (95% CI: 0.21 – 0.37), while the dose 10^6 CFU/g was slightly appetitive
211 (Figure 1; Table S1.1). The oviposition preference of the YW double mutant also decreased
212 significantly below 0.5 at 10^9 CFU/g but with a smaller amplitude (average preference of
213 0.37, 95% CI: 0.24 – 0.50) (Figure 1; Table S1.1). For all the four *D. melanogaster* strains,
214 female motivation for egg laying in the presence of *Btk* bioinsecticide was similar to that
215 without bioinsecticide and was similar between *Btk* doses (Figure 2; Table S1.1).

216 Over the course of the 18-h choice test, the oviposition preference of the control
217 Canton-S females unexposed to *Btk* bioinsecticide did not differ from the “neutral” preference
218 0.5 despite random variation between time points. In contrast, when offered the choice
219 between a *Btk* substrate at 10^9 CFU/g and a control substrate, the female preference for *Btk*
220 was already below 0.5 at 2 h and further decreased at 4 h to remain down to ~0.2 until the end
221 of the choice test (Figure 3A; Table S1.2). The motivation of Canton-S females for egg laying
222 evolved similarly and regardless of the choice they were offered (Figure 3B; Table S1.2).

223

224 (b) All the *Btk* bioinsecticide fractions elicited the fly oviposition avoidance

225 While the preference after 18h of Canton-S females for both Ringer and PBS controls did not
226 differ from 0.5 (Figure 4A, Table S2), females significantly avoided the dialyzed *Btk*
227 suspension, the suspended pellet and the supernatant with a similar amplitude as the non-
228 dialyzed *Btk* bioinsecticide at 10^9 CFU/g (average preference of 0.30, 95% CI: 0.21 – 0.39;
229 Figure 4A, Table S2). The female motivation for egg laying was similar across choice
230 modalities (Figure 4B, Table S2).

231

232 (c) *The amplitude of fly avoidance of Btk bioinsecticide varied between species*

233 Females of the invasive species *D. suzukii* strongly avoided *Btk* in the choice test: their

234 oviposition preference dropped to 0.16 on average at 10^9 CFU/g of *Btk* (95% CI: 0.11 – 0.21;

235 Figure 5A, Table S3), the results being similar when including only cages with >15 eggs

236 (Figure S4). *Drosophila busckii* females' preference also dropped significantly to 0.38 on

237 average in presence of 10^9 CFU/g *Btk* (95% CI: 0.28 – 0.49; Figure 5C, Table S5). The female

238 motivation for egg laying of the two species was independent of the choice they were offered

239 (Figures 5B, D; Figure S4; Tables S3, S5).

240

241 **4. Discussion**

242 When offered the choice between laying eggs on uncontaminated substrates or on *Btk*

243 contaminated substrates, females of four strains of *Drosophila melanogaster* and of *D. busckii*

244 and *D. suzukii* expressed avoidance of the *Btk* bioinsecticide. These oviposition responses

245 were independent of confounding differences in the female motivation for egg laying. Since

246 only non-ageing mated females were used during a short experimental period, this excludes

247 the potential confounding effects of the female mating status and disturbance by male

248 courtship, of sensory ageing impairing the ability to discriminate between food substrates, and

249 of general ageing influencing the number of eggs laid.

250 *Drosophila melanogaster* strains avoided the bioinsecticide in a dose-dependent

251 manner, and the three wild-type strains (Canton-S, Nasrallah, and Sefra) showed a stronger

252 avoidance of the highest bioinsecticide dose than the YW double mutant strain. The smaller

253 avoidance amplitude by YW females coincides with the fact that the yellow and white

254 mutations affect the flies' non-social and social behaviour and their ability to learn with

255 olfactory cues.^[58-61] The invasive Asian species, *Drosophila suzukii*, exhibited a strong

256 avoidance as the wild-type *D. melanogaster* Canton-S, although this species underwent an
257 evolutionary shift in the bitter taste perception and oviposition preferences in the presence of
258 microorganisms compared to other frugivorous *Drosophila* species.^[62,63] The third tested
259 species, the opportunistic frugivorous *Drosophila busckii*, from the subgenus *Dorsilopha*,
260 belongs to the *Drosophila* cosmopolitan guild of domestic species along with *D.*
261 *melanogaster* and is specialized on vegetables.^[46] This species was the least avoidant,
262 indicating that, although the bioinsecticide avoidance was general to our study, there was
263 inter-species variability within the cosmopolitan guild.

264 *Drosophila melanogaster* behavioural avoidance of *Btk* bioinsecticide was detectable
265 as early as 2 hours after the choice test onset, with increasing amplitude in the following few
266 hours. The time scale of our results is consistent with previous reports of rapid learned
267 avoidance towards pathogenic bacteria previously observed in *D. melanogaster*^[12] and
268 *Caenorhabditis elegans*.^[13] The avoidance of the bioinsecticide may have started even earlier
269 during the choice test, yet counting laid eggs does not allow a fine time resolution since a
270 robust result requires substantial numbers of laid eggs. A video tracking of the fly positions in
271 the cage might indicate whether females innately avoided *Btk* bioinsecticide (*i.e.* from the test
272 onset) or were initially attracted to it and shifted their preference during the test, although
273 *Drosophila* positional and oviposition preferences do not necessarily match.^[64] Nevertheless,
274 our study showed that the expression of *Btk* bioinsecticide avoidance is rapid on a fly's
275 lifetime scale. The female decision-making for oviposition is a highly complex and dynamic
276 trait that combines several parameters: the female's genotype and experience of the
277 oviposition substrates,^[12,65-67] the presence at oviposition sites of the male-derived aggregation
278 pheromone transmitted to females during mating and emitted by recently mated females and
279 of the deterring host marking pheromone,^[68-70] the social transmission of oviposition substrate
280 preferences between females^[71-73] and of other information linked to substrate quality

281 (presence of larvae and faeces),^[70,74,75] the presence of specific commensal
282 microorganisms,^[63,76] the amplification of pheromone aggregation signal in infected flies by
283 pathogenic bacteria^[77] and the group size.^[78] In addition, the texture of the oviposition
284 substrate also plays an important role in the female oviposition decisions.^[63,79] In our study
285 system, the bioinsecticide or Ringer buffer addition to the fly medium changed similarly the
286 texture of the food substrate and did not change its pH.^[27]

287 Behavioural avoidance was observed consistently for all the strains and species at the
288 highest tested dose, 10^9 CFU/g of *Btk* bioinsecticide, and at 10^8 CFU/g for *D. melanogaster*
289 Canton-S and Nasrallah. This mirrors the recent report of development alterations upon
290 chronic exposure to these doses of *Btk* bioinsecticides, and the smaller bioinsecticide impacts
291 on the emergence rates of *D. melanogaster* YW and *D. busckii* compared to other *D.*
292 *melanogaster* strains and *D. suzukii*.^[27] While the dose 10^9 CFU/g, which is 1,000 times
293 above the manufacturer's recommendations, seems unrealistic in the field, the dose 10^8
294 CFU/g (equivalent to a field application of 1.4×10^7 CFU/cm²)^[27] is reachable under current
295 agriculture and horticulture practices where repeated applications are usually recommended
296 (up to 8 authorized applications^[80,81] www.certiseurope.fr; www.certisusa.com). Indeed, *Bt*
297 spores and toxins naturally persist and could accumulate in the field^[16,23,24,82] and
298 bioinsecticide products contain protective compounds to lengthen their activity after field
299 application.^[80,83] Very recently, doses close to 10^8 CFU/g have been measured in honey bee
300 matrices and flowers after the field application of the maximum recommended *Bt*
301 bioinsecticide dose and concentrations up to 10^7 CFU/g still persisted two days after
302 application.^[84]

303 Expression of behavioural avoidance toward *Btk* bioinsecticide was observed with the
304 regular suspension, as well as with the dialyzed suspension and each of its fractions
305 independently. This excludes a role in the avoidance of small molecular weight compounds of

306 the formulation,^[85] and suggests that of spores, toxins, or residual bacterial fragments. Since
307 *Bt* spores persist longer in the field than toxins,^[16,23,24] our results might indicate that the
308 presence of spores in the environment may be sufficient to mediate bioinsecticide avoidance
309 expression by non-target *Drosophila* females. Moreover, it was demonstrated that the
310 nematode *C. elegans*^[86] and *D. melanogaster* males and females^[14] exhibit bacteria avoidance
311 based on the presence of bacterial cell wall components. Here, the fractions of the
312 bioinsecticide suspension after dialysis may contain cell wall components of the vegetative
313 bacteria from the bioinsecticide manufacturing, which presence and role in the oviposition
314 avoidance remain to be evaluated. When foraging, larvae and adult *Drosophila* naturally
315 avoid specific harmful compounds or nutritionally unsuitable food based on the sensory
316 perception of olfactory cues,^[87-89] gustatory cues,^[62,90-92] or the physiological consequences of
317 ingesting virulent bacteria.^[12] In our case, it seems unlikely that female avoidance of *Btk*
318 bioinsecticides for oviposition relies only on olfactory cues, as this would likely result in
319 stronger oviposition avoidance early during the test. The involvement of gustatory cues when
320 assessing the oviposition site (e.g., bitter taste) and/or on physiological consequences of
321 ingesting *Btk* bioinsecticide remains to be assessed.

322 From the point of view of females' offspring, the oviposition avoidance of *Btk*
323 bioinsecticide alleviates the cost of developing under chronic bioinsecticide exposure. Indeed,
324 the growth and gut physiology of *D. melanogaster* larvae is already dramatically disturbed at
325 5×10^7 CFU/g of *Btk* bioinsecticide.^[28] In addition, emergence rates of *D. melanogaster* strains
326 developing on 10^8 CFU/g of *Btk* bioinsecticide dropped by up to 81% compared to unexposed
327 controls and groups exposed to only 10^6 CFU/g.^[27] The development success was even null at
328 the highest tested dose of *Btk* bioinsecticide, 10^9 CFU/g.^[27] Avoidance of *Btk* bioinsecticide
329 by females while searching for oviposition sites would thus increase their inclusive fitness,
330 since more of their progeny would have a chance to develop and reach the adult stage and

331 reproduce. Given that *Drosophila* females both feed and lay eggs on food substrates, the
332 avoidance of *Btk* contaminated oviposition sites would also reduce the adult fly exposure to
333 bioinsecticide, although adults do not seem to be impacted.^[27]

334 From an ecological point of view, variations in avoidance amplitude between *D.*
335 *melanogaster* genotypes and *Drosophila* species may modify their competitive interactions in
336 *Btk*-treated areas. Interestingly, variations in avoidance strength have already been observed
337 for carbon dioxide and other odorants indicating the stage of the fruit ripeness. These
338 observations reflect the biological differences in feeding, mating, and oviposition between
339 *Drosophila* species specialized on overripe fruits (several genotypes of *D. melanogaster*, *D.*
340 *yakuba*, *D. pseudobscura*, *D. virilis*) and *D. suzukii* specialized on ripening fruits.^[87,88] In our
341 study, the smaller amplitude of *Btk* bioinsecticide avoidance of the opportunistic *D. busckii*,
342 combined with its lower developmental susceptibility to chronic bioinsecticide exposure^[27]
343 suggest that *Btk* applications might not dramatically affect the field presence of this species in
344 the *Drosophila* community. By contrast, the high susceptibility of *D. suzukii* to developmental
345 alterations upon chronic exposure to bioinsecticide^[26,27] combined with the females'
346 amplitude of oviposition avoidance, suggest that developmental alterations could be alleviated
347 by avoidance of *Btk*-treated areas. Despite the fact that *D. melanogaster* and *D. suzukii* have
348 different niche specializations, their potential indirect interactions would be displaced mostly
349 to untreated areas since both species avoid strongly the bioinsecticide for egg laying. The
350 population dynamics of their natural enemies (predators and parasites) would be impacted by
351 the changes in the location of their prey/host populations, in addition to be impacted directly
352 by the bioinsecticide as previously reported for two species of *D. melanogaster* parasitoids.^[93]
353 Indirectly, our results further suggest that *Btk* bioinsecticide application may be useful as a
354 repellent to *D. suzukii* in orchards and gardening, but it may not be an efficient tool to control
355 populations of this invasive fly and comes with side-effects on other non-target species.

356 In summary, females of several *Drosophila* species and genotypes expressed
357 oviposition avoidance of food substrates contaminated with *Btk* bioinsecticide. The avoidance
358 appeared rapidly after the onset of choice tests, for all the fractions of the bioinsecticide
359 suspension and was independent of female motivation for egg laying. Our study extends the
360 assessment of *Btk* bioinsecticide chronic effects previously reported in multiple *Drosophila*
361 species to behavioural aspects, and highlights the need for multi-component assessments
362 (development, physiology, life history, behaviour) of the potential effects of bioinsecticides
363 on non-target invertebrates.

364

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369

370 **Author contributions**

371 AB: conceptualization, data curation, formal analysis, investigation, methodology, writing-
372 original draft, writing-review and editing; JLG: conceptualization, writing-review and editing;
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374

375 **Competing interests**

376 We declare no competing interests.

377

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385

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657

658 **Figure legends**

659 **Figure 1.** Female oviposition preference in the 18-hour choice test as the proportion of eggs
660 laid on one food substrate (quartiles, median and mean preference in red points) of
661 *Drosophila melanogaster* wild-type strains Canton-S, Nasrallah and Sefra, and the YW
662 double mutant strain, with three doses of *Btk* bioinsecticide (10^6 , 10^8 , and 10^9 CFU/g of fly
663 medium) and the no-*Btk* Ringer control (0). Significance of *post hoc* pairwise comparisons of
664 the control with each *Btk* dose: *** $P < 0.0001$. $N = 10$ replicate cages per treatment for each
665 fly strain.

666

667 **Figure 2.** Fly motivation for egg laying during the 18-hour oviposition choice test as the total
668 number of eggs laid on both food substrates offered (quartiles, median and mean of the total
669 number of eggs in red points) of *Drosophila melanogaster* wild-type strains Canton-S,
670 Nasrallah and Sefra, and the YW double mutant strain, with 3 doses of *Btk* bioinsecticide
671 (10^6 , 10^8 , and 10^9 CFU/g of fly medium) and the no-*Btk* Ringer control (0). $N = 10$ replicates
672 cages per treatment for each fly strain.

673

674 **Figure 3.** Dynamics of *Drosophila melanogaster* Canton-S female (A) oviposition preference
675 as the proportion of eggs laid on one food substrate, and (B) motivation for egg laying as the
676 total number of eggs laid on both food substrates (quartiles, median and mean per treatment in
677 red points) recorded at 2h, 4h, and 18h in the oviposition choice test with 10^9 CFU/g of *Btk*
678 bioinsecticide and the no-*Btk* Ringer control (0). Significance of *post hoc* pairwise
679 comparisons of the control with the *Btk* bioinsecticide: * $P < 0.05$, ** $P < 0.01$ *** $P <$
680 0.0001 . $N = 15$ replicate cages per treatment and test duration.

681

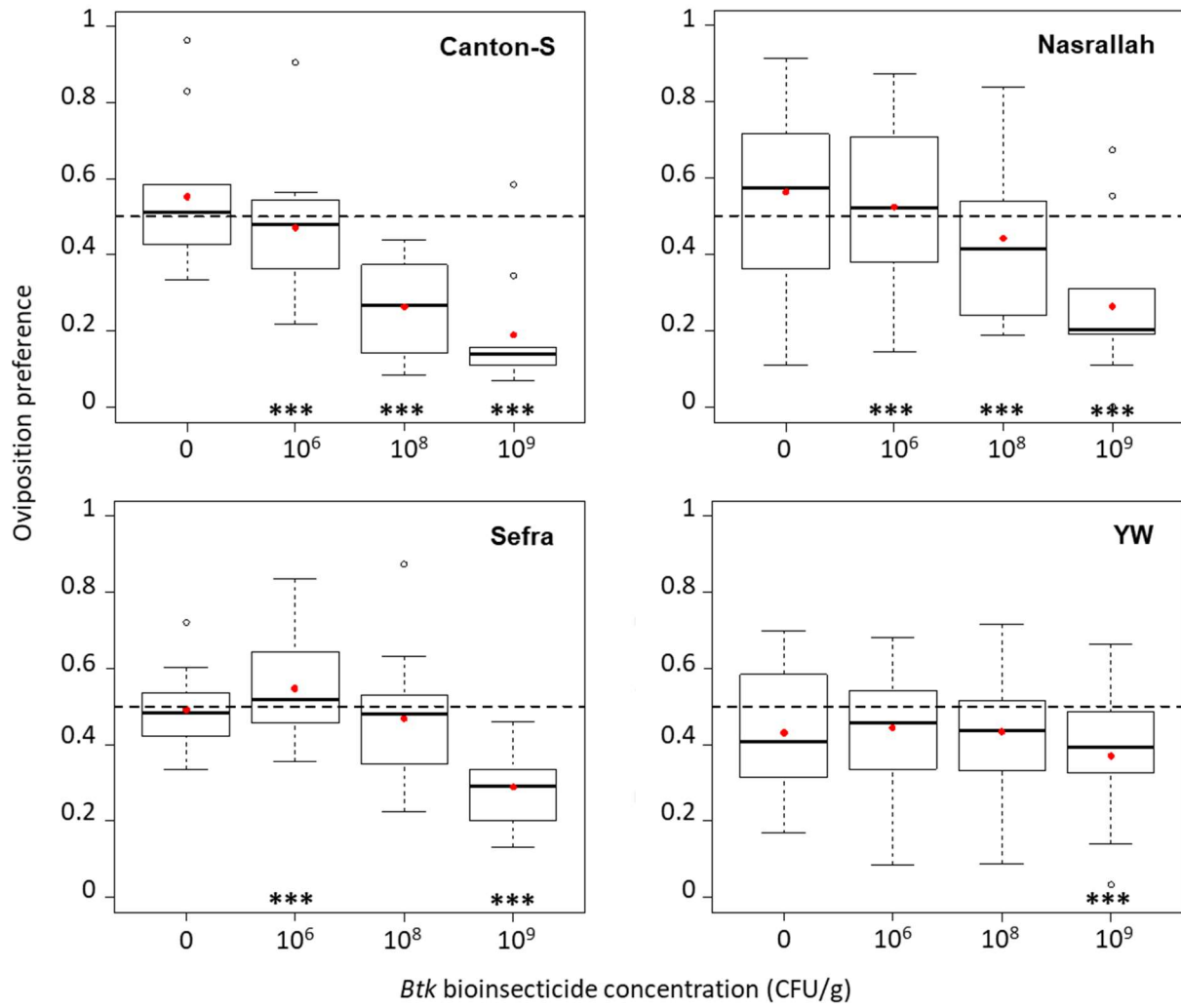
682 **Figure 4.** *Drosophila melanogaster* Canton-S female (A) oviposition preference as the
683 proportion of eggs laid on one food substrate, and (B) motivation for egg laying as the total
684 number of eggs laid on both food substrates (quartiles, median and mean per treatment in red
685 points) in the 18-hour oviposition choice test with *Btk* bioinsecticide at 10^9 CFU/g of fly
686 medium (*Btk*-ND), dialyzed *Btk* bioinsecticide (*Btk*-D) and the pellet (Pellet) adjusted to the
687 same concentration, the supernatant (Supernat.) after centrifugation, and the Ringer and PBS
688 controls. Significance of *post hoc* pairwise comparisons of the Ringer control with each of the
689 other treatment modalities: *** $P < 0.001$. $N = 15$ replicates cages per treatment.

690

691 **Figure 5.** *Drosophila suzukii* (A) and *Drosophila busckii* (C) female oviposition preference as
692 the proportion of eggs laid on one food substrate, and their respective motivation for egg
693 laying (B, D) as the total number of eggs laid on both food substrates during the 24-hour
694 oviposition choice test with *Btk* bioinsecticide at 10^9 CFU/g and the no-*Btk* Ringer control (0)
695 (quartiles, median and mean per treatment in red points). Significance of *post hoc* pairwise
696 comparisons of the control with the *Btk* bioinsecticide: * $P < 0.05$ and *** $P < 0.0001$. $N = 25$
697 replicate cages per treatment for *D. suzukii* (all cages) and $N = 15$ replicate cages for *D.*
698 *busckii*.

699

700 **Figure 1.**

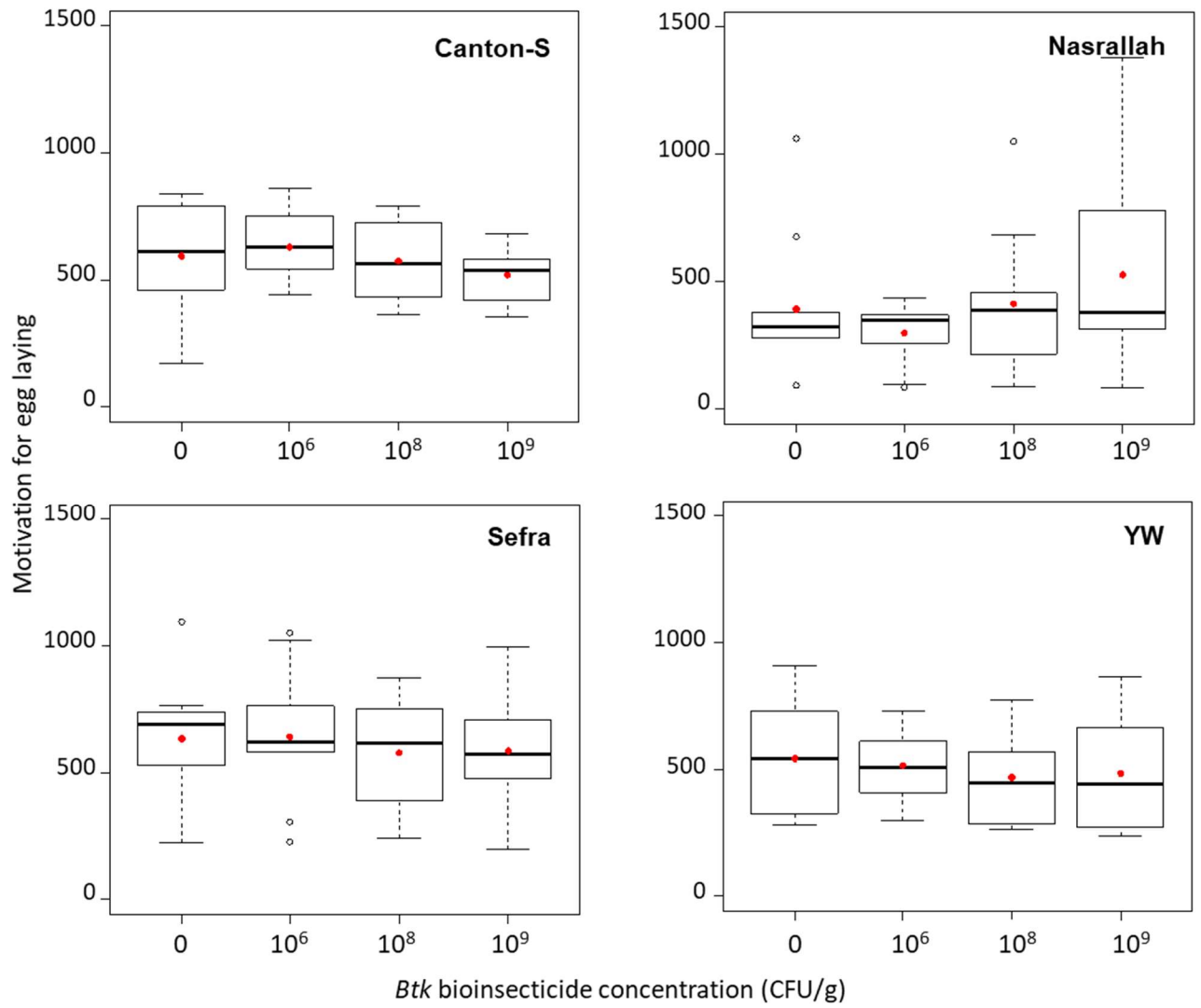


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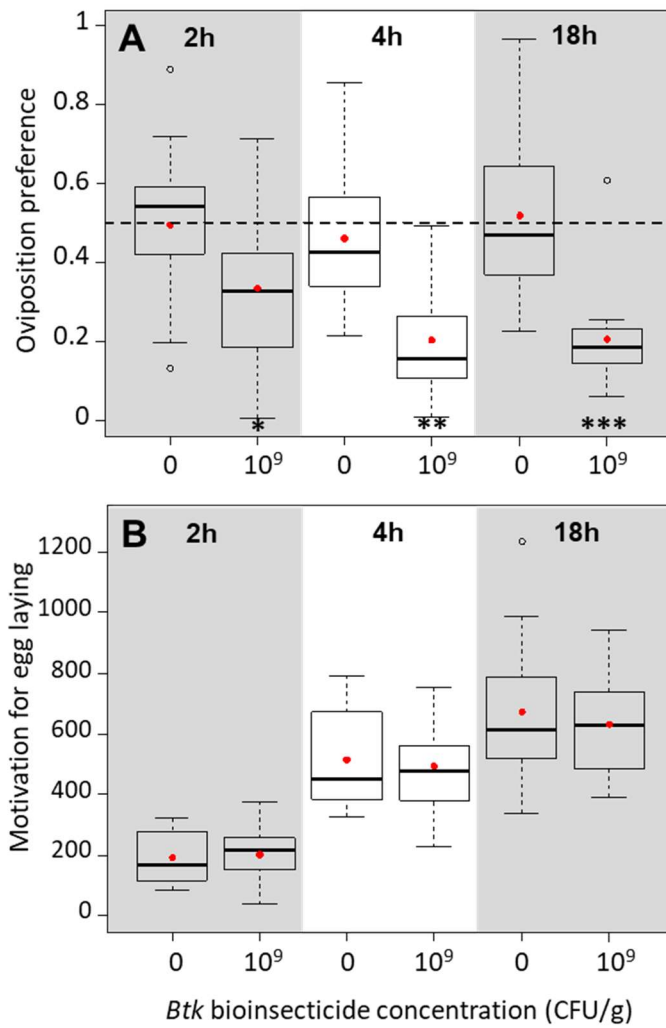
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704 **Figure 2.**



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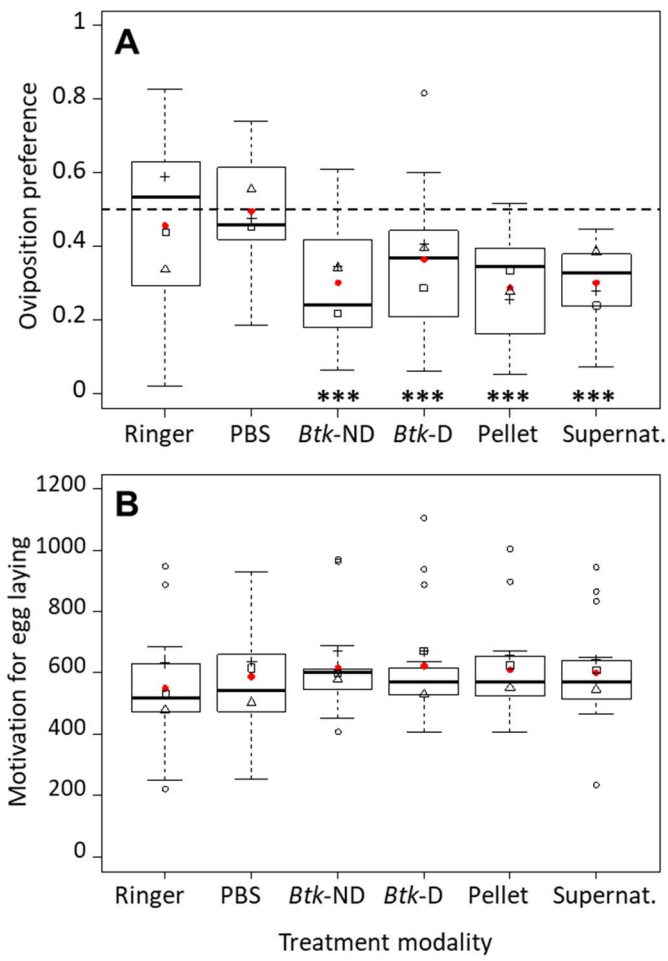
706 **Figure 3.**



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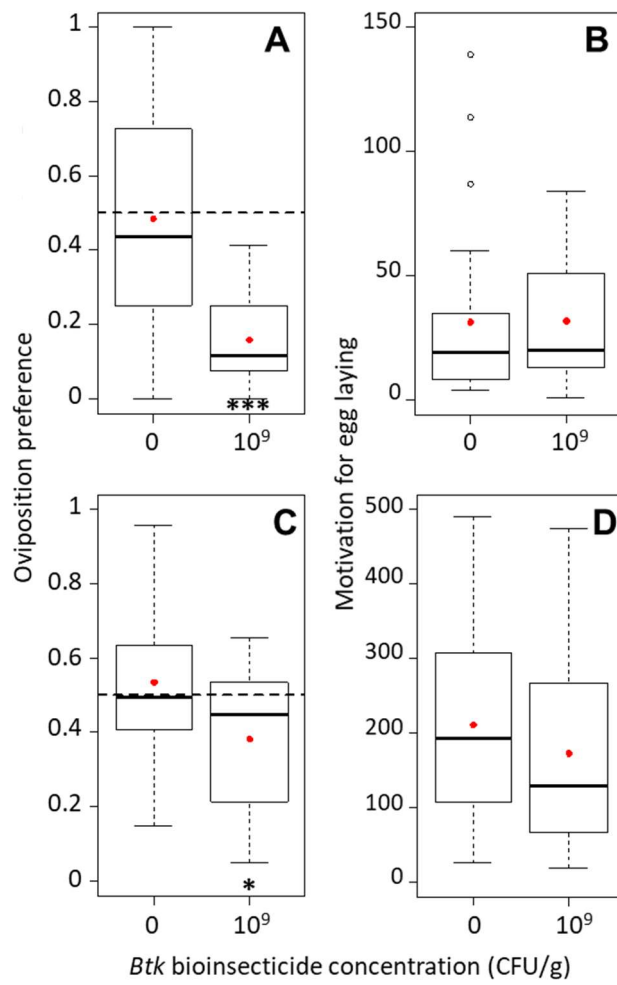
709 **Figure 4.**



710

711

712 **Figure 5.**



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714