Entomologia Generalis, Vol. XX (2022), Issue X, XXX–XXX
Published online February 2022

PrePub-Article

Effects of *Bacillus thuringiensis kurstaki* bioinsecticide on two non-target *Drosophila* larval endoparasitoid wasps

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With 3 figures

Abstract: *Bacillus thuringiensis kurstaki* (*Btk*) is among the most used microbial agent worldwide to control lepidopteran pests in organic and non-organic crops. The extensive use of this bioinsecticide and its environmental accumulation may become a major issue for integrated pest management (IPM) programs that include *Btk* and non-target species (parasitoids and predators) if side effects on these organisms and their associated food-webs occur. Here, chronic effects of *Btk* bioinsecticide were explored in two *Drosophila* larval endoparasitoids, *Leptopilina heterotoma* and *L. boulardi* (Hymenoptera: Figitidae), indirectly on their development in the exposed host and directly on their adult longevity. Emergence rates of *D. melanogaster* "Canton-S" and "Sefra" host strains fed *Btk* bioinsecticide throughout larval development decreased at the highest concentration (10 times the recommendations), and no significant effect was observed on the parasitisation behaviour of female parasitoids toward *Btk*-fed larval hosts. Parasitoids' developmental time in *Btk*-exposed hosts remained unchanged while emergence rate decreased as a function of bioinsecticide concentration, host fly strain and parasitoid species. In adult parasitoids *Btk*-fed, the longevity of *L. heterotoma* males and *L. boulardi* females were reduced even at recommended spraying concentrations. Our study reports that the *Btk* bioinsecticide may affect non-target parasitoids through chronic indirect host exposure and directly through food poisoning. In this context, since closely related parasitoid species may respond differently to bioinsecticide exposure, species-specific studies must be conducted when IPM strategies combine these natural enemies with the *Btk* bioinsecticide.

Keywords: endoparasitoid wasps, *Leptopilina*, Hymenoptera, Figitidae, *Bacillus thuringiensis*, bioinsecticides, non-intentional effects, *Drosophila*

1 Introduction

The increasing global food demand requires the control of insect pests that cause significant agricultural losses, through novel and effective pest management solutions (Pimentel & Burgess 2014). The effectiveness of chemical pesticides has been reduced by the emergence of pest resistance, and their extensive use raises concern about their side effects on the environment, the biodiversity and human health (Cocco 2016, Kim et al. 2017). Hence, there is a need for environmentally sound pest management alternatives as main components of Integrated Pest Management (IPM) programs.

Biopesticides have been developed as a more specific and safer alternative to chemicals. Most bioinsecticides against insect pests are microbial formulations of viable spores and toxins of the Gram-positive bacterium *Bacillus thuringiensis* (*Bt*) (Sanchis & Bourguet 2008, Lacey et al. 2015). During sporulation, *Bt* synthesizes spores and toxins as parasporal crystalline inclusions including the most abundant and best-studied Cry δ-endotoxins (Adang et al. 2014, Crickmore 2017). Specific cocktails of Cry toxins produced by *Bt* subspecies delineate potential targets, primarily insects but also protozoa and nematodes (Palma et al. 2014; Mendoza et al. 2020). *Bt kurstaki* (*Btk*) is among the most commercialized *Bt* subspecies that produces Cry1 and Cry2, effective against Lepidoptera and black flies (Diptera: Simuliidae) (Ben-Dov et al. 1997). After spraying, *Bt* spores and toxin crystals are ingested by insect larvae. Toxin crystals are dissolved in the larval alkaline midgut, releasing protoxins that are cleaved by gut proteases into active toxin fragments that bind to specific epithelial cell receptors (Bravo et al. 2007, Heckel 2020). Intra-membrane assembly of these fragments forms pores, eliciting cell lysis and epithelium disorganization (Bravo et al. 2011). This can entail the entry of gut bacteria into the hemocoel, including *Bt*, and the insect death by septicaemia within days (Obata et al. 2009, Caccia et al. 2016).

DOI: 10.1127/entomologia/2022/1452
www.schweizerbart.de
0171-8177/2022/1452 $ 4.50

Many studies concluded that *Bt* bioinsecticides are safe or have limited impacts on non-target organisms and associated communities (Glare & O'Callaghan 2000). However, concern has recently raised about their potential unintended impacts when low and sub-acute concentrations are permanently present in the environment (Desneux et al. 2007). Growing evidence indicate a partially specific targeting with effects of *Bt* formulations and toxins across orders and even phyla (Venter & Bøhn, 2016, van Frankenhuyzen 2017). Concern has also raised about the potential environmental accumulation of *Bt* spores and active toxins due to the spore and toxin persistence, linked to protective compounds in the formulations, and the repeated sprayings required for the desired level of pest control (Duchet et al. 2014, Hung et al. 2016, Enger et al. 2018). All this points to a possible environmental accumulation of *Bt* bioinsecticides, which could result in chronic exposure (*i.e.,* extended and increasing exposure) of non-target natural enemies of crop pests when simultaneously involved in IPM programs.

Parasitoids and predators of crop insect pests are crucial biological control agents in IPM programs (Pennacchio & Strand 2006, van Lenteren 2007). So far, the study of possible unintended effects of pure Cry toxins or *Bt* Cry-expressing transgenic plants on the hosts or preys of these beneficial arthropods has not provided a clear consensus on their toxicity or innocuity. The reported effects on parasitoid developmental traits, adult fitness-related traits, and parasitisation behaviour, ranged from none to strong negative effects (rarely positive effects), with large differences even between species from the same genus (Weseloh et al. 1983, Schuler et al. 1999, Glare & O'Callaghan 2000, Chen et al. 2008, Momanyi et al. 2012, Tanaka & Minakucki 2012, Amichot et al. 2016, Stemele 2016, De Bortoli et al. 2017). Although it would provide a comprehensive overview of potential long-term consequences, a thorough investigation of the potential effects of exposure to *Btk* bioinsecticides on most parasitoid species remains lacking. So far, these effects have only been tested on the adults of a few parasitoid species by exposing them to recommended or acute toxic concentrations. The effects of a chronic exposure during parasitoid development over longer periods at concentrations just above recommended ones have remained poorly explored so far.

Here, the potential effects of a chronic exposure to a commercial formulation of *Btk* bioinsecticide on endoparasitoids (*i.e.*, parasitoids that develop inside other insects, causing their death) were explored on the parasitoid development via the host contamination, and on the adult stage. The focus is on two well-studied species of *Drosophila* larval endoparasitoids, *Leptopilina boulardi* (Hymenoptera: Figitidae, Barbotin et al. 1979) and *L. heterotoma* (Hymenoptera: Figitidae; Carton et al. 1986) and the *Drosophila melanogaster* host (Diptera: Drosophilidae; Meigen, 1830). These are closely-related parasitoids that are sympatric over most of their geographic distributions. Yet they differ in their competitive ability and the width of their host range: *L. heterotoma* is known as less competitive and a generalist parasitoid (successfully developing in multiple *Drosophila* species and a few non-*Drosophila* hosts), while *L. boulardi* is a specialist parasitoid that mainly exploit *D. melanogaster* and a few related species (Allemand et al. 2002, Fleury et al. 2009). Females of both species inject venom when parasitizing, disturbing the host's cellular immune defences, but their parasitic success relies on two strategies: *L. heterotoma* maximizes the host attack number, while *L. boulardi* optimizes this number and develops faster (Schlenke et al. 2007, Lee et al. 2009). The two parasitoids have ecological and agronomic importance in controlling *Drosophila* swarms that can damage some crops (Barata et al. 2012, Ramirez-Camejo et al. 2017, Rombaut et al. 2017), possibly including the fruit pest *D. suzukii* (Matsumura, 1931) (Asplen et al. 2015). Although the two parasitoids are not capable of using *D. suzukii* to reproduce (Stacconi et al. 2015, Iacovone et al. 2018), many *D. suzukii* larvae die after being parasitized (Iacovone et al. 2018, Kruitwagen et al. 2021).

Btk is not expected to target the *Drosophila* host (Diptera: Brachycera) or the two parasitoids. However, since *Btk* bioinsecticides affect the *Drosophila* development and metabolism at concentrations closely above those used in crops (Cossentine et al. 2016, Babin et al. 2020, Nawrot-Esposito et al. 2020), the effects of chronic exposure at sub-lethal concentrations of *D. melanogaster* hosts on the parasitoid developmental time and emergence rate were first investigated. Second, as adults, *Leptopilina* parasitoids feed pollen, nectar and exudates of plants (Jervis et al. 1993) and spend time grooming (Zhukovskaya et al. 2013), exposing them directly to *Btk* bioinsecticide. The direct effects of chronic ingestion of *Btk* bioinsecticide on adult longevity of the two parasitoids were thus also investigated.

2 Material and methods

2.1 Parasitoid and fruit fly rearing

L. boulardi (strain "G431", Gif-sur-Yvette, Dupas et al. 1998) and *L. heterotoma* (strain "Gotheron") parasitoids were reared on the wild-type *D. melanogaster* Nasrallah (strain "1333"). After emergence, adult parasitoids were maintained on agar jelly with honey *ad libitum*. Parasitoid development in host exposed to *Btk* formulation was performed using two wild-type strains of *D. melanogaster* to control for a host genotype effect: the laboratory strain "Canton-S" (Bloomington Drosophila Center) and the strain "Sefra" derived from flies collected in South-East France in 2013. The fly strains were reared on a protein-rich medium (100g yeast, 100g corn flour, 20g agar, 60ml of methyl 4-hydroxybenzoate 10% in ethanol 70%: qsp 1000 ml water). All insect rearing, maintenance, and experiments were performed under controlled laboratory conditions (25°C, 60% relative humidity, 12:12 L:D photoperiod).

2.2 *Btk* bioinsecticide and contamination procedure

The *Btk* formulation used is commercialized as wettable granules (brand Delfin®, *Bacillus thuringiensis* subsp. *kurstaki* strain SA-11; 32000 UI/mg; AMM 9200482; https://ephy.anses.fr/ppp/delfin). The strain *Btk* SA-11 contains genes in plasmids encoding six insecticidal Cry proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ia, Cry2Aa, Cry2Ab), two Cry-like proteins, one vegetative insecticidal protein (VIP 3Aa10), but no ß-exotoxins (EFSA 2020). The commercial formulation contains spores and toxins (850g/kg of product), and additives (*e.g.*, surfactants). Viable *Btk* spores were counted by plating serial dilutions of a formulation suspension on LB agar and overnight incubation at 30°C. Colony Forming Units (CFU) estimations averaged 5×10^7 CFU/mg throughout the experiments, in agreement with the formulation technical information (4.85×10^7 CFU/mg). Recommended concentrations vary depending on the crop, from 0.15 to 1.5 kg/ha for a single treatment, which corresponds to 7.5×10^4 to 7.5×10^5 CFU/cm^2 (Supplementary material S1). For the experiments, formulation granules were dissolved in Ringer buffer (7.5 g NaCl, 0.1 g $NaHCO_3$, 0.2 g KCl, 0.2 g $CaCl_2$, qsp 1000 ml water) to reach the desired CFU concentrations, then mixed with the food (fly host medium for parasitoid larvae, honey for adult parasitoids; see below). Control groups received Ringer buffer alone.

2.3 Parasitoid development in hosts exposed to *Btk* bioinsecticide

Indirect effects of *Drosophila* host exposure on parasitoid development were tested on groups of 50 eggs from each *D. melanogaster* strain collected from the 4-hour oviposition of a large population. Eggs were transferred to a dish containing 6g of fresh protein-rich fly medium mixed with either Ringer buffer (100µl/g of fly medium) or concentrations of *Btk* formulation (in 100 µl/g of fly medium) and let to develop into larvae. Three formulation concentrations were applied: 5×10^5 CFU/g and 5×10^6 CFU/g of fly medium that fall within the range recommended by manufacturers (Supplementary material S1), and a 10-fold higher concentration, 5×10^7 CFU/g, which slightly affects *D. melanogaster* larval survival and emergence rate (Babin et al. 2020).

Forty-eight hours later, at the onset of the second *D. melanogaster* larval instar which is the best stage for parasitism (Fleury et al. 2009), 3 mated females *L. boulardi* or *L. heterotoma* aged 5–10 days were let parasitize fly larvae for 5 h. After parasitoid removal, fly larvae were maintained until the last parasitoid wasp emerged. Three replicate vials of 50 eggs were initiated for each combination of *D. melanogaster* host strain, parasitoid species, and controls or *Btk* formulation concentrations. Emerged male and female parasitoids were counted daily to calculate the parasitoid emergence rate (number of emerged parasitoids divided by number of initial fly eggs), average developmental time (average numbers of days from the onset of parasitism to parasitoid emergence, per replicate), and sex-ratio (proportion of males, see Supplementary material S5).

To monitor the effect of *Btk* on *D. melanogaster* development, three replicates of 50 eggs for each *D. melanogaster* strain were exposed to the three formulation concentrations or to Ringer buffer and let to develop unparasitized. Emerged flies were counted daily to calculate the fly emergence rate and average developmental time per replicate.

2.4 Adult parasitoid longevity upon feeding *Btk* bioinsecticide

The potential direct chronic effect of *Btk* formulation on longevity of *L. boulardi* and *L. heterotoma* was evaluated on sex-mixed groups of 10 freshly emerged females and 5 freshly emerged males. Agar jelly was provided as moisture source and renewed weekly. Food was provided on a 10-cm^2 piece of Whatman® paper renewed every 2–3 days, wetted with 200 µl of a 1:1 mixture of honey and *Btk* formulation for exposed groups or Ringer for controls. Three concentrations of *Btk* formulation were tested: two within the range of recommended spraying, 10^5 CFU/cm^2 and 10^6 CFU/cm^2 (respectively, 10^6 CFU and 10^7 CFU on 10 cm^2), and one at 10^8 CFU/cm^2 (10^9 CFU on 10 cm^2), equivalent to 100 times the highest recommended concentration. Dead parasitoids and their sex were recorded daily until the last parasitoid died. Data were collected from two independent experimental batches of three replicate groups for each combination of parasitoid species and *Btk* concentration.

2.5 Data analysis

For parasitoid development in *Btk*-exposed hosts, data on fly emergence rates in non-parasitized controls, and parasitoid emergence rates and parasitoid sex-ratios in the parasitism treatment were analysed using generalized linear models with binomial distribution and logit link, including *Btk* formulation concentration, *D. melanogaster* strain, and parasitoid species as fixed effects and replicate vial as a random effect. Data on average fly and parasitoid developmental times were analysed with linear models, including the same fixed and random effects where appropriate. The significance of post-hoc Tukey tests is indicated as letters in the figures.

A proportional hazard Cox's regression model was applied to data on parasitoid longevity exposed to bioinsecticide, including *Btk* formulation concentration and species as fixed effects, and replicate vials (and experimental block when necessary) as a random effect.

In all the analyses, the significance of effects and interactions was evaluated by model comparisons. Statistical analyses were performed with the R software (R Development Core Team 2008), using the packages lme4 (Bates et al. 2015), multcomp (Torsten et al. 2008), survival (Terry et al. 2000) and coxme (Terry & Therneau 2015).

3 Results

3.1 Development of *D. melanogaster* hosts upon exposure to *Btk* bioinsecticide and parasitism

Exposure to *Btk* formulation impacted the fly emergence rates (fER) similarly for both fly strains (Fig. 1A, Table S2). The fER at 5×10^7 CFU/g of *Btk* formulation decreased by 14% for the "Canton-S" strain and by 20% for the "Sefra" strain compared to their respective controls. At the two *Btk* concentrations within the range of field recommendations, 5×10^5 CFU/g and 5×10^6 CFU/g, fER decreased either with a smaller amplitude, or marginally significantly (significance of pairwise comparisons in Fig. 1A). Fly development time (fDT) was longer for "Sefra" than "Canton-S" and not influenced by exposure to *Btk* formulation (Fig. 1B, Table S2).

Upon parasitisation, fER dropped, with high variability, and without clear general pattern related to *Btk* concentrations, except the dose-dependent increase in fER for "Sefra" flies parasitized by *L. heterotoma* (Supplementary material S3). Interestingly, proportions of fly eggs that did not develop into an insect (fly for the unparasitized controls and fly or parasitoid for the parasitism treatment) were similar without and with *L. boulardi* parasitism across *Btk* concentrations, while they varied without clear pattern with *L. heterotoma* parasitism (Supplementary material S4).

3.2 Development of parasitoids in *Drosophila* hosts exposed to *Btk* bioinsecticide

Parasitoid emergence rate (pER) varied with the parasitoid species and host strain, with higher pER for *L. boulardi* (Fig. 2A, Table S2). pER also depended on the *Btk* concen-

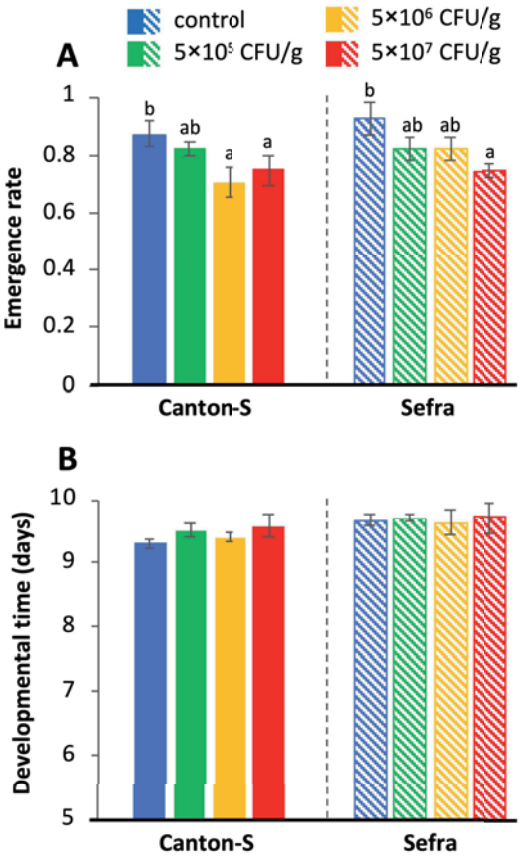

Fig. 1. Emergence rate **(A)** and developmental time **(B)** of the *Drosophila melanogaster* host strains "Canton-S" and "Sefra", under chronic exposure to *Btk* formulation from egg stage to adult emergence. All values are means ± se. *N* = 3 replicate vials per concentration and fly strain. Statistical significance of post-hoc pairwise comparisons with Tukey's tests for each fly strain within parasitoid species is indicated by different letters for two groups compared. Concentrations used: 5×10^5 (green), 5×10^6 (yellow), and 5×10^7 CFU (red) per gram of fly medium; blue, control no *Btk*.

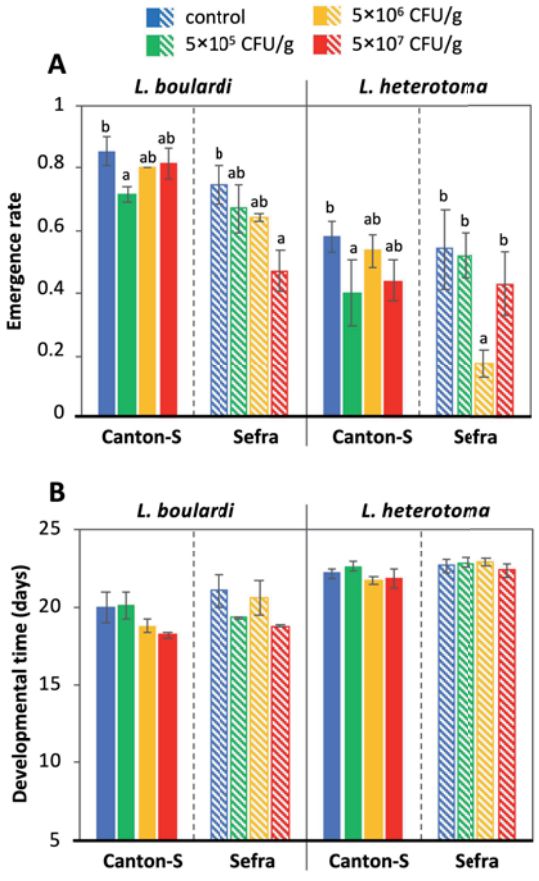

Fig. 2. Emergence rate **(A)** and developmental time **(B)** of the two parasitoids, *Leptopilina boulardi* and *L. heterotoma*, after development into *Drosophila melanogaster* host strains "Canton-S" and "Sefra" chronically exposed to *Btk* formulation. All values are means ± se. *N* = 2-3 replicate vials per concentration, fly strain, and parasitoid species. Statistical significance of post-hoc pairwise comparisons with Tukey's tests for each fly strain within parasitoid species is indicated by different letters for two groups compared. Concentrations used: 5×10^5 (green), 5×10^6 (yellow), and 5×10^7 CFU (red) per gram of fly medium (in blue, control no *Btk*).

tration applied to the host, in interaction with the fly strain and parasitoid species (Table S2). For *L. boulardi*, pER in "Canton-S" fly larvae exposed to 5×10^5 CFU/g decreased by 16% compared to the control; pER decreased also at the two other *Btk* concentrations but not significantly relative to the control (Table S2, Tukey pairwise comparisons on Fig. 2A). The pER of *L. boulardi* reared in "Sefra" host decreased with increasing *Btk* concentration compared to the control, but only significantly by 37% at 5×10^7 CFU/g of *Btk* (Table S2, Tukey pairwise comparisons on Fig. 2A).

For *L. heterotoma*, the concentration of *Btk* formulation impacted pER in both host strains (Fig. 2A, Table S2). pER of *L. heterotoma* that developed in "Canton-S" larvae decreased compared to the control, but only significantly by 31 % at 5×10^5 CFU/g of *Btk* (Tukey pairwise comparisons on Fig. 2A). When reared in "Sefra" larvae, *L. heterotoma* showed a 68% decrease in pER only when fly hosts were exposed to 5×10^6 CFU/g of *Btk* formulation (Fig. 2A, Table S2).

In a similar way for the two parasitoids and regardless of their *D. melanogaster* host strain, the parasitoid developmental time (pDT) was not significantly influenced by the *Btk* formulation (Fig. 2B, Table S2). pDT was generally longer for *L. heterotoma* than for *L. boulardi*, and longer in "Sefra" hosts than in "Canton-S" hosts (Fig. 2B, Table S2). Note that there was substantial variation in the pDT, especially for *L. boulardi* (Fig. 2B). The parasitoid sex-ratio (pSR) depended on the interaction of the *Btk* concentration with the host fly strain and parasitoid species, but no clear dose-response emerged when parasitoid species and host fly strains were analysed separately (Supplementary material S5).

3.3 Longevity of parasitoids upon exposure to *Btk* bioinsecticide

Parasitoids were fed with three concentrations of *Btk* formulation, two within the range of spraying recommendations (10^5 and 10^6 CFU/cm^2) and one concentration 100-fold higher (10^8 CFU/cm^2). While generally lower for *L. boulardi*, parasitoid longevity varied with *Btk* concentration, parasitoid species and sex (Fig. 3, Table S6.1). For *L. boulardi* control, longevity differed between sexes consistently between experimental blocks (Fig. 3A-D, Table S6.1). Exposure to *Btk* formulation did not influence the longevity of *L. boulardi* females (Fig. 3A, B, Table S6.1), but the longevity of males decreased at 10^8 CFU/cm^2 in both experimental blocks, and to a lesser extent, at 10^6 CFU/cm^2 in one experimental block (Fig. 3C, D, Tables S6.1 and S6.2 for details).

The *Btk* formulation influenced the longevity of *L. heterotoma* males and females differently, with a shorter male lifespan as observed for *L. boulardi* (Fig. 3E-H). However, by contrast with the effects observed for *L. boulardi*, the longevity of *L. heterotoma* females exposed to *Btk* formulation decreased in a dose-dependent manner at 10^6 and 10^8 CFU/cm^2, and already at 10^5 CFU/cm^2 in the 2nd experimental block (Fig. 3E, F, Tables S6.1 and S6.2 for details). Note that the effect amplitude at 10^8 CFU/cm^2 of *Btk* for *L. heterotoma* females was similar to that for *L. boulardi* males. For *L. heterotoma* males, longevity was impacted only at 10^8 CFU/cm^2 in one experimental block, and with a smaller effect amplitude than for *L. heterotoma* females (Fig. 3G, H, Tables S6.1 and S6.2 for details).

4 Discussion

In the endoparasitoid life cycle, host death during parasitoid larval development due to the exposure to *Btk* bioinsecticide would have the greatest impacts on parasitoid populations as it would prevent development completion and negate adult reproduction. Here, consistently with recent studies on several *D. melanogaster* strains (Babin et al. 2020, Nawrot-Esposito et al. 2020), the developmental traits of both *D. melanogaster* hosts without parasitism were not influenced by the presence of *Btk* bioinsecticide at concentrations falling within the range of spraying recommendations (5×10^5 and 5×10^6 CFU/g) and were slightly impacted at a ten-fold higher concentration potentially reachable in the field upon repeated sprayings (5×10^7 CFU/g, see EFSA 2012). Overall, the selected *Btk* concentrations allowed the complete development of parasitoid larvae until imago emergence, without the confounding effect of high host mortality. Therefore, our study system was deemed appropriate to investigate the effect of chronic exposure to *Btk* bioinsecticide on the endoparasitoid larval development.

Upon parasitism, there are three possible outcomes for a fly host egg: emergence of a parasitoid (parasitic success, no fly emerged), emergence of a fly (parasitism did not occur or failed), and death of the parasitized host. Here, as expected, the emergence rates of both fly hosts dropped sharply after parasitisation, validating the parasitism protocol efficiency and excluding the possibility of low parasitoid emergence due to low parasitism rates. For parasitism by *L. boulardi* in both hosts, missing fly emergences were replaced by parasitoid emergences without interaction with the applied *Btk* concentrations. This is not exactly the case for parasitism by *L. heterotoma*, but without a clear *Btk* dose-response. These results suggest no strong interaction between absence of development and exposure to *Btk* bioinsecticide. For both parasitoids, the lower emergence rates in "Sefra" fly hosts indicate a less successful parasitism in this host, perhaps because of the more recent laboratory history of this strain. With the exception of "Sefra" flies parasitized by *L. heterotoma*, no clear difference in fly emergence rates was observed between control and *Btk* treatments, suggesting that the *Btk* bioinsecticide has no influence on the oviposition behaviour of female parasitoids. Following parasitism of "Sefra" by *L. heterotoma*, a dose-dependent increase in fly emergence rates corresponded to lower parasitism rates. This may be partially explained by the lower parasitism success in "Sefra" hosts and the higher rates of no emergence.

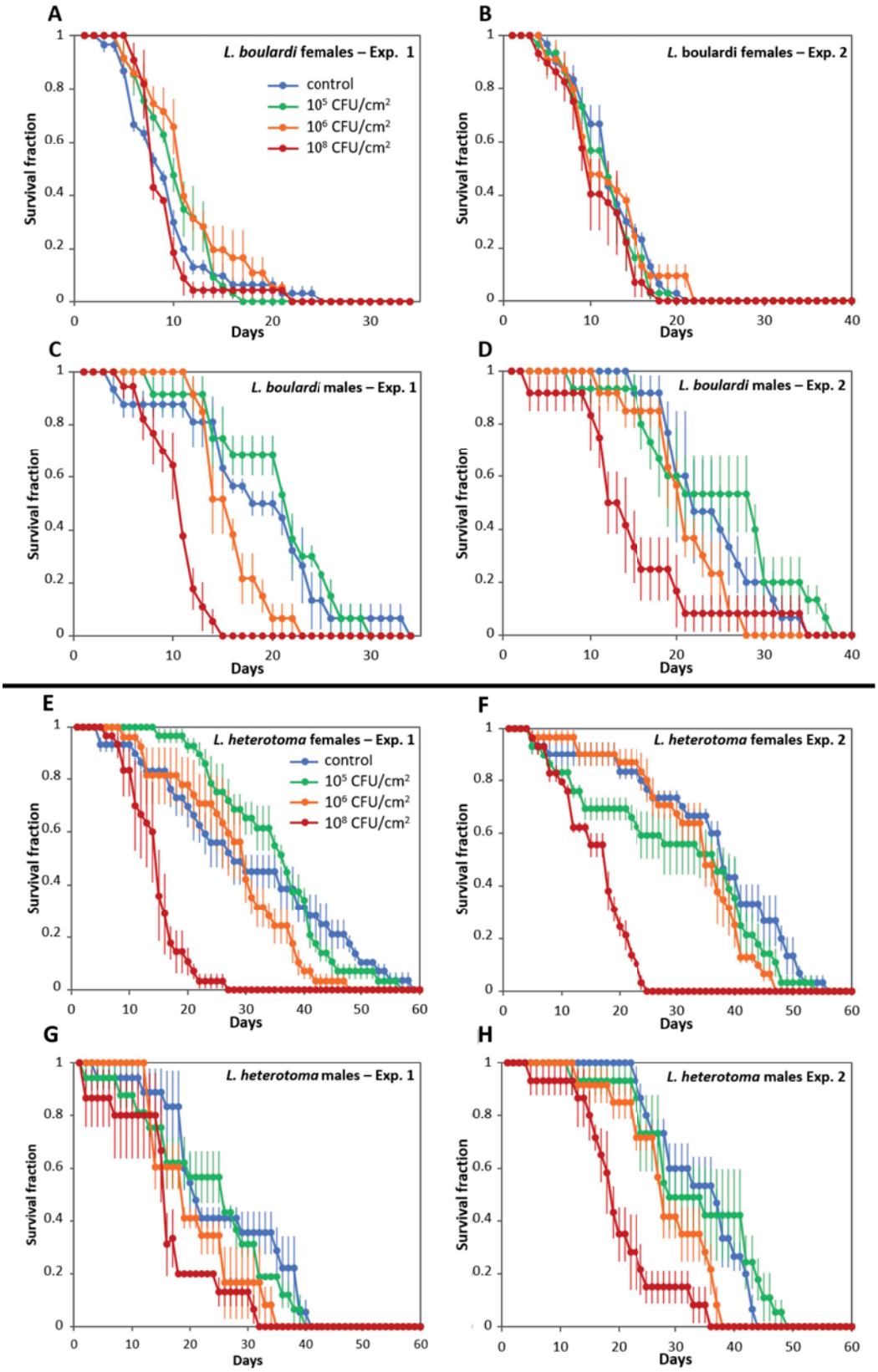

Fig. 3. Longevity of adult *L. boulardi* **(A-D)** and *L. heterotoma* **(E-H)**, females **(A, B, E, and F)** and males **(C, D, G and H)** under chronic exposure to *Btk* formulation. Two replicate experiments were performed. All values are means ± se. *N* = 3 replicate vials per concentration and sex. Concentrations used: 10^6, 10^7 and 10^9 CFU for 10 cm^2 (control, no *Btk*).

But this also suggests that the presence of *Btk* bioinsecticide in the fly larval environment might influence the oviposition behaviour of *L. heterotoma* females, although this should have been observed on "Canton-S" hosts as well. So far, one study has reported no influence of the presence of *Bt* bioinsecticide on the oviposition behaviour of *Cotesia plutellae* (Hymenoptera: Braconidae; Haliday, 1834), an endoparasitoid of the cabbage diamondback moth (Haseeb et al. 2004). Further experimental investigations will be needed to confirm our observations.

Regarding the parasitoid development, *L. heterotoma* exhibited general lower emergence rates and longer development than *L. boulardi*. These two closely related parasitoids live mainly in sympatry and differ in their competitive ability, host ranges, and parasitism strategies: *L. heterotoma* being less competitive, maximizing the number of host attacks, developing more slowly, and being considered more generalist than *L. boulardi* (Allemand et al. 2002, Schlenke et al. 2007, Fleury et al. 2009, Lee et al. 2009). Our results agree with these reported biological and ecological differences. When exposed to *Btk* bioinsecticide, the developmental time of both parasitoids did not significantly change under our experimental conditions. By contrast, their emergence rates were impacted in both hosts, suggesting an impact of *Btk* bioinsecticide extended to the genus *Leptopilina*, yet at a single *Btk* concentration sometimes falling within the range of spraying recommendations. The maximum impact was moderate for *L. boulardi* (about 30% decrease), and greater for *L. heterotoma* (about 60% decrease). The impact at low field-realistic concentrations of *Btk* bioinsecticide suggests that *Leptopilina* parasitoids are susceptible organisms, and that indirect exposure during larval development through their host represents an environmental stressor *per se*. Since a *Drosophila* larva cannot process 1g of medium during its development until pupation, the total quantity of *Btk* bioinsecticide ingested by the host larva (and indirectly the endoparasitoid larva) is certainly much less than that applied.

No clear consensus has emerged from previous studies on feeding sub-acute *Btk* concentrations to parasitoid hosts. Some reported deleterious effects varying with parasitoid genus and species, *Bt* subspecies, and type of bioinsecticides tested (e.g., Glare & O'Callaghan 2000, Tanaka & Minakucki 2012, Amichot et al. 2016, De Bortoli et al. 2017). Others reported an increase in parasitic success due to a delay in host growth that lengthens the parasitoid development period, but many studies reported no marked effect on the parasitoid developmental time or emergence rate (see reviews: WHO 1999, De Bortoli et al. 2017). However, most studies used Lepidoptera hosts, *Btk* targets, which limits the range of testable *Btk* concentrations. Here, we demonstrate that chronic feeding of *Btk* non-target hosts with sub-acute concentrations of *Btk* bioinsecticide impacts the development of their *Btk* non-target endoparasitoids.

In the adult stage, *L. boulardi* lifespan was shorter than that of *L. heterotoma*, in addition to its shorter development. Yet, no study has established a clear relationship between parasitoid developmental time and lifespan (Fleury et al. 2009). For both parasitoid species, chronic ingestion of *Btk* formulation influenced differently male and female longevity. *L. boulardi* males appeared to be more susceptible to the presence of *Btk* formulation in their food than females, which showed no change in longevity. For *L. heterotoma,* both sexes were impacted but females appeared more susceptible than males. For both parasitoids, the highest *Btk* concentration decreased strongly the parasitoid longevity by 2.5 up to 13 times compared to the control, while the two lower *Btk* concentrations decreased moderately the parasitoid longevity (by 1.8 to 2.5 times). Since one sex was less or not significantly impacted than the other, and both sexes were housed together, these observations did not result from starvation due to food avoidance. Although environmental accumulation of our highest bioinsecticide concentration seems hard to reach (100-fold the recommended concentrations), the two lower concentrations are either used or can be reached by repeated sprayings in crop fields. In the literature, no clear consensus on the effects of feeding directly *Btk* on parasitoid longevity has emerged so far. A shortening of adult lifespan was observed for some species such as *Cotesia glomerata* (Hymenoptera: Braconidae; Linnaeus, 1758) or *Hyposoter exiguae* (Hymenoptera: Ichneumonidae), while no effect was observed for others (*Trichogramma cacaeciae* (Hymenoptera: Trichogrammatidae; Marchal, 1927)*, Pimpla turionellae* (Hymenoptera: Ichneumonidae; Linnaeus, 1758) (reviewed in WHO 1999; De Bortoli et al. 2017) suggesting a species-specific susceptibility.

To conclude, we showed that *L. boulardi* and *L. heterotoma* parasitoids were impacted by chronic exposure to the *Btk* bioinsecticide, both indirectly through their developing host environment and directly through adult food resources. Impacts were moderate and differed between the two species, but were observed at bioinsecticide concentrations reachable in the crop fields. Differences in developmental and longevity responses between the two parasitoid species indicate that the chronic effects of *Btk* bioinsecticide may be partially species-specific, which may affect the competition of parasitoids with overlapping host ranges (Carton et al. 1986, 1991, Allemand et al. 2002, Fleury et al. 2009). The greater susceptibility of *L. heterotoma* may thus have consequences for the competitive relationships between the two *Leptopilina* species (and with other *Drosophila* endo- and ecto-parasitoids), for the dynamics of their sympatric populations, as well as for the population dynamics of their hosts and hence on a long term for parasitoid-host coevolution. Although *Btk* bioinsecticides are not used against *Drosophila*, they are sprayed in fruit orchards where flies are present. By impacting the parasitoid community, *Btk* bioinsecticide may indirectly increase the threats and damage linked to *Drosophila* species as vectors of plant pathogens

and fruit pests. For example, *D. melanogaster* is responsible for the spreading of the grape sour rot disease (Barata et al. 2012, Rombaud et al. 2017) and has been suggested to carry various microorganisms that represent potential threats to plant and public health (Ramirez-Camejo et al. 2017, Black et al. 2018). In addition, *D. melanogaster* and its relative species (*e.g., D. simulans*) are viewed as a potential reservoir for local or introduced parasitoids to control the fruit pest *Drosophila suzukii* (Girod et al. 2018, Wang et al. 2019, Wang et al. 2020). As an example, the *Drosophila* generalist pupal parasitoid *Trichopria drosophilae* (Hymenoptera: Diapriidae; Perkins) is already sold to fight *D. suzukii* and *D. melanogaster* (Mazzeto et al. 2016, Wang et al. 2016) and the introduction of the Asian endoparasitoid *Ganaspis cf. brasiliensis* (Hymenoptera: Figitidae, Ihering, 1905) to North America and Europe is planned for the near future. Our data thus emphasize that more specific studies are needed under controlled laboratory and field conditions for the safe use of *Btk* bioinsecticides in IPM programs to maintain or increase the effectiveness of the parasitoid spreads.

Acknowledgements: We thank Dr A. Gallet for helpful discussions. This work was funded by the French "Agence Nationale de la Recherche" (ANR-13-CESA-0003-01) and the "Investments for the Future" programs LABEX SIGNALIFE ANR-11-LABX-0028-01 and IDEX UCAJedi ANR-15-IDEX-01.

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The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement: Supplements S1–S6

Manuscript received: 27 September 2021
Revisions requested: 14 October 2021
Modified version received: 2 November 2021
Accepted: 6 November 2021