Bacillus thuringiensis var. kurstaki bioinsecticide induces growth defects and developmental delay of the non-target organism *Drosophila melanogaster* by disturbing homeostasis of the larval intestine

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Running Head: *Bt* bioinsecticides impacts on *Drosophila* development

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Abstract

Bioinsecticides made of the bacterium *Bacillus thuringiensis* (*Bt*) are the bioinsecticide best sale worldwide. Due to the governmental policies and the public opinion, the use of such products has increased exponentially over the last decade. Among *Bt* bioinsecticides, those based on the strain *Bt var. kurstaki* (*Btk*) are widely used in farming to fight lepidopteran pests. Although there is much evidence of the short-term safety of *Btk* products on non-target animals, only scarce data are available on their long-term unintended effects. Here, we aimed to characterize the putative unintended effects of the ingestion of environmental doses of *Btk* products on the larval development of the non-target dipteran *Drosophila melanogaster*. We showed that *Btk* products impair growth and developmental time of *Drosophila* larvae. These effects are mediated by the synergism between *Btk* bacteria and *Btk* insecticidal toxins, which induces significant apoptosis of enterocytes, resulting in a decreased intestinal capacity to digest proteins. The harmful effects can be compensated by a protein-rich diet or the addition of the probiotic bacterium *Lactobacillus plantarum* in the food. Finally, we uncovered the ability of the larval midgut to maintain its integrity upon *Btk* aggression thanks to both the generation of new immature cells arising from adult midgut precursors and the flattening of old enterocytes. Our work demonstrates that unintended effects of *Btk* products impede gut physiology but can be mitigated by the regenerative capacity of the gut, the commensal flora composition and the diet composition.

IMPORTANCE Spores of *Bacillus thuringiensis* are broadly used biopesticides to kill lepidopteran pest. Although lethal toxicity of *Bt* products is restricted to target organisms, many gaps in our knowledge remains regarding the putative unintended effects of *Btk* biopesticides on non-target organism. Our study focuses on the impacts of *Btk* products on *Drosophila melanogaster* development and growth upon the ingestion of a food contaminated with doses of *Btk* spores detected on vegetable after spraying. Our results highlight the

importance of the nutritional environment and the composition of the commensal flora to endure the harmful effects of *Btk* products on growth and development.

Keywords

Bioinsecticides, *Bacillus thuringiensis*, *Drosophila melanogaster*, *Lactobacillus plantarum*, Developmental defects, Gut homeostasis

INTRODUCTION

The use of conventional synthetic chemical pesticides is controversial mainly because of their detrimental effects on human health and ecosystem. Public opinion along with governmental policies encourage to reduce their use (1, 2). However, because of the world's population expansion, the use of pesticides will still be necessary to produce enough food to feed the expected 9.5 billion of humans in 2050 (https://population.un.org/wpp/). An emerging solution to cut the use of conventional pesticides is their replacement by biopesticides. Based on the nowadays consumption and anticipating the continuation of organic farming growth, the question about the putative impacts of chronic exposure to biopesticides on both health and environment is of prime interest.

Among bioinsecticides, *Bacillus thuringiensis* (*Bt*) products are more and more sprayed in both organic farming and conventional agriculture (3). *Bt* products are the second most used insecticides in the world with 32 000 tons sold in 2017 (2). *Bt* is a Gram-positive sporulating bacterium belonging to the *Bacillus cereus* group (4). *Bt* was first identified and characterized for its specific entomopathogenic properties due to the presence of specific Cry toxins, which are produced under a crystal form during the sporulation of the bacteria (5). Seventy-eight different strains of *Bt* are currently inventoried (http://www.bgsc.org/), altogether producing more than 300 distinct Cry toxins (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) having a toxicity spectrum ranging from nematodes to human cells (6, 7). Nevertheless, only four strains are commercially used as bioinsecticides thanks to the specific acute toxicity of their Cry toxins toward pest larvae: *Bt var. kurstaki*, *Bt var. aizawai*, *Bt var. israelensis*, and *Bt var. morrisoni* (also named *tenebrionis*) (http://sitem.herts.ac.uk/aeru/bpdb/index.htm). Upon ingestion of commercial formulations made of spores, crystals and additives, Cry toxins are released from the crystal, bind to specific receptors at the surface of intestinal cells, inducing their death and ultimately the destruction of the gut epithelium. The spores germinate and, thanks to the holes

made by Cry toxins in the gut lining, invade the internal body cavity, provoking a septicemia. The targeted pest dies in 2 or 3 days (6). Each variant of *Bt* produces between 1 and 6 different Cry toxins. In general, all Cry toxins contained in one *Bt* variant target a specific phylogenetic order (*e.g.* lepidopteran, coleopteran…) (8). The most used *Bt* variant is *Bt var. kurstaki* (*Btk*), which produces 5 different Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab), all targeting lepidopteran larvae (9). *Btk* is widely used in forestry and organic farming.

The increasing environmental dispersion of *Btk* bioinsecticides has raised the question of their putative long-term impacts on both health and environment. Indeed, although there are many data asserting that Cry toxins produced by *Btk* do not display harmful effects against non-target organisms (10, 11), there are also studies suggesting that Cry toxins and/or *Bt* products may display unintended impacts on non-target animals. For example, the presence in water streams surrounding maize fields of Cry1Ab toxins produced by GM maize delays the growth and increases the mortality of trichopteran species whose larvae have aquatic lifestyle (12, 13). More recently, Amichot and colleagues have shown that *Btk* bioinsecticides displayed acute toxicity against *Trichogramma chilonis* females, a tiny egg parasitoid hymenoptera used as a biocontrol agent in integrated pest management (14). Also, *Btk* spores and toxins could induce lethality in the early larval stage of many *Drosophila* species (dipterous) (15, 16). On vertebrates, Grisolia and colleagues observed that exposure of *Danio rerio* fry to different *Btk* Cry toxins induced lethality and developmental delay (17).

Based on these data, we decided to accurately investigate the putative adverse effects of *Btk* bioinsecticides on the development and growth of a non-target organism: the dipteran model *Drosophila melanogaster*. While the acute toxicity of the agricultural doses of *Btk* toxins efficiently kills lepidopteran larva, *Drosophila* larvae, being insensitive to those doses, allow studying non-lethal impacts. Furthermore, *D. melanogaster* has been successfully used

for many studies deciphering insecticides toxicology and is a toolkit widely used to identify perturbed cellular and molecular mechanisms (18).

In the present study, using *Btk* commercial products at a dose similar to the amount detected on vegetables after spraying and at a dose only tenfold higher, we observe that *Btk* bioinsecticides induce a developmental delay and a reduced growth of *D. melanogaster* larvae. We further show that *Btk* bioinsecticides trigger intestinal cell death and alter protein digestion. Interestingly, we demonstrate that a protein-rich diet or complementation of the food with the probiotic *Lactobacillus plantarum* (*L. plantarum*), known to enhance protein uptake (19), mitigate *Btk* bioinsecticide negative effects on growth and development. We also found that *Btk* products do not interfere with the commensal flora of the larvae. Finally, we unraveled new mechanisms of intestinal integrity maintenance mounted by the larval midgut epithelium in response to *Btk* aggression, particularly the flattening of enterocytes and the production of new cells from the adult midgut precursors. Altogether, our data suggest that *Btk* bioinsecticides should be used with care to avoid any long-term detrimental impacts on non-target organisms.

RESULTS

Btk bioinsecticide induces developmental delay and growth defect

We have previously shown that *Btk* bioinsecticides coming from diverse manufacturers induced larval lethality and delayed adulthood emergence of many different *Drosophila* species at doses ranging from $5x10^7$ to $1x10^9$ Colony Forming Units (CFU) of *Btk* spores/g of fly food medium (16). It has been estimated by the European and the French Food Safety Agencies (Efsa and Anses respectively) that the amount of *Btk* spores on fruits and vegetables after treatment can reach $5x10^6$ CFU/g (20, 21). This quantification may be underestimated since up to 12 successive treatments can be applied by farmers (21) (see also the European directives SANCO/1541/08 – rev. 4 and SANCO/1543/08 – rev. 4). Therefore, we decided to

assess the impacts of the ingestion of two *Btk* bioinsecticides (i.e. Delfin and Dipel) on *Drosophila* larval growth and development using two different doses: $5x10^6$ CFU/g (close to the dose observed after spraying) and $5x10^7$ CFU/g (a dose that could be reached upon repetitive treatments), named in the present study as "1X" and "10X" respectively. We first compared the impacts of Delfin to water (the diluent of the commercial products). We contaminated 2g of standard fly medium on which we deposited 20 eggs aged of 0-4h. We measured the timing of larval development by performing a pupation curve. In the control experiment (H_2O), 90% of larvae were pupating at 169h after eggs laying (AEL) (this threshold will serve as a point of comparison throughout the rest of our study, Table 1 summarizes all the data at 10%, 50% and 90% of the pupae entry). The 1X and 10X doses of Delfin postponed pupation by 10.5h (+6.2%) and by 16h (+9.5%) respectively (Figure 1A and Table 1). We then assessed the impact of the second bioinsecticide Dipel. Because the developmental delay was marked with Delfin at the 10X dose, we also used this dose as a benchmark for Dipel. Strikingly, the pupation delay was increased to reach 25h (+14.8%). The commercial products are made of spores, toxin crystals and additives (see Materials And Methods). Dipel contains 46% of unknown components, while Delfin contains 15% of naphthalene sulfonic acid. To assess the potential involvement of the unknown additives present in Dipel in the augmented delay observed, we plated the commercial product on LB agar, isolated a colony and prepared our own mixture of spores and toxins (the *Btk* strains used in Dipel is named *ABTS-351*). When larvae were fed with the 10X dose of the homemade preparation, we obtained developmental delays similar to that obtained with Delfin treatments (Figure 1A and Table 1), suggesting that the unknown additives present in the commercial preparation of Dipel might contribute to the increased delay we observed. On the contrary, these data suggested that the additive present in Delfin (15% of naphthalene sulfonic acid) had no impact on the developmental timing. We verified this hypothesis by feeding developing larvae with an amount of naphthalene sulfonic acid equivalent to a 10X

dose of Delfin and confirmed that developmental timing was unaffected (Figure 1B and Table 1). Then, we tested whether the developmental delay was due to the spores alone, the crystals of toxins alone, or both. We fed the developing larvae with the 1X and 10X doses of spores of a *Btk* strain devoid of Cry toxins (*Btk*$^{\Delta Cry}$). Whatever the dose, no developmental delay was observed (Figure 1B and Table 1). Finally, we fed the developing larvae with an equivalent 10X dose of toxin crystals considering that crystals represent 30% of the dry weight of bioinsecticides (22-24) (Supfigure 2). We did not observe any developmental delay (Figure 1B and Table 1). Therefore, we assume that the developmental delay we observed was mainly due to the spores and toxins of *Btk* contained in the commercial products.

Since growth defects have a well-documented impact on developmental timing (25), we next measured the pupal volume, a known readout to estimate growth defects (26). Interestingly, we could observe growth defects only when larvae were fed with Delfin and Dipel (Figure 1C). We also detected a slight decrease in size with *ABTS-351*, suggesting that unidentified components present in the commercial products (Delfin and/or Dipel) could indeed induce growth defect. Together, our data show that the ingestion of food contaminated with commercial *Btk* products induces a developmental delay and a growth defect on the model organism *D. melanogaster*, at doses that can be recovered in treated areas.

Since i) the composition of Delfin is clearly provided by the manufacturer compared to Dipel (see Materials And Methods), ii) Delfin is composed of 85% of spores and toxin crystals while only 54% for Dipel, and iii) the naphthalene sulfonic acid has no effect on the developmental timing and growth, we decided to use only Delfin in the rest of the present study.

Btk bioinsecticide does not alter feeding behavior and food intake

Btk load in the midgut could explain the differences observed between *Btk*$^{\Delta Cry}$ and Delfin. When we estimated the amount of *Btk* cells in the midgut of the L3 larvae (Figure 2A), no

difference was observed for 1X or 10X doses of Delfin compared to the equivalent doses of *Btk*$^{\Delta Cry}$. Therefore, the quantity of *Btk* bacterial cells in the midgut could not explain the perturbed developmental timing and growth observed with Delfin.

Adult flies and larvae can sense the presence of harmful microbes and display an aversion for contaminated food (27-30). Therefore, we tested whether the feeding behavior was affected by the presence Delfin. We added a blue dye in the food and counted the number of feeding larvae containing blue food in their gut, either in L3 (Figure 2B) or in L2 stage (Figure 2C). Although we observed a weak decrease in L2 larvae reared in Delfin 1X dose condition, we did not detect any drastic changes in the feeding behavior. We then quantified the food intake and did not detect any significant modification in L3 larvae (Figure 2D), unlike in L2 larvae, which were affected whatever the condition (excepted for the *Btk*$^{\Delta Cry}$ 10X condition that was not significant) compared to the control (Figure 2E). Therefore, although food contaminated by *Btk* spores (with or without crystal containing Cry toxins) disturbed food intake of L2 larvae but not of L3 larvae, these data do not explain the specific impact of Delfin on larval developmental time and growth.

Btk bioinsecticide impairs protein metabolism

It has been extensively shown that proteins are a key nutrient to drive normal developmental timing and growth. Increasing protein content in the diet accelerates the developmental time and/or stimulates growth, while an increase in sugar content delays development and a change in lipid amounts has only a marginal impact (31-36). Because we have previously shown that the presence of opportunistic bacteria in the adult midgut impede protein digestion (37), we wondered whether this could also be the case in larvae in the presence of *Btk* bioinsecticide. While the 10X dose of *Btk*$^{\Delta Cry}$ had no consequence, we observed a decreased capacity of the midgut of L3 larvae to digest proteins in a Delfin dose-dependent manner (Figure 3A).

We next wondered whether increasing the protein content of the diet could rescue the developmental delay and the growth defects caused by the ingestion of Delfin. Our rearing medium contains 2% yeast as protein source (see Materials And Methods). We therefore performed a pupation curve with larvae raised on a medium containing 10% yeast with the 1X or 10X doses of Delfin (Figure 3B). Compared to the control, no difference in the developmental time was now observed for the 1X dose of Delfin and only a slight delay for the 10X dose of Delfin (Table 1). Noteworthy, the developmental time for all the conditions on the protein-rich medium was shortened compared to the control on our conventional medium (light green curve in Figure 3B and Table 1). Similarly, the pupal volume (Figure 3C) was completely restored for the 1X dose of Delfin and partially rescued for the 10X dose of Delfin (5.9% volume loss instead of 11.9% in poor-protein diet). Taken together, our data suggest that Delfin impairs protein digestion, and thus gut function, that can be rescued by adding extra dietary protein.

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

It has been recently shown that the commensal bacterium *L. plantarum* by stimulating proteins digestion and absorption promotes juvenile systemic growth of *Drosophila* larvae and infant mice, and protects child against sepsis upon undernutrition conditions (19, 38-41). Therefore, we wondered whether complementing our conventional medium with *L. plantarum* would compensate for the impacts of the 10X dose of Delfin. First, we observed that adding *L. plantarum* in the conventional medium shortened the developmental time of control larvae compared to the control without *L. plantarum* (Figure 4A, purple curve compared to green curve and Table 1). Interestingly, the presence of *L. plantarum* fully counteracted Delfin-dependent developmental delay (Figure 4, pink curve compared to red curve and Table 1). We next observed that the presence of *L plantarum* in the conventional medium reduced the pupal size of the control larvae by 4.4% compared to the control without *L. plantarum* (Figure 4B,

purple bar compared to green bar) but fully rescued the growth defects induced by the 10X dose of Delfin (Figure 4B pink bar). Because ingestion of *L. plantarum* and its establishment in the midgut (37) might compete for the presence of *Btk*, and not act on protein intake as expected, we also controlled the number of *Btk* CFUs in the absence or in presence of *L. plantarum* in the midgut of L3 larvae and we found no difference (Figure 4C). Taken together, our data demonstrated that the stimulation of protein digestion and absorption by the commensal bacterium *L. plantarum* helps to overcome the adverse effects of the *Btk* bioinsecticides on larval development.

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

L. plantarum is a widespread commensal bacterium of the *Drosophila* midgut (42-44). Because complementing the larval diet with *L. plantarum* rescued the developmental delay and growth defects induced by *Btk*, we wondered whether the indigenous commensal flora of larvae reared on our conventional medium (i.e. poor in protein) was inhabited by *L. plantarum*. Only few *L. plantarum* and/or *L. brevis* (belonging to the family of *Lactobacillaceae*) were present in the commensal flora of our larvae (Figure 4D). The dominant bacteria family we identified belonged to *Acetobacteraceae* known to be common in flies feeding on sugary, acidic and alcoholic food (42, 45). This observation was in agreement with the composition of our conventional rearing medium poor in protein but quite rich in simple sugar (i.e. sucrose, see Materials And Methods) and it suggested that the *Btk* effects we observed were independent of a putative deleterious impact on the indigenous *L plantarum* of our larvae.

However, we could not exclude an impact of the *Btk* bioinsecticide on other members of the commensal flora. To test this hypothesis, we generated an axenic fly strain (see Materials And Methods). Axenic larvae (Figure 4D) were further fed on our conventional medium treated or not with the 10X dose of Delfin. The developmental time of control axenic

larvae was slightly shorter than that of conventionally reared (CR) larvae (i.e. non-axenic) (Figure 4E and Table 1). Nevertheless, axenic larvae infected with Delfin 10X displayed a 19h developmental delay compared to the control axenic larvae (+11.7%) (Figure 4E and Table 1), which was similar to the delay observed with treated CR larvae (see Figure 1). Then, while axenic larvae gave rise to pupae with a similar volume to that of CR larvae (Figure 4F), Delfin 10X still induced a reduction of pupal volume in axenic larvae compared to the axenic control (Figure 4F) in a magnitude similar to what we observed for Delfin 10X with CR larvae (see Figure 1). Taken together our data strongly suggest that Delfin ingestion affects developmental time and growth independently of the commensal bacteria.

Btk bioinsecticide induces midgut perturbations

Because we had previously showed, in adult *Drosophila*, that ingestion of *Bt*k vegetative cells reduced lipid accumulation in enterocytes (37), we wondered whether commercial *Btk* product did the same in larvae. In unchallenged L3 larvae, lipid droplets strongly accumulate in enterocytes in the anterior most of the midgut and weakly in the posterior midgut (Figure 5A) (46). Instead of a lipid droplet depletion, feeding larvae with Delfin 10X provoked an accumulation of lipid droplets in the posterior larval midgut (Figure 5E). Furthermore, lipid droplets accumulated apically, facing the lumen (Figure 5F and F' compared to 5B and B'). Such an accumulation was not observed when developing larvae were fed with $Btk^{\Delta Cry}$ spores (Figure 5C, D and D'). Interestingly, it has been recently demonstrated that the pore-forming toxins hemolysin and monalysin produced respectively by the enteropathogenic bacteria *Serratia marcescens* (*Sm*) and *Pseudomonas entomophila* (*Pe*) can induce an apical accumulation of lipid droplets in adult enterocytes of *Drosophila*, honeybees and mice as well as in epithelial Caco-2 cells in culture. This phenomenon precedes the expulsion of cytoplasm required to get rid of toxins from the enterocytes (47). Since the Cry toxins produced by *Btk* are also pore-forming toxins (6), we fed developing larvae with purified toxin crystals and

checked for the accumulation of lipid droplets. Unexpectedly, we did not observe any lipid droplets accumulation (Figure 5G, H and H'). However, feeding our developing larvae with the naphthalene sulfonic acid (the additive present in the commercial products, see Supplemental Materials) provoked an accumulation of apical lipid droplets posterior to the acidic domain (Figure 5I, J and J'). To further assess whether such a lipid accumulation in the midgut would impair lipid metabolism in the whole larva, we quantified the circulating lipids (the diacylglycerides - DAG) in the hemolymph and the stored lipids (triacylglycerides - TAG) (48) in mid-third instar larvae. We did not notice any significant perturbation in lipid metabolism (Figure 5K) though a downward trend was visible for circulating DAG in larvae fed with Delfin 10X. Thereby, it is possible that such an apical accumulation of lipid droplets serves for the trap and elimination of the naphthalene sulfonic acid similarly to what happens for the elimination of the *Sm* and *Pe* pore-forming toxins (47).

In adult *Drosophila*, pathogenic and opportunistic bacteria can induce enterocyte apoptosis upon ingestion (37, 49). Therefore, we verified the appearance of dying cells in the midgut using the Caspase3::GFP sensor to visualize apoptosis in living tissue (50) (see Materials And Methods). While unchallenged and $Btk^{\Delta Cry}$ fed larvae did not display any obvious apoptosis, the developing larvae fed with Delfin harbored apoptotic enterocytes mainly in the posterior part of the intestine (Figure 5L-M and Supfigure 3). Because in target lepidopteran larvae, Cry toxins make holes in the gut lining, breaking down the gut barrier and allowing *Btk* cells to penetrate inside the internal milieu (6), we checked whether the sealing of the gut epithelium was still maintained even in presence of widespread apoptosis. We fed our developing larvae with the small fluorescent molecule Dextran-FITC that is unable to cross the midgut epithelium (Figure 5O) unless the intestinal barrier is broken allowing the fluorescence to spread throughout the larva like in the case of *Pe* infection (Figure 5P). Interestingly, $Btk^{\Delta Cry}$ spores as well as Delfin did not induce the loss of gut barrier function (Figure 5Q and R). Thus, our data show that *Btk* bioinsecticide hampers lipid

homeostasis and induces enterocyte apoptosis without causing the rupture of the intestinal barrier. Such physiological perturbations may be responsible for the developmental delay and the growth defect observed.

Enterocyte flattening and incomplete differentiation of Adult Midgut Precursors help to maintain intestinal integrity

Intestinal physiology can also be impeded by a disturbance of the apico-basal polarity of the gut epithelium. In the control L3 larval midgut, Armadillo (Arm, β-Catenin), a component of the adherens junction, marks the basolateral compartment of the enterocytes and Disc Large (Dlg), a component of the septate junction, marks the sub-apical compartment. Phalloidin that binds to F-Actin reveals both the apical brush border (facing the lumen) and the basal visceral muscles that surround the midgut lining (Figure 6A and A'). This organization is similar to the adult midgut (37, 51). Moreover, Arm also delineates the islets of the adult midgut precursors (AMPs) which will give birth to the adult midgut during metamorphosis (Figure 6A) (52-54). When larvae were fed with the 10X dose of $Btk^{\Delta Cry}$ spores, we did not observe any major perturbations of the architecture of late L3 midguts (Figure 6B and B'). However, we observed an increased number of AMPs per islet (Figure 6D) correlated with an increase in AMP division (Figure 6E). When larvae were fed with Delfin 10X, we also observed an increased number of AMPs by islet (Figure 6D) and a huge increase in AMP proliferation (Figure 6E) though at the expense of the number of islets (Figure 6F). Moreover, we also observed an increase in the enterocyte surface (Figure 6C and G). Interestingly, such an increase in enterocyte surface did not rely on a higher ploidy (Figure 6J) but rather on a change in cell shape, i.e. enterocyte flattening (Figure 6C' and H). Finally, we noticed the appearance of uncharacterized cells having an “intermediate” size. These cells, intercalated between large enterocytes and the islets, were neither mature polyploid enterocytes nor diploid AMPs or enteroendocrine cells (Figure 6I and I'). This observation was confirmed by

analyzing the ploidy of the nuclei. In the control larvae, 89% of the polyploid cells underwent 4 endocycles of replication (32C nuclei, C corresponding to chromatin copy number) and 10% reached 5 cycles of endoreplication (64C) (Figure 6J). When larvae were raised on medium contaminated with *Btk*$^{\Delta Cry}$ spores, 74% corresponded to 32C nuclei and 25% to 64C nuclei (Figure 6J). When larvae were fed with Delfin 10X, a new population of cells with 16C, 8C and 4C nuclei appeared, representing 37% of the population of polyploid cells (Figure 6J). While the adult midgut quickly regenerates upon an oral infection thanks to the proliferation of adult intestinal stem cells and the differentiation of their daughter cells (the enteroblasts) into new enterocytes which repair the damages (55), such a mechanism has never been observed in *Drosophila* larvae. Indeed, the number of enterocytes composing the L3 intestine is defined during embryogenesis (56) and the larval gut grows without cell division but by increasing the ploidy and consequently the size of enterocytes. However, our data suggested that upon Delfin ingestion, larval gut homeostasis is perturbed and the apparition of undefined cells is promoted. In the larval intestine, AMPs are the only cells capable of division (52-54). Therefore, we hypothesized that the unknown cells having a nucleus ranging from 4C to 16C (Figure 6J) and adjacent to the AMP islets (Figure 6I) could arise from the differentiation of those AMPs. To test our hypothesis, we used the lineage tracing genetic tools developed in *Drosophila* named ReDDM (57). The principle of this lineage tracing is based on the expression of both a labile GFP and a stable RFP within the AMPs thanks to the use of a promotor specifically expressed in AMPs and inducible at will (see Materials And Methods). Accordingly, AMPs express both GFP and RFP, while the progenies, which are not AMPs, only express RFP. In control larvae, GFP and RFP were only present in the islet cells (Figure 6K) (52-54). When larvae were fed with *Btk*$^{\Delta Cry}$ spores, GFP and RFP were also only expressed in the same AMP cells (Figure 6L). In contrast, when larvae were fed with Delfin 10X, cells harboring only RFP were now detectable. Furthermore, the newborn cells had nuclei of intermediate size ranging from 4n to 16n (Figure 6M). This

result demonstrated that upon aggression by *Btk* bioinsecticides, the larval midgut is able to produce new cells, most probably immature enterocytes arising from AMPs. Altogether, our data suggest that the larval intestine maintains its integrity upon aggression through two mechanisms, i) enterocyte flattening which increases the cell surface without new cycles of endoreplication and ii) producing new immature enterocytes. Both mechanisms may allow the intestine to maintain the intestinal barrier, avoiding the bacteria to penetrate into the internal milieu and therefore septicemia.

Discussion

The long-term effects of chronic exposure to low doses of potential noxious products cannot be easily revealed by conventional epidemiological studies (which are long lasting and expensive) or conventional (eco)toxicological approaches based on acute toxicity. The use of amenable and cheap laboratory models becomes of prime importance to pinpoint discrete chronic adverse effects. *D. melanogaster* fulfills all the required criteria to be such a model. Indeed, *Drosophila* is easily handling, cheap to rear and the availability of genetic, cellular and molecular tools allows to detect tenuous effects and to decipher the underlying physiological mechanisms impaired (18). Moreover, the main physiological functions and genetic networks governing the *Drosophila* gut physiology are well conserved among the animal kingdom (58), allowing to provide informative outputs for both human health and wildlife.

We previously identify that *Btk* commercial products could induce developmental delay in many *Drosophila* species (16). In this study, we investigated the physiological mechanisms disturbed by *Btk* products responsible for developmental delay and the growth defects using *D. melanogaster* and doses close to those potentially present in the field after agricultural spraying. The lower dose we used corresponds to the amount deposited on the vegetables after one treatment. The highest dose reflects the amount that may be encountered

after multiple treatments, even though data regarding *Bt* accumulation in the fields remain scarce. Indeed, the half-life of the entomopathogenic Cry1 toxins being estimated at ten days in the field (59), weekly sprays are recommended by the manufacturer and up to 12 are authorized. Regarding the persistence of spores, it has been shown that 72 hours after spraying of chards with *Btk* bioinsecticides, almost all of the *Btk* was still present at the surface of the leaves (60). Ten days later, the amount of *Btk* bioinsecticides corresponds to 50% of the initial dose sprayed on the leaves. Only 28 days after treatment, the amount of *Bt* found on the leaves approaches normal values ($<10^1$ CFU/g) (61). Furthermore, once *Bt* has reached the soil, it persists more than a year in the treated soil (62). Our study highlights that such a persistence and accumulation of *Btk* bioinsecticides may affect the development of the non-target dipteran *D. melanogaster*. Nonetheless, the impacts are mainly due to the synergism between *Btk* spores and crystals since either the spores ($Btk^{\Delta Cry}$) or the purified crystals alone do not promote detectable developmental delay or growth defects in *Drosophila* larvae. These data are in agreement with the study analyzing the safety of *Btk* purified Cry toxins on *Drosophila* larval development (11).

Interestingly, the effects we characterized here are reminiscent to those observed on the target organisms *Helicoverpa armigera* and *H. zea*, *Spodoptera frugiperda* and *Choristoneura fumiferana*. Indeed, when fed with sublethal doses of *Btk* spores, all these target organisms display developmental delays and/or reduced pupal size (5, 63-65). Moreover, a recent work has also shown that the potent pathogens *P. entomophila* and *P. aeruginosa* or huge amounts of the opportunistic bacterium *Erwinia carotovora carotovora* induce developmental delay and growth defect in *Drosophila* larvae (66). These data suggest that developmental delay and/or growth defects must be general consequences of intestinal infections. The compromised gut digestive capacity of the infected larvae (whatever the insect) is likely the main reason for such developmental defects. Indeed, we and others have shown in the adult (19, 37, 67-69) or in larva (this work) that allochthonous bacteria induce a

more or less widespread apoptosis of enterocytes leading to a decreased digestive and absorptive capacities. The developmental delay and the growth defect likely reflect the degree of damages suffered by the intestine. Houtz and colleagues, using strong pathogens, have observed a pupation delay of up to 4 days (66) while, in our study, we did see a delay between 16 hours (Delfin) and 24 hours (Dipel) with *Btk* products. Therefore, while *Btk* behaves as a strong pathogen on target organisms, ultimately leading to death (in fact the expected effect), consistently with the "opportunistic" status of bacteria belonging to the *B. cereus group* (70), it has milder but non-negligible impacts on the development of non-target insect.

Our work demonstrates that the composition of the diet is important to overcome *Btk* intestinal infection. Increasing the protein content of the nutritive medium rescues both growth defects and developmental delay. Our data are in accord with previous observations in mammals and insects infected with various pathogenic bacteria or viruses showing that protein intake is important to withstand attacks by pathogens, to support an efficient immune defense and to promote growth, while intake of carbohydrates is deleterious for all these functions (34-36, 71-73). Moreover, our data show that the commensal bacterium *L. plantarum* also participates to the protection of larvae from *Btk* intestinal aggression in accordance with previous works involving commensal bacteria in gut protection against pathogens (74, 75). In *Drosophila*, *L. plantarum* has been shown to protect adult flies from *Sm*- or *P. aeruginosa*-induced mortality and this protection is specific since the commensal bacteria *Enterococcus faecalis* or *B. subtilis* do not operate as such (76). Our work strongly suggests that the benefit brought by *L plantarum* to larvae to endure *Btk* aggression passes through an enhanced protein digestion and absorption by the gut in line with previous observations (19). Finally, we did not observe a decrease in food intake suggesting that spores and commercial products do not induce avoidance behavior. Together, our data support the model that the impact of the *Btk* products on the development of the non-target organism *D. melanogaster* (and probably many other arthropods) depends on both the nutritional

environment and the performance of its commensal flora. Consequently, the spraying of *Btk* products when the environmental conditions are stressful would amplify the adverse effects of *Btk* products. Our work also sheds light on the pathogenic opportunism of *Btk* on a broad range of organisms owing to the conservation of the digestive tract architecture and functions (58). For instance, the impacts of *Btk* on animals or humans suffering from dietary deficiencies (very often associated with dysbiosis) may be more deleterious than on individuals with a well-balanced diet. Of note, bacteria of the *B. cereus* group are well-known opportunistic pathogens responsible for foodborne poisoning (77, 78). Therefore, a more accurate monitoring of the presence of *Btk* in food and environment is needed to avoid any setbacks, especially in the current context where organic farming is gaining market share.

Finally, our investigations have unraveled two concomitant mechanisms allowing *Drosophila* larvae to overcome an intestinal infection. It was thought so far that the *Drosophila* larval gut was unable to regenerate due to the short period covering the larval stages (about 5-6 days) and the inability to give birth to news cells to replenish the damaged ones (56). Only two choices were thus offered to the larvae: *Live or let die*. However, we have uncovered a third choice for the infected larvae: *Die another day*. First, we demonstrate that the adult midgut precursors (AMPs) can be diverted from their original fate. Indeed, upon aggression of the larval intestinal epithelium, the AMPs can engage toward a process of differentiation to give birth to enterocyte-like cells, which are intercalated in between AMP islets and enterocytes. We can assume that such newborn cells could plug the holes caused by the death of enterocytes, thus avoiding the entry of luminal bacteria into the internal milieu. Unfortunately, newborn cells do not fully differentiate and probably do not participate in digestive functions as efficiently as well-differentiated enterocytes. The addition of these misdifferentiated cells to dying enterocytes can explain the reduced protease activity of the intestine of infected larvae and the subsequent developmental delay and growth defect. The huge increase in AMP proliferation we observed at the end of L3 upon *Btk* spore ingestion

could be explained by the necessity to replenish the right number of AMPs before pupation to achieve a complete adult gut metamorphosis and therefore produce a viable adult fly. These AMPs replenishment could also participate to the developmental delay we observed. The work very recently published by Houtz and colleagues reaches to similar conclusions (66).

We have also uncovered a second unexpected mechanism involved in the maintenance of gut integrity: the change in enterocyte shape. So far, it has been shown in many species (insects, mammals or plants) that the loss of cells in response to stressors can be compensated by the rapid increase in the size of neighboring cells thanks to successive endocycles (79). Here, we evidence an increase in enterocyte area upon *Btk* spore ingestion but concomitantly we have observed a thinning of the epithelium. Above all, we did not observe an increase in the ploidy of the enterocyte nuclei. Interestingly, it has been shown that old enterocytes in adult *Drosophila* midgut cannot re-enter the endocycle (80). Therefore, as for old adult enterocytes, L3 enterocytes (born during embryogenesis 7 days earlier) are probably unable to re-enter a cycle of endoreplication to promote cell growth. Hence, changing their shape through flattening allows to increase the area covered by one enterocyte and to participate in maintaining epithelium sealing. Here again, the absence of endoreplication likely participates to the reduced digestive capacities of the intestine and therefore to the developmental delay and growth defects.

It has been proposed that lepidopteran larvae succumb to *Btk* spores and toxins because they are unable to maintain their gut integrity. Conversely, one mean for a lepidopteran larva to acquire resistance to *Btk* bioinsecticides may rely on its capacity to maintain/regenerate its intestinal integrity (81-83). Non-target organisms are likely as such because they are able to regenerate their gut lining faster than *Btk* products destroy it. However, increasing the amount of *Btk* products provided to *Drosophila* larvae ultimately leads to their death (16), suggesting that the harmfulness of *Btk* products relies on both the amounts of *Btk* products ingested and the affinity of Cry toxins for specific receptors.

Altogether, these data suggest that *Btk* spores used as bioinsecticides may be not as safe as expected on non-target organisms. Moreover, the opportunistic virulence of *Btk* for many organisms should not be overlooked, especially for those suffering from nutritional stress, dysbiosis or with a compromised innate immunity. A situation that more and more species will encounter with the climatic changes occurring worldwide. Many investigations remain to be done to improve the use and efficiency of *Btk* products and to make their use as safe as possible for both handlers and consumers (wildlife or humans).

MATERIALS AND METHODS

Fly Stocks and genetics

Canton S (WT) (Bloomington #64349); *w, dlg::GFP cc01936* (FlyTrap) was provided by A. Spradling; *w; myo1A-Gal4* was a gift from N. Tapon; *w, UAS-GC3Ai*^{G7S} were kindly provided by Magali Suzanne and described in (50). Apoptosis was monitored on F1 progeny *w/w; myo1A-Gal4/+; UAS-GC3Ai*^{G7S}/+ L3 larvae. The ReDDM fly stock to trace cell progenies in the midgut was kindly provided by Maria Dominguez (57). To perform lineage tracing, synchronized eggs (*w/+; esg-Gal4 UAS-GFP/+; tub-Gal80*^{ts} *UAS-H2B::RFP/+*) were hatched on contaminated food (see below for the method) and maintained for 24h at 25°C. Then, L1 larvae were transferred and maintained at 29°C to alleviate the inhibitory effect of Gal80^{ts} on the Gal4 transcription factor. Intestine were dissected and observed at mid L3 (between days 4 and 5).

Bacterial strains

L. plantarum^{WJL} that has been described in (84) and sequenced in (85) was kindly provided by Bernard Charroux (IDBM, Marseille, France) and by Renata Matos (F. Leulier's Lab, IGFL, Lyon, France). *Pseudomonas entomophila* (86) was kindly provided by Bruno Lemaitre's Lab (EPFL, Lausanne, Switzerland). *Pe* and *L. plantarum*^{WJL} were grown as described in (37). The *Btk*$^{\Delta Cry}$ strain (identified under the code 4D22) (87) was obtained from the *Bacillus* Genetic

Stock Center (www.bgsc.org). Delfin and Dipel formulated bioinsecticides were bought in an agricultural shop center. The strain *Btk* ABTS-351 was isolated from Dipel on LB agar.

Spore preparation

Btk vegetative cells were let to grow and sporulate during two weeks at 30°C with shaking in 2L of PGSM medium. The culture was then heated 1h at 70°C to eliminate vegetative cells and centrifuged for 15 min at 5000g. The pellet was first washed with 1 L of 0.15M NaCl and twice with sterile water. The final pellet was suspended in 30 mL of sterile water, aliquoted and lyophilized 24h-48h. The spore titration was determined by serial dilution of a known weight of lyophilized spores and counting the number of "colony forming unit" (CFU) on LB agar after incubation at 30°C for 18h.

Crystal purification

Two grams of the commercial product were suspended in 60 mL of sterile water, dissolved for 5h at 4°C with agitation and then sonicated 4 times 15s with a frequency set at 50% (Fisherbrand™ Model 505 Sonic Dismembrator). Ten milliliters of the homogenate were deposited on a step density gradient of 67/72/79% sucrose and centrifuged at 100 000g for 18h at 4°C. Crystals were collected on the interfaces between the different sucrose phases and washed twice with sterile water. The final pellet was suspended in 12 mL of sterile water, aliquoted and lyophilized 24h-48h.

Diet composition

Drosophila were reared at 25°C on conventional medium (referred as the "poor-protein" medium in this study): 8% cornmeal (AB Celnat), 2% yeast (Springaline® BA10/0–PW, Amcan), 2.5% sucrose, 0.8% agar (ref 20768-361, VWR) and 0.6% methylparaben (Tegosept,, ref 789063 Dutscher). The protein rich medium contains 10% yeast instead of 2%.

Intestinal CFU counting

Colony forming unit counting were described in (37).

Commensal flora estimation

Ten flies or ten mid-third instar larvae were washed first with ethanol 70% for 30 seconds, then washed with PBS and crushed in 500 µL of LB medium with a Tissue Lyser (Qiagen ref. 85600) at 50Hz for 5 minutes. The homogenate was plated on different selective media to identify the common commensal bacteria. MRS agar (Sigma ref 69964 + 1 mL of Tween 20/L) at 37°C in anaerobic was used to identify *Lactobacillus plantarum* and *brevis*. BHI agar (Sigma ref 70138) at 37°C allowed the identification of *Enterococcus faecalis*. LB agar (Fisher ref BP1426-2) at 37°C allowed the identification of *Escherichia coli* and *Staphylococcus aureus*. Mannitol medium (Bacto peptone 3g/L, Yeast extract 5g/L, D-Mannitol 25g/L, agar 15g/L) at 30°C allowed the identification of *Commensalibacter intestini* and *Acetobacter pomorum*.

Axenic flies

Adult Canton S flies were let to oviposit eggs for two days at 25°C and 12h/12h day/night cycle on our conventional medium supplemented with a cocktail of four antibiotics: ampicillin, kanamycin, tetracyclin and erythromycin (50 µg/mL each). On the next generation, newly hatched adults were immediately removed and transferred on fresh conventional medium containing the four antibiotics. This operation was repeated until flies with axenic gut were obtained. The presence of commensal flora was checked at each fly generation (Supfigure 1). Axenic flies were obtained after 6 generations.

Oral infection of larvae

Adult flies were let to oviposit eggs for 4h at 25°C on laying nests containing grape juice medium (25% grape juice, 3% agar, 1.2% sucrose, 2% ethanol, 1% acetic acid). Twenty eggs were collected and transferred in a *Drosophila* vial containing 2g of the desired medium mixed with *Btk* spores, *Btk* crystals or the naphthalene additive. The conditions used for 2g of medium and 20 eggs were: 10^7 CFU of *Btk* spores for the 1X dose; 10^8 CFU of *Btk* spores for the 10X dose; 0.67mg of *Btk* purified crystals (equivalent to the 10X dose and considering that crystals represents 15-30% of spore weight (22-24)); 10^8 CFU of *L. plantarum* or *Pe*;

0.4mg of naphtalene-2-sulfonic acid sodium salt (ref A13066, Alfa Aesar) (equivalent to the 10X dose). Vials were incubated at 25°C with a 12h/12h day/night cycle until further processing.

Pupation curve

Immobilized larvae forming white pupae were counted. The number of pupae was expressed as the percentage of the final number of pupae. While 20 eggs were deposited in each tube, in some conditions, especially with Dipel and Delfin, we never got 20 pupae probably due to previously described larval lethality induced by the *Btk* products (16). Table 1 summarizes the developmental time at which 10%, 50% and 90% of the larvae were immobilized for all the conditions we tested. For each condition described above, the experiment was performed at least in three experimental blocks of triplicates.

Pupal size

On day 7, 8 and 9 after egg laying, pupae were gently removed from the vial, washed in water and slightly dried with tissue paper. Pupae were placed on a microscope slide and pictures were taken with a numeric microscope VHX-2000 (Keyence). Pupal length and width were measured with Image J software and pupal volume was calculated using the following formula for ellipsoid: $V= [(4/3) \times 3,14 \times (L/2) \times (l/2)^2]$ where V is the volume, L the length and l the width of the pupa.

Larval feeding behavior and Food intake

Quantification of larval food intake was performed according to (88) with the following modifications: we deposited a mix of 50% yeast/0.75% of the food blue dye Erioglaucin (ref 861146, Sigma) in the center of a Petri dish lid (diameter: 35mm) to feed 20 mid-third larvae (previously infected or not) *ad libitum*. After 2h at 25°C, larvae were collected and washed with water. The number of white larvae (indicating no feeding) and blue larvae was counted. Ten blue larvae were transferred into a microtube containing 500 µL of PBS, crushed at 50Hz for 2 minutes with a TissueLyser (Qiagen, ref 85600) and centrifuged at 10000g for 5 minutes

at 4°C. The amount of Erioglaucin in the supernatant was quantified at 629 nm with a spectrophotometer (SpectraMax® Plus384, Molecular device).

Midgut permeability

The same technique as described above was used except that the mix contained 50% yeast and 1.25 mg/ml Dextran FITC (ref 46944, Sigma) in water. Twenty previously orally infected mid-third instar larvae were placed in the mix. After 2h at 25°C, the larvae were collected, washed with water and placed at least 30 minutes at 4°C. Pictures were taken with a fluorescent stereoscopic AxioZoom V16 (Zeiss) microscope.

Metabolic assay

Intestinal protease activity assay was performed as described in (89) with 8 mid-third instar larval midguts per condition. Lipids were assayed as described in (48). The tri- and diacylglycerol (TAG and DAG) were measured on 3µl of hemolymph obtained from 5 days old larvae using the Triglyceride Reagent (ref T2449, Sigma) and Free Glycerol Reagent (ref F6428, Sigma). The metabolite quantity was reported per mg of protein or per µL of hemolymph. Protein assay was achieved with Biorad Protein Assay Dye Reagent Concentrate (ref 5000006, Biorad). All experiments were performed at least 3 times in triplicates.

Lipids labelling

Five days after infection (mid-third instar larvae), ten larval guts were dissected in PBS and fixed 40 min in 4% formaldehyde in PBS. Guts were then washed twice in PBS and further incubated for 30 min. either in 5µg/ml of BODIPY™ 493/503 (ref D3922, Invitrogen™ Molecular Probes™) or in a 0.06% Oil-red-O solution (ref 189400250, Acros Organics). Guts were then washed twice with PBS. For BODIPY™ labelling guts were mounted in Fluoroshield/DAPI (ref F6057, Sigma) and immediately observed under a fluorescent microscope (Zeiss Axioplan Z1 with Apotome 2). For Oil-red-O labelling, guts were mounted in 80% Glycerol/PBS and observed with a numeric microscope (VHX 2000, Keyence).

Immunohistochemistry, image capture and processing

Ten guts of late third instar larvae were dissected in PBS and fixed for 40 min. in 4% formaldehyde in PBS. Guts were then washed twice with PBS-0.1% Triton X100, incubated for 3h in Blotto (2.5% FBS, 0.1% Triton X-100, 0.02% sodium azide in PBS) and further incubated with the primary antibody overnight at 4°C in Blotto. The following dilutions were used: Mouse anti-Armadillo (DSHB #N2 7A1) at 1/50; Rabbit anti-Phospho-histone-3 (ref 9701S, Cell signaling) at 1/500. Guts were washed once with Blotto before incubation in the secondary antibody and/or phalloidin (2h at room temperature in Blotto). The secondary antibodies were the followings: AlexaFluor™ 546 donkey anti-Rabbit IgG (ref A10040, Invitrogen) used at 1:500; AlexaFluor™ 647 goat anti-Mouse IgG (H+L) (ref A21235, Invitrogen) used at 1/500; Alexa Fluor™ 555 Phalloidin (ref A34055, Invitrogen) used at 1/1000. Guts were finally washed several times in PBS, mounted in Fluoroshield/DAPI (ref F6057, Sigma) and observed with a Zeiss Axioplan Z1 with Apotome 2 microscope. Images were analyzed using ZEN (Zeiss) software or Photoshop. For cell death monitoring (in *myo1A-Gal4 UAS- UAS-GC3Ai*^{G7S} *larvae*), guts were dissected in PBS, immediately mounted in 80% Schneider medium/20% glycerol and pictures were rapidly taken with a fluorescent stereoscopic microscope (AxioZoom V16, Zeiss). Image acquisition was performed at the Microscopy platform of the Institut Sophia Agrobiotech (INRAE 1355-UCA-CNRS 7254-Sophia Antipolis).

Polyploidy estimation

Ploidy was calculated by measuring the area of the nuclei of late L3 larvae. Only round nuclei were analyzed. The average nucleus area of diploid adult midgut precursor cells was calculated. This average area (8µm²) was assigned to the 2C ploidy (C corresponding to chromatin copy number). The theoretical average area of 4C, 8C, 16C, 32C and 64C nuclei was calculated on the basis of the measured 2C average area. 109 nuclei for the control larvae, 184 nuclei for *Btk*$^{\Delta Cry}$ 10X larvae and 306 nuclei for Dipel 10X larvae were counted and binned into ploidy groups.

Statistics

When “n” was equal or superior to 30, statistical analysis was performed using a parametric T-test. An F-test was systematically done before applying the T-test to verify the homogeneity of variances. When “n” was inferior to 30, we used the non-parametric pairwise comparisons of the Wilcoxon-Mann-Whitney test. Significance symbols are the following: *** (P<=0.001); ** (P<=0.01), * (P<=0.05).

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Table 1

Time in hours when 10%, 50% and 90% larvae enter the pupation stage (immobilized) expressed in hours after egg laying. "n" corresponds to the number of larvae counted in each condition. Note that we compiled the controls per year of experiments (encompassing years from 2017 to 2019). The design of our experimental procedures allowed obtaining highly reproducible pupation curves from one year to another one.

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

Delfin 10X Axenic	142.0	159.0	182.0	172

Figure legends

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

(**A**) Pupation curve of larvae fed with commercial products (Delfin and Dipel) or with a homemade preparation of spores of the *Btk* strain ABTS-351 (Dipel). In this figure and in all the following figures, the cumulative percentage of pupation is shown over time. (**B**) Pupation curve of larvae fed with a *Btk* strain devoid of Cry toxins ($Btk^{\Delta Cry}$) or with Cry toxin containing crystals purified from Dipel or with Naphthalene sulfonic acid at a dose equivalent to the amount found in Delfin 10X. In A and B, red dashed lines mark 90% of the population reaching pupation. (**C**) Pupae volume in the different conditions listed on the abscissa. n= number of individual counted. For the controls, n correspond to the total number of pupae measured when one compiled all the internal controls. Errors bars represent the Standard Deviation of the Mean (SEM). *=P<0.05; ***=P<0.001 compared with controls.

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

(**A**) L3 midgut load of larvae fed with either spores of $Btk^{\Delta Cry}$ or Delfin. (**B** and **C**) Percentage of L3 (B) and L2 (C) larvae presenting blue dyed food in the intestine. (**D** and **E**) Amount of dye ingested by L3 (D) and L2 (E) larvae. Green bars: control larvae (fed with water); light blue bars: 1X dose of $Btk^{\Delta Cry}$ spores; dark blue bars: 10X dose of $Btk^{\Delta Cry}$ spores; yellow bars: 1X dose of Delfin; Red bars: 10X dose of Delfin. n= number of individuals analyzed. Errors bars represent the SEM. *=P<0.05, **=P<0.01 compared with controls.

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

(**A**) Measurement of the protease activity of the intestine of L3 larvae raised on control medium contaminated with H2O (control) or with 10X doses of spores of *Btk*$^{\Delta Cry}$ or Delfin or 1X dose of spores of Delfin. (**B**) Pupation curve of larvae raised on a protein rich medium (10% yeast instead of 2%) contaminated with 1X or 10X doses of Delfin. Note the shortening of the developmental time of the control larvae raised on the protein-rich medium (10% yeast) compared to larvae raised on the protein poor-medium (2% yeast). (**C**) Pupae volume derived from larvae raised on the protein-rich medium and in the different conditions listed on the abscissa.

Figure 4: *Btk* bioinsecticides interaction with commensal flora

(**A**) Pupation curve of larvae reared on a medium complemented with 10^6 CFU of *L plantarum* and contaminated with Delfin 10X (pink) or not (purple) compared to control larvae (green) or Delfin 10X only (red). (**B**) Pupae volume derived from larvae raised on a medium complemented with 10^6 CFU of *L plantarum* and contaminated with Delfin 10X (pink) or not (purple) compared to control pupae reared on control medium (green) or medium contaminated with Delfin 10X only (red). (**C**) *Btk* load in the midgut of L3 larvae reared on a medium contaminated with Delfin 10X and complemented (pink) or not (red) with 10^6 CFU of *L plantarum*. (**D**) Commensal bacterial load in the midgut of L3 larvae of our *Drosophila* Canton S strain reared on our conventional medium (left bar) or reared in axenic condition (right bar). The *Commensalibacter intestini* and *Acetobacter pomorum* grow on mannitol plate (orange), *L plantarum* and *L brevis* grow on MRS plate (grey), *Enterococcus faecalis* grows on BHI plate (green) and *Escherichia coli and Staphylococcus aureus* grow on LB plate (blue). (**E**) Pupation curve of axenic larvae raised on a medium contaminated with Delfin 10X (dark blue) or not (light blue, control Ax) compared to larvae conventionally reared (CR, green). (**F**) Pupae volume derived from conventionally reared larvae (green) or

from axenic larvae raised on a sterile medium contaminated with Delfin 10X (dark blue) or not (light blue).

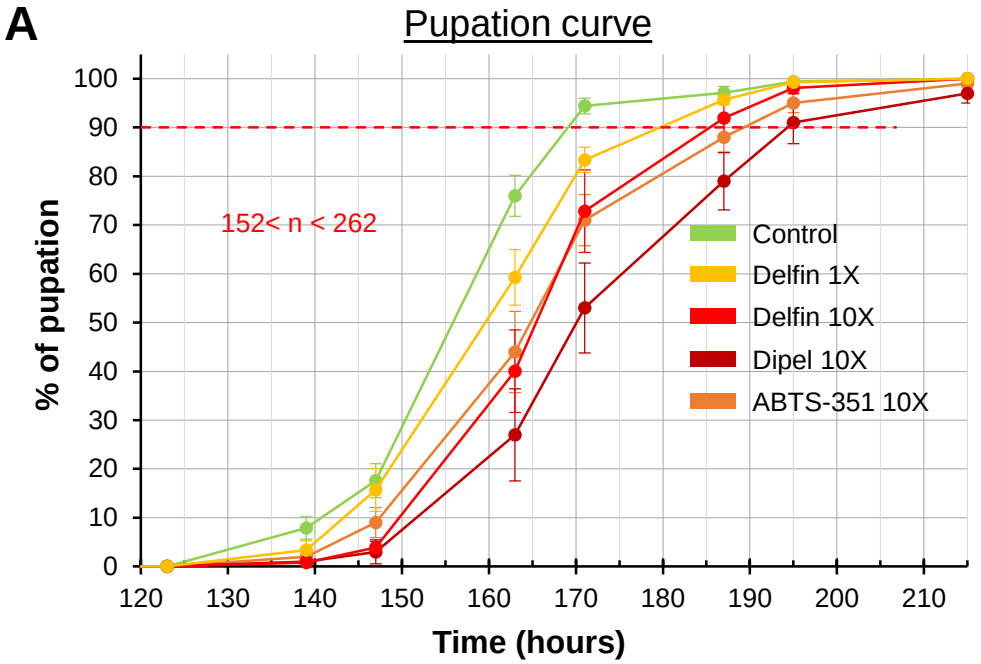
Figure 5: *Btk* bioinsecticides impacts on gut integrity

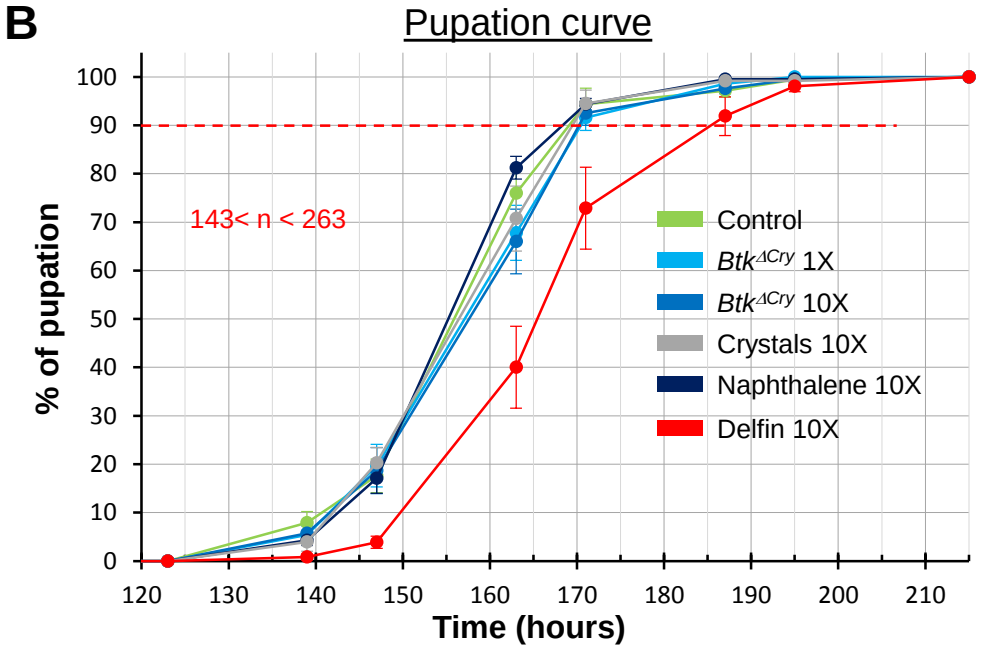
L3 larvae raised on a control medium (**A-B'**) or a medium contaminated with $Btk^{\Delta Cry}$ 10X (**C-D'**), Delfin 10X (**E-F**), purified Crystals 10X (**G-H'**), naphthalene 10X (**I-J'**). (**A-J'**) Labelling of lipid droplets with ORO (red, A, C, E, G and I) or with Bodipy (green, B, B', D, D', F, F', H, H', J and J'). DAPI staining in blue marks the nuclei. Midguts are oriented anterior to the left. Red brackets delineate the acidic region of the midgut. (B',D', F', H' and J') are x/z section with the apical pole of the epithelium facing the lumen. (**K**) Stored (TAG, upper panel) and circulating (DAG, lower panel) lipid dosages in control larvae (green bars) and in larvae raised on Delfin 10X contaminated medium (red bars). (**L-N**) *myo1A>Casp::GFP* larvae raised on control a medium (L) or on a medium contaminated with $Btk^{\Delta Cry}$ 10X (M) or Delfin 10X (N). Arrows point enterocytes in which Caspase 3 is activated. (**O-P**) L3 larvae raised on a medium complemented with $Dextran^{FITC}$ (O) or with $Dextran^{FITC}$ and contaminated with *P. entomophila* (P), with $Btk^{\Delta Cry}$ 10X (Q) or Delfin 10X (R).

Figure 6: Larval midgut regeneration upon aggression

(**A-C'**) Immunolabelling of *dlg::GFP* L3 midgut from larvae raised on control medium (A and A'), on medium contaminated with $Btk^{\Delta Cry}$ 10X (B and B') or Delfin 10X (C and C'). Midguts were stained with the adherens junction marker Armadillo (Arm, purple) that strongly delineates the Adult Midgut Precursors (AMP) (A, B and C) and marks the basolateral compartment of the enterocytes (A', B' and C'). DAPI (blue) marks the nuclei and Phalloidin (red) labels the visceral muscle surrounding the intestine and the apical pole of the enterocytes facing the lumen (in A', B' and C'). Dlg::GFP (green) marks the sub apical compartment of the enterocytes. (**D-H**) L3 midgut from larvae raised on control medium

(green bars), on medium contaminated with *Btk*$^{\Delta Cry}$ 10X (dark blue bars) or Delfin 10X (red bars). (**D**) Number of AMPs per islets, (**E**) number of dividing AMPs per intestine (mitotic AMPs are labeled by the Phospho-Histone 3 (PH3) immunostaining), (**F**) Number of AMP islets (an islet contains more than one diploid cell) per unit of surface, (**G**) enterocyte area and (**H**) epithelium thickness in the posterior midgut (3 independent measures were taken by image capture). (**I** and enlargement in **I'**) *dlg::GFP* L3 midgut from larvae raised on medium contaminated with Delfin 10X and stained for Arm (purple) and DAPI (blue). Arrows point the cells with intermediate size (smallest than enterocytes but biggest than AMPs). (**J**) Assessment of enterocyte ploidy in posterior L3 midgut from larvae raised on control medium, on medium contaminated with *Btk*$^{\Delta Cry}$ 10X or Delfin 10X (C corresponding to chromatin copy number). (**K-M**) Lineage tracing of AMP progeny in the *w; esg-Gal4, UAS-GFP/+; UAS-H2B::RFP, tub-Gal80*^{ts}*/+* (esg REDDM) L3 midgut from larvae raised on control medium (K), on medium contaminated with *Btk*$^{\Delta Cry}$ 10X (L) or Delfin 10X (M). DAPI (blue) marks the nuclei. **=P<0.01, ***=P<0.001 compared with controls.


A
Pupation curve
% of pupation
Time (hours)
152< n < 262
Control
Delfin 1X
Delfin 10X
Dipel 10X
ABTS-351 10X


B
Pupation curve
% of pupation
Time (hours)
143< n < 263
Control
Btk^ΔCry 1X
Btk^ΔCry 10X
Crystals 10X
Naphthalene 10X
Delfin 10X

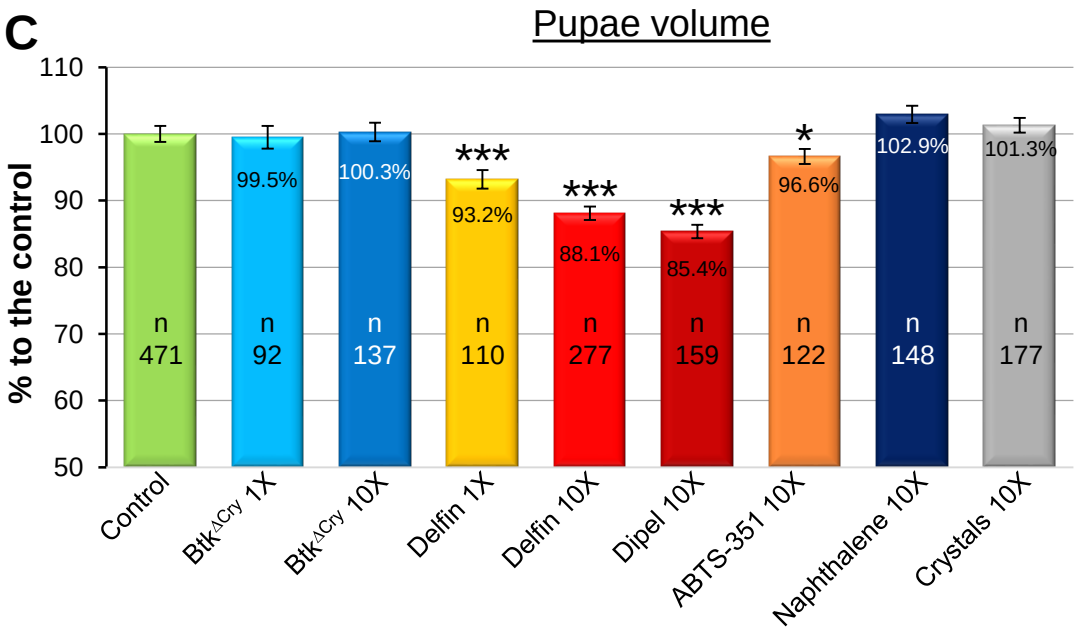

C
Pupae volume
% to the control
99.5%
100.3%
93.2%
88.1%
85.4%
96.6%
102.9%
101.3%
n 471
n 92
n 137
n 110
n 277
n 159
n 122
n 148
n 177
Control
Btk^ΔCry 1X
Btk^ΔCry 10X
Delfin 1X
Delfin 10X
Dipel 10X
ABTS-351 10X
Naphthalene 10X
Crystals 10X

Figure 1

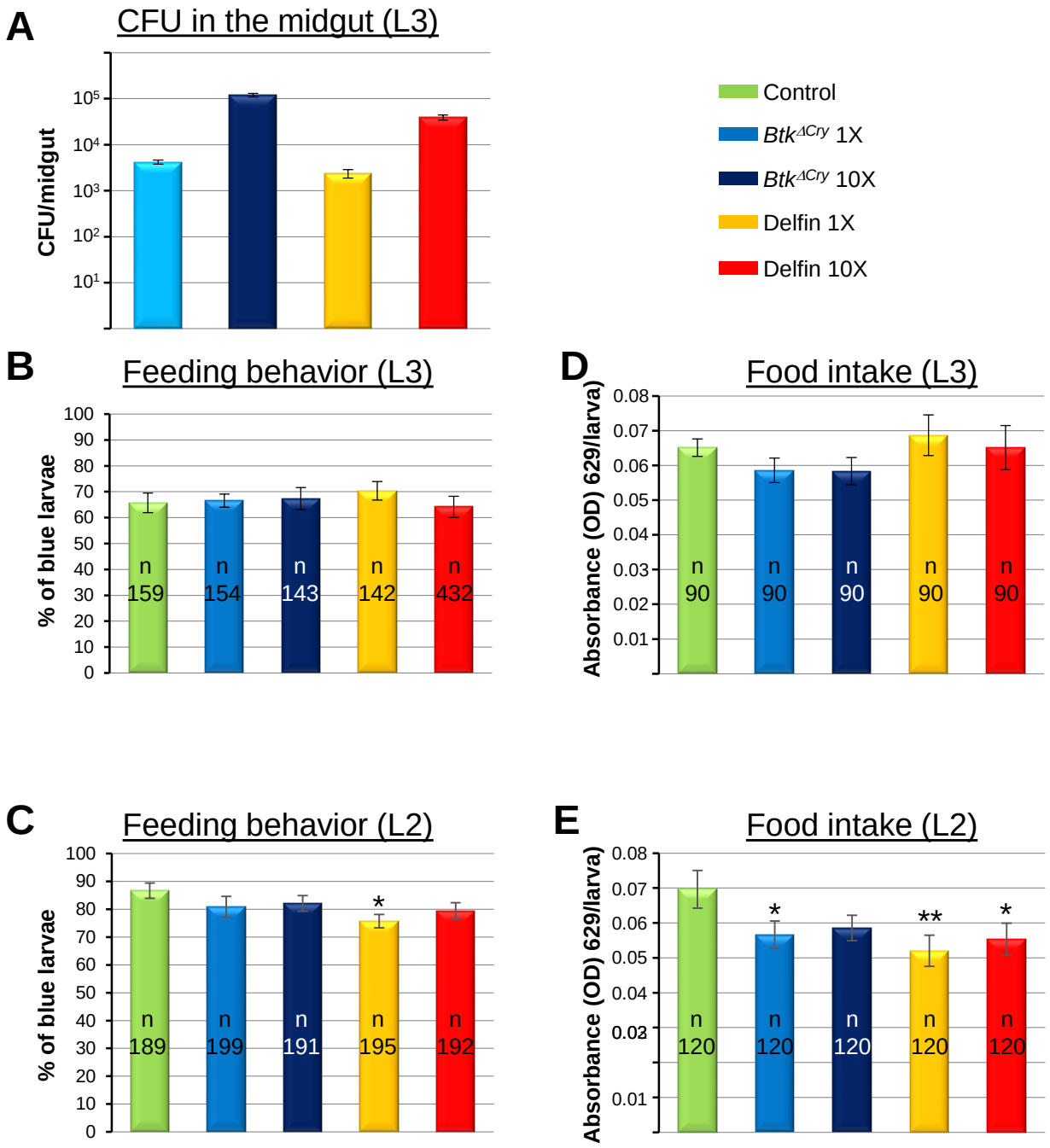

A
CFU in the midgut (L3)
CFU/midgut
10^5
10^4
10^3
10^2
10^1
Control
Btk$^{\Delta Cry}$ 1X
Btk$^{\Delta Cry}$ 10X
Delfin 1X
Delfin 10X
B
Feeding behavior (L3)
% of blue larvae
100
90
80
70
60
50
40
30
20
10
0
n 159
n 154
n 143
n 142
n 432
D
Food intake (L3)
Absorbance (OD) 629/larva)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
n 90
n 90
n 90
n 90
n 90
C
Feeding behavior (L2)
% of blue larvae
100
90
80
70
60
50
40
30
20
10
0
*
n 189
n 199
n 191
n 195
n 192
E
Food intake (L2)
Absorbance (OD) 629/larva)
0.08
0.07
0.06
0.05
0.04
0.03
0.01
*
**
*
n 120
n 120
n 120
n 120
n 120

Figure 2

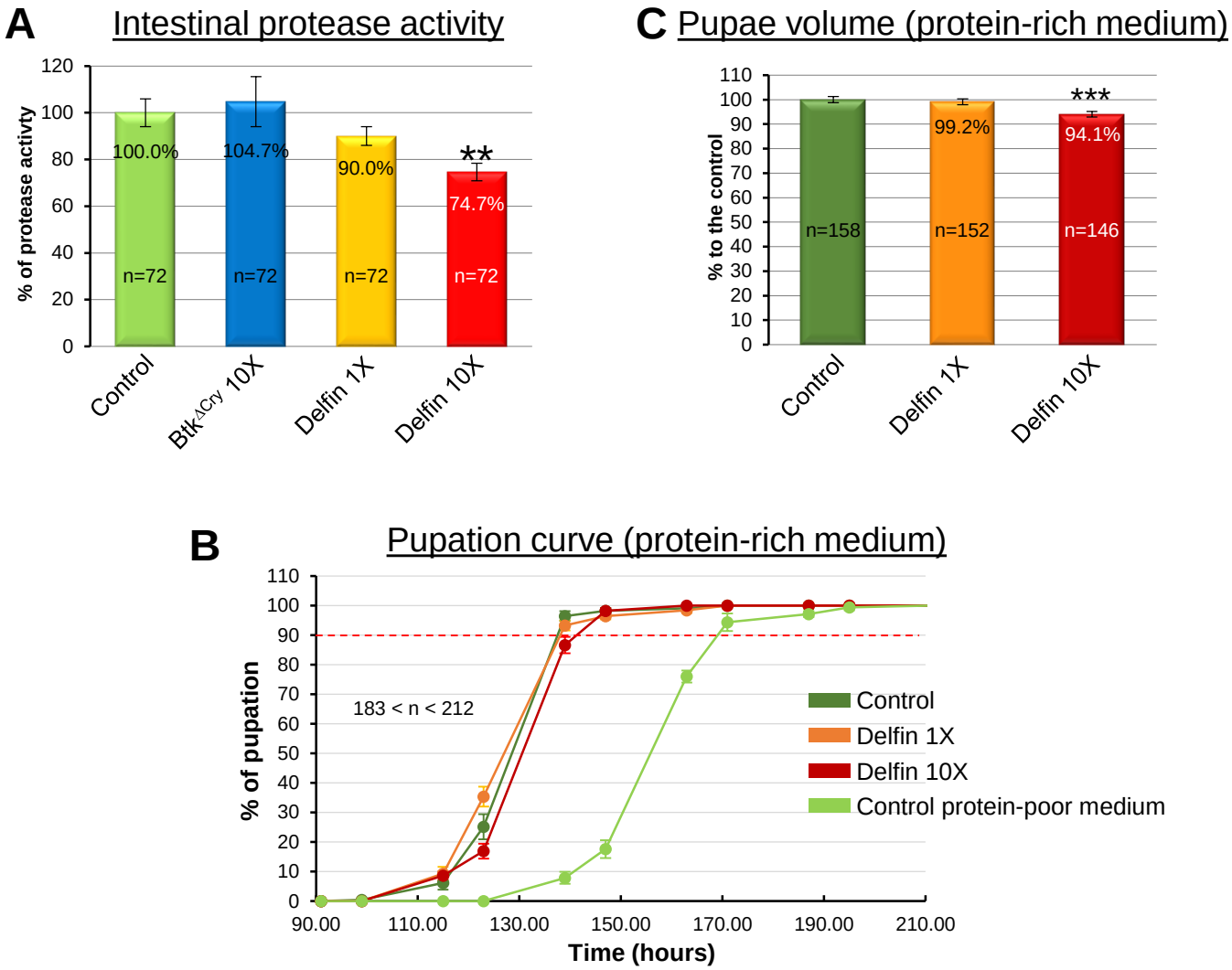

A
Intestinal protease activity
% of protease activty
120
100
80
60
40
20
0
100.0%
104.7%
90.0%
**
74.7%
n=72
n=72
n=72
n=72
Control
BtkΔCy 10X
Delfin 1X
Delfin 10X
C
Pupae volume (protein-rich medium)
% to the control
110
100
90
80
70
60
50
40
30
20
10
0
***
99.2%
94.1%
n=158
n=152
n=146
Control
Delfin 1X
Delfin 10X
B
Pupation curve (protein-rich medium)
% of pupation
110
100
90
80
70
60
50
40
30
20
10
0
183 < n < 212
Control
Delfin 1X
Delfin 10X
Control protein-poor medium
90.00
110.00
130.00
150.00
170.00
190.00
210.00
Time (hours)

Figure 3

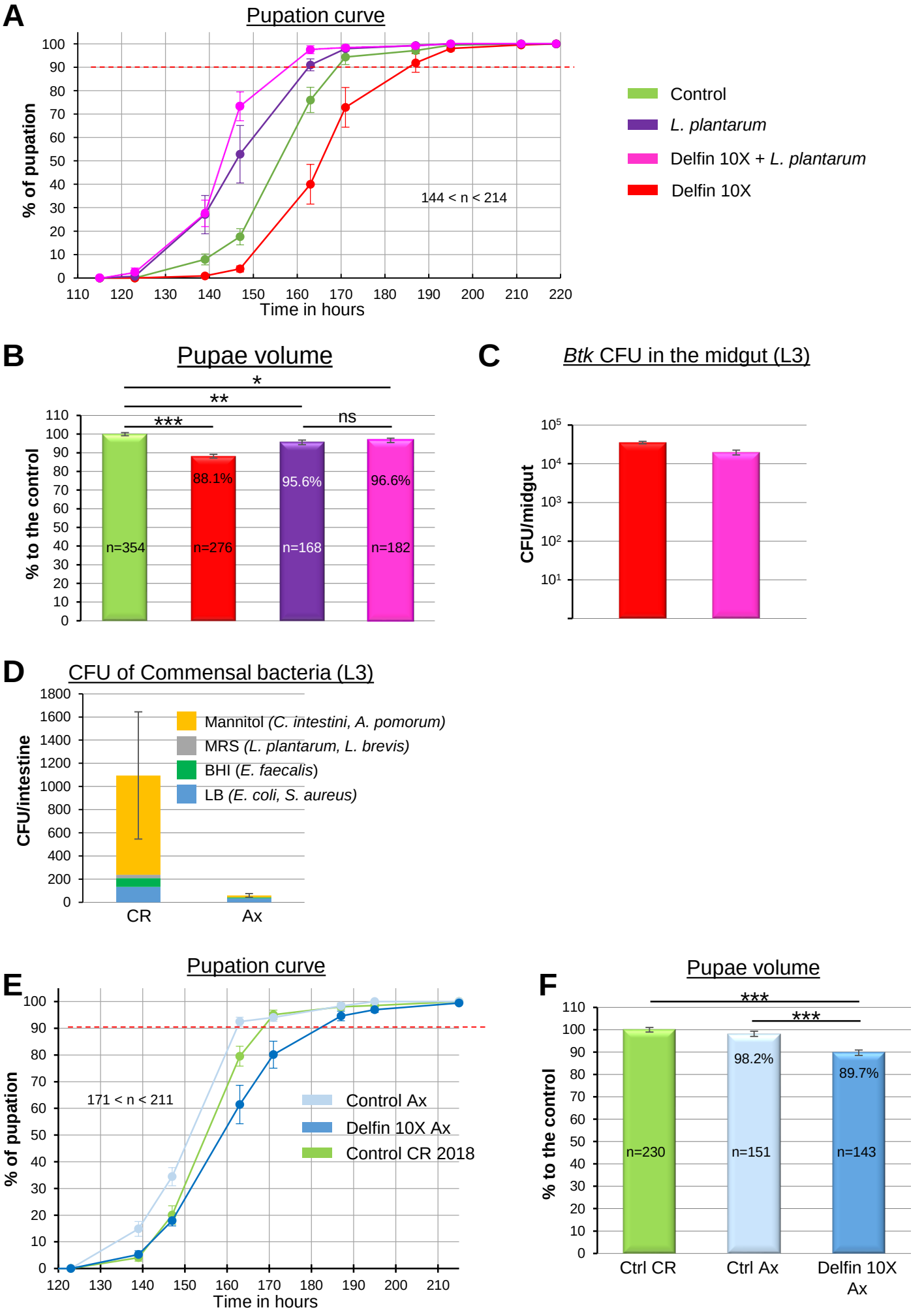

A
Pupation curve
% of pupation
Time in hours
144 < n < 214
Control
L. plantarum
Delfin 10X + L. plantarum
Delfin 10X
B
Pupae volume
% to the control
*
**
***
ns
88.1%
95.6%
96.6%
n=354
n=276
n=168
n=182
C
Btk CFU in the midgut (L3)
CFU/midgut
D
CFU of Commensal bacteria (L3)
CFU/intestine
Mannitol (C. intestini, A. pomorum)
MRS (L. plantarum, L. brevis)
BHI (E. faecalis)
LB (E. coli, S. aureus)
CR
Ax
E
Pupation curve
% of pupation
Time in hours
171 < n < 211
Control Ax
Delfin 10X Ax
Control CR 2018
F
Pupae volume
% to the control
***
***
98.2%
89.7%
n=230
n=151
n=143
Ctrl CR
Ctrl Ax
Delfin 10X Ax

Figure 4

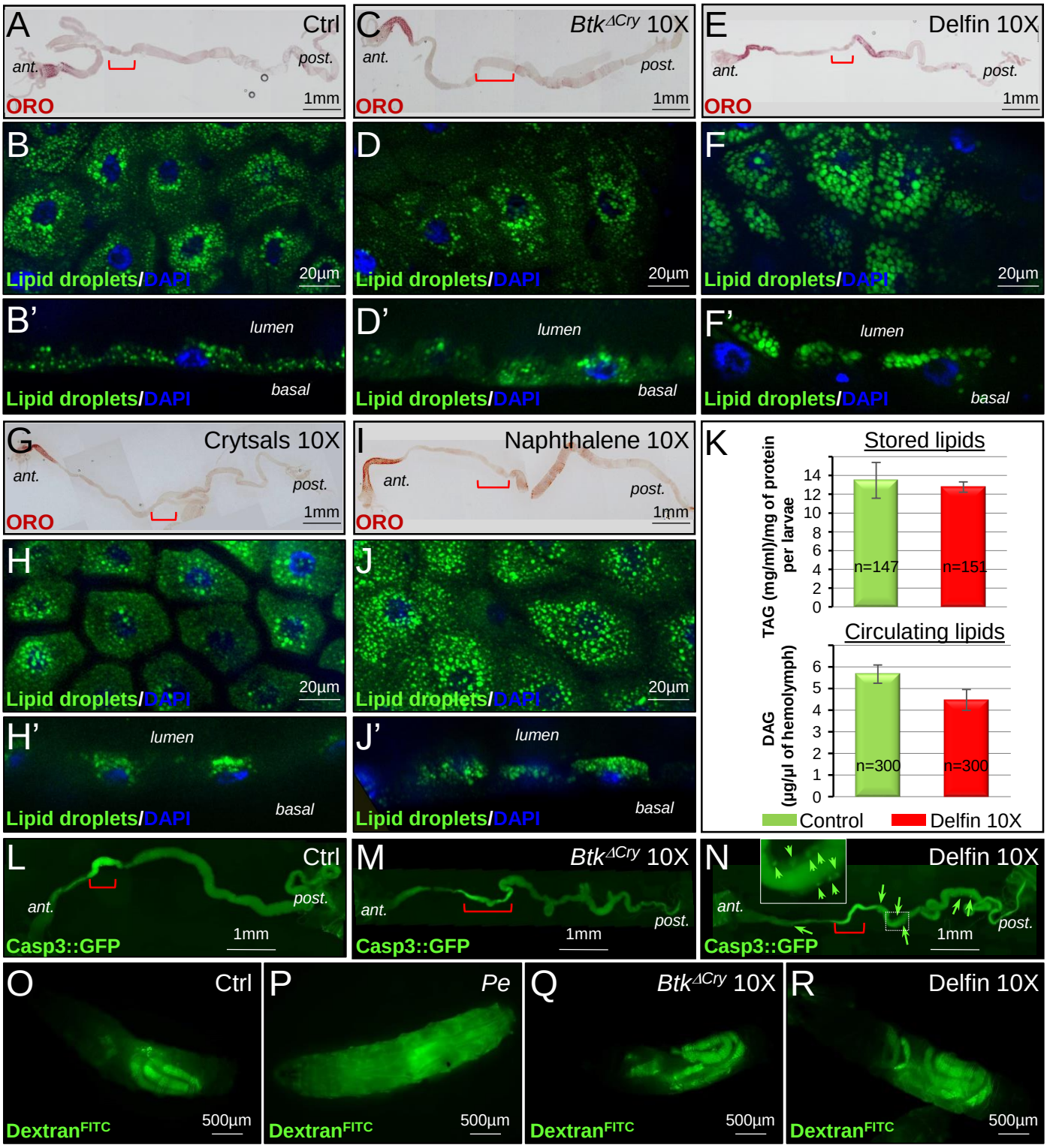

A
Ctrl
ant.
post.
ORO
1mm
C
Btk ΔCry 10X
ant.
post.
ORO
1mm
E
Delfin 10X
ant.
post.
ORO
1mm
B
Lipid droplets/DAPI
20µm
D
Lipid droplets/DAPI
20µm
F
Lipid droplets/DAPI
20µm
B'
lumen
basal
Lipid droplets/DAPI
D'
lumen
basal
Lipid droplets/DAPI
F'
lumen
basal
Lipid droplets/DAPI
G
Crytsals 10X
ant.
post.
ORO
1mm
I
Naphthalene 10X
ant.
post.
ORO
1mm
K
Stored lipids
TAG (mg/ml)/mg of protein per larvae
n=147
n=151
Circulating lipids
DAG (µg/µl of hemolymph)
n=300
n=300
Control
Delfin 10X
H
Lipid droplets/DAPI
20µm
J
Lipid droplets/DAPI
20µm
H'
lumen
basal
Lipid droplets/DAPI
J'
lumen
basal
Lipid droplets/DAPI
L
Ctrl
ant.
post.
Casp3::GFP
1mm
M
Btk ΔCry 10X
ant.
post.
Casp3::GFP
1mm
N
Delfin 10X
ant.
post.
Casp3::GFP
1mm
O
Ctrl
Dextran FITC
500µm
P
Pe
Dextran FITC
500µm
Q
Btk ΔCry 10X
Dextran FITC
500µm
R
Delfin 10X
Dextran FITC
500µm

Figure 5

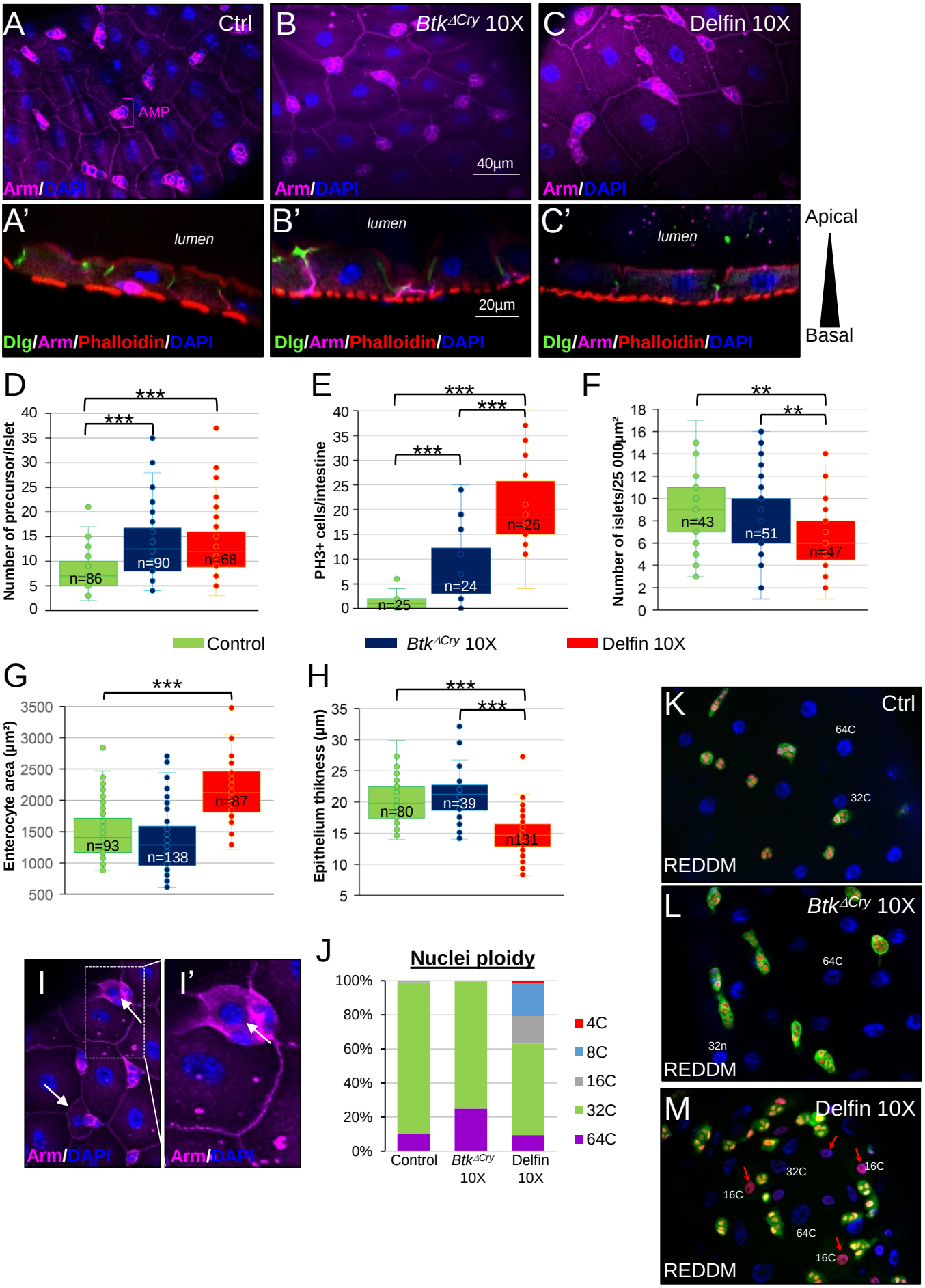

A
Ctrl
AMP
Arm/DAPI
B
Btk⊿Cry 10X
40µm
Arm/DAPI
C
Delfin 10X
Arm/DAPI
A'
lumen
Dlg/Arm/Phalloidin/DAPI
B'
lumen
20µm
Dlg/Arm/Phalloidin/DAPI
C'
lumen
Dlg/Arm/Phalloidin/DAPI
Apical
Basal
D
Number of precursor/Islet
n=86
n=90
n=68
E
PH3+ cells/intestine
n=25
n=24
n=26
F
Number of islets/25 000µm²
n=43
n=51
n=47
Control
Btk⊿Cry 10X
Delfin 10X
G
Enterocyte area (µm²)
n=93
n=138
n=87
H
Epithelium thikness (µm)
n=80
n=39
n131
I
I'
Arm/DAPI
J
Nuclei ploidy
Control
Btk⊿Cry 10X
Delfin 10X
4C
8C
16C
32C
64C
K
Ctrl
64C
32C
REDDM
L
Btk⊿Cry 10X
64C
32n
REDDM
M
Delfin 10X
32C
16C
16C
64C
16C
REDDM

Figure 6