Apidologie **Online Resource 1**

Observations on midgut of *Apis mellifera* workers (Hymenoptera: Apoidea) under controlled acute exposures to a *Bacillus thuringiensis*-based biopesticide

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Additional details regarding Materials and Methods

Bioassay and symptoms observation

The caught bees had a 2-days adaptive period to the laboratory conditions, in previously prepared aired cages (Han et al. 2010), before being used for the assay; during this period they didn't show evident symptoms of possible infectious diseases (e.g., varroasis, nosemiasis and virosis) or pesticide poisoning.

The biopesticides tested is specifically suggested to be used on the grapevine plantation to control the moths *Lobesia botrana* Denis and Schiffermüller, 1775 and *Eupoecilia ambiguella* Hübner, 1796.

Low and very-high concentrations have been found, respectively, by dividing and multiplying "field concentration" in a geometric progression (OECD guidelines 1998).

In each of the three repetitions, before the exposure to the biopesticide, the 10 bees of each group were kept without food for 2 hrs, so that all bees are equal in terms of their gut contents at the start of the test (OECD guidelines 1998).

The 10 µl dose (with or without the tested product) was administered to each specimen only once in each of the three repetitions, according to the "natural" or "artificial flower" method (Ladurner et al. 2003, 2005a). The complete consumption is according a no-choice dietary feeding protocol that should guarantee the highest exposure level to bees (Han et al. 2010). Percentage ratio of food consumption was measured by weighing the group feeder before being inserted into the cage and after the different times of observations.

The bees were considered dead when motionless for at least 10 seconds (Iwasa et al. 2004) and then rapidly removed from the cages.

All the specimens from each of the four groups (including the control one) were similarly exposed to the above-mentioned treatments, during each of the three replications of the bioassay and of the separate trial replication.

SEM, LM and TEM observations

Observations were carried out on specimens, still alive, from each of the four groups of the abovementioned separate trial replication.

Since the very high concentration would be likely unrealistic in natural conditions, it represented mainly a basis for comparison of the toxic effects detected in lower concentrations. The photographic documentation of our LM, SEM and TEM observations has been aimed to show possible differences in the midgut features among the four groups; so, when the observed features were similar among one or more groups, we opted to supply only the most descriptive photos.

Additional Results

4 hrs after the single ingestion of the doses of the biopesticide solution, in all the specimens from all the groups, midgut epithelium shows internal folds and the peritrophic membrane (PM), consisting of a dense fibrillar meshwork, uniformly covers the microvilli of the epithelial surface (Supplemental Figures 1B and C). LM observations of the epithelium (of pseudostratified type) show: *regenerative cells*, mainly organized in small groups at the bottom of the cups of the epithelial folds and not reaching the organ lumen; *digestive cells* (or *enterocytes*), higher than regenerative cells, reaching the organ lumen. The enterocytes, with long and packed microvilli, have a cytoplasm rich in vesicles, some of which containing a spheroidal granule; these cells also show signs of a more or less marked apocrine (Jimenez and Gilliam 1990) secretory activity

48, 72 and 96 hrs after the ingestion, the epithelial changes are markedly widespread in all the specimens treated with very-high concentration, if compared to 24 hrs after treatment. 96 hrs after the treatment, in particular, the midgut epithelium shows numerous enterocytes with almost entirely vacuolated cytoplasm and irregular protrusions; the apical plasma membrane of many enterocytes is open in some tracts; in several midgut areas, moreover, the epithelium does not show its typical folds (Supplemental Figures 3A and B).

TEM observations, up to 4 hrs after the trial start, show that all the specimens from the three groups have epithelial cells with: voluminous ovoid nuclei, homogeneously dispersed chromatin and few small chromatin clumps; cytoplasm with numerous vesicles of various sizes and heterogeneous content, mainly gathered in the apical cytoplasm; many vesicles containing a spheroidal granule consisting of a thin electron-dense wall and a low elctron-dense inner material; long and packed microvilli in contact, through their distal portion, with the PM fibrils, regularly arranged in undulating course (Figure 4A).

After 24 hrs, in all the specimens from very-high concentration group, alterations are more frequently observed and dramatically worsen. 96 hrs after treatment, in particular, numerous midgut areas have enterocytes showing their cytoplasm almost entirely devoid of organelles and swollen nuclei, containing only few large chromatin clumps; also the nucleus of the regenerative cells is frequently dilated, with dispersed chromatin (Supplemental Figure 3C). The introflections of the basal plasma membrane of the enterocytes are strongly reduced and the basal lamina is about 0.3 μm thick; in addition, some muscle cells with pyknotic nuclei are observed (Supplemental Figure 3D). Small vesicles with electron-transparent content are frequently gathered in their apical cytoplasm. Their cell surface, almost devoid of microvilli, is in contact with the PM fibrils, irregularly arranged in numerous midgut areas (Supplemental Figure 3E). Some enterocytes show strongly electron-dense cytoplasmic areas, apical blebs and very packed microvilli, irregularly arranged (Supplemental Figure 3F).

Additional details regarding Discussion

It should be noticed that the observed temporal trend in the worsening of the symptoms in the very-high concentration group, including a certain increase in abdomen volume of the bees and watery stools onto the walls of their cages (personal observations), is consistent with findings of previous studies on other insects (Trona et al. 2004; Brighenti et al. 2007; Ruiu et al. 2012).

The steady mortality rate of 0% and the lack of evident symptoms in almost all of the bees from low concentration group clearly indicate that single exposure to this biopesticide concentration doesn't affect both survival and behavior of the workers.

In accordance with previous studies on other species of insects (Ruiu et al. 2012), our LM, SEM and TEM analyses show that midgut changes in the *A. mellifera* workers are found only 24 hrs after the single ingestion of the biopesticide solutions, in all the specimens from all the three treated groups. Furthermore, in agreement with data on species of other insect orders treated with various

bacterial strains, including *Bt* (Singh et al. 1986a, 1986b; Trona et al. 2004; Ruiu et al. 2012), the muscular-connective changes, especially observed in very-high concentration group (personal observations), cannot be ruled out that are additional factors leading to midgut peristalsis cessation and body paralysis.

In conclusion, although biopesticides represent a category of products certainly less stressful for the environment than other agrochemicals (Gupta and Dikshit 2010). in the light of some of our observations, we deem useful to remind that evaluations of the registration procedures and monitoring of any product must be always based on a variety of parameters. Moreover, possible long-term/chronic toxicity has to be taken into account, in order to achieve greater effects on insect pests and the least damage on beneficial insects, as *A. mellifera*.