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EXPOSURE OF HONEYBEES (*APIS MELLIFERA* L.) AND OTHER HYMENOPTERA POLLINATORS TO DIFFERENT *BACILLUS THURINGIENSIS* BASED BIOPESTICIDES IN LABORATORY CONTROLLED TRIALS

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ABSTRACT

Apoidea play a fundamental and strategic role in the regulation of reproductive processes of many plants, including those of agricultural interest, due to their pollination activities. In Italy, Apoidea important for agriculture include: *Apis mellifera* Linnaeus 1758, known eusocial species with organization into castes (Tepedino, 1981; Vicens and Bosch, 2000), *Osmia cornuta*, Latreille 1805 and *O. rufa* Linnaeus 1758, two species of solitary bees that nest in gregarious form, profitable and easy to use on different crops in semi-field and open field (Accorti, 1988). *O. cornuta* is distributed in central and southern Europe, Turkey and parts of North Africa and the Middle East (Peters, 1977) and *O. rufa* can be found also in northern Europe.

However, populations of many species of Apoidea are in decline, and this phenomenon seems to be also related to the increase of the use of chemicals in agricultural practices (Oldroyd, 2007; vanEngelsdorp et al., 2009; Hamdi et al., 2011).

This decline not only representing a clear loss of biodiversity, but it has also a sensitive impact on productivity of agro-ecosystems, bringing serious economic damage to agricultural production (Porrini et al., 2003; Biesmeijer et al., 2006; Goulson et al., 2008; Gallai et al., 2009).

Biopesticides are a class of natural products used in agriculture to limit the damage caused by harmful organisms and include commercial formulations with microorganisms, entomopathogenic

nematodes, natural and semiochemichal pheromones of insects and plant extracts (Copping and Menn, 2000; Warrior, 2006).

Biopesticides should be less harmful for the environment compared to traditional chemical pesticides, because they are effective at very low concentrations and have a rapid inactivation; furthermore, these products have a lower risk of non-specificity, acting mainly on the larval stages of some species of pests (Gupta and Dikshit, 2010). In the context of the Integrated Pest Management (IPM) (Dent, 1985), biopesticides are valuable feedback associated with products of synthesis bringing benefits to both the agricultural production and in integrated control programs of phytoparasites.

Bacillus thuringiensis-based biopesticides are often used in IPM and they are dispersed in the environment (George and Crickmore, 2012). *Bacillus thuringiensis* (*Bt*) B. is one of the most common microorganisms which toxicity is due to the production of parasporal crystals exhibiting a highly specific insecticidal activity during sporulation of the bacterium (Höfte and Whiteley, 1989). These toxic crystals (*Cry*), consist of different protein families (*insecticidal crystal proteins* or ICPs) not closely related, that are released into the environment (Lambert and Peferoen, 1992) and, once arrived in the gut of the organism by via trophic, act specifically on the epithelium of the midgut. Since insects epithelial cells of midgut are involved in multiple processes such as digestion, absorption, formation of peritrophic membrane (Snodgrass, 1956), the alteration caused by the toxins of *Bt* would involve a functional impairment that could end with the death of the insect, according to the mechanisms of action

(Percy and Fast, 1983; Cavados et al., 2004).

There are different strains of the bacterium that produce several combinations of Cry toxin proteins, which are considered selective against insects of a specific order (Knowles, 1994; Gupta and Dikshit, 2010; Bravo et al., 1992).

However, the natural compounds are not necessarily safer than synthetic ones because their biological properties and activity are in function of the structure rather than the origin, the applied dose in certain situations, and in relation to the safety of the product utilization (Coats, 1994).

Bt-based products are considered harmless for species of Apoidea so far investigated, but it is believed that they could lead to sublethal effects (Coats, 1994; Han et al., 2010), thus reducing pollinator populations in the field. Despite the importance of information on this issue, investigations, are still limited (Mommaerts et al., 2010).

Given the crucial role played by both honeybees and solitary bees, not only for an ecological standpoint, but also for an agro-economic aspect, I thought it was interesting to undertake investigations leading to evaluation the toxic effects associated with the administration of various concentrations of three *Bt*-based formulations. For this purpose, I have focused my research on the effects of products at different levels: assessing the mortality of *O. cornuta*, *O. rufa* and *A. mellifera*, and, for the latter species, it was observed a possible behavior modification related to histological and ultrastructural impairment of the midgut epithelium of workers. Moreover, any biochemical alteration and gene expression was assessed in midgut of

workers after ingestion of one of biopesticides tested.

Overall, results confirm such low toxicity of the tested *Bt*-based biopesticides on workers of *A. mellifera* and *O. cornuta* and *O.rufa*, at the concentrations presumably found in field-environment conditions, confirming that the biopesticides represent a category of commercial products certainly more convenient for the environment and the human health than the other agrochemicals, although any possible long-term and chronic effects should be taking into account.

RESEARCH GOAL

Biopesticides are the most important and safe alternative to agrochemical, in particular *Bt*-based commercial formulation cause death due to septicemia in many species of susceptible insects.

Since Apoidea are beneficial arthropods playing a key role in pollinating wild and crop plants and despite the fact that *Bt*-biopesticides do not cause the death of the species of Apoidea so far investigated, the ingestion of them could represents a stress factor, from which bees must defend themselves and might affect or influence the fitness and the behavior of the individual and alter the normal microflora.

In my study I have conducted investigations aimed to the evaluation of the toxic effects following the administration of different concentrations of a *Bt*-based commercial formulation to honeybee specimens (*Apis mellifera* L.) that are generally considered as more sensitive to pesticides when compared to other bee species. The results obtained would be useful to make predictions for other species by extrapolation from the data on honeybees.

Since the insect midgut represents a target of *Bt* biopesticide, it has been detected possible midgut morpho-structural changes, by optical (OM) and electronic (SEM, TEM) microscopes, caused by *Bt* toxins, and their correlation with mortality/behavior in workers of *A. mellifera*. Furthermore, it has been evaluated the gene expression and biochemical alterations of some proteins that are potentially involved

in the response to the treatment of specimens with *Bt*-biopesticide. Moreover, I try to perform a preliminary investigation of midgut microflora of honeybee workers evaluating if a change more or less accentuated of the normal state of colonization could be occur after the ingestion of a *Bt*-based biopesticide.

The oral toxicity has been evaluated not only on *Apis mellifera* but also on two species of solitary bees, *Osmia cornuta* and *Osmia rufa*.

Finally, the goal of this investigation is to make a contribution, in the Integrated Pest Management (IPM) context, to understanding the impact of *Bt*-based biopesticides may have on the environment and, furthermore, it could be an introduction for further studies on *A. mellifera* and other Apoidea species, in order to limit the damage to this superfamily of insect of great agro-economic interest.

Chapter 1

BEES AND ENVIRONMENT

1.1 Introduction

Sexual reproduction in plants involves pollination, that is the transfer of pollen grains from the anthers of a flower to the stigma of the same flower or another flower of the same species. Then, a pollen tube is developed from the stigma and proceeds along the stylus until it reaches the ovule in the ovary where fertilization occurs. Pollen can be moved by the wind or by animal pollinators. The animals, in particular insects, largely pollinate plants with flower. It is presumed, that the success of the Angiosperms is linked precisely to the development of these relations with insects (Gullan and Cranston, 2006).

Apoidea are considered the most important group of pollinators. They collect nectar and pollen for both their offspring and own consumption. Globally, about 20.000 species of Apoidea are known and they are all antophilous. The considarable trophic specialization has as consequence an intimate and profound interaction between Apoidea and Angiosperms. Indeed, plants pollinated by Apoidea often have many morphological characteristics of the floral parts to vexillum function with very bright colors (yellow or blue), with sweet smell and petals with guidelines to nectar (often visible only in the ultraviolet) (Gullan and Cranston, 2006). Even, the eco-physiological

characters such as the synthesis of pheromones are capable of selectively attract pollinators and allow them collecting pollen to transport to other flowers as well as the nectar (Felicioli et al., 1998). In this regard, the chemical composition of the nectar varies greatly depending on the type of pollinator.

In addition, the pollinators are attracted, not only by the nectar, but also by pollen that is the vehicle through which it takes place the process of fecundation of the flower. The pollination is mediated primarily by female specimens that collect pollen as a protein source and to feed their larvae; males as well as females of the species that do not supply the nest, instead, bring with them the pollen almost involuntarily in the act to collect the nectar from the flowers that they visit and then play a less important role in pollination. An extremely diverse Apoidea fauna should have led to a general and widespread co-evolution with the different species of plants visited by them; indeed, many species gather nectar from the same flowers that provide their pollen. Most of the groups of Apoidea are defined generalists towards the plant species from which collect nectar, although they often prefer some groups that facilitate the achievement of the nectar.

The effectiveness of an Apoidea species as pollinator does not rely exclusively on the structures and characteristics of the flower itself, but also by factors related to the collection and transport of pollen by insect. Unlike social species, solitary bees living adult stage only for short periods and therefore may represent specialists for a plant species only for a few weeks every year. Apoidea generalists collect pollen from different types of flowers and are defined polylectic;

others specialize on a particular pollen taxa are defined oligolectic, although seems to be a continuation from the larger group polylectic to the narrower oligolectic group. Although many oligolectic species seem to be dependent on particular types of flowers and don't move from that flowering range, plants generally do not depend on oligolectic pollinators but they reproduce even outside of the given range in which their oligolectic pollinators are present; plants are able also to meet their reproductive needs thanks to the pollination of other polylectic Apoidea or other insects (Michener, 2007).

The role of bees in the ecosystem is, therefore, of undeniable ecological importance, mainly in tropical areas where most of the Angiosperms rely processes of pollination just to Apoidea. Instead, in temperate areas a variable part of Angiosperms (an average of 20%) has adopted anemophilous mechanisms, without a particular interaction with pollinator species. In any case, the ecological role of bees as pollinators is indisputable as well as the fact that the economic productivity of the surfaces of the crops pollinated by these insects is considerably increased (O'Toole, 1993; Klein et al. 2007).

1.2 Importance of bees for agricolture

Most of the plants of agricultural interest requires insect pollinators for their reproductive process. Honeybees are beneficial arthropods playing a key role in pollinating wild and crop plants. In the US, thanks to their pollination services in agriculture provide a contribution estimated at several billion dollars annually and the

estimated value of pollination due to wild bees can reach 5-6 billion of dollar per year (Gullan and Cranston, 2006).

The role of bees in agricultural ecosystems is even more relevant than in the recent past if you consider that the populations of the most common species, *Apis mellifera* L., have recently undergone a drastic reduction in many temperate zones. This reduction is caused by both human indirect actions, such as the introduction of the parasite mite *Varroa destructor* Anderson and Truemann in Europe and in North and South America and the massive use of pesticides in intensive agriculture. Unfortunately, during their foraging activity honeybee workers can be exposed to pesticides (Devillers J. 2002). Pesticides have induced resistances in target organism, contamination of ecosystems, and producing adverse effects on non-target species.

In addition, exist several crops for which *Apis mellifera* appears to be a weak pollinator than the other species of Apoidea. Some examples are represented by two Megachilidae: *Osmia cornifrons* Radoszkowski, 1887 pollinator of many types of fruit tree crops in Japan; *Megachile rotundata* F., which effectively pollinate alfalfa in huge areas where this plant species is cultivated; also the social Apoidea of genus *Bombus*, especially the species *Bombus terrestris* L., pollinating in greenhouse many varieties and cultivars of tomato. The choice of the most suitable species for production goals, it must take into account the characteristics of eco-ethology of the species, and in particular the social structure, which can be quite variable, from small colonies with a few individuals to society who have thousands and even millions of specimens (subsocial and eusocial Apoidea) (fig

1.1).

Fig. 1.1 – Colony of the eusocial species *Apis mellifera* L.

However, the modern industrialized agriculture, with the establishment of monoculture and the new common agricultural practices (weeding, removal of hedges, etc.), especially the indiscriminate use of chemical pesticides, above all insecticides, it made crops an inhospitable environment virtually for all insect pollinators. Although phytoiatric treatments made during the blooming are forbidden in many Italian regions, several reckless farmers still perform them, decimating numerous species of wild bees. An example is the disappearance from the fields of flowering alfalfa of *Megachile rotundata* and *Nomia melanderi* that assured pollination which manages to produce up to 2.000 kg/ha of seeds against a current

average of 220 kg/ha (Contessi, 2012). Furthermore, the introduction or increase of *Apis mellifera* populations is an important competitive factor for the ecological trophic resource (Banaszak, 1995; Matheson et al., 1996; O'Toole, 1993).

1.3 Importance of bees for ecosystem conservation

Apoidea have a significant role in the formation and preservation of the environment. About 80% of the wild plants is pollinated by several species of Apoidea and a lack in their pollination service can have drastic consequences leading in the worst case to their complete extinction (Contessi, 2012).

The extintion of a wild plant, maybe considered a weed or a pest, may have long-term consequences on the whole planet plant cover so difficult to predict. Every slightest disruption can have drastic consequences from the geological to the food levels (decrease in production areas). Whereas a number of wild plant species constitute the starting point for the life of many species of wild animals, defending and safeguarding bees means defending and protecting the vegetation and in other words the ecosystem in which we live (Contessi, 2012).

Chapter 2

BACILLUS THURINGIENSIS AND BIOPESTICIDES

2.1 Introduction

Chemical pesticides were developed during and after the Second World War and they showed immediately effective and economic damage. The 50s and 60s of last century were so real periods of success for pesticides, especially for organochlorine compounds such as DDT. However, many pesticides are now banned by Rotterdam Convention (DDT, Pentachlorophenol, Hexachlorobenzene, Toxaphene, Anthophyllite), others, such as Azadirachtin, Indoxacarb, Spinosad, though allowed and potentially active against many pests, are subject to restrictions on use for their toxicity (Gullan and Cranston, 2006).

The current market for pesticides is characterized by a significant increase (about 15% per year) of the biopesticides, as reported by Global Industry Analysts, Inc., MCP-1573 (February 2012). Microbial insecticides are considered a successful alternative to chemicals and the bacterium *Bacillus thuringiensis* (*Bt*) represents the most successful insect pathogen used for insect control (presently 2% of the total insecticidal market) (Bravo et al. 2011). The global legislative scenario, in fact, encourages the development and pre-commercial registration of substances with less environmental impact, but effective for the pests control (EC Regulation No. 1107/2009).

Moreover, it becomes compulsory the use of integrated control or Integrated Pest Management (IPM) in 2014, on international scale (EC Directive 2009/128). However, today, on the whole market of insecticides, biopesticides represent only about 3%, of which more than 90% consists of commercial products based on *Bt* (Ruiu and Floris, 2012).

2.2 *Bacillus thuringiensis* B.

Modern agriculture must incorporate the needs of companies such as productivity and profitability with food safety, respect for nature and biodiversity. For this reason, several research groups are undertake in the identification of new strategies for pests control and, in particular, in the identification of natural products with insecticidal activity. Interest is aimed to identify proteins and peptides produced by viruses, bacteria, fungi, plants, insects and their natural enemies, such as predators and parasitoids, which may interfere with the complex physiological processes of this class of arthropods (Bale et al., 2008; Dayan et al., 2009; Whetstone and Hammock, 2007).

Bacillus thuringiensis Berliner, 1915 (Bacteria, Bacilli, Bacillales, Bacillaceae) was reported, for the first time, in 1901 in Japan, from Shigetane Ishiwata on larvae of *Bombyx mori* L. (Lepidoptera, Bombicidae) and subsequently described by Ernst Berliner that isolated it in Thuringia (central Germany) on infected larvae of *Ephestia kuehniella* Zeller, 1879 (Lepidoptera, Pyralidae), the Mediterranean flour moth. Subsequently, Berliner hypothesized the

use of this bacterium in the fight against those insects**.** *Bacillus thuringiensis* B. is a Gram positive, aerobic, spore-forming bacterium found in soil and in different environments around the planet. It is a unicellular microorganism that multiplies by binary fission (Höfte and Whiteley, 1989).

As it regards the biological cycle, when the environmental conditions become unfavorable, in terms of humidity, temperature and nutrients, the cell produces endospores, becoming a sporangium. Spore, released by lysis of the cell wall, can remain vital for long periods, also for years (Dehò and Galli, 2012).

The peculiarity of *Bacillus thuringiensis* B., which it distinguishes from other species belonging to the same genus, is the production of one or more parasporal crystals in the cytoplasm of the cell, and at the same time the formation of the endospore. These parasporal corpuscles of crystalline appearance and protein nature (fig. 2.1), conferring the characteristic insecticidal activity of this microorganism (Claus and Berkeley, 1986); they are named δ-endotoxins and are highly toxic proteins for a great variety of insects and invertebrates (Feitelson, 1993), many of which are of agricultural importance. The simultaneous production of more δ-endotoxins may give rise to a higher insecticidal activity against a specific species, carrying out a synergistic effect (Gelernter and Schwab, 1993). The proteins of the crystal have a size that varies from 27 to 140 kDa and have the functional significance of protoxins. In fact, the insecticidal activity is due to their enzymatic degradation (Bravo et al., 2011).

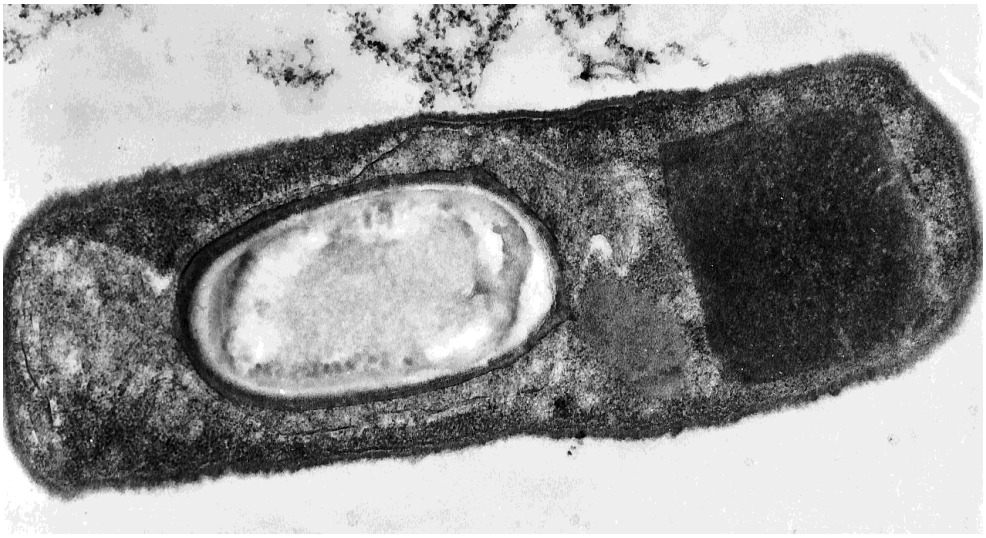

Fig. 2.1 - Electron micrograph of *B. thuringiensis* during sporulation (from Agaisse, 1995).

The δ-endotoxins are generally divided into two groups:

- Cry proteins, the most common and numerous;
- Cyt proteins, mainly active against Diptera.

Although these molecules having parts of amino acid sequence conserved and in both cases structurally related, they have phylogenetic differences. At the same time, however, those differences, in the light of current knowledge of their mechanism of action, explain the great variability observed in their insecticide power and in their activity spectrum (Lereclus et al., 1993). Since 1985, when the first gene encoding a δ-endotoxin was discovered and sequenced (Schnepf and Whiteley, 1985), the number of proteins described grew enormously: today it comes to over 500 toxins, classified based on the amino acid sequence in 60 groups and, based on the mechanism of action, in 4 families (Bravo et al., 2011).

The largest family is represented by the Cry toxins with three

domains (3d-Cry). Domain I is implicated in membrane insertion, toxin oligomerization and pore formation responsible for altering transmembrane potential; domains II and III are, however, involved in the recognition of specific proteins present in the apical membrane of absorptive cells that serve as receptors for the toxin. Therefore, it comes to domains responsible for the species specificity of the different toxins (Bravo et al., 2011).

The toxin performs its action through several steps, which can be described as follows (fig. 2.2) (Schnepf et al., 1998; Bravo et al., 2011):

- Crystal inclusions ingested by the larvae are solubilized in the intestinal lumen due to strongly reducing conditions and with highly alkaline pH;
- Protoxins, the inactive form of the toxins, are released into the lumen and they are activated by proteolytic cleavage made by intestinal proteases;
- The toxin, in monomeric form, binds proteins to the apical membrane of the cells, called primary receptors; in this case, for the protein Cry1A, mainly active against larvae of Lepidoptera, the primary receptors are proteins belonging to the family of cadherins. This has recently been demonstrated to insects belonging to the order Coleoptera and Diptera;
- The interaction ligand-primary receptor makes possible a further proteolytic cleavage of the toxin that leads to the elimination of a short peptide at the N-terminus of the protein, namely corresponding to α-helix of domain I;

- The proteolytic cleavage induces oligomerization of structures that show a high binding affinity for secondary receptors, i.e. proteins anchored to the apical membrane of absorptive cells by means of glycosylphosphatidyl-inositol residues (GPI-anchored proteins). Among the secondary receptors identified in the Lepidoptera and Diptera we can mention the aminopeptidase N or alkaline phosphatase, which has recently been shown to be an enzyme that acts as a secondary receptor for Coleoptera;
- Upon binding to the secondary receptor, the oligomers are inserted in the apical membrane of absorptive cells, at the level of membrane microdomains (lipid rafts), on which are expressed the same secondary receptors, with the consequent formation of pores selective for potassium ions;
- Pores formation is responsible for the occurrence of osmotic shock phenomena in the intestinal cells, which determines colloidal osmotic lysis.

The effects on the insect are:

- The intestinal muscles, the mouthparts and, sometimes, the entire body suffer from paralysis, for which the insect stops feeding.
- The collapse of the intestinal epithelium creates the optimal conditions for germination of spores previously ingested; *Bt* can migrate into haemocoel and cause the death of the insect by septicemia.
- Death typically occurs in a variable period, from a few hours to a few days, in relation to the susceptibility of the considered species

(van Frankenhuvzen, 1993; Dubois and Dean, 1995).

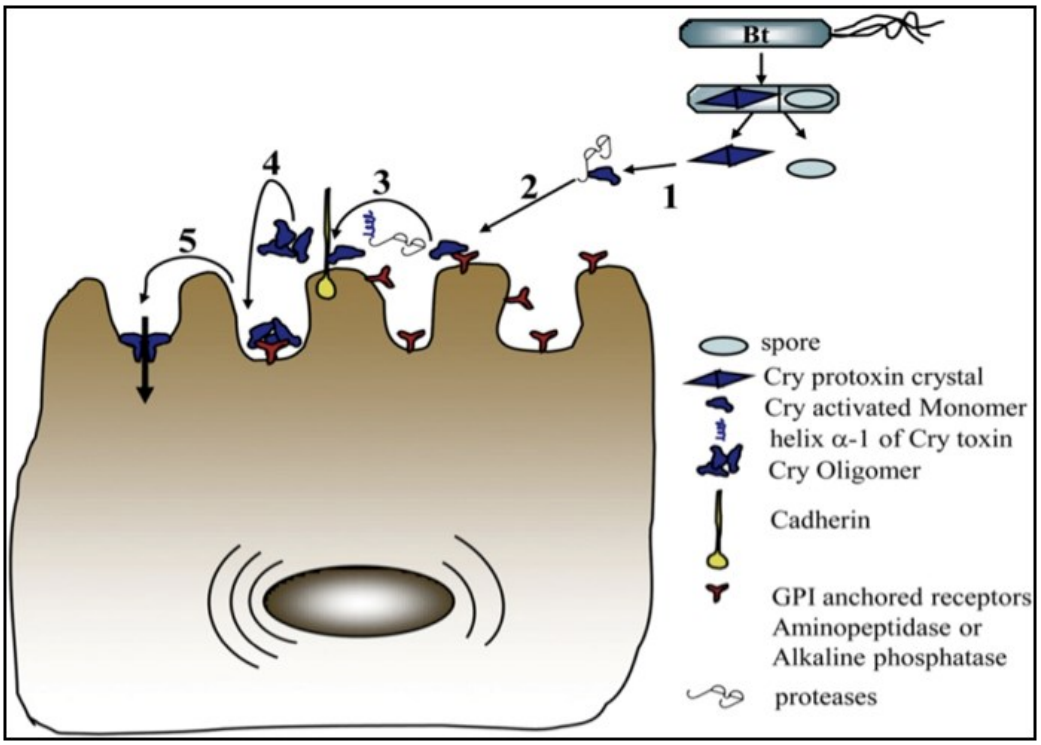

Fig. 2.2 - Mode of action of Cry1A toxins in the lepidopteran *M. sexta* (from Bravo et al. 2011).

As already said, given the fundamental physiological role played by the midgut and more in detail by the intestinal epithelium, the latter is the target for biopesticides orally administered (Casartelli, 2012). For example, a study of Rouis et al., 2007 shows histopathological effects of δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* in olive moth larvae, *Prays oleae*, that are vacuolization of the cytoplasm, hypertrophy of the epithelial cells and their nucleus, brush border membrane impairment, vesicle formation in the apical region

of cells toward the midgut lumen, and disintegration of the cells. The disintegration of the cells allows spores to pass into the lymphatic system of the insect, causing septicemic infection. The latter induces immediate paralysis of the alimentary canal resulting in interruption of trophic activity; the larva stops feeding from 30 minutes to 2 hours. The death of the larva occurs in a variable time (1-3 days) depending on the susceptibility of the species (Gullan and Cranston, 2006).

It is worth remembering the aforementioned proteins Cyt: these do not bind receptor proteins expressed on the apical membrane of intestinal cells; rather they directly interact with the lipid membrane, entering in the phospholipid bilayer and forming a pore, or by destroying the organization of the bilayer. As for the synergistic effect between certain Cry and Cyt proteins, the proposed mechanism is as follows: proteins Cyt inserted in the apical membrane of absorptive cells, exposing areas that are recognized by the Cry proteins, facilitating the oligomerization and the pore formation.

In conclusion, it can be concluded that the susceptibility of an insect to the toxic action of *Bt* depends on various factors, among which intestinal pH, presence of specific digestive enzymes, as well as specific receptors with a high binding affinity with the δ-endotoxins expressed on the apical membrane. These factors, of course, depend on the genetics of the insect and its stage of development (Schnepf et al., 1998; Bravo et al., 2011).

2.3 *Bacillus thuringiensis* biopesticide formulations: strains, serotype and subspecies

Among the various classification criteria proposed to classify the different *Bt* strains, the H antigen immunoassay, described for the first time by de Barjac and Bonnefoi in 1962, is still one of the most commonly used. Based on the antigenic properties of bacterial flagella, through serological tests it can be classified *Bt* strains in serotypes, which divided into serovarieties. Thus, *Bacillus thuringiensis* subsp. *kurstaki* belongs to serovariety H3: 3a 3b 3c of H3 serotype, while *Bacillus thuringiensis aizawai* belongs to the only serotype H7. The most used strains for biopesticides in Western countries belong to the subspecies *Bt kurstaki* and *aizawai* for the control of Lepidoptera, *Bt israelensis* for the control of hematophagous Diptera and *Bt tenebrionis* for the control of Coleoptera (Caroli et al., 1998).

In Italy, experiments with *Bt,* began in the '60s and the registration of the first product dates back to 1984 (Deseö and Rovesti, 1992). The new genetic techniques developed from Ecogen Inc. as the "conjugation" (transfer of genetic material by means of a plasmid vector between two bacterial cells) and the "plasmid curing" (selection of the most effective genes from various strains expressed in a single strain, e.g. *Bt* var. *kurstaki* and *aizawai*) have allowed a more efficacy of the new generation formulations (Caroli et al., 1998). In Italy are commercialized many formulated products, registered at Ministry of Health, in various formulations such as: wettable powder (WP), water

dispersible granules (WG) and suspension concentrate (SC).

The preparations power of *Bt*-based biopesticides is expressed in "international units" (IU), determining the power of the preparations (IU/mg) by comparison with a standard preparation adopted internationally and according to the following formula:

$$Formulation\ Power\ \left[\frac{UI}{mg}\right] = \frac{LC_{50}Standard\ \times Standard\ Power}{LC_{50}Formulation}$$

where LC_{50} is the concentration of product that kills 50% of the insects sample in a laboratory bioassay on semi-artificial substrate. The adoption of a standard in the calibration of the preparations is very important because it allows minimizing variations in the susceptibility of test insects and in the method of assay, both in the same laboratory and among different laboratories. Because of the diversity of products on the market, the calculation of IU has shown its limits; since, the formulations that differ in the range of quality and quantity of toxins Cry have a different spectrum of activity, and consequently their insecticide power is not unique but relative to the insect which is measured (Caroli et al., 1998).

2.4 Risk assessment of *Bacillus thuringiensis* biopesticide formulations on non-target organisms and environment

Indispensable prerogative for a formulation to reach commercial development is that it discharges the safety criteria regarding the

absence of toxicity and the inability to produce unwanted side effects. In addition, it must adapt to the environment and persist in field during a production season. Therefore, it is necessary to develop methods capable of monitoring the production of any toxic metabolites and to ensure effective assessment of risks resulting from large-scale use. The active components of these formulations should not be toxic or pathogenic to humans, plants or other animals, including insects that don't belong to the biopesticide targets (Caroli et al., 1998).

Actually, the data reported by the European Food Safety Authority (EFSA, 2013a), concerning different *Bt* strains, including *Bt* subsp. *aizawai* GC-91, suggest the need for further investigations in terms of ecotoxicology, since, no information is available on the persistence and multiplication in the soil of the aforementioned strain. Vettori et al., 2003 mention a resistance of about 88 months for the spores of *Bt kurstaki* and about 28 months for its toxin: this doesn't exclude that the same will occur for other strains. In addition, the lack of information on the persistence and multiplication of bacteria in the water, the degradation path, its mobility in soil and the groundwater contamination represents a *data gap* (EFSA, 2013a).

Finally, the formation of residues in the soil represents a risk factor for those species of Apoidea nesting in the soil or using the mud as nesting material in addition to being a source of biopesticide accumulation: the death of a nesting female results in the end of the reproductive activity (Arena and Sgolastra, 2014).

Chapter 3

THE ALIMENTARY CANAL OF HONEYBEE

3.1 The anatomy and digestion

In honeybees, just like all insects, the alimentary canal is a tube which extends through the entire length of the body and it is divided into three main regions: foregut, midgut and hindgut. Foregut and hindgut, being of ectodermal origin, are covered by a thin layer of cuticular intima; the midgut, instead, being of endodermal origin, has not such cuticolar layer (Gullan and Cranston, 2006).

The part of the foregut immediately following the mouth forms an enlargement, the pharynx, provided with muscles which able to make it dilate to facilitate the intake of the nutrient liquids. Esophagus follows the pharynx and, after going through the entire thorax, it enters into the abdomen where it widens to form the crop (or honey stomach) (fig. 3.1). This is where the nectar is stored in the workers during the harvest to be transported in the hive. Behind this tract there is a short, narrow, necklike division, with rigid walls constituting the proventriculus: honey stomach and proventriculus are divided by a X-shaped opening (Serrão and Cruz-Landim, 1995; Contessi, 2012). This opening is the mouth of the proventriculus, and its four triangular lips, which are thick and strong, mark four longitudinal ridges of the proventricular tube. This structure is commonly known as the "stomach-mouth" and is supposed to be an apparatus designed

especially to enable the worker to pick out pollen grains from the honey stomach and swallow them on down into the midgut or ventriculus, while the nectar is left to be stored in the hive (Snodgrass, 1956).

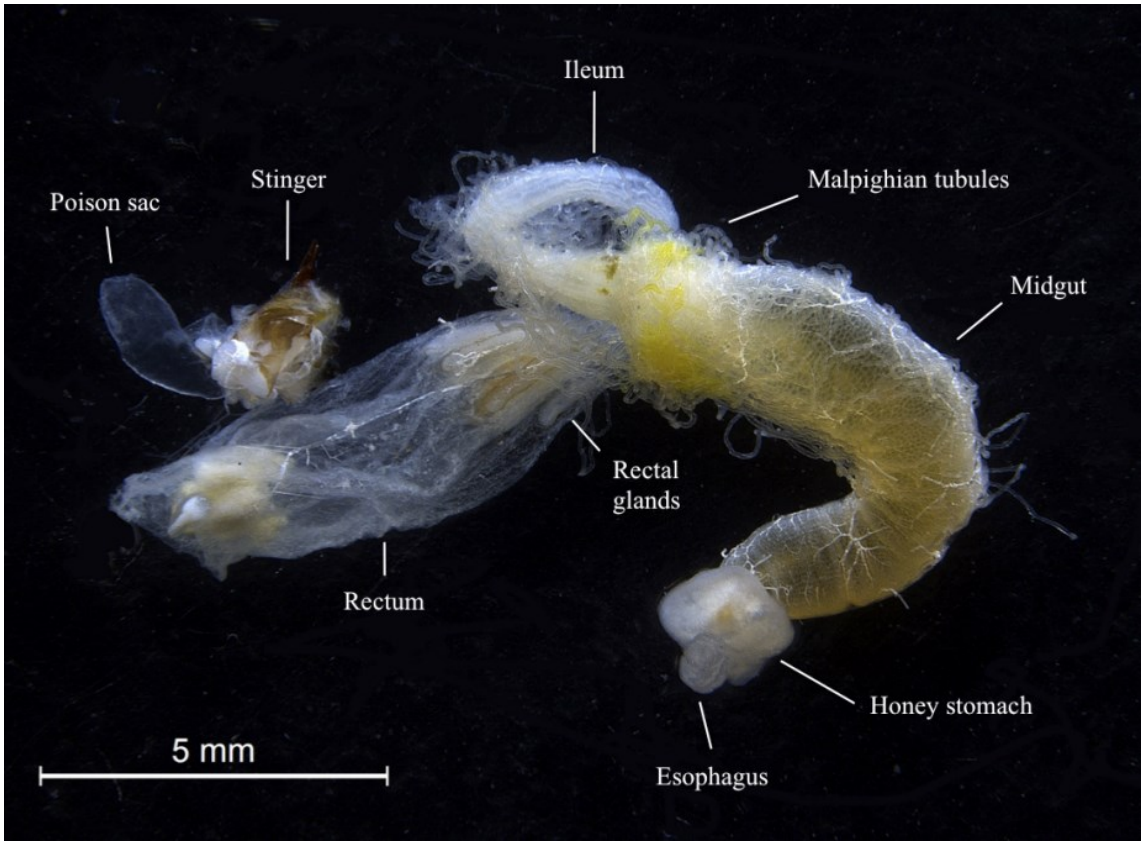

Fig. 3.1 – The alimentary canal and stinger with poison sac of honeybee (*Apis mellifera*). The image was obtained using stereoscopic microscope Leica MZ 205A equipped with the software auto-montage pro, Syncroscopy.

The midgut or ventriculus is the largest part of the alimentary canal in the bee and is bent into a U-shaped loop of which the posterior arm is dorsal (fig. 3.1). It is cylindrical and does not vary so much in shape and diameter according to its contents as do the other parts of the canal, although the numerous transverse constrictions which give it a segmented appearance are not at all constant. The ventriculus, when

examined in the natural condition in a freshly killed or asphyxiated bee, is of a dark-brown color with lighter rings corresponding to the constrictions. The latter represent internal folds where the walls are really thicker than elsewhere, the color being due to the contents which naturally show more plainly through the thin parts (Snodgrass, 1956). In this tubular structure it is possible to distinguish from the outside towards the inside two layers of musculature: the outer is longitudinal while the inner is transversal; on the transversal musculature layer laying the epithelium of the midgut consisting of columnar cells, separate from the underlying muscle fibers by a basal membrane.

The surface of the epithelium is separated from the ventricular lumen by a thin sheath called peritrophic membrane, consisting of a meshwork of chitin placed in a glycoprotein matrix. The peritrophic membrane, which originates from the underlying epithelial cells, has the function of retaining solid residues of food within the lumen. It forms concentric rings which partly remain adherent to the surface epithelium, and partly fall off completely, pouring into the lumen of the midgut. You can distinguish, therefore, an endoperitrophic space and a ectoperitrophic space (Snodgrass, 1956).

In the endoperitrophic space, secretory cells of intestinal epithelium, producing numerous enzymes, are involved in digestion of the molecules introduced with food (Terra and Ferreira, 1994). The fluid containing molecules of food partially digested and enzymes produced by cells of intestinal epithelium, flows along the alimentary canal towards the posterior end within the ectoperitrophic space,

instead, it flows towards the anterior end within the endoperitrophic space (Gullan and Cranston, 2006). In this way an endo-ectoperitrophic countercurrent flow is made which spreads the enzymes and nutrients efficiently throughout the midgut: the products of digestion can, therefore, pass through the porous wall of the peritrophic membrane and pour in the ectoperitrophic space to reach the surface of epithelial cells and to be absorbed (Jimenez and Gilliam, 1989; Giordana et al., 1998; Gullan and Cranston, 2006). The final stage of digestion usually takes place in the midgut on the surface of the microvilli, where some enzymes are trapped in a coating of mucopolysaccharides or attached to the cell membrane. Therefore, the peritrophic membrane form a permeable barrier and it helps to compartmentalize the various stages of digestion, in addition to providing mechanical protection to the cells of the midgut (Gullan and Cranston, 2006).

Following the midgut is a short, narrow, coiled ileum bringing a about one hundred of long, greatly coiled, blind, threadlike tubes opening into its anterior end (fig. 3.1). These latter are called the Malpighian tubules. Functionally they do not belong to the digestive tract, since they are excretory organs, corresponding with the nephridia of other invertebrates and with the kidneys of vertebrates. Following the ileum there is the rectum, whose lumen is coated by a cuticle that has its origin already in the ileum (fig. 3.1). The rectum is often distended by its contents into a great sac (rectal ampoule) more voluminous and extensible, in which can be accumulated for a long time the excrement, occupying a large part of the abdominal cavity.

Six whitish bands on its anterior dome-shaped end are called the rectal glands, which function is to reabsorb water and minerals from the feces (fig. 3.1). The rectum opens to the exterior through the anus, which is situated at the end of the rudimentary tenth or last segment of the abdomen (Snodgrass, 1956).

3.2 The midgut as barrier against pathogens and as mechanism of internal defense

The anatomical and physiological barriers, including the cuticle, the tracheal system and the midgut, play an important role in honeybees' defense as well as in all insects, against the penetration of microbial intruders into the haemolymph (Glinski and Jarosz, 1995). The peritrophic membrane in the midgut, acts as a sieve to rapid flow, with pores which permit the passage of small molecules, while inhibit the larger molecules, bacteria and food particles from a direct access to the intestinal epithelium (Lehane, 1997). Honeybees, during their life, are subjected to a continuous contact by different microorganisms, saprophytic and pathogenic bacteria, viruses, parasites (protozoa and metazoans) and mites. This leads them to develop a great variety of immune processes to counteract the development of infections (Dunn, 1986). Bacteria associated with bees are widely distributed in soil, water, air, stored bee food, surface plants and other living creatures. In most infections, the bacteria invade the body cavity of the bees through the gut, due to ingestion of

contaminated food (Glinski and Jarosz, 1992). Symbiotic normal microflora of the digestive tract of honeybees consists of Gram-positive bacteria, Gram-negative, Gram-variable, moulds and yeasts. Microflora develops due to normal consumption of pollen, other food, and direct contact with bacteria. Several species of the genus *Lactobacillus*, including lactic bacteria, and genera *Gluconobacter*, *Acetobacter*, *Gluconacetobacter* and *Saccharibacter*, including acetic bacteria, *Bacillus* spp., *Bifidusbacterium* spp., *Escherichia*, *Enterobacter*, *Proteus*, *Hafnia*, *Klebsiella*, *Erwinia* (Kačániová et al. 2004; Alma and Gonella, 2012) and numerous bacteria belonging to the family Enterobacteriaceae were found in the midgut of *A. mellifera*. The latter are the most representative species of the digestive system of honeybees (Rada et al., 1997), play a crucial role not only in the etiology of bacterial infections but also in the microbiological characteristics of pollen and beekeeping products.

Any injury of the alimentary canal cuticle is a potential access route for bacteria. In the adult bees, it is often reported septicemia by *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Hafnia alvei*. Moreover, saprophytic bacteria and plant pathogens can cause septicemia after invading the haemocoel. Bee body coverings and the biochemical environment of the midgut juice inhibit, thanks to bactericidal or bacteriostatic activity, the development of the most infections caused by bacterial saprophytes (Glinski and Jarosz, 1995).

Haemocytes represents the mechanisms of internal defense and the major cellular components in the immune system involved in the cell-mediated immune reactions and humoral defense, attributed to the

activity of soluble protective factors both innate and inducible (Gliński and Jarosz, 1994). Antimicrobial peptides are important components of the honeybee immune system. The majority of antimicrobial peptides is produced with infections and injuries of the midgut cuticle, and usually distinguished in the hemolymph. Defensins, for example, have a cytotoxic activity against gram-positive bacteria and several species of gram-negative bacteria (Ilyasov et al. 2012). In addition, serine proteases (SPs) present in hemolymph, in addition to their function in digestion food, could partecipate in regulatory cascade pathways in immune responses (Kanost and Clarke, 2005).

Furthermore, the production of ROS (*reactive oxygen species*) in case of bacterial infection is a key feature of the protective response besides the main molecular pathways in insects (*NF-kB*, *Toll* and *immune deficiency* pathways). ROS, which are efficient antimicrobial molecules, generally derived from oxidation-reduction process and their production is a general gut immune response to microorganism infection. A concurrent elimination of residual ROS is observed to protect the host, since the homeostasis of redox (reduction-oxidation) balance mediated by antioxidant enzymes is essential to the host survival (Dussaubat et al. 2012).

Chapter 4

MATHERIALS AND METHODS

4.1 Toxicity test

4.1.1 Honeybees (*Apis mellifera*)

The acute oral toxicity assay for honeybees has been performed according to the OEPP-EPPO guidelines (OEPP-EPPO, 2010) considering not only mortality but also behavioral and sublethal effects.

Workers of different ages of *Apis mellifera* were caught during summer (June 2014), in agreement with Ladurner et al. (2005b), from a healthy queen-right colony of a previously prepared experimental apiary of Catania (Italy), in the morning, on the flight board at the hive entrance (Ladurner et al., 2005b; OEPP-EPPO, 2010) and brought to the laboratory. The caught workers didn't show evident symptoms of diseases (e.g., varroasis, nosemiasis and virosis).

4.1.2 Solitary bees (*Osmia cornuta* and *Osmia rufa*)

As far as solitary bees are concerned, there are no official guidelines to perform oral toxicity tests on non-*Apis* species of Apoidea. In this study it was decided to adopt the guidelines drawn up by European Food Safety Authority (EFSA, 2013b) that, in fact, proposes *Omia cornuta* Latreille, 1805 and *Osmia rufa* Linnaeus,

1758 as test species for the risk assessment of solitary bees. The choice fell on these species since they have much in common with the Palearctic species in terms of evolutionary history and behavioral habits (EFSA, 2013b). Furthermore, according to EFSA, 2013b, these two species are considered as suitable test species because:

1. Species of the genus *Osmia* are already used in ecotoxicological studies and some protocols are available in literature [EFSA Panel on Plant Protection Products and their Residues (PPR), 2012].

2. These species are quite easy to rear and it is possible to obtain large populations (Krunic and Stanisavljevic L.Z., 2006; Bosch, 2008).

3. compared with other species of solitary bees, the biology of these species is well known (Bosch et al., 2008).

4. They are economically important species and management methods have been developed to use various *Osmia* species as commercial pollinators in crop pollination in Asia, North America and Europe (Bosch and Kemp, 2002).

Specimens who have been employed in this study come from male cocoons of both species, *O. cornuta* and *O. rufa*, only available by the CRA-API (Consiglio per la Ricerca e la Sperimentazione in Agricoltura – Unità di Ricerca in Apicoltura e Bachicoltura) Bologna.

The cocoons were obtained by nest-trapping which consists of a number of reed segments that once tied together in a bundle, can be hung on any support (Felicioli and Pinzauti, 1994) (fig. 4.1).

Fig. 4.1 – Nest trapping; in particular, *O.cornuta* (▲) and *O.rufa* (■) cocoon inside the reeds.

In October 2013, after the opening of the nests in Bologna at CRA-API, cocoons were put at 4°C (*wintering*) and remained at this temperature for about 150-180 days, which corresponds to the average duration of diapause of these insects (Felicioli and Pinzauti, 1994).

At the end of *wintering* (April 2014), the cocoons were individually incubated at 22°C in plastic glass (diameter 6,5 cm, height 8 cm) and sealed with lids from glass polystyrene to which has been inserted a network of durable nylon to ensure adequate ventilation. Wintering individuals (after 24 to 48 hrs of incubation), before being subjected to the test, were starved until the next morning.

4.1.3 Biopesticides

Biopesticides commonly used in Italy tested in this study were:

- Agree® (Certis) with a 50% w/w, power 25.000 UI/mg, formulation of *Bacillus thuringiensis* var. *aizawai* and *kurstaki* (GC-91 strain) (46.2% δ-endotoxin and 3.8% spores in 100 g of wettable powder). Commonly used in grapevine, pepper, apple and pear plantation against lepidopteran pest.
- DiPel® DF (Valent BioSciences) power 32.000 UI/mg formulation of *Bacillus thuringiensis* var. *kurstaki* (ABTS-351 strain) (6,4 g in 100 g of wettable granules). Commonly used in grapevine, apple and pear plantation against lepidopteran pest.
- VectoBac® DT (Valent BioSciences) power 3.400 UI/mg formulation of *Bacillus thuringiensis* var. *israeliensis* (serotype H-14) (3,4% in 100 g of formulated product). The product is in tablets commonly used for civil use against dipteran pest such as mosquitoes and it can be dissolved in the saucers.

4.1.4 Bioassay with honeybees

In the bioassay, worker bees of *A. mellifera* were subdivided into five groups, in one of which, used as control group, each specimen has been fed with a 10 µl single dose of a sugary solution [distilled water – honey, 1:1 v/v, in agreement with Bailey et al. (2005)], previously

sterilized by autoclave and devoid of the tested biopesticide; each specimen of the other four groups were fed with 10 µl of the same single dose of sugary solution mixed with different amounts of the biopesticide, so preparing different biopesticide solution concentrations; more in detail: 1) "field concentration", it is not the concentration which normally found in the field after the use of product, but it is a concentration comparable to the field-dose in the label of the tested products i.e. 100.0 g/hl for Agree and DiPel specifically suggested to be used on the grapevine plantation to control the moths *Lobesia botrana* Denis and Schiffermüller, 1775 and *Eupoecilia ambiguella* Hübner, 1796 and 0,384 g/100 ml for VectoBac; 2) “low concentration” (40.00 g/hl for Agree and DiPel and 0,154 g/100 ml for VectoBac), assumed to be a possible concentration in field-environment conditions, of the aforementioned biopesticide concentration; 3) "very-high concentration" (24400,0 g/hl for Agree and DiPel and 37,5 g/100 ml for VectoBac), lethal to more than 50% of the tested foraging bees; 4) "medium-high concentration" (9765,00 g/hl for Agree and DiPel and 6,0 g/100 ml for VectoBac), between field and very-high concentrations. Low, medium-high and very-high concentrations, in particular, have been found, respectively, dividing (for the low one) and multiplying, by a factor of 2.5 fold, the field concentration, in a geometric progression. Furthermore, these concentrations were chosen in a suitable range in order to provide a regression line and LD_{50} oral i.e. the single dose of a substance that can cause death in 50% of specimens when administred by the oral route.

In bioassay, the trials were performed in triplicate for each group (including the control one) and product: 10 specimens were used in each group, for each of the three repetitions (total of 150 specimens for each product tested, 50 for each repetition), strictly in agreement with the recommendations of the same OEPP-EPPO guidelines. Before the exposition 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 213 guidelines, 1998), then anesthetized 30 minutes at 4 °C and placed individually in maintenance wooden cages (37x15x15 cm) divided into 10 cells (7x7x15 cm) created by removable dividing walls (fig. 4.2), at 25 ± 2 °C, 60% RH, in agreement with Ladurner et al. (2005b).

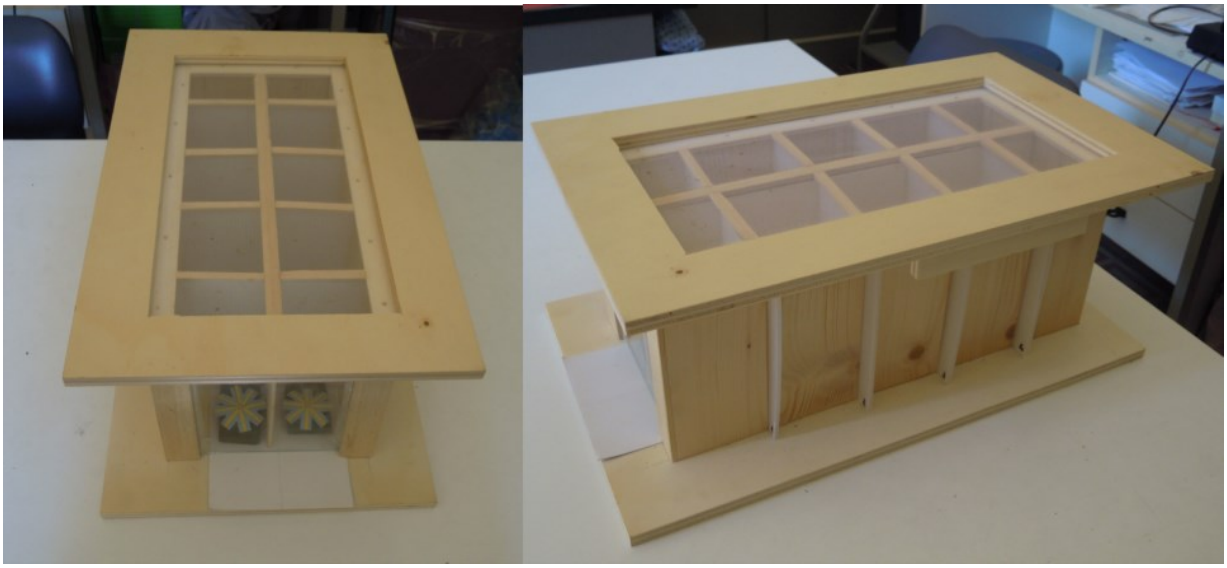

Fig. 4.2 - Wooden cages with artificial flower inside each cell.

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The dose of the sugary solution (10 µl), without the biopesticide (for the control group) or with the biopesticide at the different concentrations (group-specific), was administered to each specimen only once in each of the three repetitions, according to the "natural flower" method (Ladurner et al., 2003) or the "artificial flower" method (Ladurner et al., 2005a) for *A. mellifera* until its total intake (fig. 4.3).

The "natural flower" method consist in the modification of a flower from which the reproductive structures were removed with the help of tweezers and entering into the calyx a small plastic ampoule (diameter 0.3 cm, height 0.5 cm) formed by the terminal part of a test tube, subsequently filled with the test solution. The flowers used, belonged to the species *Convolvus althaeoides* L. and *Ipomoea acuminata* Vahl as constituting the most abundant wild flowers during the study period.

In the “artificial flower” method, however, an artificial flower is made artificially, as described by Ladurner et al., 2005: eight petals of blue cardboard (2 x 1 cm), on which were pasted yellow cardbord guidelines nectar (2 x 0.5 cm) arranged in symmetrical order around a small ampoule of plastic (diameter 0.3 cm, height 0.5 cm), inside which the test solution was pipetted. Finally, both the natural and the artificial flower were mounted on a cube of green florists’ dry foam (5 x 3 x 1 cm). This no-choice dietary feeding protocol should guarantee, especially for the low and field concentrations administered in this assay, the highest exposure level to bees potentially occurring in natural conditions (worst case scenario) (Han et al., 2010).

Fig. 4.3 – The two methos of feeding: the "natural flower" method (Ladurner et al., 2003) and the "artificial flower" method (Ladurner et al., 2005a).

Each bee, exposed to an artificial light (cool white 15W), was kept isolated into the cell (about 1 hr) until the complete consumption of the 10 µl dose of solution on the flower (Ladurner et al., 2005b). The dividing walls were then removed, and a plastic container (group feeder), consists of two small Petri dishes and overlapping of different dimensions, was added with the sugary solution (5 ml), devoid of the tested biopesticide and replaced every 24 hrs (OEPP-EPPO, 2010). Food consumption was measured weighing the group feeder before being inserted into the cage and after the time observations, the amount of sugary solution remaining was estimated weighing the group feeder and by difference and percentage ratio was obtained the value of food consumed. The workers were kept in the maintenance cages for the entire period of observation at 25 ± 2 °C, 60% RH and 0L:24D (Ladurner et al., 2005b). The bees considered dead, when

motionless for at least 10 seconds (Iwasa et al., 2004), were removed from the cages.

In accordance with the guidelines of OEPP-EPPO and OECD (OECD guidelines 213, 1998) for whichever acute toxicity oral assay, all the observations (about mortality, symptoms, behaviour and sublethal effects), made in the trials, on specimens from both the control group and the treated ones, were carried out at different time intervals after the start of the same trials (4, 24, 48, 72 and 96 hrs); in particular, although 48 hrs is suggested as a significant deadline as result of acute toxicity (OECD guidelines 213, 1998), the observations until 96 hrs after the start of the trials (possible recommended deadline by the same guidelines) have been aimed to assess any long-term effect.

4.1.5 Bioassay with solitary bees

Unlike honeybees, male specimens of *O. cornuta* e *O. rufa* were subdivided into four groups: field concentration, medium-high concentration, very high concentration and control group (see above).

The product tested was only DiPel and the low concentration was not tested because of the small number of cocoons available.

As reported above, wintering individuals of *O. cornuta* and *O. rufa* individually have been incubated at 22°C in plastic glass, before being subjected to the test, and starved overnight after emergence of their cocoon (Ladurner et al. 2005b).

The dose of the sugary solution (10 µl) was administered to each

specimen only once in each of the three repetitions using only the "natural flower" method until its total intake (fig. 4.3). This method was chosen because it was considered by Ladurner et al. 2003 highly effective in studies of oral toxicity in laboratory with solitary bees.

Similar to bioassay with *A. mellifera*, the trials with *O. cornuta* and *O. rufa* were performed in triplicate for each group (the low concentration was not tested): 10 specimens were used in each group, for each of the three repetitions (total sum of 120 specimens, 40 for each repetition). Also solitary bee was exposed to an artificial light (cool white 15W) at 22 ± 2 °C, 60% RH, and each of them was kept isolated into the plastic glass (about 1 hr) until the complete consumption of the 10 µl dose of solution on the natural flower (Ladurner et al., 2005b). Specimens of *O. cornuta* and *O. rufa* bees have been transferred into maintenance cages (600 ml clear plastic containers appropriately drilled and sealed with lids made from Petri dishes which have been inserted a network of durable nylon to ensure good ventilation), and within them was inserted the artificial feeder with the control solution (fig. 4.4).

Fig. 4.4 – Test with solitary bees: maintenance cages, plastic glass with natural flower and specimens inside.

Specimens of *O. cornuta* and *O. rufa* were kept in the maintenance cages for the entire period of observation at 22 ± 2 °C, 60% RH and 12L:12D (Ladurner et al., 2005b).

As for honeybees, the observation of mortality made in the trials, on specimens from both the control group and the treated ones, were carried out at 4, 24, 48, 72 and 96 hrs after the start of the trials.

4.1.6 Statistical analyses and symptoms observation

Overall mortality analysis has been made by calculating the mortality rates (Malone et al., 1999; Ladurner et al., 2005b) observed

in the different groups and species. Data on mortality rates were arcsine square root transformed and analyzed by means of an *one-way* ANOVA, followed by LSD test (Least Significant Difference) for homogenous groups to separate the mean obtained (Siscaro et al., 2006; Ruiu et al., 2007). Homogeneity of variance was assumed in all the assays. All the statistical analyses were performed with the STATISTICA software (Statsoft®). Mortality rates have not been corrected with the Abbott's formula (1925), since the death of the specimens of the control group was 0% (Ladurner et al., 2005b).

For each insecticide, regression lines, LD_{50} values, 95% fiducial limits (FL) were determined using application tools for Microsoft Excel® software.

Direct observations on the behavior and symptoms of honeybees, treated specimens and control ones have been made at the considered time intervals (4, 24, 48, 72 and 96 hrs) as result of acute toxicity.

Furthermore, survival curves have been made to assess the survival in the different groups and species after 7 days from the treatment.

4.2 Morphological, histological and ultrastuctural analysis

4.2.1 Preparation of honeybees workers for SEM, OM and TEM observations.

Apis mellifera workers were collected and tested as described in par. 4.1.1 and par. 4.1.4 using the product Agree at four different concentration (low, field, medium-high and very high concentration).

The observations of morphology, histology and ultrastructure made in my study, on specimens from both the control group and the treated ones, were carried out at five different time intervals after the total intake of the test solution (4, 24, 48, 72 and 96 hrs).

The observations conducted to detect possible alterations in the midgut epithelium were carried out on five specimens, still alive, from each of the five groups, including the control one. The alimentary canal was extracted by decapitating the individual by bistoury with disposable sterile blade, removing the last abdominal segments using sterile scissors and with the help of tweezers, the alimentary canal was totally extract. Each alimentary canal was dissected in a Ringer's solution and the various midgut samples were prepared according to the following methods for SEM, OM and TEM observations.

4.2.2 Scanning electron microscopy (SEM) preparation

Each midgut sample was fixed in 2.5% glutaraldehyde in a 0.1 M Sorensen's phosphate buffer (SB), pH 7.4, for 4 hrs at room temperature (r.t.). The samples were then washed several times in the same buffer, dehydrated in ethyl alcohol, immersed in hexamethyldisilazane (HMDS) and air dried; finally, the samples were mounted on SEM stubs, metal coated and then observed by a ZEISS EVO LS10 microscope.

4.2.3 Transmission electron microscopy (TEM) preparation

Each midgut sample was fixed in 2.5% glutaraldehyde and 3% sucrose, in a 0.1 M Sorensen's phosphate buffer (SB), pH 7.4, for 4 hrs at room temperature (r.t.). The samples were then washed several times in the same buffer, post-fixed in 2% osmium tetroxide in the same buffer, at r.t. for 1 hr. their dehydration in ethyl alcohol, then immersed in propylene oxide and embedded in Embed 812. Ultra-thin sections (50-70 nm), placed on copper-rhodium grids (200/300 mesh), were contrasted in uranyl acetate and lead citrate (Reynolds, 1963) and then observed by a JEOL 1220 microscope.

4.2.4 Optical microscopy (OM) preparation

For OM observations of workers midgut sections (5-7 μm) were used three different fixatives and no obvious difference between them was shown. Furthermore, in order to prepare semi-thin sections (400-700 nm) for OM observations, midgut samples were fixed in 2.5% glutaraldehyde and 3% sucrose, in a 0.1 M Sorensen's phosphate buffer (SB), pH 7.4 for 4 hrs at room temperature (see par. 4.2.3).

Three fixatives used are:

- Formalin: after fixation in 4% formalin for 24 hrs at 4°C, the samples were washed with tap water and dehydrated in a series of increasing gradation ethanol (35°, 50°, 70°, 80°, 95°, absolute) and finally placed in xylene for 1 hr. Following the infiltration in paraffin

for 4 hrs at 60°C.

- Carnoy: fixation in Carnoy is faster than the formalin. Midgut samples, therefore, were placed in Carnoy for 4 hrs at room temperature (r.t.) and then directly dehydrated with 95° ethanol and absolute. Finally, samples are immersed in xylene and infiltrated in paraffin.

- Bouin: it is one of the best fixatives. Being very penetrating, it serves to fix pieces of organs, even bulky, and allows the use of almost all staining methods. Fixation according to the thickness of the pieces from 12 to 48 hrs. After fixation with Bouin for 24 hrs at 4 ° C, the samples were washed with 50° ethanol up to eliminate the yellow color typical of the fixative. The samples were then dehydrated before in 70° ethanol then in 95° ethanol. Finally, samples are immersed in xylene and infiltrated in paraffin.

Samples included in paraffin blocks were cut with microtome REICHERT-JUNG; 1150/AutoCut, in 5-7 µm sections and the histological characterization was performed using the hematoxylin-eosin (HE) staining.

As far as semi-thin sections are concerned, these were cut with an Ultratome III LKB then mounted on microscope slides, deresined with a saturated solution of sodium hydroxide in absolute ethanol and stained with 0.5% Toluidine blue in SB.

4.3 Gene expression and biochemical analysis

4.3.1 Preparation of biological samples

Apis mellifera workers were collected and tested as described in par. 4.1.1 and par. 4.1.4 using the product Agree at two different concentration: 1) "field concentration", a concentration comparable to the field-dose in the label of the tested product i.e. 1 g/l and 2) “high concentration”, five times the field concentration corresponding to 5 g/l and represents an unlikely concentration in field established in order to make a comparison with specimens treated with field concentration and control.

The observations for the control group and for the two groups treated with the biopesticide were conducted at 4 hrs and 24 hrs after the single administration of the test solution. Specimens were taken from the maintenance wooden cages, where the toxicity test had been performed, and the midgut was completely extract as describe in par 4.2.1.

For each treatment (control, field concentration and high concentration) 15 midguts were extracted and grouped into 3 pools (total sum of 90 specimens, 45 for each observation), of which we proceeded to proteins and total RNA extraction.

4.3.2 Protein extraction, one-dimensional SDS gel electrophoresis and protein identification by mass spectrometry (LC-MS)

Midguts for proteins extraction were transferred to dry in a eppendorf tube and they were added 60 µl of extraction buffer (NP40), containing protease inhibitors. The samples were homogenized and kept on ice for 30 minutes, after which they were centrifuged at 13.000 rpm for 10 minutes at 4°C. The protein lysates were dosed following the Bradford method with a spectrophotometer (NanoDrop 1000). Bovine serum albumin (BSA) was used as standard, and absorption was measured at 595 nm.

20 µg di protein e Laemmli buffer 1X of each sample was boiled at 95°C for 5 min and loaded with the marker See Blue Plus 2 Prestained in SDS electrophoretic gel.The electrophoretic run was performed at 100-150V for about 1h.

The gels were stained with Novex® Colloidal Blue staining kit (Invitrogen) Coomassie Blue according to manufacturer's protocol, and bands differentially expressed between control and treated groups were manually excised, digested and subjected to analysis by mass spectrometry (LC-MS) using Nano IC Orbitrap™ based system (Thermo Scientific). Proteome Discoverer Software was used to retrieve tandem mass spectra. The protein characterization data were searched against sequence database generated from protein sequence of *Apis mellifera*. Protein identification was accepted if it contained at least two unique peptides and taking into account the score, the

percentage of coverage of the protein identified and the molecular weight.

The identified proteins by mass were annotated by searching against the Uniprot database (www.uniprot.org).

4.3.3 Total RNA extraction and evaluation of gene expression by RT-PCR (Reverse Transcriptase)

For selected proteins, the level of gene expression in honeybee midguts was assessed by RT-PCR (Reverse Transcriptase). Total RNA extraction was carried out by PureLink™ RNA Mini Kit (Ambion) following the kit instructions. The concentration and purity of total RNA extracted was determined using the NanoDrop 1000 (Thermo Scientific).

Total RNA extracted was converted to double stranded cDNA using M-MLV Reverse Transcriptase kit (Invitrogen) in the presence of Random hexamer Primer (Invitrogen) and used as template in subsequent amplifications by polymerase chain reaction (PCR) in 2720 Thermal Cycler (Applied Biosystems), for which were used the primers shown in table 4.1. Primers was selected using “Primer” software v.1.1 which analyzes the different parts of RNA to be amplified with a melting temperature comprised between 60-70°C and eliminating those too rich in GC and/or exhibit repeats of bases or groups of bases. PCR assay was performed with the following amplification program: denaturation, 1 cycle (3 min, 95°C), 30 cycles

(1 min 95°C, 30 s 65°C, 1 min 72°C) and final elongation, 1 cycle (7 min 72°C). The presence of PCR product was checked by electrophoresis in 1% agarose gel stained with syber safe (Invitrogen) and viewed by UV transilluminator.

Primers choice was performed on gene sequences present in the NCBI Honeybee database and by Blast it was evaluated the specificity based on the ability to hybridize selectively with the region of interest. The gene *elongation factor 1 - alpha* (EF1 - α) was used as housekeeping gene (Lourenço et al., 2008) and the modulation of gene expression was assessed by comparing the expression of genes of interest with the housekeeping gene by densitometry using ImageJ software.

Statistical analysis were performed with application tools for Microsoft Excel® software and statistical significance of differences among treatments was determined using Student's t-test.

Tab. 4.1– Primers used.

Gene primers	**Sequences**	**Amplicon (bp)**
Alpha-glucosidase – FW	5' – GTCCTGAGCGAATTCACTG - 3'	645
Alpha-glucosidase – RV	5' – TCGAGACGATTTATAATACCCT - 3'	
Defensin-1 – FW	5' – CCTTCTCTTCATGGCTATGG - 3'	203
Defensin-1 – RV	5' – TTCTCGCAATGACCTCCA - 3'	
EF1 – α - FW	5' – CGTTATTGGACACGTCGACT - 3'	456
EF1 – α – RV	5' – AGAATACGGTGGTTCAGTGG - 3'	
Eukaryotic translation initiation factor 6 – FW	5' – GCGGTACGTGTGCAATTC - 3'	243
Eukaryotic translation initiation factor 6 – RV	5' – CTGTAATTCTGTGTCCGTCG - 3'	
Serine Protease 36 – FW	5' – ACTGCATACGGTGTTCCTGA - 3'	596
Serine Protease 36 – RV	5' – ACCGACTTGTAGATCACCGA - 3'	
Superoxide dismutase - 2 – FW	5' – GGCTTACTCGCATTCGCA - 3'	463
Superoxide dismutase - 2 – RV	5' – TTAAGAAGTGCAGCGTCTGG - 3'	
Thioredoxin reductase – FW	5' – CTCTCCACGAGGTAGCACTT - 3'	361
Thioredoxin reductase – RV	5' – GTCTACCACCTACAGCAATCAG - 3'	

4.3.4 Western blot analysis and immunoblotting

To verify the results of differently expressed SOD-2 protein, for each treatment (control, field concentration and high concentration) 3 midguts were extracted (total of 18 specimens, 9 for each observation), and proteins was separated by SDS-PAGE. 30 µg of protein was run under denaturating and reducing conditions and transfered from the gel to nitrocellulose paper by western blotting transfer system (Bio Rad Trans-Blot® Turbo™ Transfer System). The marker used was SeeBlue®2 Plus Pre-Stained Standard (Novex, Life technologies™). The primary antibody used was: rabbit polyclonal anti-SOD2 antibody (ab13534 Abcam®) at dilution 1:2000 and rabbit polyclonal anti-β-Actin (sc-10731 Santa Cruz BioTechnology) was used as a marker for protein loading at a dilution of 1:200. The secondary antibody was anti-rabbit at a dilution of 1:10000. Proteins were detected by using Novex® HRP Chromogenic substrate (TMB) (Invitrogen).

4.4 Preliminary investigation on microflora

4.4.1 Preparation of honeybees workers

Apis mellifera workers were collected and tested as described in par. 4.1.1 and par. 4.1.4 using the product Agree at two different concentration: 1) "field concentration", a concentration comparable to

the field-dose in the label of the tested product i.e. 1 g/l and 2) "high concentration" corresponding to 97,65 g/l, this concentration was eastablished as describe in par. 4.1.4 and represents an unlikely concentration in field established in order to make a comparison with specimens treated with field concentration and control.

The study were carried out on five specimens, still alive, from each groups: field concentration, high concentration and control. After 96 hrs from treatment, specimens were removed from maintenance cage and washed in sterile 0.9% NaCl solution (two washes in vortex for approximately 1 min) to remove any contamination arising from the external environment and stored at -4 ° C for 24 hrs.

To ensure the best possible condition of sterility, the following sterility protocol was adopted: three washes were performed in 70% ethanol of the outer surface of the bees, before proceeding with the extraction of the alimentary canal.

The midgut was extract as describe in par. 4.2.1 and put into 1.5 ml tubes and the contents weighed. Once weighed, the intestine was mechanically homogenized with an inoculating loop inside the test tube containing 1 ml of BHI 10% of glycerol (9 ml BHI and 100 µl glycerol) and the tubes agitated in a vortex for 2 min. Finally the mixture was stored at -80°C.

From each sample was prepared a 1:10 dilution of the initial bacterial suspension (900 µl distilled water and 100 L of the initial suspension). 100 µl of the dilution were streaked on the different media.

4.4.2 Culture media

The investigation was carried out using general and specific media:

- MH - Mueller-Hinton agar: the media for the culture of aerobic bacteria used at 37 ° C and at room temperature;

- Blood Agar: the media for the culture of anaerobic bacteria used at room temperature;

- MSA - Mannitol Salt Agar (37°C, 18 – 24 hrs, aerobically): media used for the isolation of pathogenic staphylococci. It inhibits the growth of many bacterial species, except halophilic bacteria;

- EMB - Eosin Methylene Blue Agar (37° C, 18 – 24 hrs, aerobically): media for the isolation and identification of Enterobacteriaceae. It is prepared starting from the formula specified by APHA, for the identification and differentiation of the coliform group;

- BEA - Bile Aesculin Agar (37°C, 18 – 24 hrs, aerobically): media for the isolation of enterococci and streptococci of D group. These bacteria are capable of hydrolyzing the esculin, forming esculetin and dextrose. The esculetin combines with ferric citrate of the media forming a dark or black-brown sediment which is indicative of a positive reaction. The bile, instead, inhibits the growth of other Gram-positive bacteria;

- Mc - Mac Conkey Agar (37°C, 18 – 24 hrs, aerobically): selective media that allows the distinction between coliforms and non-lactose

fermenting bacteria, with inhibition of the growth of Gram-positive micrococci;

- S - Sabouraud Agar (35°C, 72 hrs, aerobically): media for the isolation of dermatophytes, fungi and yeasts.

All culture media (Oxoid), weighed and dissolved in distilled water, were subjected to sterilization in autoclave 121°C, 1 bar, for 15 minutes (Ebrahimi and Lotfalian, 2005; Rada et al., 1997; Kačániová et al., 2009; Bridson, 2006).

Chapter 5

RESULTS

5.1 Toxicity test

5.1.1 Mortality of honeybees (*Apis mellifera*)

Overall mortality analysis shows that the mortality rate of the low concentration group of three pesticides is 0% and remains unchanged for the entire period of observation (96 hrs after treatment).

Products Agree and DiPel tested at the field concentration show the mortality rates arises after 48 hrs (it doesn't exceed 6.67% after 96 hrs), while after the administration of VectoBac, mortality rates arises at 96 hrs (tabs. 5.1 - 5.3); in fact, although these values are very low, comparing the mortality mean at 96 hrs of individuals treated at the field concentration of all three products, a significant difference exists between them and the control ($F = 31.7726687066$; $df = 3$; $P = 0.0000856763$); moreover, the mortality mean at 96 hrs is lower in VectoBac than the other two products (fig 5.1).

Tab. 5.1 – Mortality rates (%) of *A. mellifera* treated with Agree at field concentration

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	1	0	1	2	8	0	0	10	0	10	
R2	0	0	0	1	0	1	9	0	0	0	10	0	
R3	0	0	0	0	1	1	9	0	0	0	0	10	
Tot	0	0	1	1	2	4	26	0	0	3,33	3,33	6,67	13,33±4,58

Tab. 5.2 – Mortality rates (%) of *A. mellifera* treated with DiPel at field concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	1	1	0	2	8	0	0	10	10	0	
R2	0	0	0	0	1	1	9	0	0	0	0	10	
R3	0	0	0	1	1	2	8	0	0	0	10	10	
Tot	0	0	1	2	2	5	25	0	0	3,33	6,67	6,67	16,67±4,88

Tab. 5.3 – Mortality rates (%) of *A. mellifera* treated with VectoBac at field concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	0	1	1	9	0	0	0	0	10	
R2	0	0	0	0	1	1	9	0	0	0	0	10	
R3	0	0	0	0	1	1	9	0	0	0	0	10	
Tot	0	0	0	0	3	3	27	0	0	0	0	10	10±4,14

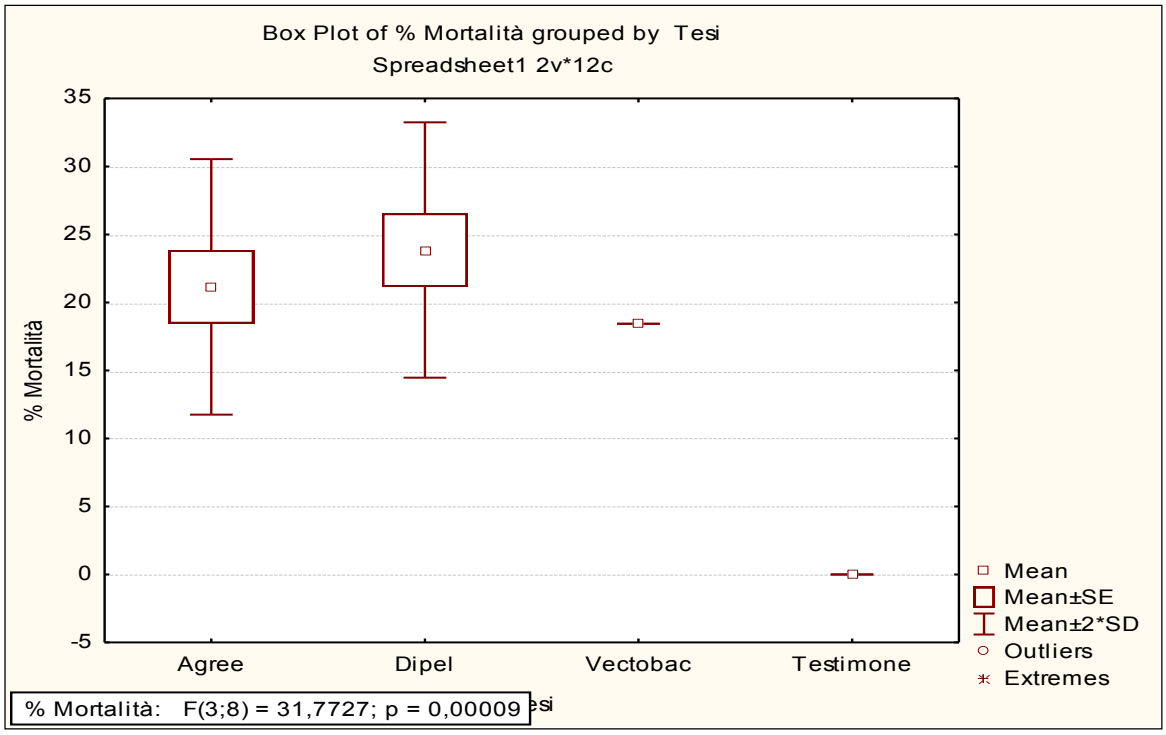

Fig. 5.1 – Results of *one-way* ANOVA between all products tested at the field concentration.

The mortality rate of specimens treated with Agree and DiPel markedly increases at 72 hrs and 96 hrs after the treatment, in the groups treated with medium-high and very-high concentration if compared to the respective times after treatment with field concentration (tabs 5.4 - 5.7).

Tab. 5.4 – Mortality rates (%) of *A. mellifera* treated with Agree at medium-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	0	2	2	8	0	0	0	0	20	
R2	0	0	0	1	1	2	8	0	0	0	10	10	
R3	0	0	2	2	3	7	3	0	0	20	20	30	
Tot	0	0	2	3	6	11	19	0	0	6,67	10	20	36,67±10,33

Tab. 5.5 – Mortality rates (%) of *A. mellifera* treated with DiPel at medium-high concentration

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	3	1	4	6	0	0	0	30	10	
R2	0	0	0	0	1	1	9	0	0	0	0	10	
R3	0	0	1	1	2	4	6	0	0	10	10	20	
Tot	0	0	1	4	4	9	21	0	0	3,33	13,33	13,33	30±9,10

Tab. 5.6 – Mortality rates (%) of *A. mellifera* treated with Agree at very-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	5	4	9	1	0	0	0	50	40	
R2	0	0	0	3	4	7	3	0	0	0	30	40	
R3	0	0	2	0	3	5	5	0	0	20	0	30	
Tot	0	0	2	8	11	21	9	0	0	6,67	26,67	36,67	70±18,82

Tab. 5.7 – Mortality rates (%) of *A. mellifera* treated with DiPel at very-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	3	4	7	3	0	0	0	30	40	
R2	0	0	0	2	3	5	5	0	0	0	20	30	
R3	0	0	1	2	2	5	5	0	0	10	20	20	
Tot	0	0	1	7	9	17	13	0	0	3,33	23,33	30	56,67±11,95

Instead, the mortality rate of honeybees treated with VectoBac at medium-high and very-high concentration quickly changes at 48 hrs and 24 hrs after the treatment (tabs 5.8 – 5.9), if compared to the respective times after treatment with field concentration, such as specimens treated with Agree and DiPel.

Tab. 5.8 – Mortality rates (%) of *A. mellifera* treated with VectoBac at medium-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	1	0	2	3	7	0	0	10	0	20	
R2	0	0	1	1	2	4	6	0	0	10	10	20	
R3	0	0	0	2	1	3	7	0	0	0	20	10	
Tot	0	0	2	3	5	10	20	0	0	6,67	10	16,67	33,33±8,16

Tab. 5.9 – Mortality rates (%) of *A. mellifera* treated with VectoBac at very-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	2	4	1	2	9	1	0	20	40	10	20	
R2	0	0	4	3	2	9	1	0	0	40	30	20	
R3	0	0	3	2	1	6	4	0	0	30	20	10	
Tot	0	2	11	6	5	24	6	0	6,67	36,67	20	16,67	80±14,54

Comparing the mortality mean at 96 hrs of individuals treated at the medium-high concentration (F = 7,9336294719; df = 3 ; P = 0,0088057875) and very-high concentration (F = 27,9672227956; df = 3 ; P = 0,0001362950) of all three products, a significant difference exists between them and the control; in particular concerning the specimens treated with the medium-high concentration, the mortality mean of Agree is the highest compared to DiPel and VectoBac, the latter with the lowest value (fig. 5.2). Instead, at very-high concentration, the products has the same more or less mortality means (DiPel with the lowest value) (fig. 5.3).

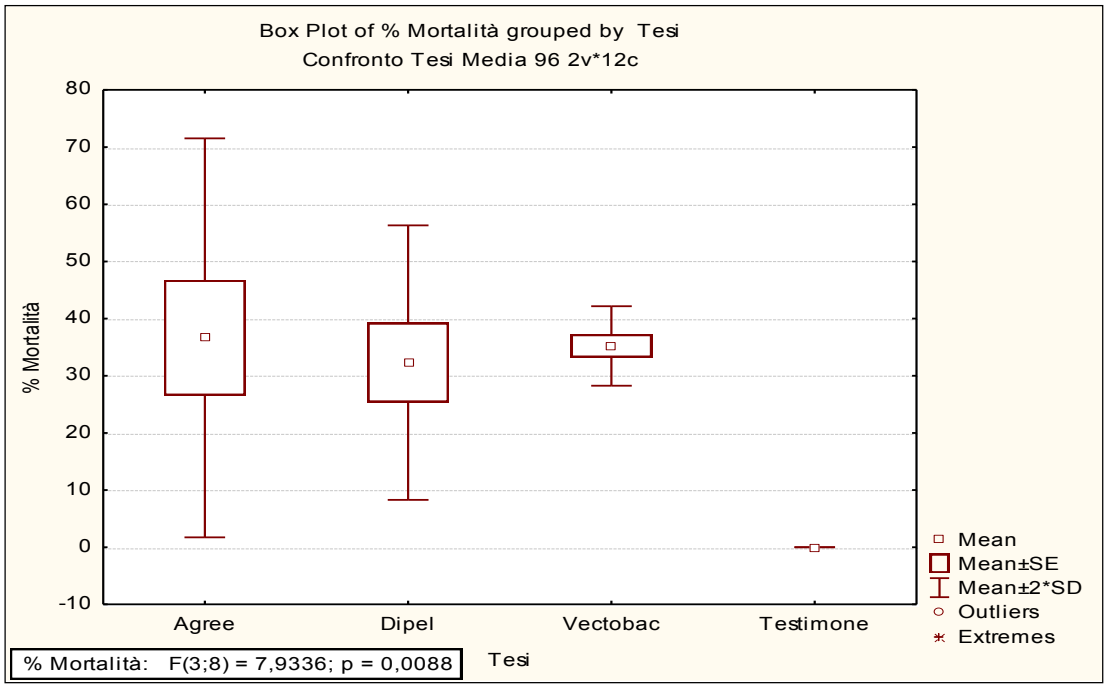

Fig. 5.2 - Results of *one-way* ANOVA between all products tested at the medium-high concentration.

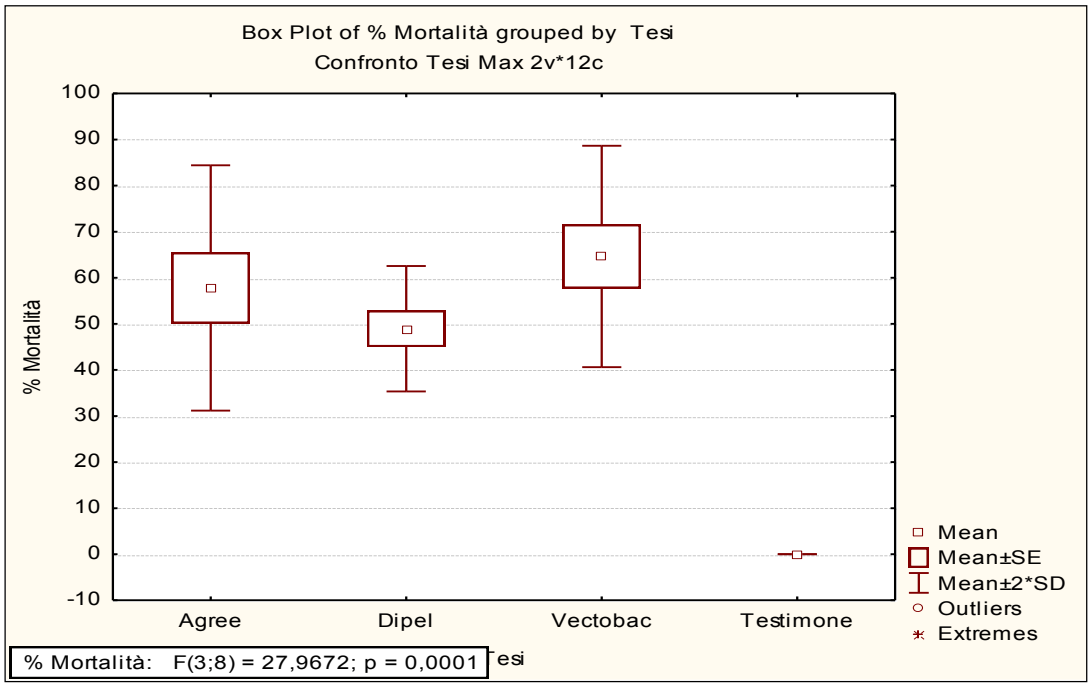

Fig. 5.3 - Results of *one-way* ANOVA between all products tested at the very-high concentration.

The graph shown in fig. 5.4 denotes an increase in the percentage of total mortality at 96 hrs in relation to the concentration of product tested, regardless of its formulation. It can be noted as the mortality rate of individuals treated with medium-high concentration is below of 50%. Although, this percentage is exceeded 50% at very-high concentration of each product tested, DiPel appears to be less toxic.

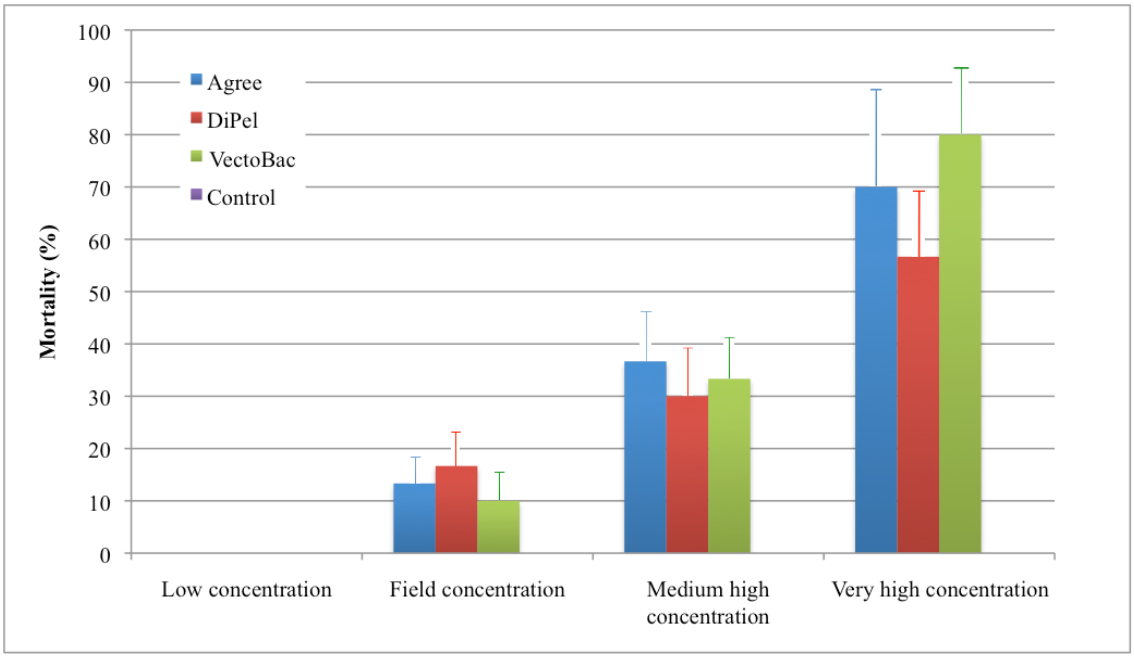

Fig. 5.4 - Total mortality at 96 hrs (%±s.d.) of *Apis mellifera* treated with three products at different concentrations.

The *one way*-ANOVA followed by LSD test on mortality mean at 96 hrs of individuals treated with Agree (F = 18,2951032875; df = 4 ; P = 0,0001355813) and DiPel (F = 31,7236044043365; df = 4 ; P = 0,0000117205) both show for field, medium-high and very high

concentration treatments a significant difference between them and low concentration group if compared with control; in particular the mortality mean of specimens treated with the field concentration and medium-high concentration, not significantly differ between themselves, but there is a difference between these and the very high concentration group if compared with control. Instead, the mortality mean of the specimens treated with VectoBac at different concentrations (F = 71,0227353101386; df = 4 ; P = 0,0001452961), show a significant difference between them and the control with the exception of the low concentration, just like Agree and DiPel low concentration group.

Fig. 5.5 shows the comparison of mortality mean (%) of honeybees treated with different concentrations of three biopesticides, each of them compared to control at the different time interval (4, 24, 48, 72, 96 hrs) and, as said previously, the mortality markedly increases at 72 hrs and 96 hrs after the treatment, in the groups treated with medium-high and very-high concentration for all products and, in particular, VectoBac show a high response already after 48 hrs.

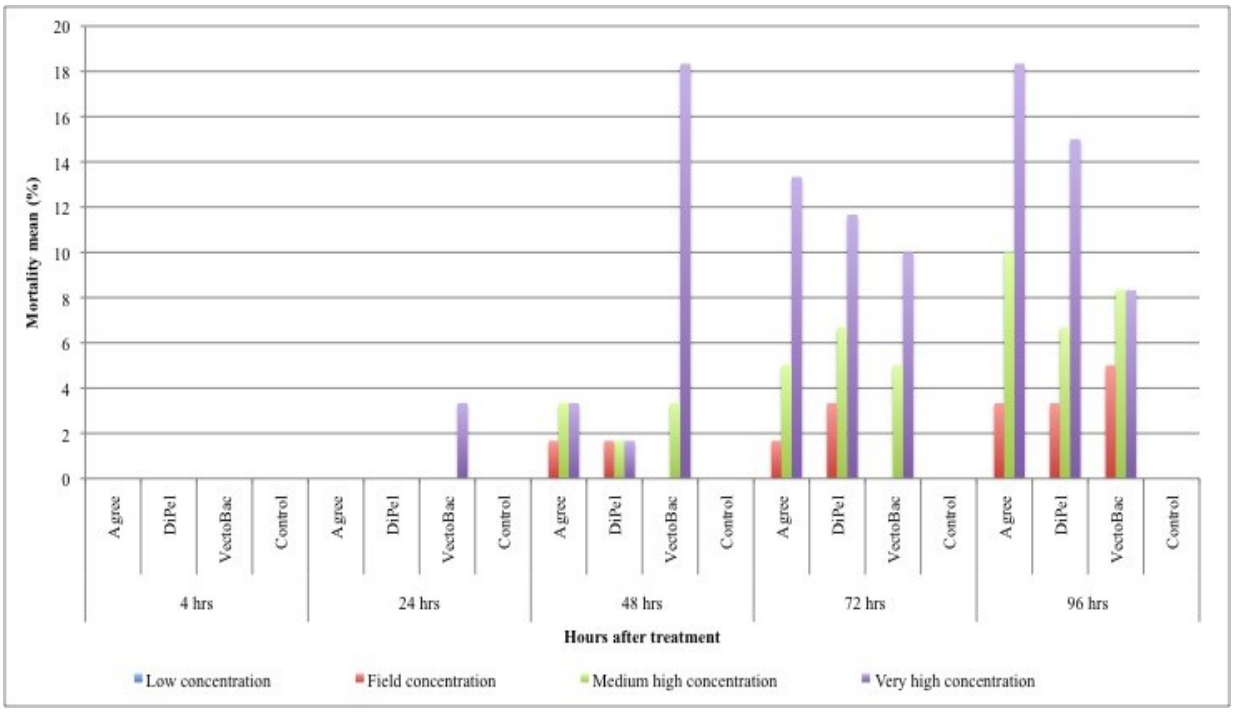

Fig. 5.5 – Comparison of mortality mean (%) of *Apis mellifera* at 4, 24, 48, 72 and 96 hrs.

Results of regression line are shown in figs. 5.6-5.8. In particular, LD_{50} value for Agree and DiPel are respectively, 162 g/l and 207 g/l, showing a lower toxicity of the latter, while for VectoBac is 21 g/100ml.

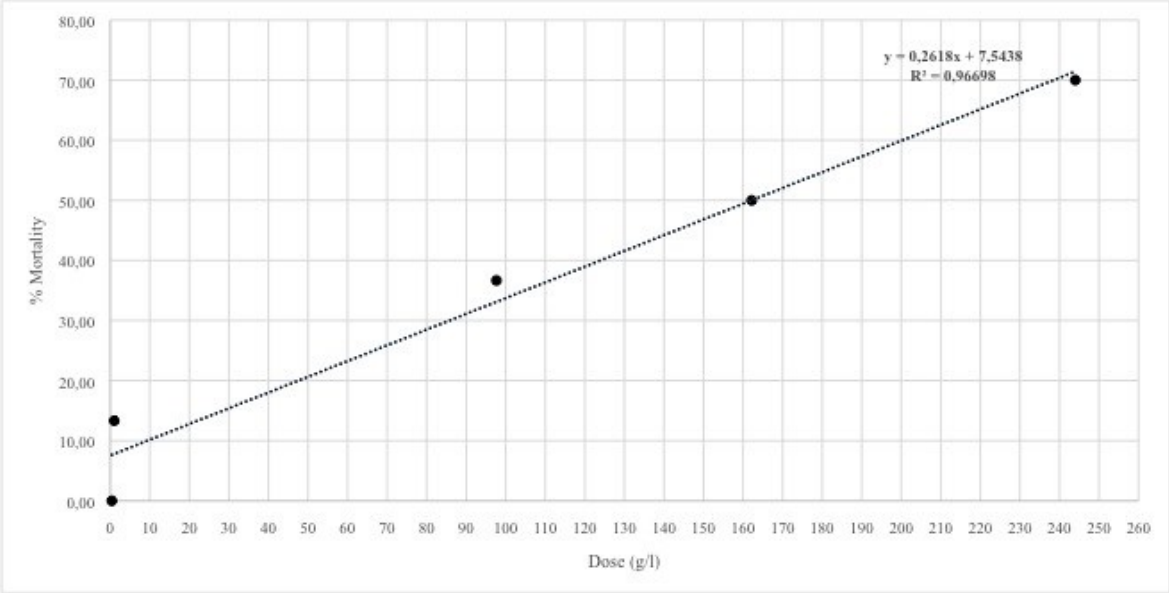

Fig. 5.6 – Regression line and LD_{50} of honeybees treated with Agree.

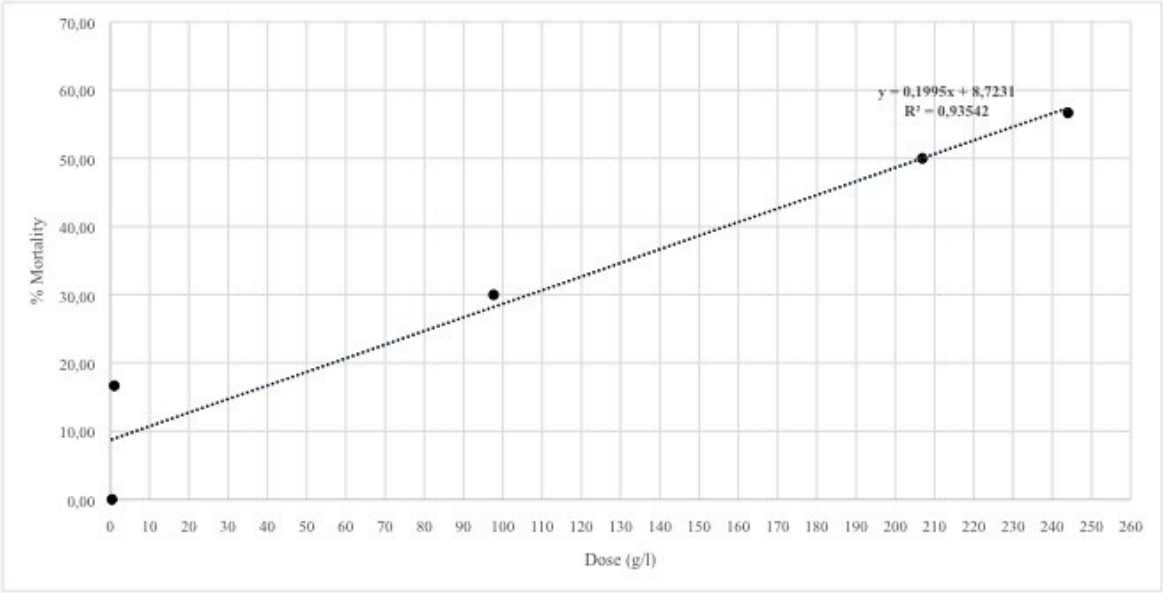

Fig. 5.7 – Regression line and LD_{50} of honeybees treated with DiPel.

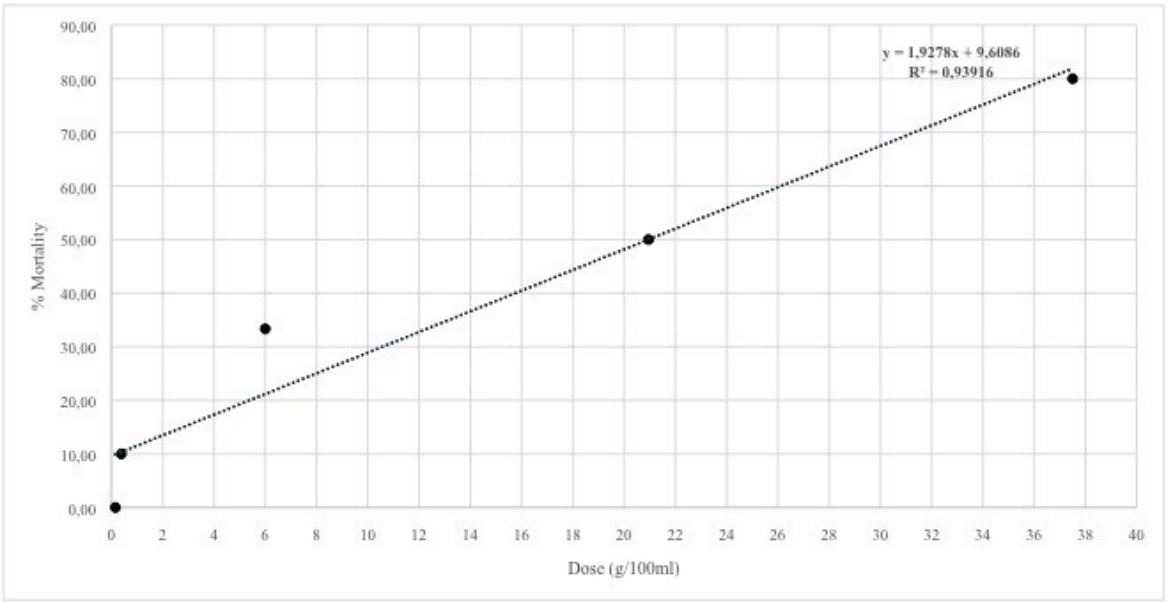

Fig. 5.8 – Regression line and LD_{50} of honeybees treated with VectoBac.

5.1.2 Survival of honeybees (*Apis mellifera*)

The survival curves show a reduced longevity of treated individuals, particularly at high concentrations.

In particular, survival curve of specimens treated with Agree show that, 96 hrs after the single ingestion of the biopesticide solution, 63,33% of the bees survive after treatment with medium-high concentration and 30% of bees survive after very-high concentration. 7 days after treatment with the single biopesticide solution at medium-high concentration, the survival rate (36,67%) approximates the rate at 96 hrs after treatment with very-high concentration (30%) (fig. 5.9).

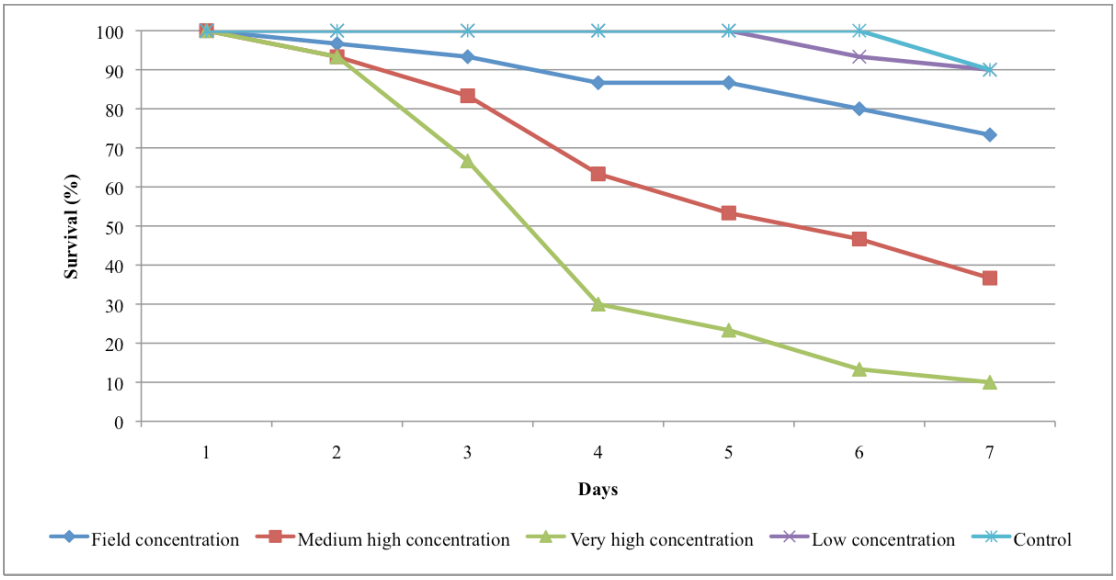

Fig. 5.9 - Survival curves of *A. mellifera* treated with Agree.

The survival of honeybees treated with DiPel is similar to specimens treated with Agree. In particular, 96 hrs after the single ingestion of the biopesticide solution, 70% of the bees survive after treatment with medium-high concentration and 43,33% of bees survive after very-high concentration. Furthermore, 7 days after treatment at medium-high concentration, the survival rate decreases rapidly (26,67%) than the group treated with very-high concentration, much more constant (fig. 5.10).

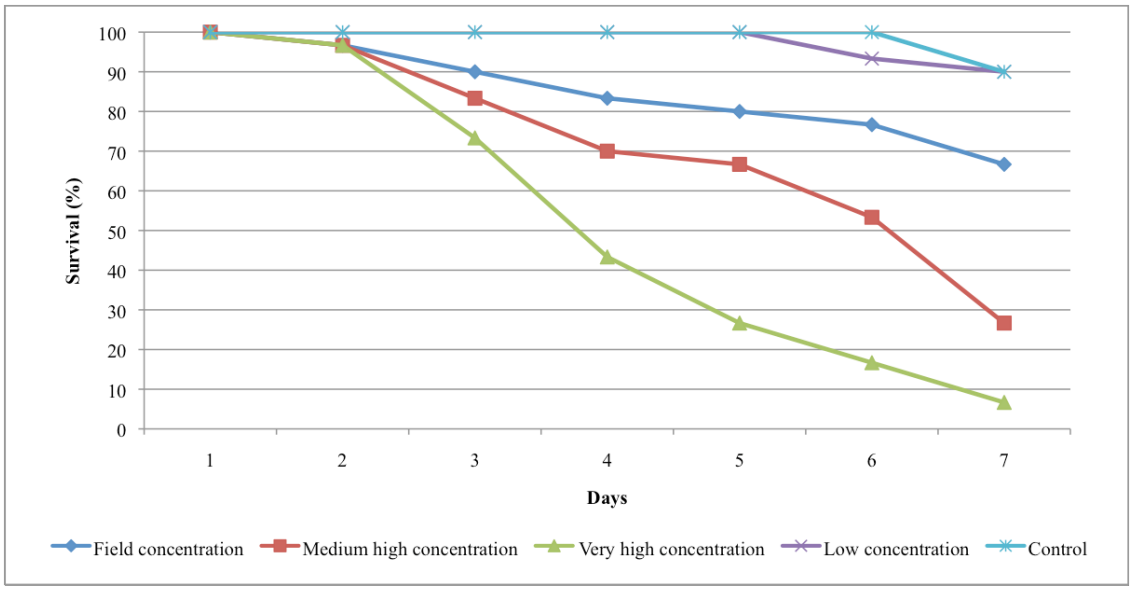

Fig. 5.10 - Survival curves of *A. mellifera* treated with DiPel.

The survival curves of specimens treated with VectoBac show that, 7 days after the treatment, 66,67% of the bees survive after treatment with field concentration and the same percentage of bees survive at 96 hrs after medium-high concentration treatment. Furthermore, already at 48 hrs, in very-high concentration treatment survival was reduced by almost 50% (fig. 5.11).

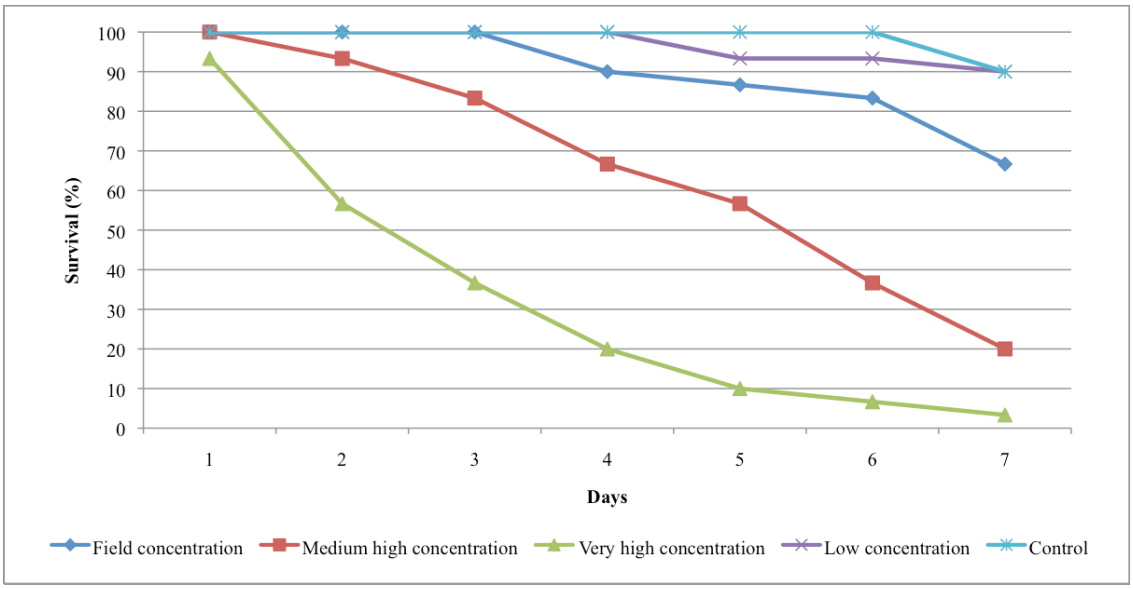

Fig. 5.11 - Survival curves of *A. mellifera* treated with VectoBac.

5.1.3 Behavior and symptoms observations on honeybees (*Apis mellifera*)

Symptoms observations and the effects on the behaviour of honeybees after the sole and not repeated ingestion of the four tested concentrations is similar for the three biopesticides.

Indeed, 4 hrs after ingestion of the four tested concentrations of the biopesticide solution, only the specimens from low concentration group don't show evident sickness symptoms, keeping these conditions up to the 96 hrs after treatment. Specimens from the groups treated with field, medium-high and very-high concentrations, instead, show an evident hypoactivity (workers are motionless, close to the walls or at the bottom of the cage) compared to the control group. This phase, called stationary in agreement with Medrzycki et al. 2003, is

also accompanied by a lower consumption of food after the treatment. Specimens from control and low concentration groups, in fact, eat on average respectively about 50,41% and 48,81% of the sugary solution devoid of biopesticide, whereas the treated bees eat on average, respectively, about 30,85% (field concentration group), 31,25% (medium-high concentration group) and 30% (very-high concentration group).

24 hrs after treatment with the single dose of the biopesticide solution, only the bees treated with the field concentration reduce their hypoactivity, although the food consumption is lower than specimens of the control and low concentration groups (33,75%, compared with 47,10% and 46,90%, respectively). As for the specimens treated with the other two concentrations, however, the food consumption is markedly reduced if compared with the control group (31,67% and 30,83%, for medium-high and very-high concentration, respectively).

48, 72 and 96 hrs after treatment, the specimens from the field concentration group show almost total recovery in their general behavioral activities, if compared to all the specimens of the control and low concentration groups. In the other two groups, instead, the specimens show a trophic and behavioral marked hypoactivity: slow and uncoordinated movements, inability to maintain proper posture, subsequent paralysis ending with death of the specimens. Moreover, 63% of treated bees show an increase in the abdomen volume (the control only 30%) and watery stools onto the walls of their cages are evident.

5.1.4 Mortality of solitary bees (*Osmia cornuta* and *Osmia rufa*)

The mortality rate of *Osmia cornuta* specimens treated with field concentration is 13,33%, at 48 hrs (tab. 5.10); the mortality rate of *Osmia rufa* specimens is 10% at 72 hrs and 16,67%, at 96 hrs (tab. 5.11). Results of *one-way* ANOVA followed by LSD test on mortality mean at 96 hrs shows a significant difference for field, medium-high and very high concentration treatments with the control group of *O. cornuta* ($F = 702,065860507$; $df = 3$; $P = 0,000256575$) and *O. rufa* ($F = 281,6347675227$; $df = 3$; $P = 0,019541463$).

Tab. 5.10 – Mortality rates (%) of *O. cornuta* treated at field concentration.

Rep.	**4h**	**24h**	**48h**	**72h**	**96h**	**Tot. died 96h**	**Tot. alive 96h**	**% mortality**					
								4h	**24h**	**48h**	**72h**	**96h**	**Tot ± s.d**
R1	0	0	2	1	1	4	6	0	0	20	10	10	
R2	0	0	1	1	1	3	7	0	0	10	10	10	
R3	0	0	1	1	1	3	7	0	0	10	10	10	
Tot	0	0	4	3	3	10	20	0	0	13,33	10	10	33,33±6,17

Tab. 5.11 - Mortality rates (%) of *O. rufa* treated at field concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	0	1	2	3	7	0	0	0	10	20	
R2	0	0	0	0	2	2	8	0	0	0	0	20	
R3	0	0	0	2	1	3	7	0	0	0	20	10	
Tot	0	0	0	3	5	8	22	0	0	0	10	16,67	26,67±8,34

The response of *Osmia cornuta* specimens treated at medium-high concentration start at 24 hrs with a mortality rate of 13.33% (tab. 5.12); while the first response of *Osmia rufa* specimens is at 48 hrs, with a mortality rate of 16.67% (tab. 5.13). In both cases the total mortality at 96 hrs exceed 50%.

Tab. 5.12 – Mortality rates (%) of *O. cornuta* treated at medium-high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	1	2	2	2	7	3	0	10	20	20	20	
R2	0	2	2	1	2	7	3	0	20	20	10	20	
R3	0	1	1	1	3	6	4	0	10	10	10	30	
Tot	0	4	5	4	7	20	10	0	13,33	16,67	13,33	23,33	66,67±8,99

Tab. 5.13 – Mortality rates (%) of *O. rufa* treated at medium high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	1	4	2	7	3	0	0	10	40	20	
R2	0	0	2	2	1	5	5	0	0	20	20	10	
R3	0	0	3	2	2	7	3	0	0	30	20	20	
Tot	0	0	6	8	5	19	11	0	0	20	26,67	16,67	63,33±12,80

The response of *Osmia cornuta* specimens treated at very high concentration is immediate already at 24 hrs with a mortality rate of 50%, (tab. 5.14) instead the mortality rate of *Osmia rufa* exceed 50% at 72 hrs (tab. 5.15). Total specimens of both species died at 96 hrs (100%).

Tab. 5.14 - Mortality rates (%) of *O. cornuta* treated at very high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	6	2	1	1	10	0	0	60	20	10	10	
R2	0	5	3	1	1	10	0	0	50	30	10	10	
R3	0	4	3	2	1	10	0	0	40	30	20	10	
Tot	0	15	8	4	3	30	0	0	50	26,67	13,33	10	100±18,52

Tab. 5.15 - Mortality rates (%) of *O. rufa* treated at very high concentration.

Rep.	4h	24h	48h	72h	96h	Tot. died 96h	Tot. alive 96 h	% mortality					
								4h	24h	48h	72h	96h	Tot ± s.d
R1	0	0	1	8	1	10	0	0	0	10	80	10	
R2	0	0	3	6	1	10	0	0	0	30	60	10	
R3	0	1	4	2	3	10	0	0	10	40	20	30	
Tot	0	1	8	16	5	30	0	0	3,33	26,67	53,33	16,67	100±24,20

The graph in fig. 5.12 shows a range of total mortality at 96 hrs of individuals treated with field concentration of 26,67% (*O. rufa*) and 33,33% (*O. cornuta*) and this range of total mortality exceed 50% for specimens treated at medium-high concentration. Overall we can deduct a certain sensitivity of the two species initially greater for *O. cornuta*.

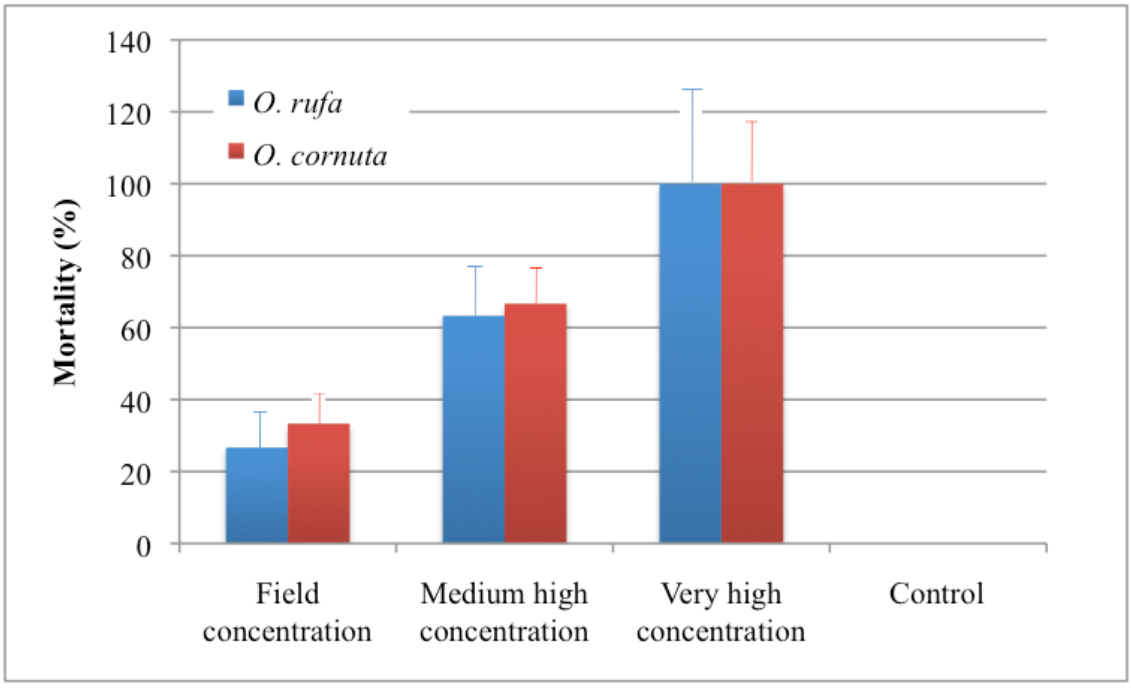

Fig. 5.12 - Total mortality at 96 hrs (%±s.d.) of *O. rufa* and *O. cornuta* treated with DiPel at different concentrations.

Furthermore, in figs. 5.13 and 5.14 are shown the comparison of mortality mean of *O. cornuta* and *O. rufa* specimens treated with different concentrations of DiPel, each of them compared to control, at the different time (4, 24, 48, 72, 96 hrs).

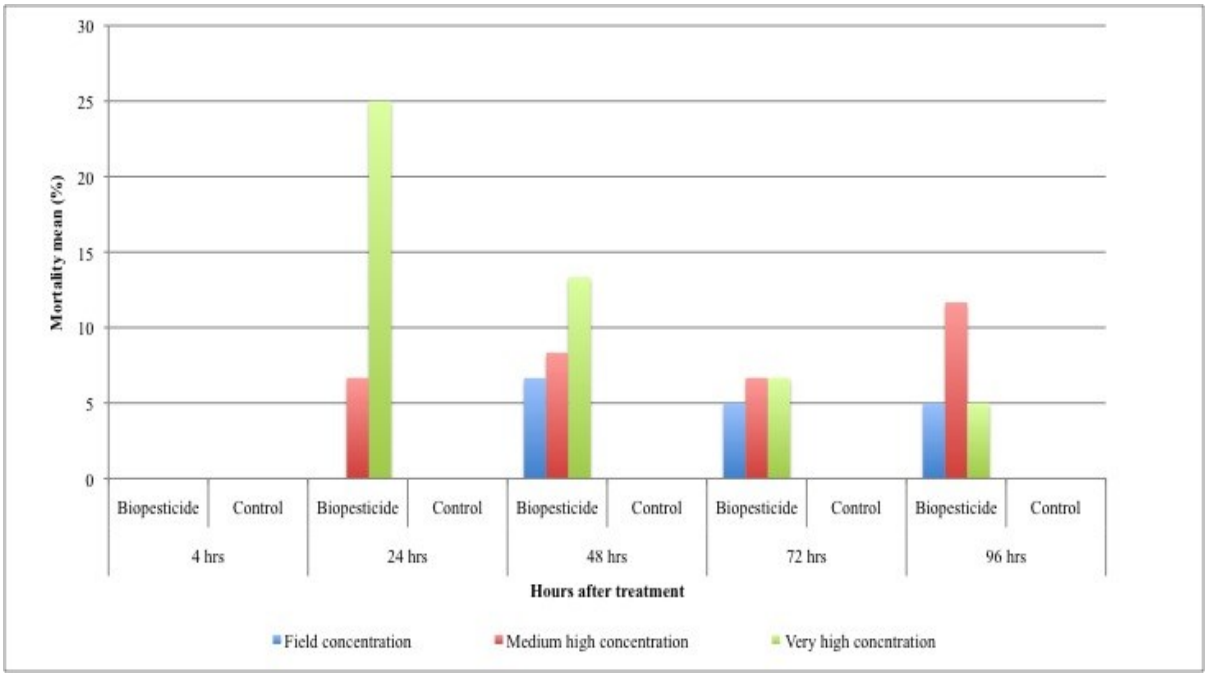

Fig. 5.13 - Comparison of mortality mean (%) of *Osmia cornuta* at 4, 24, 48, 72 and 96 hrs.

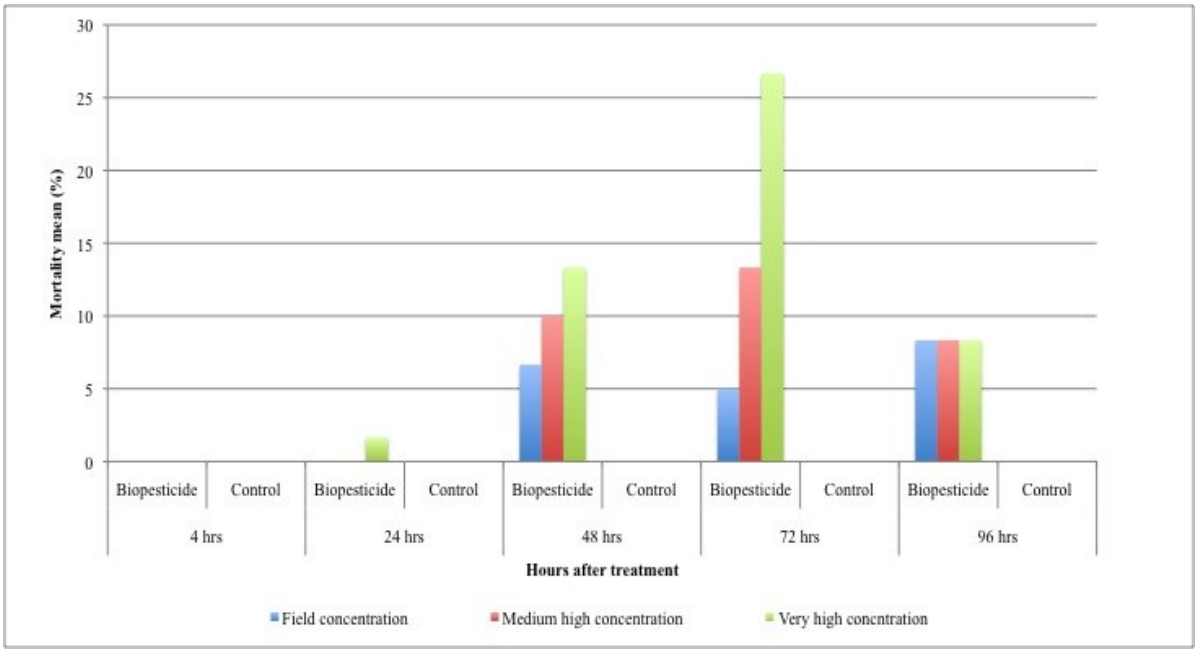

Fig. 5.14 - Comparison of mortality mean (%) of *Osmia rufa* at 4, 24, 48, 72 and 96 hrs.

Results of regression line are shown in figs. 5.15-5.16. In particular, LD_{50} value for *O. cornuta* and *O. rufa* are respectively, 53 g/l and 69 g/l.

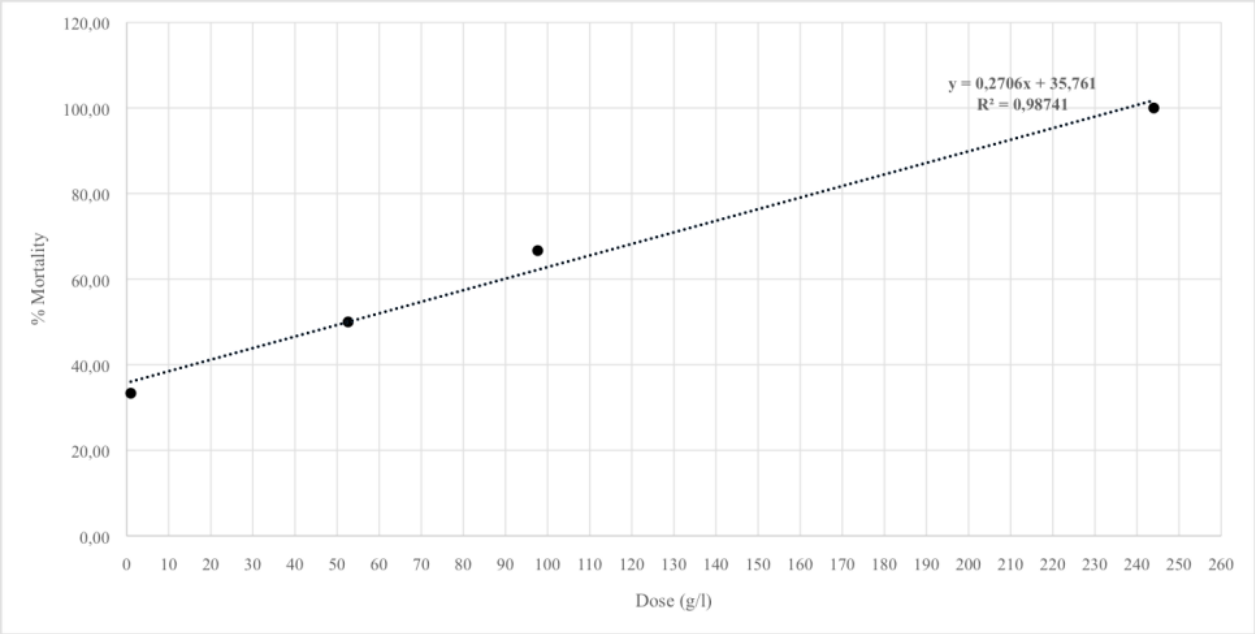

Fig. 5.15 - Regression line and LD_{50} of *Osmia cornuta* treated with DiPel.

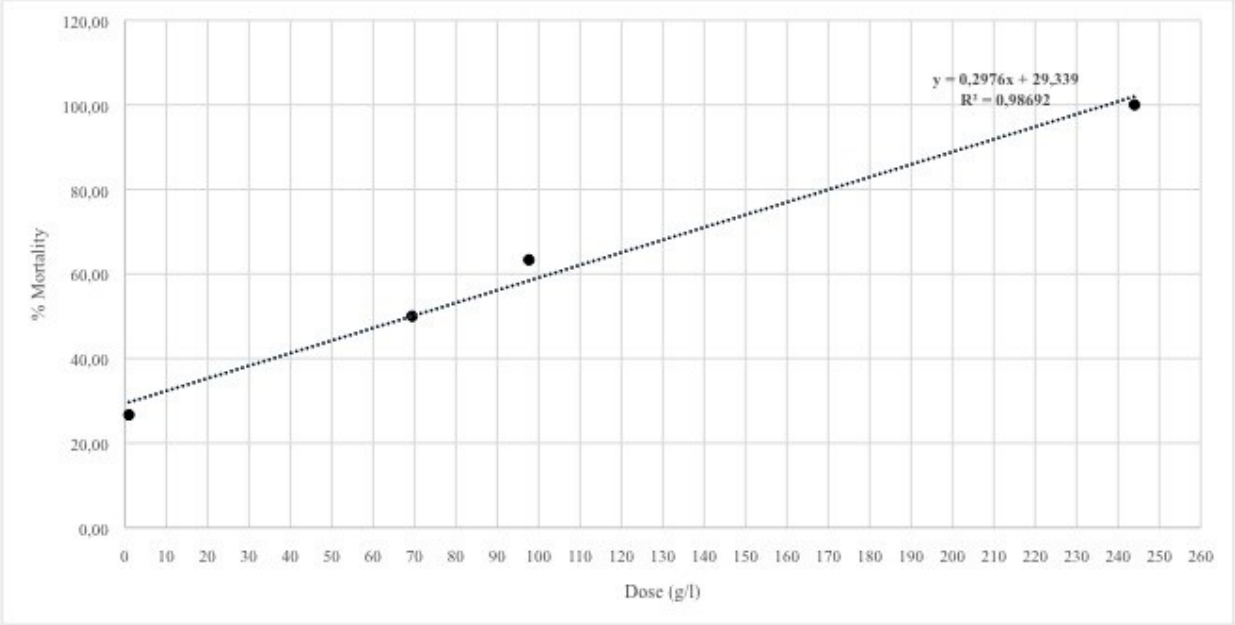

Fig. 5.16 - Regression line and LD_{50} of *Osmia rufa* treated with DiPel.

5.1.5 Survival of solitary bees (*Osmia cornuta* and *Osmia rufa*)

Survival curves for both species show a clear decrease with the death of almost all of the individuals at medium-high concentration. In particular *O. cornuta* specimens treated at field concentration shown a very low values of survival at 7 days after treatment (less than 50%) compared to *O. rufa* (53.3%) that nonetheless suffers a sharp decline (figs. 5.17 and 5.18).

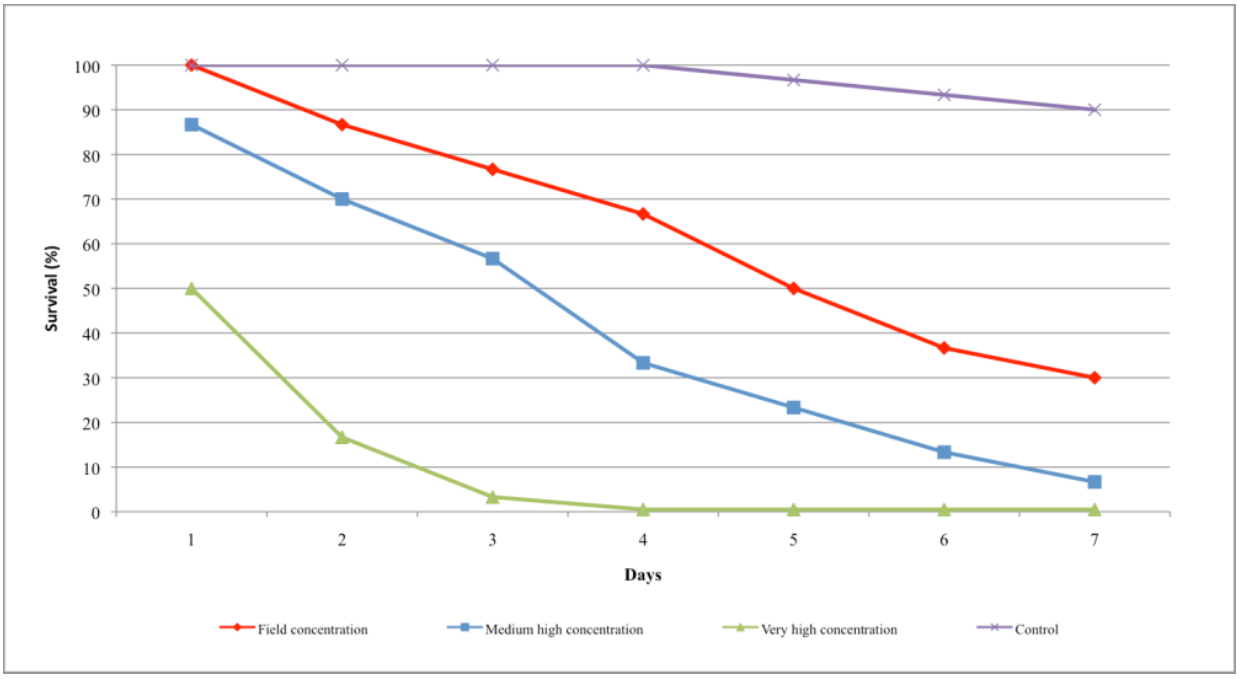

Fig. 5.17 - Survival curves of *O. cornuta* treated with DiPel.

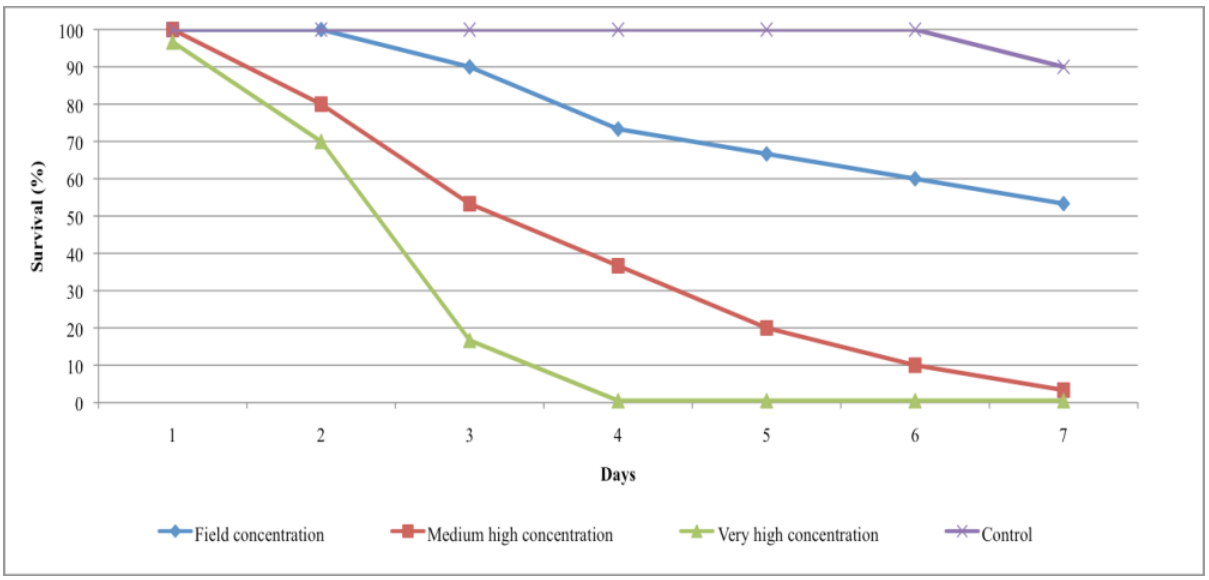

Fig. 5.18 - Survival curves of *O. rufa* treated with DiPel.

5.2 SEM, OM and TEM observations of the honeybees midgut

Morphological and histological SEM observations of the midgut (appendix table I fig. 1), 4 hrs after single ingestion of the biopesticide-solution, do not show significant differences in the specimens of the four groups treated if compared to untreated specimens. The midgut epithelium shows folds (appendix table I fig. 2). The peritrophic membrane, consisting of a dense fibrillar meshwork, is evident in the midgut lumen (appendix table I fig. 3) and visible also in OM obervations (appendix table II fig. 1); this membrane adheres to the epithelial surface, appearing detached in the most posterior tracts of the midgut, so delimiting the endoperitrophic

space from the ectoperitrophic one. Portion of the proventriculus protrudes into the most anterior tract of the midgut lumen (appendix table I fig. 2).

OM observation of the pseudostratified epithelium of the midgut wall shows *regenerative cells* and *digestive cells* (or *enterocytes*): the former do not reach the surface and are mainly organized in small groups; the latter are higher and reach the lumen of the organ (appendix table II fig. 1). Long, densely packed microvilli are evident in the apical region of the enterocytes (appendix table II fig. 2). The latter have a cytoplasm rich in vesicles and show a more or less marked secretory activity (Jimenez and Gilliam, 1989); some globular cellular fragments, with an almost similar diameter, are in the lumen of the organ, frequently interspersed with the fibrils of the peritrophic membrane (appendix table II fig. 3). The abovementioned morphological and histological features remain unmodified up to 96 hrs after treatment only in the untreated specimens.

24 hrs after treatment, in specimens treated with the four different concentrations of the formulation, instead, the midgut epithelium shows alterations in several tracts: numerous enterocytes, in fact, show a mostly vacuolated cytoplasm, nuclear swelling or piknotyc nuclei, increased apocrine secretion and increased cell elimination These alterations are more widespread in specimens treated with medium high and very high dose whereas limited at some areas in the low concentration group (appendix table II figs. 4 and 5; appendix table III fig. 1).

48, 72 and 96 hrs after administration of the formulation, the

vacuolation of the cytoplasm and alteration in general are confined in a few areas decreases gradually in specimens treated with field and low concentration groups, more rapidly in the low concentration group compared with 24 hrs after treatment. In specimens treated with medium high and very high concentration, instead, the vacuolation is markedly widespread compared with 24 hrs after treatment; 96 hrs after treatment, in particular, the apical plasma membrane of many enterocytes is open in some tracts and numerous cellular fragments of different sizes are in the lumen of the organ (appendix table III fig. 3); the muscle cells, furthermore, are not regularly arranged. In the specimens treated with very high dose, in addition, the epithelium has numerous irregular protrusions, does not show folds and its thickness is much reduced compared with the epithelium of the specimens from the other groups (appendix table III figs. 3 and 4).

As for the TEM observations, up to 4 hrs after the trial start, all the specimens from the different groups (including the control one) have similar ultrastructural midgut features that remain unmodified, up to 96 hrs after the trial start, in all the specimens from the control group, similarly to the abovementioned SEM and OM observations: significant differences in the features of the midgut wall are evident only starting from 24 hrs after the administration of the formulation to the four different concentrations, if compared with control specimens. The latter, in particular, show enterocytes having a cytoplasm with numerous vesicles of various sizes and heterogeneous content; many vesicles, in particular, contain a spheroidal granule consisting of a thin electron-dense wall and a low electron-dense inner material (appendix

table III figs. 5 and 6; appendix table II fig. 2). The vesicles are more gathered in the apical cytoplasm, beneath the long microvilli that are in contact with the fibrils of the peritrophic membrane through their distal portion (appendix table III figs. 5 and 6; appendix table IV figs. 1 and 2).

48, 72 and 96 hrs after treatment, the observation frequency of these ultrastructural differences with the control group gradually decreases in all the specimens from the low and field concentration groups, although some similar changes are detectable even 96 hrs after treatment. These changes include, in particular, some enterocytes that have a more compact and electron-dense cytoplasm, rich in vesicles with the spheroidal granules (appendix table IV fig. 3). In other enterocytes, instead, the cytoplasm shows areas devoid of organelles and mitochondria with dilated cristae; the mitochondria are more gathered in the apical cytoplasm (appendix table IV figs. 4 and 5).

In individuals treated with medium-high and very high concentration, instead, these alterations are more frequently observed and dramatically worsen after the 24 hrs. The cytoplasm of numerous enterocytes, after 96 hrs, is almost devoid of organelles and the nucleus is pyknotic (appendix table IV fig. 6). Small vesicles with electron-transparent content are gathered in the apical cytoplasm of these cells and the microvilli are reduced or even absent: cell fragments of different sizes, with pyknotic nucleus and microvilli, interspersed with a meshwork with fibrils irregularly organized than other groups (appendix table V fig. 1, 2 and 3). The nucleus of the regenerative cells is frequently dilated, with dispersed chromatin

(appendix table IV fig. 6 and appendix table V fig. 4).

The basal plasma membrane of the enterocytes of the control specimens and in most of the midgut areas of the bees treated with low concentration, 96 hrs after treatment in particular, shows numerous deep introflections where the fibrillar basal lamina, about 0.6 μm thick, is evident (appendix table V fig. 5).

In some areas of the specimens from the low concentration group and in most of the midgut epithelium of the specimens treated with field concentration, instead, many enterocytes show numerous small vesicles, with electron-transparent content, close to the introflections, less regular and more dilated, of the basal plasma membrane (appendix table V fig. 6). Furhermore, the meshwork that covers the epithelium appears, at different points, less homogeneous in its organization and distribution (appendix table VI fig. 1), compared to the control group.

In specimens treated with medium-high and very high concentration, instead, the basal lamina of the epithelium is about 0.3 μm thick; the introflections of the basal plasma membrane of the enterocytes are reduced (even absent in individuals treated with very high concentration) and frequently close to large vesicles with a mainly electron-transparent content (appendix table VI fig. 2, 3 and 4; appendix table IV fig. 6). The plasma membrane appears even broken in some places, pouring the cytoplasmic contents into the lumen of the organ; the latter also presents a material intensely basophilic, of granular nature, in which are intermingled with the fibrils of a less compact peritrophic membrane (appendix table VI figs. 5 and 4.6).

In addition, in all the specimens treated with field, medium-high and very high concentrations, particularly the latter two cases, some muscle cells have pyknotic nuclei and others muscle cells have a cytoplasm devoid of organelles (appendix table VI fig. 6).

Finally, midgut microflora, including unspecified bacteria and fungi not uniformly spread, is constantly observed during our SEM and TEM observations of the midgut lumen of all the specimens examined from the five different group (including the control one) during the 96 hrs of our observations (appendix table VII figs. 1, 2 and 3). 96 hrs after treatment, in particular, the SEM and TEM findings of the midgut microflora is much higher in all the specimens treated with field, medium-high and very-high concentrations, if compared to the control and the low concentration groups.

Furthermore, in all the specimens treated with medium-high and very high concentrations, the muscular-connective sheath underlying the basal lamina is frequently also rich in bacteria (appendix table VII fig. 4). These features are more frequent in individuals treated with medium high and very high concentration.

5.3 Gene expression and biochemical analysis

By mass spectrometry analysis were identified some proteins and from these were selected 6 proteins (recover to 4 hrs) considered interesting for their biological significance and potentially involved in the response to the treatment of bees with biopesticide on the basis of their description (http://www.uniprot.org/). Proteins identified are:

1) Alpha-glucosidase (hbg1), catalysis of the hydrolysis of terminal, non-reducing alpha-(1->4)-linked alpha-D-glucose residues with release of alpha-D-glucose. The chemical reactions and pathways involving carbohydrates, any of a group of organic compounds. Includes the formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule;

2) Defensin-1, found in royal jelly and in hemolymph, potent antibacterial protein against Gram-positive bacteria at low concentration. Reactions triggered in response to the presence of a bacterium that act to protect the cell or organism. Innate immune responses are defense responses mediated by germline encoded components that directly recognize components of potential pathogens;

3) Eukaryotic translation initiation factor 6 (eIF6), binds to the 60S ribosomal subunit and prevents its association with the 40S ribosomal subunit to form the 80S initiation complex in the cytoplasm. May also be involved in ribosome biogenesis. The aggregation, arrangement and bonding together of the large and small ribosomal subunits into a functional ribosome. A cellular process that results in the biosynthesis

of constituent macromolecules, assembly, and arrangement of constituent parts of a large ribosomal subunit; includes transport to the sites of protein synthesis;

4) Serine Protease 36 (SP36), catalysis of the hydrolysis of internal, alpha-peptide bonds in a polypeptide chain by a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine);

5) Superoxide dismutase (SOD-2), destroys radicals which are normally produced within the cells and which are toxic to biological systems;

6) Thioredoxin reductase (Trxr-1), interacting selectively and non-covalently with nicotinamide-adenine dinucleotide phosphate, a coenzyme involved in many redox and biosynthetic reactions; binding may be to either the oxidized form, NADP+, or the reduced form, NADPH.

The results obtained by RT-PCR, were negative for the genes defensin-1 and α-glucosidase because the primer used didn't permit a clear amplification. Regarding *eIF6*, *TrxR-1* and *SP 36* genes normalized to the housekeeping gene EF1-α, the densitometric analysis and Student's t test didn't reveal a clear and significant difference in expression between the samples, while for the *SOD-2* gene also normalized to the *EF1-α* gene it was observed a dose-dependent increase of the gene expression after 4 hrs from the treatment in treated groups compared to the control; this difference was no longer observable at 24 hrs (fig. 5.19 and 5.20).

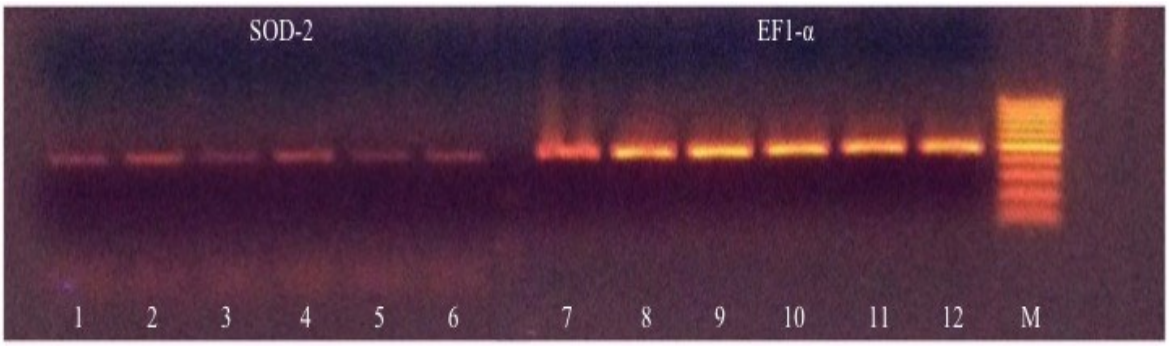

Fig. 5.19 - Electrophoresis of SOD-2 gene and EF1-α gene after amplification; lines 1-3 and 7-9 represents specimens after 4 hrs of treatment while lines 4-6 and 10-12 after 24 hrs; lines 1,4,7,10: control; lines 2,5,8,11: field concentration; lines 3,6,9,12: high concentration; M: 100bp DNA marker.

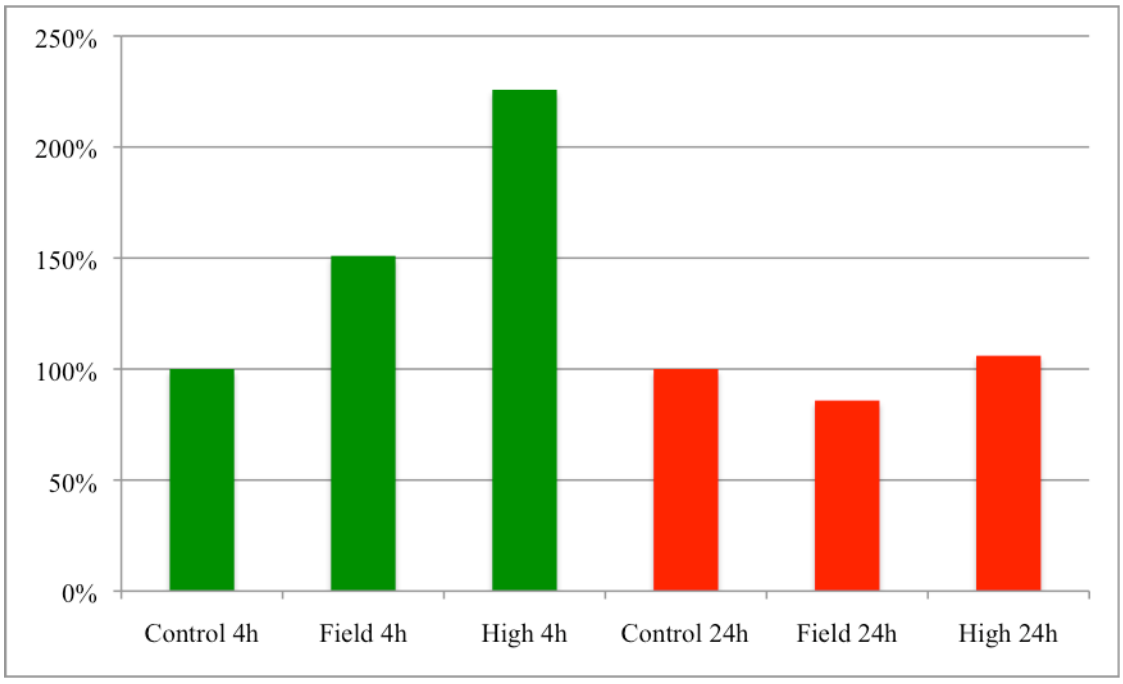

Fig. 5.20 – Densitometric expression (%) of SOD-2 gene. Student's t test $P<0.05$.

In light of these results, we proceeded with the validation of the protein characterization data and gene expression of SOD-2 gene by Western blotting both on control and treated samples with the specific primary antibody anti-SOD2 (Abcam®). The results obtained by Western blotting show the same dose-dependence increase expression. SOD-2 data were normalized to the β-Actin. The difference in SOD-2 production showed by densitometric analysis was observable after 4 hrs from the treatment in treated groups compared to the control; at 24 hrs this difference was no longer evident (fig. 5.21, 5.22 and 5.23).

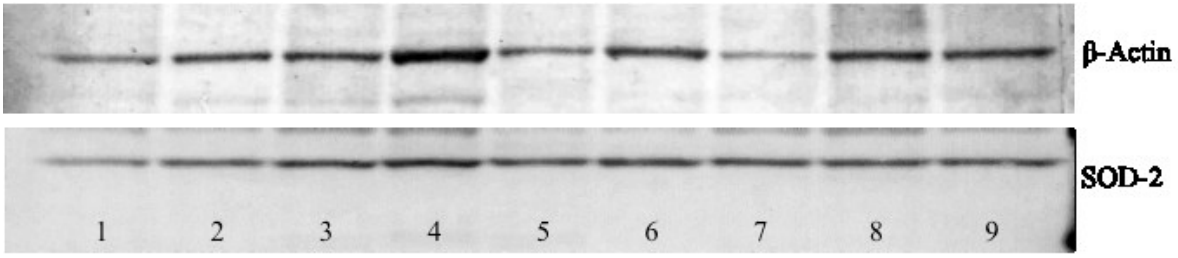

Fig. 5.21 – Western blot analysis of SOD-2 (25kDa) and β-Actin (43 kDa) after 4 hrs from the treatment; lines 1-3: control; lines 4-6: field concentration; lines 7-9: high concentration.

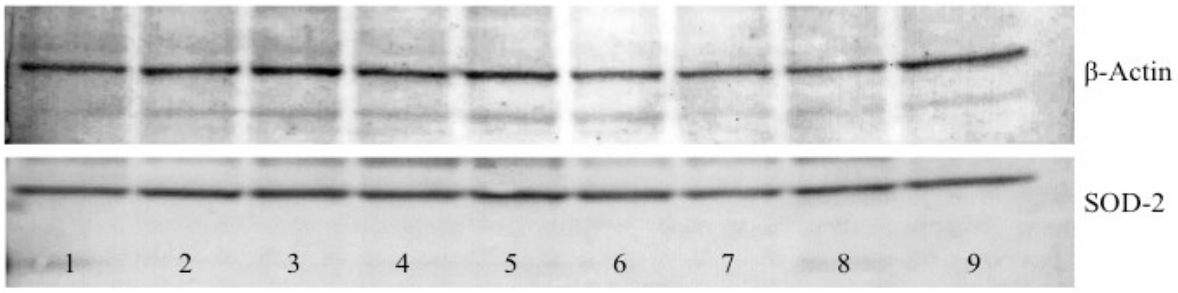

Fig. 5.22 – Western blot analysis of SOD-2 (25 kDa) and β-Actin (43 kDa) after 24 hrs from the treatment; lines 1-3: control; lines 4-6: field concentration; lines 7-9: high concentration.

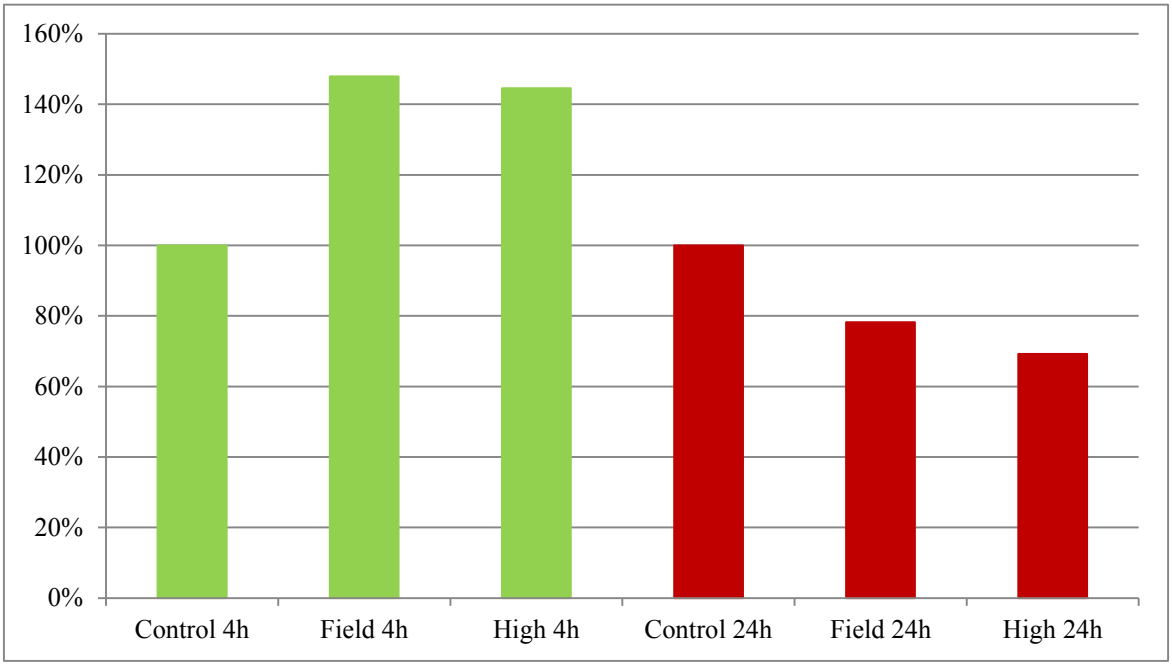

Fig. 5.21 – Densitometric expression (%) of SOD-2. Student's t test $P<0.05$.

5.4 Preliminary investigation on microflora

Midguts extracted for the preliminary investigation of microflora had an average weight of 0,02354 g (tab.5.16) and the results of both on control and treated honeybees are: a conspicuous growth of aerobic bacteria on Mueller-Hinton (MH) agar, (10^{-1}) both at 37°C and at room temperaure, after 24 hrs. Also anaerobic bacteria growth on Blood Agar, (10^{-1}) at room temperature, after 72 hrs was conspicuous.

Tab. 5.16 - Midguts weight [g] of *Apis mellifera* specimens

Sample	Midgut weight [g]
C1	0,0238
C2	0,0203
C3	0,0249
C4	0,0197
C5	0,0197
A1	0,0278
A2	0,0234
A3	0,0195
A4	0,0232
A5	0,0288
B1	0,0218
B2	0,0164
B3	0,0327
B4	0,0229
B5	0,0282

Legend:

C: Control

A: Honeybees treated with field concentration (1 g/l)

B: Honeybees treated with high concentration (97,65 g/l)

As far as the growth on the other media are concerned from the incubation at 37°C, 24 hrs, aerobically: both BEA (Bile Aesculin Agar) and MSA (Mannitol Salt Agar) was negative. Instead, a lactose-fermenting coliforms (red) and non-fermenting (white) was grown on Mac Conkey Agar (Mc) and *E. coli* colonies (dark) and other non-fermenting bacteria (clear) was grown on EMB (Eosin Methylene Blue Agar) (fig. 5.24).

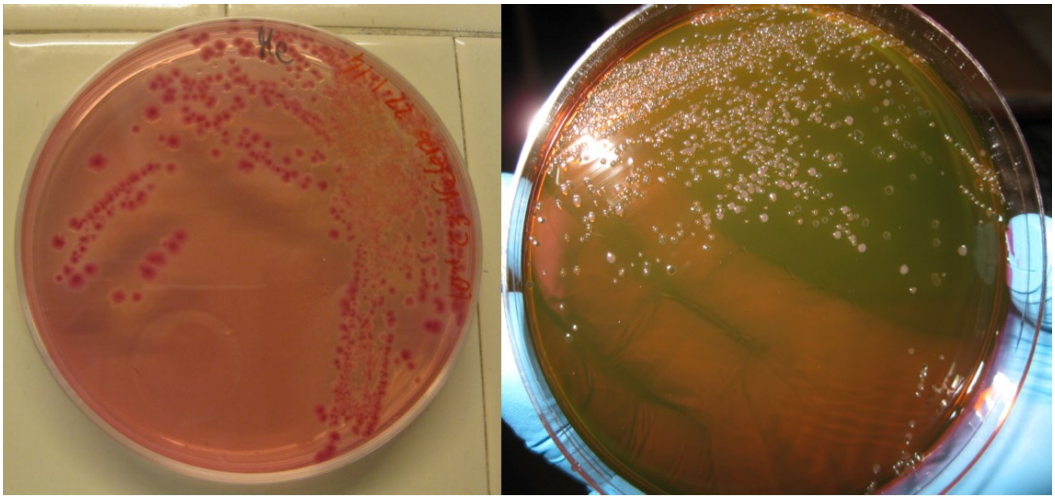

Fig. 5.24 – Lactose-fermenting coliforms (red) and non-fermenting (white) grown on Mac Conkey agar (dx) and *E. coli* colonies (dark) and other non-fermenting bacteria (clear) grown on EMB (sx) at 37°C, 24 hrs, aerobically, from specimens treated with high concentration.

The presence of yeasts was confirmed by the growth on Sabouraud Agar (72 hrs) of colonies of at least three different species of yeasts.

Although the sterility protocol adopted has proved to be suitable for this preliminary study since on the plates it was not detected the presence of inhibitors of growth arising from environmental contamination, but only the presence of intestinal bacteria.

Overall, during the preliminary investigation of microflora, the growth was abundant both in individuals treated and in the control group, therefore, a further dilution (at least 10^{-4}) is required to establish a change in the normal state of colonization of the midgut.

Chapter 6

DISCUSSION

In our laboratory controlled trials the groups of workers of *Apis mellifera* treated with four different concentrations (low, field, medium-high and very-high) of a solution containing commercial Bt-based biopesticides, display different degrees of pathological effects (mortality, behavior and morphostructural midgut alterations), during the 96 hrs of observations after treatment, if compared to the control workers.

The analyses of overall mortality and survival curves, in particular, imply obvious concentration dependence between the single ingestions of the biopesticide solution and the variation of the mortality rates in the treated groups of workers of *A. mellifera*. The observed temporal trend of the mortality rates is also congruent with the findings of similar previous studies on Diptera larvae (Trona et al., 2004; Ruiu et al., 2012).

The mortality rates of honeybees are similar in that specimens treated with Agree and DiPel, in fact, arising 48 hrs after the ingestion of a single dose of the solution (at field, medium-high and very-high concentrations), markedly increase at 72-96 hrs only in the groups treated with medium-high and very-high concentrations, if compared to the respective times after treatment with field concentration. Instead, specimens treated with VectoBac at field concentration show

a response at 96 hrs, not before, with a mortality rate of 10%. The mortality rate in the groups treated with medium-high and very-high concentration of VectoBac quickly changes at 48 hrs and 24 hrs after the treatment, such as specimens treated with Agree and DiPel.

Furthermore, it has to be noticed that, 7 days after treatment, the survival rate in the group treated with medium-high concentration of Agree, DiPel and VectoBac interestingly approximates the rate at 96 hrs after treatment with very-high concentration, so indicating a presumable long-term effect on mortality beyond 96 hrs after treatment with the tested solution at the medium-high concentration.

Regarding the investigation about the biopesticides influence on the behavior the specimens from low concentration group didn't show evident sickness symptoms, while a decrease in locomotor and trophic activity has been observed in all the specimens from field, medium-high and very-high concentration groups, starting from 4 hrs after the treatment with the respective solution concentrations. These symptoms, however, decrease during the 96 hrs only in all the specimens from the groups treated with field concentrations. The specimens treated with medium-high and very-high concentrations, instead, show worsening of the symptoms, e.g., both increase in the abdomen volume of the bees and watery stools onto the walls of their cages, similarly to what has been observed by Brighenti et al. (2007) in similar trials on adults of *A. mellifera* treated with *B. thuringiensis* var. *kurstaki*; the symptomps worsen until their complete paralysis and/or death, as reported, e.g., for *Bombyx mori* larvae (Endo and Nishiitsutsuji-Uwo, 1980) treated with the *B. thuringiensis* var.

kurstaki and *aizawai* or for house fly larvae treated with *Brevibacillus laterosporus* (Ruiu et al., 2012).

Finally the high value of LD_{50} for the products and the absence of evident sickness symptoms in the group treated with low concentration (a possible dilution of the biopesticides occurring in the field) suggest that are non-toxic in acute toxicity.

As far as *O. cornuta* and *O. rufa* are concerned a concentration dependence between the single ingestions of the biopesticide solution and the variation of the mortality rates exists also in this two species of solitary bees, indeed, they show greater sensitivity to the concentrations tested (the product tested is only DiPel) compared to *Apis mellifera*. In fact, looking at the overall mortality rate of *Apis mellifera* to 96 hrs to medium-high concentration that is 30% while it is 66.67% for *O. cornuta* and 63.33% for *O. rufa*. This percentage is similar for specimens of *Apis mellifera* treated with test solution at very-high concentration (56.67% after 96 hrs from treatment); the latter concentration, instead, causes the death of 100% of the specimens in both species of solitary bees. In addition, the mortality rate of solitary bees for the field concentration group is higher than that of the same treated group of honeybees (16.67% compared to 33.33% for *O. cornuta* and 26.67% for *O. rufa*). DiPel seems to be the least toxic product for *Apis mellifera* of three biopesticides tested at high concentrations. Comparing the results of the toxicity test with DiPel in honeybees and in *Osmia cornuta* and *Osmia rufa* it is shown that, although the value of LD_{50} is high for all three tested species, a less mortality for *A. mellifera* could indicate a probable more

sensitivity of the solitary bee species that could reduce their long-term vitality.

In this regard some considerations can be made: feeding pre-adaptation and sociality level could explain the different susceptibility between species. In fact, the complex and evolved social immune systems in honeybees, can prevent exposure of the colony to disease and xenobiotics with behavioural, physiological and spatial mechanism, such as the removal of contaminated individuals even if these mechanisms could make the single bees more sensitive to pesticides (Arena and Sgolastra, 2014).

Furthermore, solitary bees, such as *O. cornuta* and *O. rufa*, use mud as nesting material, thus, they are highly exposed to pesticide residues in the soil. Moreover, non-*Apis* larvae are much more exposed to pesticide residues in pollen because they consume large provisions of unprocessed pollen. Overall, many life-history traits influencing the vulnerability to pesticides suggest that solitary bees could be more susceptible compared honeybees because their shorter nesting period, limited foraging range, floral specialization, and in particular when the application of a pesticide coincides with the nesting period (Arena and Sgolastra, 2014).

However, this study, conducted on male specimens of *O. cornuta* and *O. rufa*, represents a preliminary investigation on susceptibility at *Bt* biopesticide from those species because a comparative study should be done also on female specimens. Thus, further investigations are needed in order to confirm this difference in susceptibility at *Bt* biopesticide from *A. mellifera* and *O. cornuta* and *O. rufa*.

As regards the morphostructural (OM, SEM and TEM) investigation on midgut of honeybee workers, shows alterations of the enterocytes, in all the four groups of specimens tested (low, field, medium-high and very high concentration), only 24 hrs after treatment; those results agree with previous studies on other species of insects (Ruiu et al., 2012).

The observations up to 96 hrs after treatment, showed that the extension degree of the alterations affecting the midgut morphostructure is related to the specific concentration of the biopesticide solution and decreases only in the specimens treated with low and field concentration, whereas in the specimens treated with medium-high and very high concentration the alterations are markedly widespread and affect also regenerative cells, involved in the epithelium renewal; in all the specimens treated with very-high concentration, furthermore, the epithelium of the entire midgut wall loses its typical folds.

Therefore, the epithelium damage is so widespread that the physiological turn-over would be unable to ensure the restoration of the tissue integrity, beyond a certain concentration. It should be also noted that these findings are in agreement with the results of the analyses of the overall mortality.

The morphostructural features of the changes observed in the midgut epithelium of the treated specimens, are closely related to the action of *Bt* Cry-toxins, following the ingestion of the biopesticide solution. Similar alterations are reported in previous investigations concerning the action of bacterial toxins, also of *Bt*, on the midgut of

insect larvae of different orders (Bravo et al., 1992, 2011; Lambert and Peferoen, 1992; Knowles, 1994; Trona et al., 2004; Rouis et al., 2007; Ruiu et al., 2007, 2012; Ribeiro et al., 2013). As already demonstrated by Bravo et al., 2011, also in my study on honeybees, the morphostructural changes mainly involve not only the plasma membrane of the midgut epithelial cells causing an osmotic shock of the same cells, but also some alterations of the muscular-connective sheath (pyknotic nuclei) in workers treated with medium-high and very-high concentrations. This muscular-connective morphostructural alterations could be an additional factors leading to blocking of the peristaltic movements of the midgut, followed by altered motoric skills, as noticed in behavior and symptoms observations on honeybees. General paralysis of the body preceded by blocking of the peristaltic movements of the midgut has been similarly observed in previous investigations on species of other orders of insects treated with various bacterial strains, also of *Bt* (Singh et al. 1986a, 1986b; Trona et al., 2004; Ruiu et al., 2012).

A peculiar feature found in this investigation is the numerous SEM and TEM observations of the midgut microflora of the specimens treated with the three different concentration (field, medium-high and very-high concentrations), if compared to the control and the low concentration groups. In particular, this microflora has been found both in the ectoperitrophic space of the midgut lumen and in the muscular-connective sheath, therefore in proximity of the haemocoelic compartment. The death of specimens treated with *Bt*-toxins has been related, in previous studies on other insects, to a septicemia caused by

physiological midgut microflora: epithelial alterations, in fact, allow their invasion inside of haemocoel (Graf, 2011).

In summary, integrating the different mortality rates observed in the oral toxicity assays they could be related to the extension degree of the midgut morphostructural alterations observed, so that going beyond a certain biopesticide concentration, the epithelium damage is so widespread that the physiological turn-over would be unable to ensure the restoration of the tissue integrity.

It is also probable that, before the first appearance of the midgut epithelium damage (24 hrs), the action of *Bt*-toxins could modify almost immediately the midgut physiology of the specimens in the groups treated with field, medium-high and very-high concentrations, so affecting their behavior 4 hrs after treatment. However, in the groups treated with low and field concentration, the epithelium rapidly restore its structural integrity and function beyond the 24 hrs after treatment differently from the specimens treated with medium high and very high concentration, indeed, the continuous action of *Bt*-toxins, could justify the persistence of the symptoms 24-96 hrs after treatment and high mortality rate 7 days after treatment for groups treated with the high concentrations. These results are in line with the numerous previous investigations that have clearly shown the high specificity of *Bt* toxins only for larvae of species of Lepidoptera and Diptera (Lambert and Peferoen, 1992; Knowles, 1994; Caroli et al., 1998; Gupta and Dikshit, 2010).

Mass spectrometry analysis of protein profiles became from control and treated specimens which has led identify different protein was

confirmed for the SOD-2 gene, both by validation with western blotting and by analysis of gene expression.

The detection of SOD-2 is interesting, since it one of the most immediate immune response of the insect gut involves the production of ROS (*reactive oxygen species*) in case of bacterial infection. ROS, which are efficient antimicrobial molecules, generally derived from oxidation-reduction process and their residual can cause inflammatory phenomena in the midgut, therefore, a balance between synthesis of ROS and elimination of residual via antioxidant is necessary to protect the affected tissue (Dussaubat et al. 2012).

Thus, in case of bacterial infection caused by *Bt*-toxin, as the microflora observed in proximity of the haemocoelic compartment during the SEM and TEM observations, the antioxidant system may play an essential role (Dussaubat et al. 2012) and the growing presence of SOD-2, one of the major antioxidant enzymes, would indicate a cellular response to biopesticide. This cellular response could represents a stress factor, from which bees must defend itself and might affect or influence the fitness of the individual despite the fact that the biopesticide not cause the death of specimens.

Furthermore, it cannot be ruled out that the highly frequent SEM and TEM observations of the midgut microflora are possible signs of midgut dysbiosis, more or less related to the action of *Bt* toxins (Babendreier et al., 2007) and it could be an aggravating factor of the abovementioned altered symptomatological conditions (Hamdi et al. 2011). However, further investigations are needed in order to confirm this hypothesis regarding alterations of honeybee midgut microflora

after ingestion of *Bt*-based biopesticides.

The findings from this investigation show some persistent morpho-structural alterations even up to 96 hrs after the treatment both at field and low concentrations, in all the specimens examined, even if at lower degrees, especially for the low concentration group; these detections lead us to reflect on the probable long-term acute toxic effects hypothesized as the worst case scenario i.e. the possibility of a single ingestion of the highest concentration level to bees potentially occurring in field-environment conditions (the field concentration of this study). Moreover, these observations might indicate a chronic toxicity of the *Bt*-toxins on the midgut of the workers of *A. mellifera,* also at concentrations recommended on the label of the products, altering morphostructural features of its epithelium, the fitness and behavior of the workers, similarly to what has been observed in our controlled trials. The possible case of chronic toxicity could occur by following repeated ingestions, within 96 hrs, at probable field-environment dilutions. In agreement with previous investigations on *Apis mellifera* about foraging/learning activity changes and bioaccumulation following the exposition to the *Bt*-toxins (Babendreier et al., 2005; Ramirez-Romero et al., 2005, 2008).

According to Mommaerts et al. (2010), about adults of *Bombus terrestris*, it should also be taken into account the possibility that certain concentrations of *Bt*-based biopesticides could affect not only the survival of the specimens, but also the reproductive rate of the species, by acting, more or less directly, on the reproductive systems.

However, in contrast with larval stages of insect plagues that feed

on foliage, workers bees especially foragers, do it with pollen and nectar. Furthermore, blooming of a determined plant occurs a single time during the year. Thus, only in this restricted time window workers are likely exposed to the insecticide. Before this period, there is no risk of exposure and after that, the effect of *Bt* toxin decreases as it degrades confirming the very low toxicity of the biopesticides both the beneficial insects and the environment.

Chapter 7

CONCLUSION

Overall, comparing results from mortality and symptoms analysis to what is known in the literature on several species of Apoidea tested with *Bt*-based formulations (Malone et al., 1999, 2001; Mommaerts et al., 2010), the mortality rate of 0%, the absence of evident sickness symptoms during the 96 hrs after treatment in the low concentration group, and the very low mortality rate at field concentration, clearly indicate that sole and not repeated ingestions of the tested biopesticides don't affect both survival and behavior of the workers of *Apis mellifera*. Moreover, histological and ultrastructural investigation and biochemical analysis confirm such low toxicity of the tested *Bt*-based biopesticide on workers of *A. mellifera*, at the concentrations presumably found in field-environment conditions, so confirming one more time the fact that the biopesticides represent a category of commercial products certainly more convenient for the environment and the human health than the other agrochemicals (Gupta and Dikshit, 2010).

However, in the light of some observations from this study and considering the environmental and economic importance of the decrease of the biodiversity within the Apoidea and the colony losses of *Apis mellifera*, it is considered useful to remind that in the registration procedure and monitoring of any pesticide product, the

evaluations of the risk must be always based on a variety of parameters, not only on acute toxic effects but also taking into account any possible long-term and chronic effects in a worst case scenario also in the light of the "monarch butterfly controversy" about presumed toxic effects of *Bt* in controlled trials (Losey et al. 1999) and according to Arena and Sgolastra, 2014, the need to include more species of bees in the standard ecotoxicity data set required for the authorization of pesticides in order to achieve the same level of protection for honeybees and wild bees alike: in this way it is possible to achieve greater effects on parasites and the least damage on beneficial insects, especially on the colonies of eusocial insects, as *A. mellifera*.

Since the economic and agricultural impact of honeybees and other wild bees is enormous, further studies are needed ensuring protection and conservation of managed and wild bees.

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http://www.uniprot.org/

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APPENDIX TABLES

TABLE I

Fig. 1. General gut organization of the workers of the control group, 4 hrs after treatment. SEM. es, esophagus, hs, honey stomach; mg, midgut; mt, Malpighian tubules; il, ileum; rt, rectum; st, stinger.

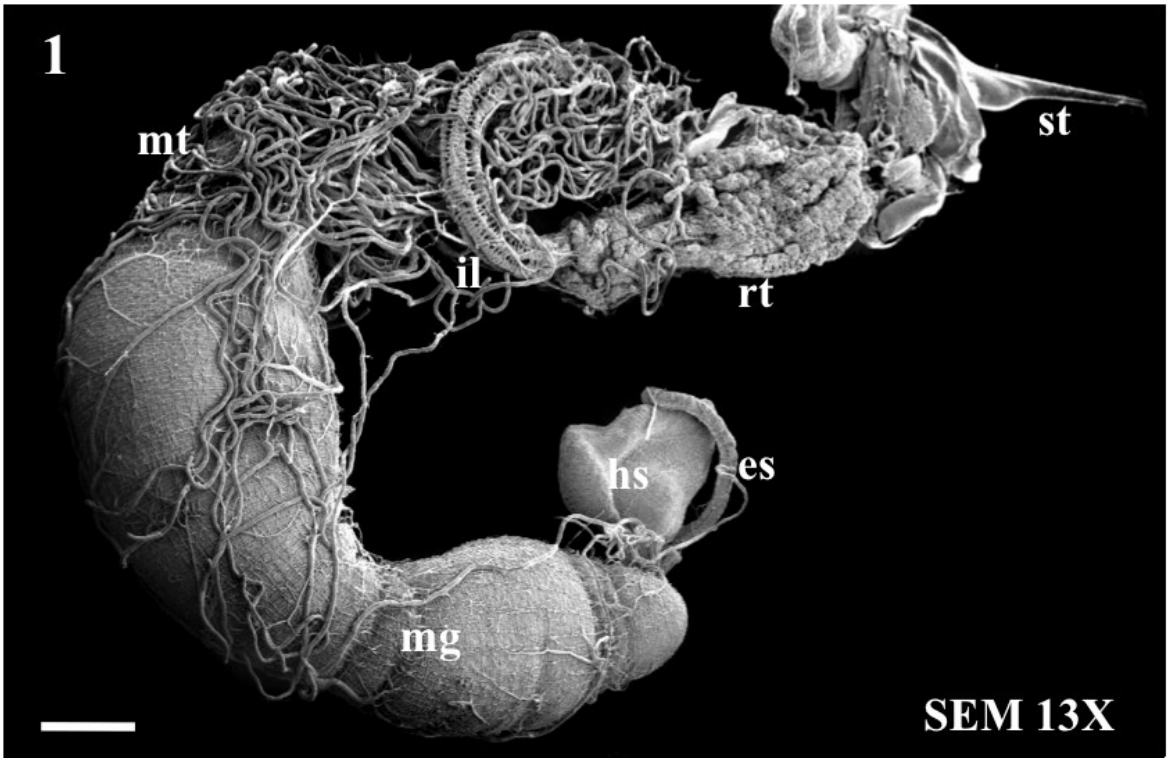
Scale bar: 1 mm

Fig. 2. Morpho-structural organization of the midgut workers of group treated with field concentration, 4 hrs after treatment; B. Organization of the midgut wall. SEM, longitudinal section. Arrows, peritrophic membrane delimiting the endoperitrophic space (en) of the lumen from the ectoperitrophic one (ec); ep, epithelium showing folds; pv, proventriculus protruding into the most anterior tract of the midgut lumen; ms, muscular sheath.

Scale bar: 150 μm.

Fig. 3. Morpho-structural organization of the midgut workers of group treated with field concentration, 4 hrs after treatment. Fibrillar meshwork (mw) of the peritrophic membrane. SEM.

Scale bar: 3 μm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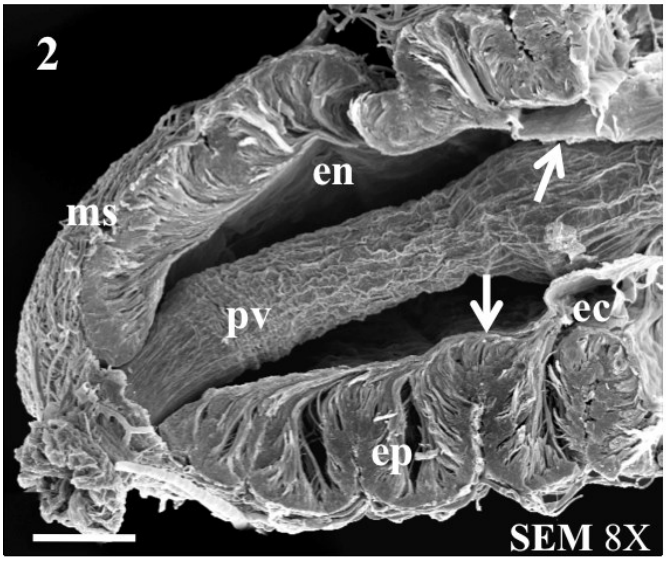
rt
hs
es
mg
SEM 13X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en
ms
pv
ec
ep
SEM 8X

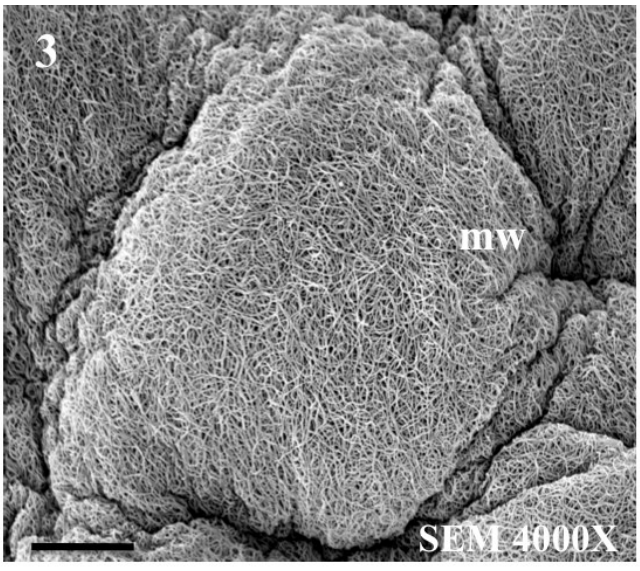

3
mw
SEM 4000X

TABLE II

Fig. 1. Morpho-structural organization of the midgut workers of the control group, 4 hrs after treatment. Enterocytes of the midgut epithelium (ep). MO, Toluidine blue. Arrow, vesicle with a spherical granule; bl, basal lamina; mc, muscular-connective sheath; mi, microvilli; n, enterocyte nucleus; pm, peritrophic membrane in the organ lumen.

Scale bar: 3 μm

Fig. 2. Morpho-structural organization of the midgut workers of group treated with very high concentration, 4 hrs after treatment. Microvilli (mi) of an enterocyte. ac, apical cytoplasm; arrowheads, vesicles with spherical granules. SEM.

Scale bar: 1 μm.

Fig. 3. Morpho-structural organization of the midgut workers of the control group, 4 hrs after treatment. Globular cellular fragments (arrows) in the lumen of the organ. Picture in the upper black frame is a magnification of the particular in the lower frame. SEM. pm, peritrophic membrane.

Scale bar: 15 μm

Fig. 4. Morpho-structural organization of the midgut workers of group treated with field concentration, 24 hrs after treatment. Epithelium (ep) showing enterocytes with a mostly vacuolated

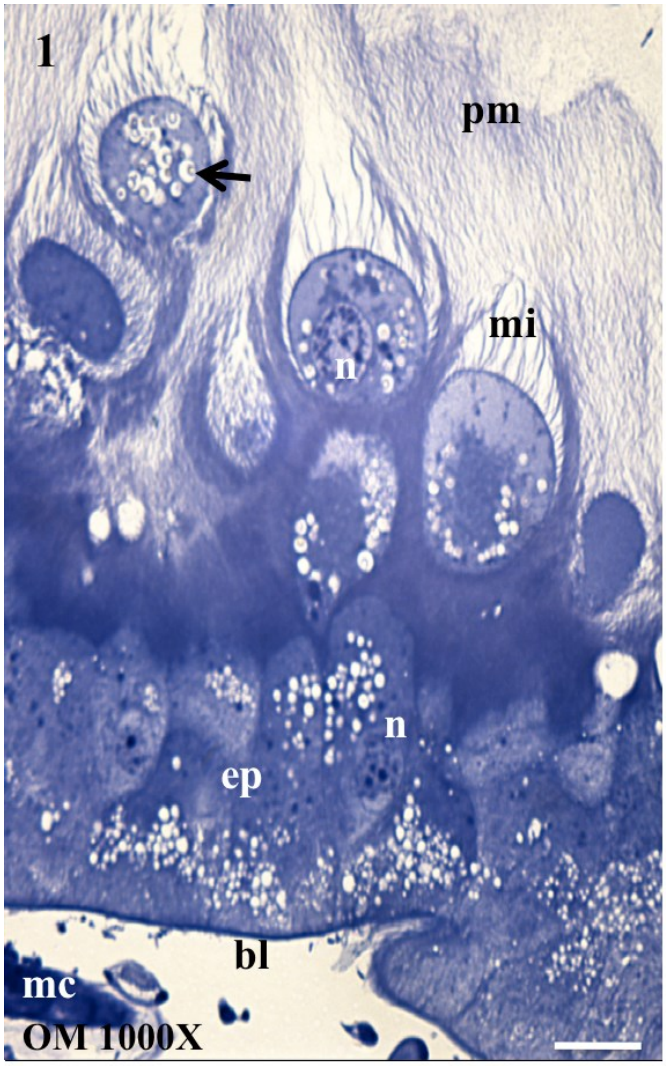
cytoplasm (arrowheads). OM, Toluidine blue. pm, peritrophic membrane; mi, microvilli.

Scale bar: 10 μm

Fig. 5. Morpho-structural organization of the midgut workers of group treated with field concentration, 24 hrs after treatment.

Epithelium (ep) showing some enterocytes with vacuolated cytoplasm (arrowhead). OM, Toluidine blue. l, lumen with peritrophic membrane (pm) and eliminated cells (arrows); mc, muscle sheath.

Scale bar: 10 μm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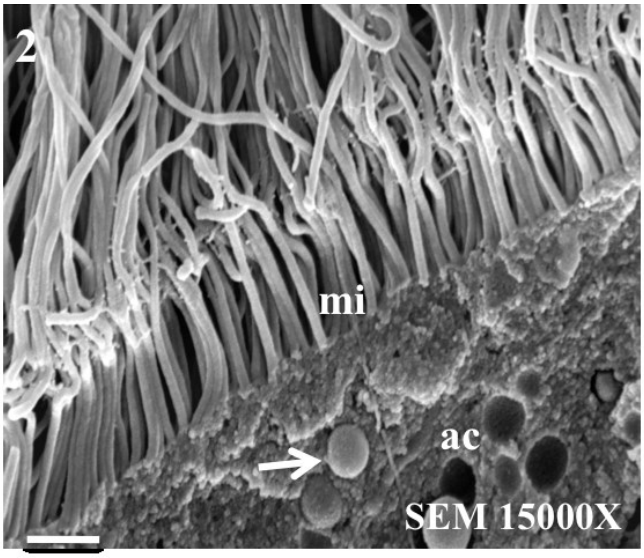
n
n
ep
bl
mc
OM 1000X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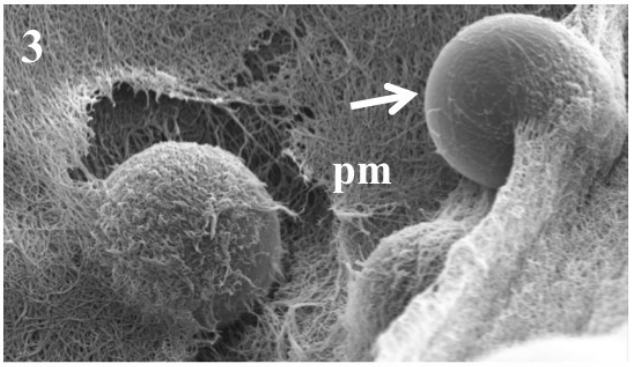
ac
SEM 15000X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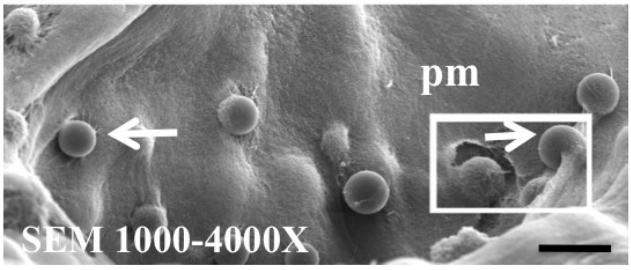
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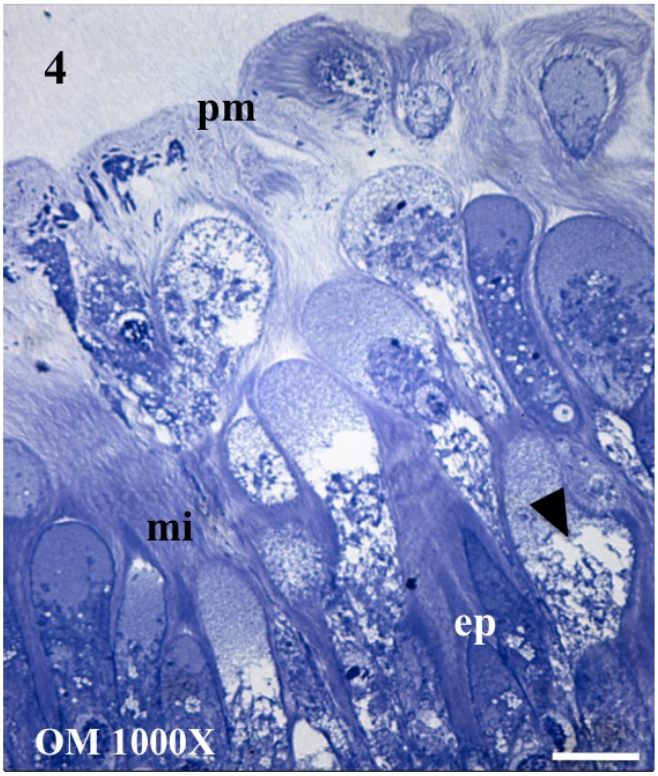
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mi
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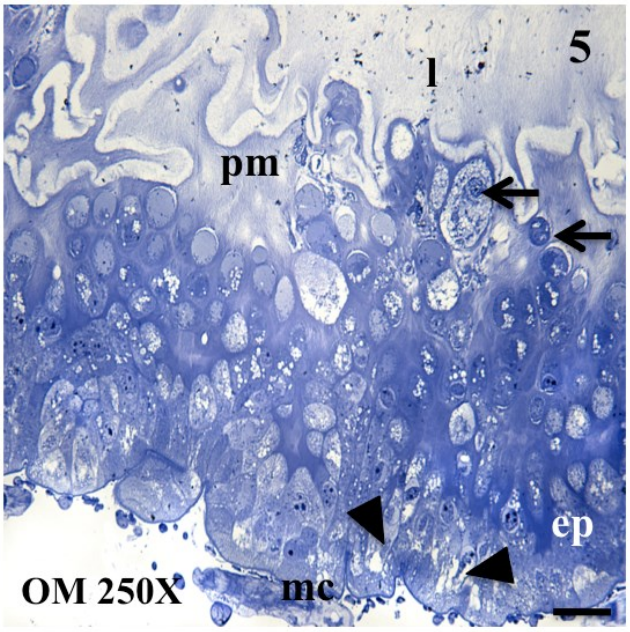

5
l
pm
ep
OM 250X
mc

TABLE III

Fig. 1. Morpho-structural organization of the midgut workers of group treated with field concentration, 24 hrs after treatment. Enterocytes with vacuolated cytoplasm (arrowhead) in apical zone. OM, Toluidine blue. bl, basal lamina; n, nucleus.

Scale bar: 10 μm

Fig. 2. Morpho-structural organization of the midgut workers of group treated with medium-high concentration, 96 hrs after treatment.

Epithelium (ep) showing some enterocytes with their apical plasma membrane opened (arrows) and vacuolated cytoplasm (arrowhead). OM, Toluidine blue. l, lumen; mc, muscle sheath.

Scale bar: 20 μm

Fig. 3. Morpho-structural organization of the midgut workers of group treated with very high concentration, 96 hrs after treatment. Epithelial surface showing irregular protrusion (ip) into the lumen of the organ. SEM. pm, peritrophic membrane.

Scale bar: 10 μm

Fig. 4. Morpho-structural organization of the midgut workers of group treated with very high concentration, 96 hrs after treatment. Epithelium (ep) showing irregular protrusions (arrows), without folds and with a very reduced thickness. MO, Toluidine blue. Arrowheads, muscular sheath; l, lumen of the organ with cellular fragments (cf).

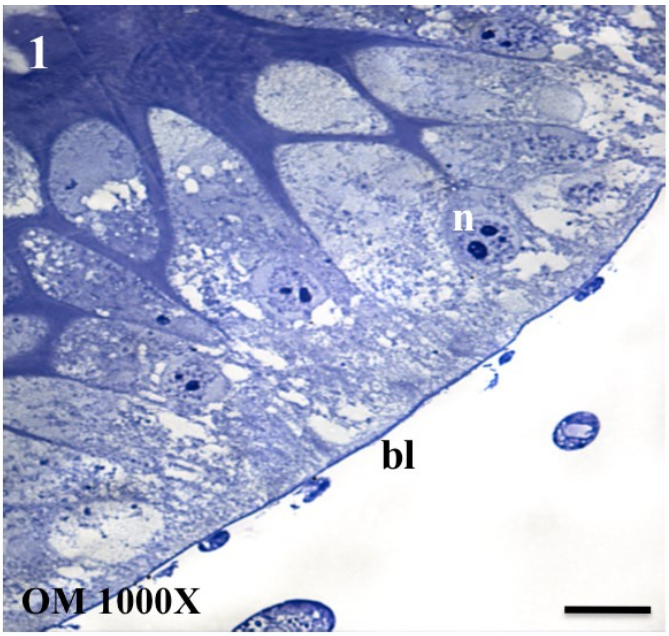
Scale bar: 40 µm

Fig. 5. TEM micrographs of the midgut wall, 96 hrs after treatment, in workers of the control group. Apical cytoplasm (cy) of two enterocytes with vesicles (v), some of which containing a spheroidal granule (arrows). mi, microvilli.

Scale bar: 2 µm

Fig. 6. TEM micrographs of the midgut wall, 96 hrs after treatment, in workers of the control group. Crossing section of microvilli (mi) in contact with fibrils of the peritrophic membrane (pm).

Scale bar: 0.5 µm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bl
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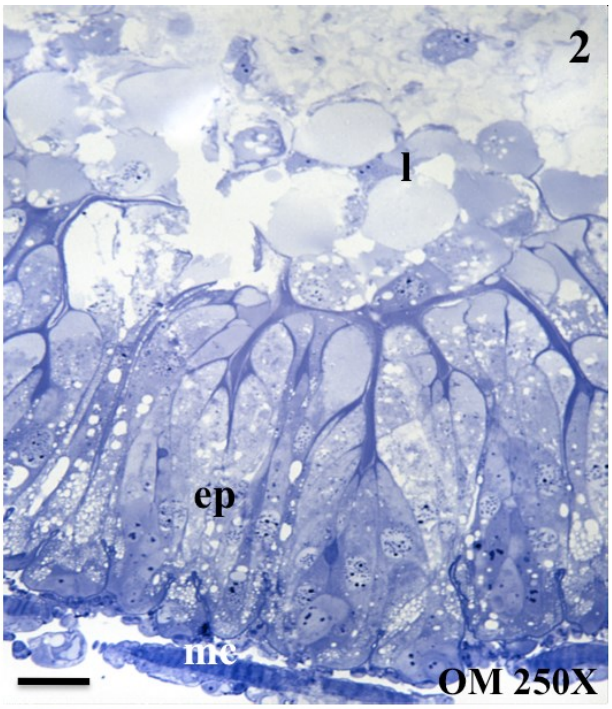
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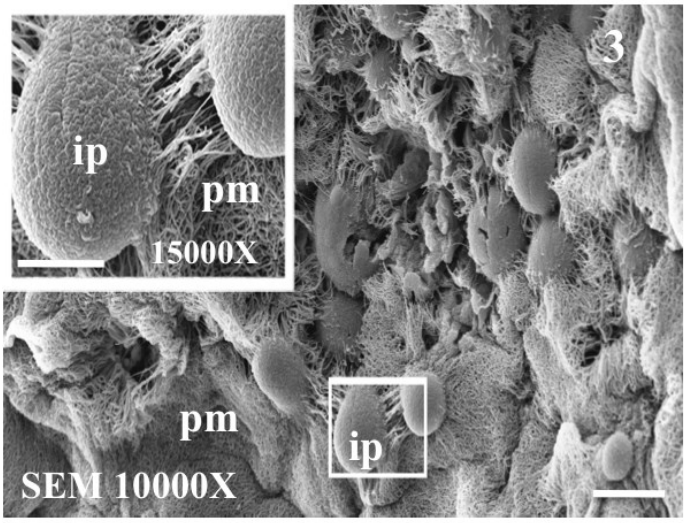
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pm
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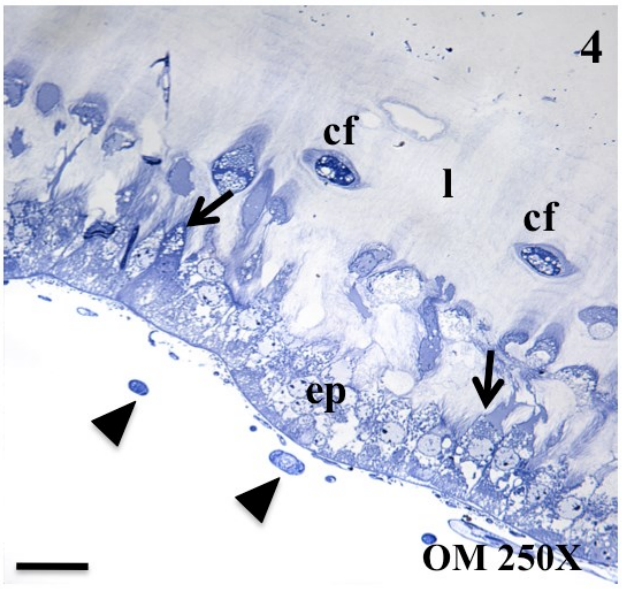
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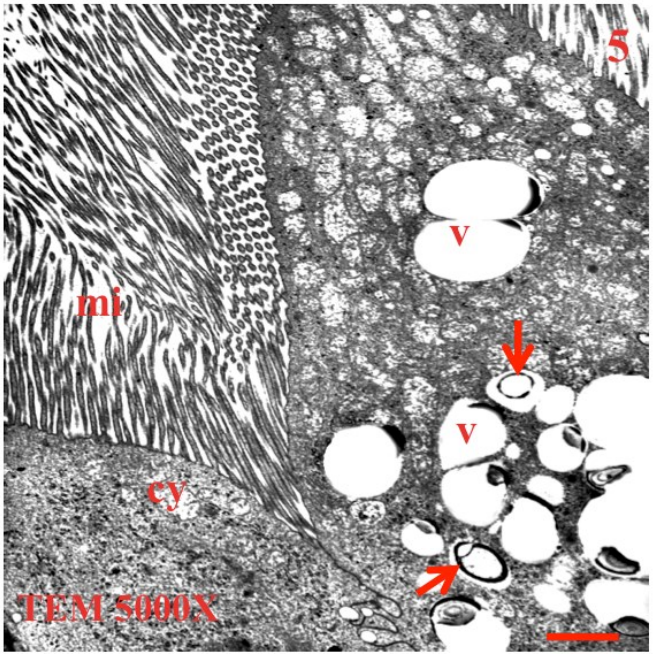
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mf
v
cy
TEM 5000X

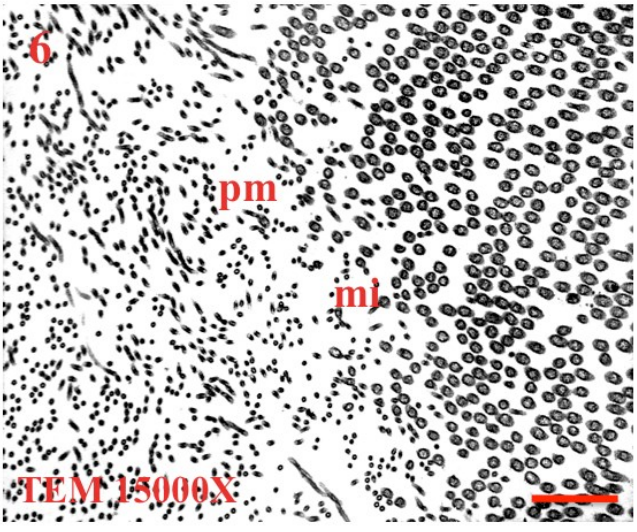

6
pm
mf
TEM 15000X

TABLE IV

Fig. 1. TEM micrographs of the midgut wall, 96 hrs after treatment, in workers of the low concentration group. Nucleus (n) of the epithelial cell. v, vesicle with grain (g) of heterogeneous appearance.

Scale bar: 1 μm

Fig. 2. TEM micrographs of the midgut wall, 96 hrs after treatment, in workers of the low concentration group. Remaining in the lumen of the organ cell nucleus with (n), appearance pyknotic, and microvilli (mi) mixed at peritrophic membrane (pm).

Scale bar: 2 μm

Fig. 3. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with field concentration. Two contiguous enterocytes, one of which having a more compact and electron-dense cytoplasm (cy). arrows, vesicles with the spheroidal granules; mi, microvilli.

Scale bar: 1.5 μm

Fig. 4. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with field concentration. Cytoplasm (cy) of an enterocyte, devoid of organelles and with mitochondria (arrowheads) showing dilated cristae. n, nucleus.

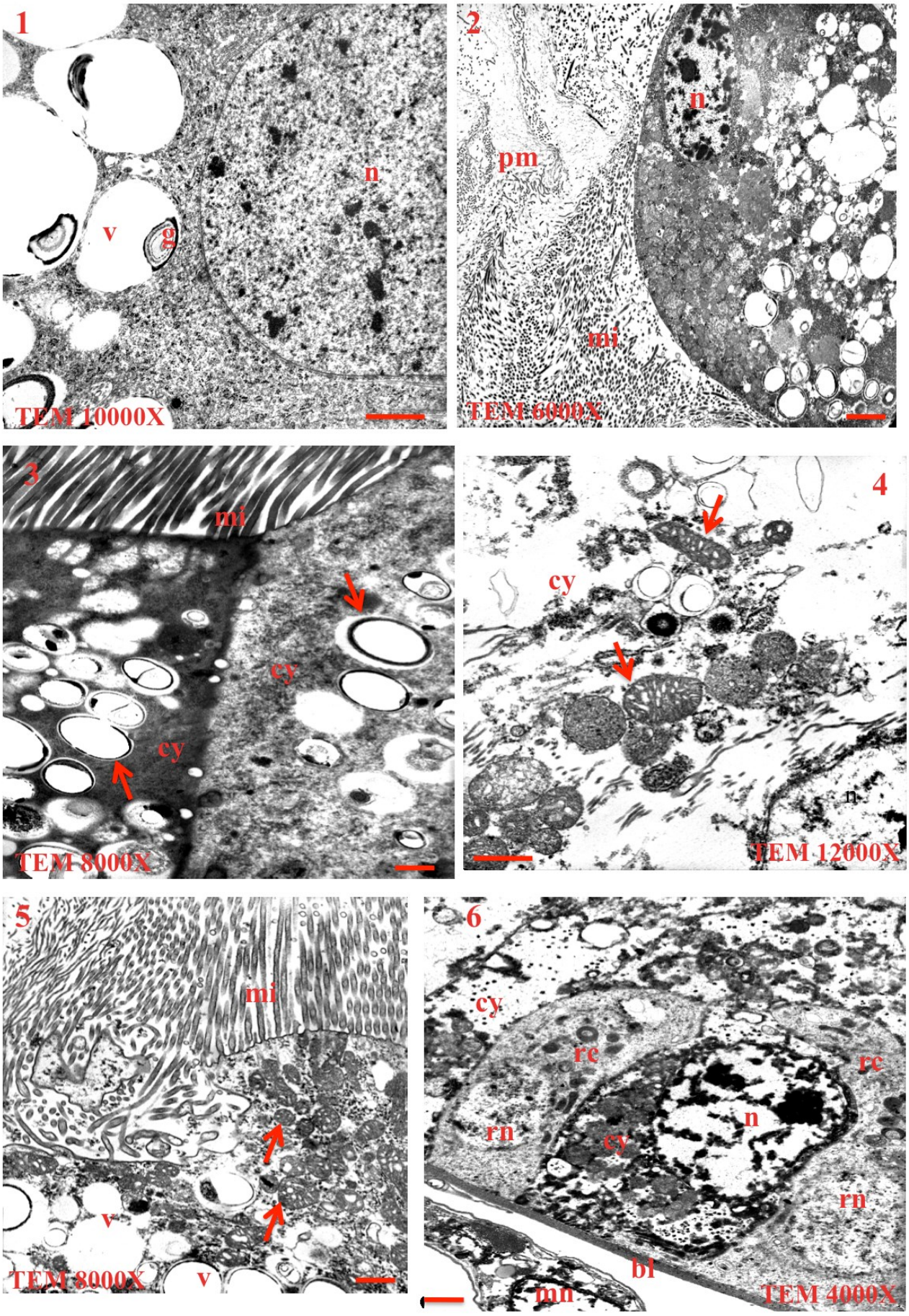
Scale bar: 1 μm

Fig. 5. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with field concentration. Mitochondria (arrows), with dilated cristae, gathered in the apical cytoplasm of an enterocyte. v, vesicles; m, microvilli.

Scale bar: 1.5 μm

Fig. 6. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with very high concentration. Basal region of the midgut wall with enterocytes showing their cytoplasm (cy), almost devoid of organelles, and pyknotic nuclei (n). rc, two regenerative cells showing their dilated nuclei (rn) with dispersed chromatin. mn, pyknotic nucleus of a muscle cell; pm, peritrophic membrane.

Scale bar: 2 μm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n
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n
pm
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TEM 8000X
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cy
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5
mi
v
TEM 8000X
v
6
cy
rc
rc
n
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cy
rn
bl
mn
TEM 4000X

TABLE V

Fig. 1. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with medium-high concentration. Small vesicles (arrow), with an electron-transparent content, gathered in the apical cytoplasm (cy).

Scale bar: 1.5 μm

Fig. 2. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with medium-high concentration. Enterocyte with cytoplasm (cy) devoid of microvilli. fibrils of peritrophic membrane (pm) in the lumen of the organ (l).

Scale bar: 1 μm

Fig. 3. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with medium-high concentration. Cell fragment, with pyknotic nucleus (n) and microvilli (I), mixed with the fibrils of peritrophic membrane (pm) in the lumen of the organ. cy, cytoplasmic expansion.

Scale bar: 2 μm

Fig. 4. TEM micrographs of the honeybee workers midgut wall, 96 hrs after treatment with very high concentration. Two enterocytes with cytoplasm (cy) strongly emptied. n, nucleus strongly dilated and with a few clumps of heterochromatin.

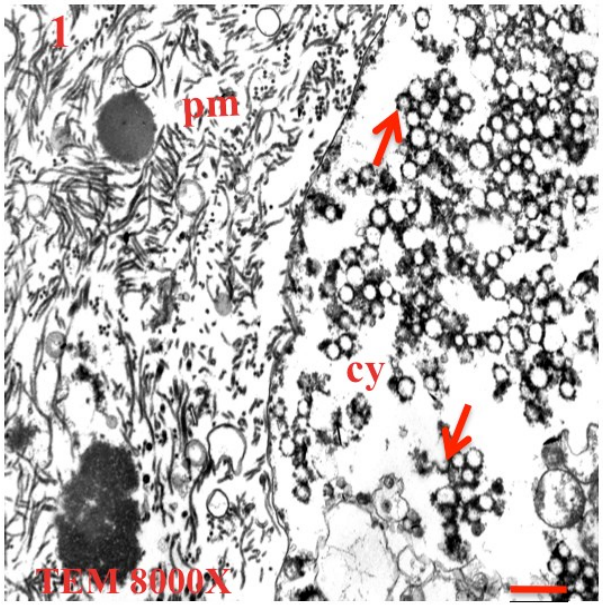
Scale bar: 1 μm

Fig. 5. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from control group. Deep introflections (arrowheads) of the basal plasma membrane of an enterocyte. bl, basal lamina; t, tracheole.

Scale bar: 1 μm

Fig. 6. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from field concentration group. Invaginations of the basal plasma (arrowhead) of some enterocytes. bl basal lamina; m, mitochondria; t, tracheola; v, vesicles with electron-transparent content.

Scale bar: 1 μm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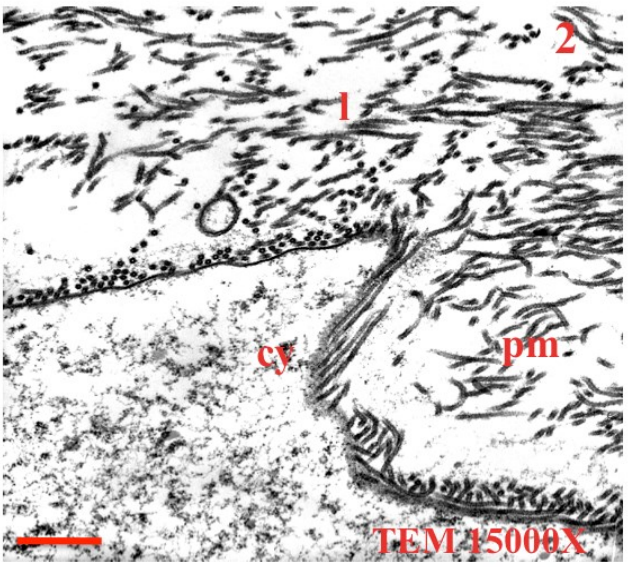
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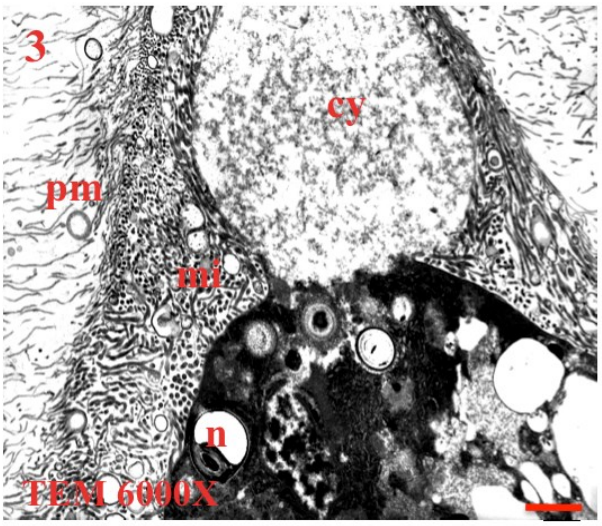
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mi
n
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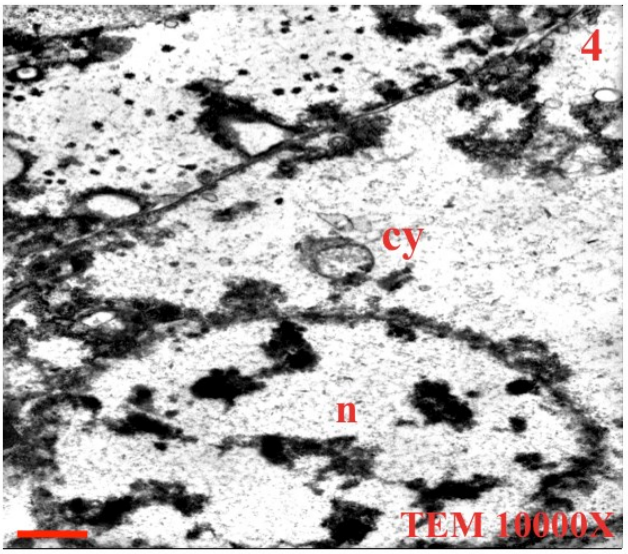
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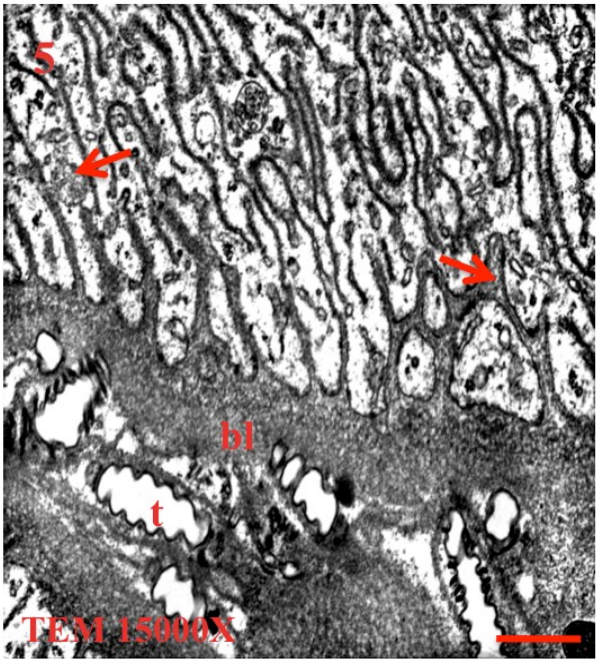
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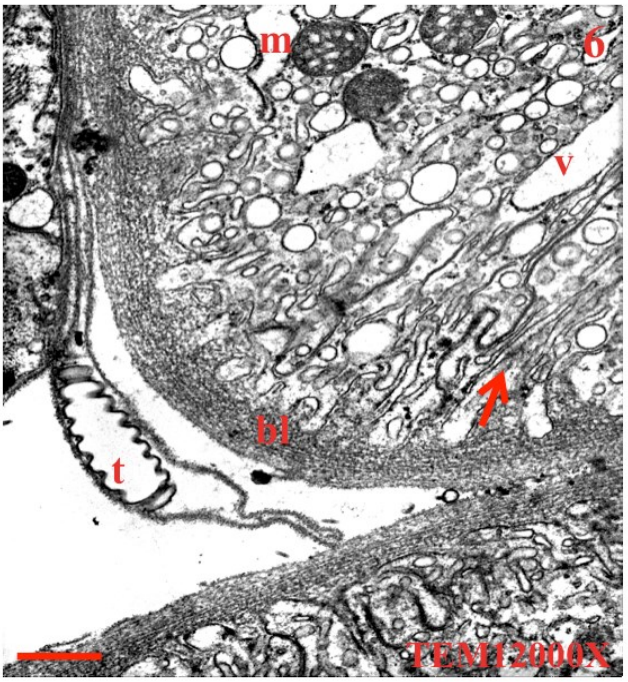
m
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TABLE VI

Fig. 1. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from field concentration group. Peritrophic membrane (pm) in the lumen (l) of the organ of the organ in contact with the microvilli (mi). cy,apical cytoplasm of enterocytes.

Scale bar: 1 μm

Fig. 2. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from medium-high concentration group. Numerous large vesicles (v) close to some reduced introflections (arrowheads) of the basal plasma membrane of an enterocyte. arrow, tracheole; bl, basal lamina.

Scale bar: 2 μm

Fig. 3. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from very high concentration group. Basal cytoplasm (cy) of contiguous enterocytes with strongly reduced introflections of the basal plasma membrane (arrow). bl, basal lamina; mc, muscle cell.

Scale bar: 0.5 μm

Fig. 4. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from very high concentration group. Enterocytes with dilated and eucromatic nucleus (n).

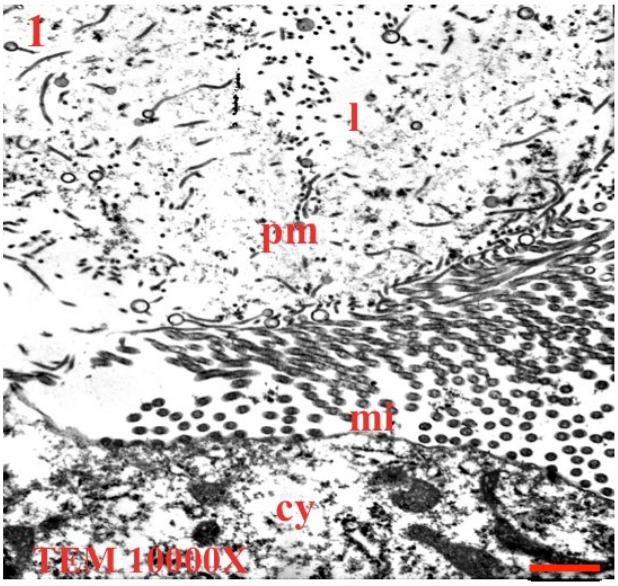
Scale bar: 2 μm

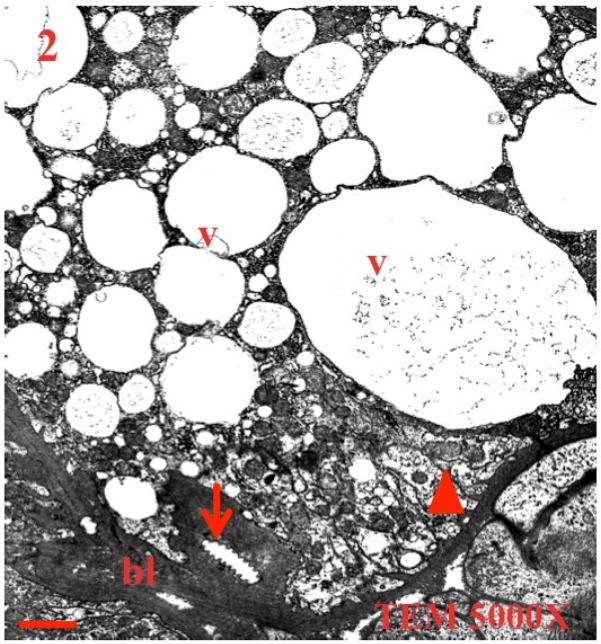
Fig. 5. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from medium-high concentration group. Apical area of an enterocyte, in which the plasma membrane is broken (arrow) from which pours the cytoplasmic content (cy) in the lumen (l) of the organ in which material is present in granular appearance (gr). pm, fibrils of the peritrophic membrane.

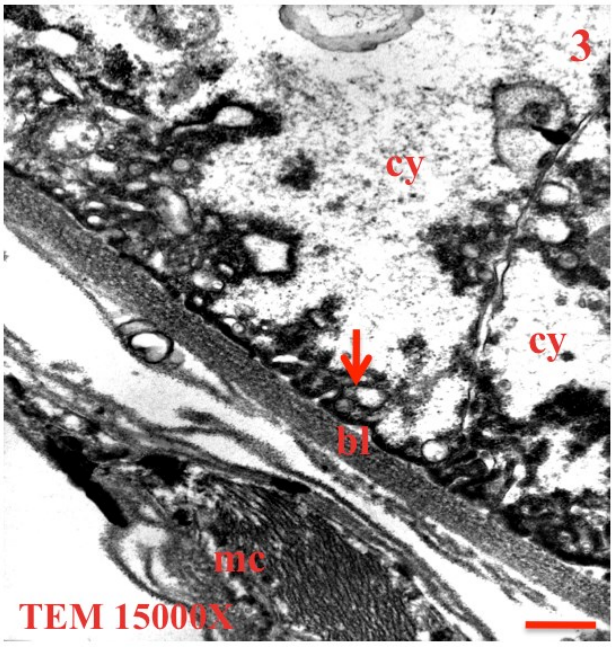
Scale bar: 1 μm

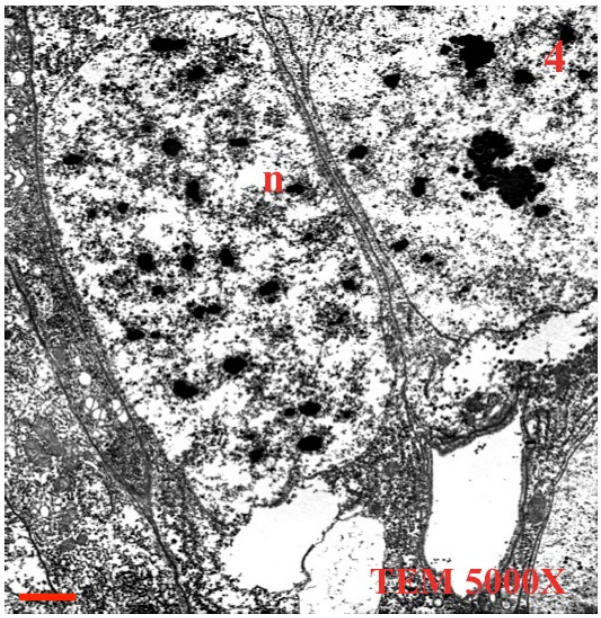
Fig. 6. TEM micrographs of the basal region of the midgut wall, 96 hrs after treatment, in workers from medium-high concentration group. Apical cytoplasm (cy) area of an enterocyte with microvilli (mi) that penetrate between the fibrils of peritrophic membrane (pm).

Scale bar: 2 μm


1
l
pm
mi
cy
TEM 10000X


2
v
v
bl
TEM 5000X


3
cy
cy
bl
mc
TEM 15000X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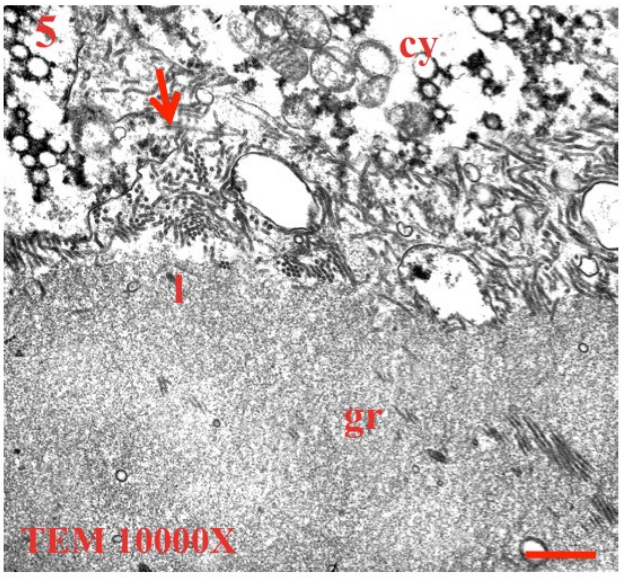
TEM 5000X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cy
l
gr
TEM 10000X

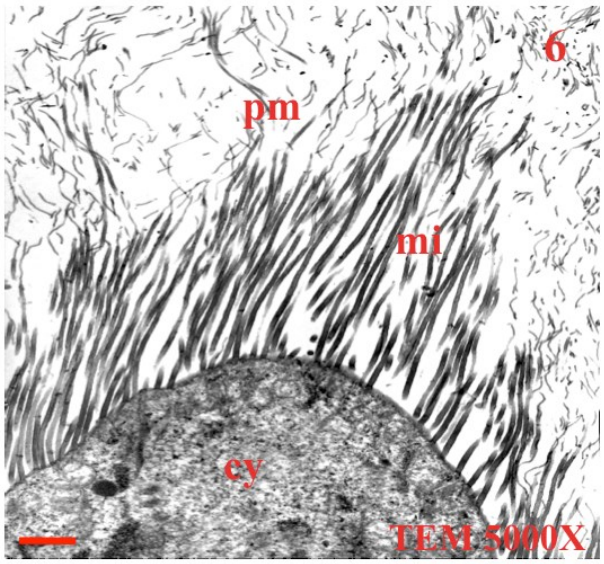

6
pm
mi
cy
TEM 5000X

TABLE VII

Fig. 1. SEM observations of midgut microflora, 96 hrs after treatment in workers from field concentration group. Bacteria (ba) on the epithelial surface (ep). Picture on the left side is a magnification of the particular in the lower white frame. arrow, bacterium undergoing binary fission.

Scale bar: 2.5 μm

Fig. 2. SEM observations of midgut microflora, 96 hrs after treatment in workers from field concentration group. Yeasts (y) on the epithelial surface (ep). Arrows, yeasts undergoing gemmation.

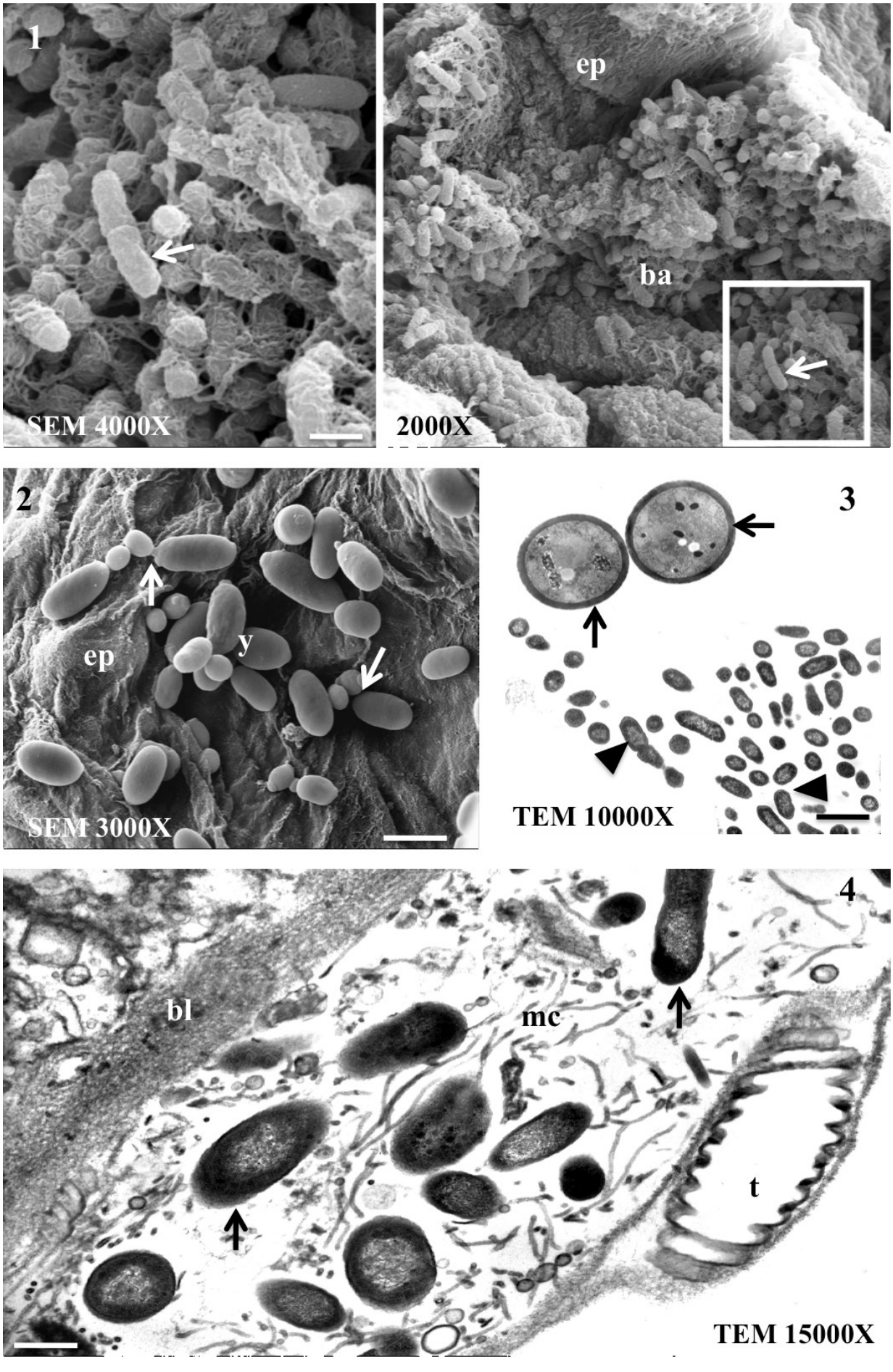
Scale bar: 5 μm

Fig. 3. TEM observations of midgut microflora, 96 hrs after treatment in workers from medium-high concentration group. Bacteria (arrowheads) and yeasts (arrows) in the lumen (l) of the midgut

Scale bar: 2.5 μm

Fig. 4. TEM observations of midgut microflora, 96 hrs after treatment in workers from very high concentration group. Muscular-connective sheath (mc) with several bacteria (arrowheads). bl, basal lamina; t, tracheole.

Scale bar: 1 μm


1
SEM 4000X
ep
ba
2000X
2
ep
y
SEM 3000X
3
TEM 10000X
4
bl
mc
t
TEM 15000X