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Effects of pesticides on honey bees (*Apis mellifera* L.): study of a specific route of exposure and evaluation of biochemical-physiological changes in the assessment of the pesticides toxicity

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**Effects of pesticides on honey bees (*Apis mellifera* L.): study of a
specific route of exposure and evaluation of biochemical-physiological
changes in the assessment of the pesticides toxicity**

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TABLE OF CONTENTS

1 INTRODUCTION

1.1	The honey bee.....	1
1.2	Honey bees and the environment: a double-sided relationship.....	2
1.3	Routes of exposure of honey bees to pesticides.....	3
1.3.1	Exposure <i>via</i> direct contact.....	3
1.3.2	Exposure <i>via</i> indirect contact.....	4
1.3.3	Exposure <i>via</i> ingestion.....	4
1.3.4	Combined exposure to multiple pesticides.....	6
1.4.	Pesticide effects and toxicity	6
1.4.1	Lethal effects.....	7
1.4.2	Sublethal effects.....	7
1.4.3	Effects on biochemical-physiological changes.....	8
1.5	Aims of the research.....	9

2 STUDY OF THE HONEY BEE EXPOSURE TO NEONICOTINOIDS AND FIPRONIL CONTAMINATED DUSTS

	Preface.....	12
2.1	Effects of clothianidin contaminated dusts – laboratory and semi-field study	13
2.1.1	Introduction.....	15
2.1.2	Materials and methods.....	17
2.1.3	Results.....	19
2.1.4	Discussion and conclusions.....	23
2.2.	Effects of indirect contact with neonicotinoids and fipronil contaminated dusts – laboratory study.....	26
2.2.1	Materials and methods.....	26
2.2.2	Results and discussion.....	27
2.3	Effect of indirect contact with clothianidin contaminated dusts on orientation –field study.....	29
2.3.1	Materials and methods.....	29
2.3.2	Results and discussion.....	30

3 STUDY OF THE EFFECTS OF DIFFERENT PESTICIDES ON BIOCHEMICAL-PHYSIOLOGICAL CHANGES

	Preface.....	33
3.1	Chronic effect of three Cry toxins and combined effect of <i>Bt</i> and fipronil on adult honey bees: toxicity and physiological changes.....	34

3.1.1	Introduction.....	35
3.1.2	Materials and methods.....	37
3.1.3	Results.....	40
3.1.4	Discussion and conclusions.....	48
3.2	Honey bees combined exposure to <i>Bt</i> spores and deltamethrin: toxicity and physiological changes.....	53
3.2.1	Introduction.....	54
3.2.2	Materials and methods.....	56
3.2.3	Results.....	58
3.2.4	Discussion and conclusions.....	65
3.3	Physiological changes induced by a combined treatment with difenoconazole and deltamethrin.....	69
3.3.1	Materials and methods.....	69
3.3.2	Results and discussion.....	70
4	GENERAL DISCUSSION AND CONCLUSIONS.....	74
5	REFERENCES.....	80
6	ANNEX – EXPERIMENTAL PROCEDURES	
6.1	Protein extraction.....	92
6.2	Glutathione-S-Transferase (GST) activity assay.....	92
6.3	Catalase (CAT) activity assay.....	93
6.4	Superoxyde dismutase (SOD) activity assay.....	94
6.5	Alkaline phosphatase (ALP) activity assay.....	94
6.6	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity assay.....	95
6.7	Glucose-6-Phosphate dehydrogenase (G6PDH) activity assay.....	96
6.8	List of abbreviations.....	97

1. INTRODUCTION

1.1 The honey bee

The domestic honey bee (*Apis mellifera* L.) is an eusocial insect, belonging to the order of *Hymenoptera* and the superfamily *Apoidea*. At present, honey bees are considered to constitute one of the most complex societies among invertebrates, with a strict caste division and a highly developed communication capacities. Caste and sexual dimorphism are well pronounced, so that within the colony we can easily distinguish the queen bee, the drones and the worker bees. The queen bee, characterized by a well-developed abdomen, is the only fertile female of the colony, mating once in life with different drones. The female progeny will originate from fecundated eggs, whereas non fecundated eggs will give origin to males.

All the other females in the colony are worker bees. They accomplish different tasks through their life, depending on age and colony necessity. In particular, newly emerged bees spend the first 3 days cleaning the cellules; afterwards they take care of brood by producing the nourishment for larvae (nurse bees). Later in life, worker bees become nesters and guardians. Foraging is the last task in honey bee polyethic development, that is accomplished generally after 14 days from the emergence (Michener, 1969); however, this indication may be variable in function of the colony's need in food storage (Schulz *et al.*, 1998).

Drones are present in the hive only for a few months a year, beginning to emerge at the end of the winter in order to accomplish mating. They are incapable to feed for the first period of their life, when worker bees take care of their nourishment.

Bee colonies can be constituted of 20.000 to more than 70.000 individuals that communicate each other with a complex network of chemical signalling. As far as we know, pheromones have a major role in communication between castes and through individuals of the same caste. The queen bee produces a complex blend of odorous stimulus that worker bees transmit through the whole colony via trophallaxis. The queen mandibular pheromone has the major role to indicate the presence of an active queen and to maintain the cohesion of the colony, mainly preventing any other female to develop the reproductive system (Slessor *et al.*, 2005). Other pheromone based communications have been discovered among worker bees, and between larvae and nurse bees.

The chemical signalling are also used for colony defence purposes and recruitment of worker bees for foraging (Breed *et al.*, 2004). The information about food source to forage are communicated to other bees through a specific code of movements, called "waggle dance", whose interpretation by Karl von Frisch (1946), gave the most amazing example of the honey bee social complexity.

Several theories refer to the honey bee colony as a superorganism (Moritz and Southwick, 1992), considering that the strict labour division among individuals provides the well functioning of the colony, at the same way the various component of a body collaborate to the survive of the organism. Furthermore, communications among individuals, mainly accomplished through pheromones, permit the coordination of the whole colony, so that a highly adaptation capacity to environmental conditions is attained.

1.2 The honey bees and the environment: a double-sided relationship

Honey bees have a key role in agriculture and in environmental preservation. Beekeeping is a fundamental agricultural activity, not only for providing hive products as honey, pollen, wax, royal jelly etc., but also for assuring the pollination of a large number of crops. The major part of cultivated plants, in fact, needs insect pollination in order to be fecundated. It has been calculated, that 35% of the world food sources derive from insect pollinated crops. Moreover, honey bees together with other pollinators provide the pollination of the spontaneous and wild vegetation, thus playing a major role in landscape and natural resource preservation and domestic honey bees strongly contributed to that (Klein et al., 2007).

Counterparty, the intense foraging activity of honey bees, that assure pollination, is the most relevant behavioural trait that exposes them to agricultural pesticides and contaminants in general. A honey bee can perform numerous foraging flights per day, with a distance from the hive that may vary between 1,5 and 6 km, even though long-range flights of more that 10 km have been recorded also (Beekman and Ratnieks, 2000). During their foraging activity, honey bees collect considerable quantities of pollen and nectar that they bring back to the hive to constitute food storage for adults and larvae. Therefore, the contamination with plant protection products might not only occur in the field, but also within the hive, so affecting adult bees and brood. In this framework, the massive pesticide presence in the environment, especially due to intensive agricultural practices is considered a relevant threat to bee and colony health.

Even though honey bee intoxication accidents don't represent a recent issue, the risks posed by pesticides to bees have become particularly relevant in the last decade. Two phenomena have in fact raised the attention on this problem. In Europe, important spring bee losses have been registered since 2000 and attributed to the extensive use of neonicotinoid insecticides in maize and sunflower seed dressing (Comité Scientifique et Technique, 2003; Greatti *et al.*, 2003). Moreover, in 2006 the first case of a "mysterious" colony collapse has been reported in the United States (Frazier *et al.*,

2008), rapidly becoming the beginning of a worldwide bee decline, whose causes are still largely unknown (vanEngelsdorp *et al.*, 2008; vanEngelsdorp *et al.*, 2009; Neumann and Carreck, 2010).

Researchers agree on the fact that there is probably not one causing agent of this phenomenon and the origin of colony collapse should be reasonably considered as multifactorial, including pathogens, climate changes and pesticides.

Further than being the object of this recent research issue, the domestic honey bee represents the reference organism for pollinators and beneficial arthropods in pesticide risk assessment. The evaluation of side-effects on honey bees is in fact essential to estimate the environmental danger of pesticides. At present, the standard toxicity tests (OEPP/EPPO, 2010), take into consideration only a few toxicological endpoints that seem no longer sufficient to adequately estimate the risk. In the last decade, in particular, new chemical classes of pesticides that have been released on the market, have raised awareness about the complexity of the lethal and sub lethal effects that may threaten honey bees. To the aim of risk assessment, two fundamental issues are to be considered: the risk of exposure of honey bees to pesticides and the assessment of the toxic or detrimental effects of these products on honey bee health. Here, a brief overview of the different routes of exposure to pesticides and of the principal toxic effect on honey bees, is provided.

1.3 Routes of exposure of honey bees to pesticides

A fundamental issue of pesticides risk assessment to honey bees is the evaluation of the exposure scenarios. Different routes of exposure can be described, reviewing the existent literature on this matter. In general, it could be said that the possibility for a honey bee to be exposed to a determined pesticide depends both on the product formulation and on its intrinsic characteristic. In fact, these two features contribute to determine the environmental persistence, the spatial dispersal and the eventual contamination of food sources for honey bees.

1.3.1 Exposure *via* direct contact

Aerial spray contamination is one of the most common ways of exposure of bees to plant protection products. Honey bees can be directly contaminated while flying in a field during a spray treatment; even though mostly all regulations forbid pesticide use during crops flowering and with unfavourable weather conditions, this way of exposure cannot be excluded. Moreover, the grass-covering in the field and the spontaneous vegetation in close proximity may result attractive for foragers, so that contamination can occur even when the spray treatment is performed out of the flowering period of the main crop. Considering that spray treatments are influenced by wind drift,

the vegetation surrounding the treated field may be contaminated as well, representing an additional source of residual contamination for bees. When using systemic products, they may also penetrate through the plant foliar tissue and reach the phloem, so that the residual contamination would be spread into the whole plant.

1.3.2 Exposure via indirect contact

The indirect contact exposure to pesticides can mainly occur when bees go foraging a previously treated area. Residues of pesticides may in fact persist on the aerial portion of the plant and can completely maintain their toxic characteristics after the treatment. The product persistence depends on the physical characteristics of the pesticide that define the time of degradation (for example, photosensitive active ingredients have rapid breakdown) and on meteorological conditions, as precipitations, that could determine a wash off of the product. However, the persistence on the foliage, measured as the half-life, may vary from some hours to several days after the treatment (Edwards, 1975).

Another way of indirect contact exposure was highlighted in regards to insecticide seed treatments formulations and the risk of a relevant dust dispersal during maize sowing was proposed (Comité Scientifique et Technique, 2003; Greatti *et al.*, 2003). The use of a pneumatic machine determines, in fact, the abrasion of seed dressing and therefore causes the dispersal of contaminated dusts in the air. The dust deposit on the spontaneous vegetation surrounding the sowed field, can contribute to expose honey bees to a further hazard during their foraging activity. Seed treatment formulations generally contain a fungicide and a neonicotinoid insecticide, as imidacloprid, clothianidin or thiamethoxam. Fipronil formulated seeds have been released, as well. Given that these insecticides are systemic, the solubilisation of contaminated dusts fallen down on vegetation may result in additional exposure likelihood for honey bees. Pesticide residues may in fact reach pollen and nectar, and eventually be present in guttation droplets (Girolami *et al.*, 2009).

Even though seed treatment is employed in a variety of crops, researches on honey bees contaminations during sowing operations have been taken into account mainly for maize and sunflower.

1.3.3 Exposure via ingestion

The systemic characteristic of several plant protection products provides the translocation of the active ingredient through the phloem towards all the plant tissues; as a consequence, pollinators and among them, honey bees, are likely to be exposed to these products by feeding nectar and pollen. In particular, the pesticide presence in pollen has been proven to be a consequence of field treatments

both for aerial spray and seed treatments. In the first case, for example, a field case study reported the presence of insecticides diazinon and thiacloprid and fungicide difenoconazole in pollen loads 10 days after the treatment in an apple orchard (Skerl *et al.*, 2009).

Similarly, pesticides used for seed dressing can be transported through the plant after the emergence and contaminate the pollen, as well. The presence of small amounts (3 µg/Kg) of imidacloprid in pollen, coming from Gaucho[®] seed dressed sunflower, has been first proven in 2003 (Bonmatin *et al.*, 2003); similar results have been achieved with maize, where pollen contamination with imidacloprid was about 2.1 µg/Kg (Bonmatin *et al.*, 2005). Most systemic pesticides used in seed dressing can also be persistent in soil and be absorbed by successive crop, via root uptake. The presence of imidacloprid has been documented in untreated sunflower plants one year after the seed treatments; in the floral parts, the concentration of active ingredient was about 1.5 µg/Kg (Bonmatin *et al.*, 2003).

Several monitoring campaigns have evaluated the pesticide presence in pollen at the hive, proving a multiple product contamination. Pollen collected in traps has been analyzed in a monitoring campaign in France, showing the presence of imidacloprid (49% of the 81 analyzed samples) and his metabolite nicotinic acid, fipronil (12% of 81 analyzed samples) and his metabolites fipronil sulfone and fipronil disulfonile and several fungicides (Chauzat *et al.*, 2006). Freshly stored pollen and bee bread are considered to be the principal sources of in hive contamination for adults and larvae (Krupke *et al.*, 2012). Miticides are the most well represented class in stored pollen chemical residues, followed by fungicides, mostly azoles, and insecticides as pyrethroids and neonicotinoids. (Bernal *et al.*, 2010; Genersch *et al.*, 2010; Mullin *et al.*, 2010). These evidences demonstrate that forager bees bring back to the hive contaminated pollen that can exhibit a high contamination, even after being processed and stored as bee bread.

Bacillus thuringiensis toxins represent another important source of pollen contamination. The extensive use of *Bt* in genetically modified crops, causes in fact the contamination of food sources for honey bees and other non-target insects (Malone and Burgess, 2009). The presence of *Bt* toxins residues in pollen has been demonstrated (Fearing *et al.*, 1997; Wraight *et al.*, 2000) and the founded concentrations resulted extremely variable, depending mainly on the type of genetically modified event and on the type of toxin.

The in-hive exposure to pesticides has to be evaluated with reference to the feeding behaviour of the different honey bee castes. Worker bees that are involved in the most energy-consuming tasks needs higher quantities of sucrose. As a consequence, forager bees and winter bees are mostly exposed to contaminants in nectar and honey, respectively. Nurse bees feed large amounts of pollen, consuming up to 65 mg of pollen in the first 10 days of their adult life. In fact, a diet high in

protein supply is required to produce royal jelly to feed larvae. For that reason, nurse bees and larvae are the most exposed to residues in pollen and bee bread (Rortais *et al.*, 2005; Halm *et al.*, 2006). Since pollen was demonstrated to be one of the most contaminated substances in the hive, it can be hypothesized that queen bees, both larvae and adult, are also intensively exposed to repeated and low pesticide quantities.

1.3.4 Combined exposure to multiple pesticides

Honey bees can be exposed in the field to combination of pesticides both whenever an area is involved in consecutive treatments with different products, and when a mixture of products is used for a single treatment. Depending on the type of the treatment, the ways of exposure could also be different. The in-hive contamination occurs *via* pollen, wax and nectar and, as highlighted by several researches (Chauzat *et al.*, 2006; Mullin *et al.*, 2010), honey bees can come in contact with a large number of active ingredients, among which, synergies are possible.

Nevertheless, few researches focussed their attention on the effects of combination of pesticides. One of the most studied synergies is between azoles fungicides and pyrethroids, particularly deltamethrin and cypermethrin. Azoles fungicides are capable to inhibit the biosynthesis of fungal cell wall by blocking P450 monooxygenases activity, thus also reducing the main detoxification pathway against pyrethroids, in insects (Vandame and Belzunces, 1998). This synergistic action has been demonstrated both on mortality (Chalvet-Monfray *et al.*, 1995) and sublethal physiological effects, like thermoregulation (Vandame and Belzunces, 1998). A similar synergy with azoles fungicides is expressed by neonicotinoids insecticides, in particular cyano-substituted compounds. The contact exposure with propiconazole strongly augmented thiacloprid and acetamiprid toxicity towards adult honey bees (Iwasa *et al.*, 2004).

The risk posed by these associations must be considered as realistic, since numerous registered products for plant protection do include combination of pyrethroids and azoles fungicides in different spray formulations. Moreover, seed treatments are usually proposed as a mixture of fungicide active ingredients and neonicotinoid insecticides.

1.4 Pesticides effects and evaluation of toxicity

Together with the possible routes of exposure, the study of pesticides effects permits a correct evaluation of the risk posed by a certain product on honey bees. The effects provoked by a substance represent the result of the intrinsic toxic characteristic of the active ingredient and the administered dose or concentration. Furthermore, the duration of the exposure represents an important parameter to take into account.

The intrinsic toxicity is a well known feature of all active molecules used as pesticides. Through time, chemical pesticides have been expressing an increasing acute toxicity, thus requiring lower quantities to be effective. Physic characteristics are also relevant as they allow, for example, the systemic translocation of several molecules through the plant phloem, thus presenting a more complex risk assessment. Systemic insecticides represent at the same time the most effective type of plant protection products and one of the most important threat to honey bees as they contaminate essential food sources for pollinating insects. Moreover, the duration of the exposure plays a central role in determining the effect of a product. A repeated (i.e. chronic) intoxication may in fact cause delayed effects, that could be difficult to predict and quantify.

Here, we briefly review the principal pesticides effects to honey bees, dividing them into lethal effects, sublethal effects and subcellular physiological modifications.

1.4.1 Lethal effects

According to the European regulation on risk assessment of pesticides to honey bees (OECD, 1998b, a; OEPP/EPPO, 2010), the toxicity is evaluated by calculation of acute LD₅₀ (lethal dose) and CL₅₀ (lethal concentration). These two parameters aim to estimate a product toxicity by considering the number of individuals that dies after 48 hours from an acute exposure. Concerning insecticides, the acute toxicity of active ingredients has been augmenting together with the introduction of new molecules; the most recently released products, neonicotinoids and phenyl pyrazoles, in fact, exhibit a significantly higher toxicity compared to all the other chemical classes (Casida, 2011). This fact contributes to give evidence to the hazard posed by neonicotinoids to honey bees, since very small doses may involve a considerable effect on mortality. Moreover, a main characteristic of neonicotinoids, is the high variability of the acute toxicity. For instance, the contact LD₅₀ for imidacloprid may vary between 18 and 104 ng/bee (Iwasa *et al.*, 2004; Nauen and Denholm, 2005) and between 4 and 60 ng/bee when administered *via* ingestion (Suchail *et al.*, 2001; Decourtye *et al.*, 2003).

Sublethal doses or concentrations are defined as the quantities of substance that do not entail a significant mortality effect. Nevertheless, the study of chronic effects of sublethal doses on mortality has well demonstrated that the duration of exposure may strongly influence the mortality effect. For example, the ingestion of imidacloprid sublethal concentrations for 10 days or 40 days, might lead to a high mortality, ranging from 50 to 100 % (Suchail *et al.*, 2001; Dechaume Moncharmont *et al.*, 2003).

1.4.2 Sublethal effects

Customarily, a dose lower than 1/10 of the LD₅₀ for a certain substance, is considered as sublethal, thus not involving mortality events. Sublethal effects may include a large number of physiological perturbations that are usually considered for all non target insects, whereas other sublethal endpoints have been specifically developed with regard to honey bees.

With respect to neural effects, that are the most widely investigated sublethal impairments, a distinction can be done between cognitive effects, behavioural effects and physiological function related effects (Belzunces *et al.*, 2012). The impact of pesticide on cognition has been mainly evaluated, testing the effects on olfactory and visual learning performances. Some pyrethroids, as deltamethrin, have been shown to have an effect on olfactory learning (Decourtye *et al.*, 2005), but the most important effects have been related to neonicotinoids exposure. Sublethal doses of imidacloprid have caused a reduction of honey bee capacity in reacting to an odorous stimulus both in laboratory and semi field conditions (Guez *et al.*, 2001; Decourtye *et al.*, 2004b; El Hassani *et al.*, 2008). The impairment of learning performances has been evidenced both *via* direct contact and *via* ingestion. Recently a similar effect on olfactory learning has been demonstrated following the indirect contact with clothianidin contaminated dusts (ApeNet, 2010).

The effects on foraging activity and orientation capacities represent other relevant behavioural endpoints. It has been proposed, in fact, that in certain cases an important mortality in the field could be attributed to a disorienting effect of pesticides on forager bees. This hypothesis has been verified in particular for neonicotinoids and fipronil. Imidacloprid has been the first active ingredient to be proven to affect honey bee homing flight in open field (Bortolotti *et al.*, 2003). Lately, the RFID (Radio-Frequency Identification) technology has allowed to perform this kind of experience with a larger number of individuals and to ensure a reliable recording of data. Thus, fipronil and thiamethoxam (Decourtye *et al.*, 2011; Henry *et al.*, 2012) have been demonstrated to be detrimental for spatial orientation capacities in forager bees.

Some of the most relevant effects on physiological function, are then represented by thermoregulation and muscle activity modifications (Belzunces *et al.*, 2012).

Furthermore, sublethal effects might also be evaluated at a colony level. Since the colony has to be considered as a superorganism, the toxic effects on individuals can have a crucial role in influencing the hive development. This phenomenon might be particularly important if pesticide exposure impairs, for example, queen bee or brood health. The opposite situation can also be expected: behavioural effects that can be observed in laboratory conditions are likely to be mitigated at a colony level, where a more pronounced adaptability is present.

1.4.3 Effects on biochemical physiological changes

Sublethal effect of pesticides may be studied at different levels of biological organisation, from troubles at colony level to sub cellular modifications. Biochemical assays that, so far, have been mainly employed for environmental biomonitoring purposes, represent a valuable tool to investigate pesticide exposure and effects. Bioindicator organisms have the characteristic to be particularly exposed and sensitive to pollutants. Therefore, the analysis of different ecotoxicological biomarkers in these species gives important information about the contamination of the environment. Biomarkers are thus defined as measurable modifications in bioindicator organisms that can be related to the exposure to contaminants (Lagadic *et al.*, 1994); to this extent, a biochemical biomarker approach in ecotoxicological studies, consists in investigating the response of a battery of enzymes that gives relevant information on the health status of the organism.

Until now, honey bees have been employed for biomonitoring mainly through chemical analysis of residues of pesticides or other pollutants present on their body or in honey and pollen. Those materials are collected up to 12 km from the hive, thus representing a good survey of pollutants present in a wide territory surrounding the hive. Moreover, the morphological conformation of the honey bee body, in particular the hairs covering their thorax and abdomen, contributes to collect granules of pollen and other particles that we could find back in the hive or on the body of dead bees (Porrini *et al.*, 2002). The use of biochemical markers in biomonitoring have been well developed especially in aquatic ecosystems, and only recently the honey bee has been considered for a similar approach in terrestrial environments.

However, some authors highlighted the opportunity of improving the study of biochemical changes in indicator organisms with a different approach than biomonitoring. The correct evaluation of the environmental risk of pesticides, in fact, requires as much information as possible on the toxicity at different levels of biological organization (Van der Oost *et al.*, 2003). Therefore, the study of biochemical changes might be useful as an additional sublethal toxicity endpoint, at a subcellular level. Moreover, it could be used to reveal the impact of very low doses or concentrations of pesticides, helping to ranking this products according to their hazardous potential (Jemec *et al.*, 2009).

1.5 Aims of the research

The present research aimed to examine two different aspects of honey bees and pesticide relationship. First, the investigation of the ways of exposure: in this study, we examined more in depth the different aspects of the exposure to contaminated dusts, with a focus on indirect contact exposure.

Second, we addressed the topic of toxicity endpoints development: in this framework, the study of some effects at a physiological level, through the use of biochemical indicators, was evaluated.

The first part of the experimentations has been guided by the hypothesis of a relevant exposure of honey bees to pesticides employed in seed treatment formulations. In particular, as it has been proposed before by Greatti *et al.* (2003), the sowing operations in maize fields represent a critical situation in which honey bees could be exposed to neonicotinoids and fipronil contaminated dusts. In order to investigate this specific phenomenon, an Italian national project (Apenet) has been developed and funded by the Italian Ministry of Agriculture, from 2009 to the end of 2010. In the framework of that project, we focussed our attention on evaluating the hazard posed by the exposure to neonicotinoids and fipronil contaminated dusts to forager bees.

More in particular, we addressed the following issues:

- developing an effective methodology to assess the effects of contaminated dusts on honey bees;
- evaluating the acute toxicity, *via* indirect contact, of clothianidin contaminated dusts, in comparison with the correspondent liquid formulation, in laboratory conditions;
- evaluating the acute toxicity, *via* indirect contact, of dusts contaminated with neonicotinoids imidacloprid, thiamethoxam and clothianidin and phenyl pyrazole fipronil to honey bees, in laboratory conditions;
- evaluating the short-term and long-term effects of clothianidin contaminated dusts, in semi-field conditions;
- evaluating the effect of clothianidin contaminated dusts, *via* indirect contact, on honey bees orientation capacities in open field.

The second part of this study aimed to improve a biochemical based tool in order to better estimate the sublethal toxicity of certain substances, investigating the effects of pesticides at a “sub-individual” level. To this extent, we chose to test different products and different treatment modalities, dividing the experimental study as follows:

- In the first experimentation, we tested the effect on different enzymes of the combination of a chemical insecticide, fipronil and a biological insecticide, *Bacillus thuringiensis*. Considering the

possible exposure to pesticide residues *via* ingestion, we administered both products chronically for 10 days, then evaluating the honey bee survival until 25 days;

- In the second experimentation, we considered once again the combination between a chemical insecticide, deltamethrin and a biological product, *Bacillus thuringiensis*, testing the hypothesis of a not simultaneous exposition. In particular, a sensitization effect of *Bt* towards deltamethrin was evaluated, with respect to the enzymatic response;

- In the third experimentation, we evaluated the enzymatic variations caused by a combined treatment with the fungicide difenoconazole and the insecticide deltamethrin.

2 STUDY OF THE HONEY BEE EXPOSURE TO NEONICOTINOIDS AND FIPRONIL CONTAMINATED DUSTS

Preface

The hypothesis of a bee exposure to pesticides contained in seed treatment, and mainly caused by the relevant dust dispersal phenomenon observed during the sowing operations, was proposed by several authors (Greatti *et al.*, 2003; Halm *et al.*, 2006; Alix *et al.*, 2009; Pistorius *et al.*, 2009). As previously explained, the possible hazard to forager bees lays in direct contact while flying, in indirect contact and in ingestion of residues of pesticides present in the dusts drifted and fallen in the proximity of the sowed field. This occurrence has been evidenced mainly in sunflower and maize crops, even though the seed treatment is widely diffused in other crops as oil seed rape and cereals. However, the extent of the dust dispersal and contamination has never been assessed before and a more accurate risk assessment related to this way of exposure has scarcely been investigated.

A high number of mortality cases was recorded in the North of Italy, through the regional monitoring networks, in 2008 (Bortolotti *et al.*, 2009). The possible causes of colonies weaken and mortality with particular reference to the hypothesis of intoxications during the maize sowing was investigated within the nation project Apenet (2009-2010). In the framework of this project, we carried out different experimentations focussing on the hazard posed to forager bees by the indirect contact with contaminated dusts, while foraging on spontaneous vegetation or cultivated crops surrounding a treated maize sowed field.

In order to do that, we particularly collaborated with the Agricultural Engineering Research Unit (CRA-ING) and the Plant Pathology Research Centre (CRA-PAV) of the Agricultural Research Council, to acquire a reference value of pesticide contamination that would permit a correct evaluation of the risk. Different experimental sowings were then performed, employing different seed drill machines, with the application of a deflector device in order to reduce dust dispersal. Dust amounts were measured by means of Petri dishes at 5 meters form the field edge, and active ingredients concentration was evaluated (Pochi *et al.*, 2012) (ApeNet, 2009, 2010).

The so obtained data were then used to perform three experimentations with the aim of developing a useful methodology to test the dust contamination and toxicity: (i) we evaluate clothianidin contaminated dusts toxicity in semi-field conditions, with respect to short-term and long-term effects. (ii) We conducted, in laboratory conditions, an assessment of the acute toxicity of four active ingredients widely employed in seed dressing formulations (imidacloprid, clothianidin, thiamethoxam and fipronil). (iii) Finally, the capacity of orientation in open field conditions was assessed in clothianidin treated bees.

2.1 Effects of clothianidin contaminated dusts – laboratory and semi-field study

Effects of neonicotinoid dust from maize seed-dressing on honeybees

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Abstract

In the last years bee and colony losses have been reported in numerous countries worldwide and many factors were taken into account to explain these phenomena. However, time-space differentiation of bee mortality factors needs to be considered. In Northern Italy from 2000 to 2008, many spring bee mortalities were clearly linked to sowing of maize seeds dressed with insecticides. In fact, pesticides used in maize seed coating may be dispersed as dust from the pneumatic drilling machine and drift to surrounding areas. Subsequently bees may enter in contact with these contaminated dusts in several ways: by direct contact (when bees fly through the toxic cloud in the sown field), by indirect contact (when bees walk on contaminated leaves of the vegetation surrounding the sown field) or by ingestion (when bees collect nectar or dew from the vegetation contaminated with the dispersed dusts). The pesticides used for maize seed dressing are extremely toxic for bees with lethal and sublethal effects depending on the level of exposure. In Italy, the high bee mortality during the sowing of coated seeds resulted in the suspension of use of the active ingredients: imidacloprid, clothianidin, thiamethoxam and fipronil for seed coating (Ministerial Decree 17/09/2008). At the same time a research project “Apenet monitoring and research in apiculture” was financed in order to establish the causes of bee mortality (external and internal to the hive) and the possible ways of mitigation. In the present study, within the framework of the Apenet project, we investigated the effects on honey bees of clothianidin derived from maize seed dressing (Poncho®) in laboratory (test by indirect contact) and in semi-field conditions. Despite the reduction of dust dispersion due to the application of the best available sowing techniques (pneumatic seeder equipped with deflector, improvement of seed dressing quality) our results showed negative effects on honey bees at individual level. In semi-field study, no effect was observed at the colony level despite the high bee mortality rate for 2-3 days after dust application. However, we can expect a colony decline if this high forager mortality rate lasts for longer than 10 days. Such a situation is possible if the sowing period lasts several days as in the Po Valley, where the landscape is characterized by extended maize cultivation.

Despite the recent implication of contaminated dust in bee mortality phenomena in several countries around the world, specific methodologies to assess the effects of dust have never been included in the official guidelines for the evaluation of side-effects of plant protection products on honeybees. For this reason, the aim of this study was also to develop suitable and standardized methods for testing in laboratory and in semi-field conditions the effects on honey bees of contaminated dust dispersed during sowing.

2.1.1 Introduction

In the last years, bee and colony losses have been reported in numerous countries worldwide and many factors, acting singularly or simultaneously, were taken into account to explain these phenomena (Neumann and Carreck, 2010). Factors contributing to the bee decline include: viruses (Berthoud et al., 2010; Martin et al., 2010; Neumann and Carreck, 2010); *Nosema ceranae* (Higes et al., 2007; Paxton, 2010; Santrac et al., 2010); *Varroa destructor* (Dahle, 2010; Martin et al., 2010; Neumann and Carreck, 2010); agrochemicals (Maini et al., 2010; Medrzycki et al., 2010; Chauzat et al., 2011); acaricides (Harz et al., 2010); loss of genetic diversity (Meixner et al., 2010) and habitat loss and fragmentation (Potts et al., 2010). Many scientists agree that bee decline is a multifactorial process in which a particular mechanism seems to be more important in a given period of the year than in another, and different mechanism may predominate in another period or in other environmental conditions. For these reasons, a time-space differentiation of bee mortality factors needs to be considered (Maini et al., 2010). In Italy, the bee mortality follows a clear seasonal pattern: a) during spring and summer colonies loose many foragers due to agrochemicals (bee losses); b) from late summer to winter, the impact of pests and pathogens becomes more important (colony losses). In Northern Italy from 2000 to 2008, many spring bee mortalities were clearly linked to sowing of maize seeds dressed with insecticides (Bortolotti et al., 2009). In 2008, over 700 beekeepers with around 12,000 hives in the Rhine Valley, Germany, were affected by contaminated dust during sowing of maize and similar incidents were observed also in France, Slovenia and US (Alix et al., 2009; Pistorius et al., 2009; Krupke et al., 2012). Greatti et al. (2003; 2006) showed that pesticides used in maize seed coating may be dispersed as dust from the pneumatic drilling machine and drift to surrounding areas. Subsequently bees may enter in contact with these contaminated dusts in several ways. The first way of exposure occurs during sowing when the bees are flying over the maize field to reach a foraging site. In this case, bees enter in direct contact with the dusts dispersed into the air from the pneumatic machine (Girolami et al., 2011; Marzaro et al., 2011). Another way of exposure occurs within few days after sowing operation when forager bees collect pollen, nectar or dew from the vegetation surrounding the sown field (Greatti et al., 2003; Greatti et al., 2006). In this case, bees are exposed both by ingestion (pollen, nectar and dew) and by indirect contact (walking on contaminated vegetation). In Italy, the high bee mortality during the sowing of coated seeds resulted in the suspension of use of the active ingredients: imidacloprid, clothianidin, thiamethoxam and fipronil for seed coating (Ministerial Decree 17/09/2008). At the same time a research project “Apenet monitoring and research in apiculture” was financed in order to establish the causes of bee mortality (external and internal to the hive) and the possible ways of mitigation. In particular, a specific objective within Apenet

project was to investigate whether the application of the best available sowing techniques (pneumatic seeder equipped with deflector, improvement of seed dressing quality) can reduce the dust dispersion below a negligible effect to bees.

The pesticides used for maize seed dressing (clothianidin, imidacloprid, thiametoxam and fipronil) are extremely toxic for bees with lethal and sublethal effects even at very low doses. Effects on orientation and foraging activity were observed in foraging bees fed *ad libitum* with 50-100 µg/L of imidacloprid (Bortolotti *et al.*, 2003; Yang *et al.*, 2008), 1.34 ng/bee of thiametoxam (Henry *et al.*, 2012) and 0.3 ng/bee of fipronil (Decourtye *et al.*, 2011). In laboratory conditions, bees fed with low concentrations (100-500 µg/L) of imidacloprid showed a reduction in the activity (Medrzycki *et al.*, 2003) and in olfactory learning performances (with 12 ng/bee) (Decourtye *et al.*, 2004a). Similar effects on learning performance were observed in honeybees exposed by contact at low doses (0.5 ng/bee) of fipronil (Bernadou *et al.*, 2009). In the Apenet project, the amount of active ingredients deposited on the ground during sowing at 5, 10, 20m distances from the field edge was measured. It was shown that during the maize sowing operation bees can be exposed to variable pesticide contamination levels. This exposure depends on many factors, as: way of contact with the active ingredient, time from the sowing operation, size of the sown area, quality and quantity of vegetation in the margin of the field, meteorological conditions, and of course seed dressing quality and the application of deflector in the pneumatic seeder.

In the present study, within the framework of the Apenet project, we investigated the effects on honey bees of clothianidin derived from maize seed dressing (Poncho®). The study was carried out in laboratory (test by indirect contact) and in semi-field conditions. We decided to consider not the active ingredient but the commercial compound, thus in our trials we applied the contaminated dust extracted by abrasion from dressed maize seeds.

We address the following questions: 1) Is the amount of contaminated dust dispersed at 5 meters from a maize field harmful for forager bees? 2) Is the dust containing Poncho® more toxic than the liquid formulation of the same active substance (Dantop®)? 3) Can the contaminated dust affect the colony at medium and long terms, including its sociophysiological parameters?

Despite the recent implication of contaminated dust in bee mortality phenomena in several countries around the world, no particular indication on how to assess the effects of dust to bees is taken into account in the official guidelines (OECD, 1998b, a; OEPP/EPPO, 2010). For this reason, the aim of this study was also to develop suitable and standardized methods for testing in laboratory and in semi-field conditions the effects on honey bees of contaminated dust dispersed during sowing.

2.1.2 Materials and Methods

Contaminated dust was extracted from maize seed dressed with Poncho® using Heubach cylinder, the dust was sieved and the fraction <45µm was used. The choice of the particle dimension was made in order to reflect field conditions where the major part of the dispersed particles during sowing operation was smaller than 45µm (Apenet, 2011). The dust was analyzed to assess the percentage of clothianidin and the tested dose (5.12 µg/m²) was chosen basing on the results of field studies. In fact, this quantity reflects the amount deposited on the ground at 5m distance from the edge of the field during maize sowing using a Gaspardo Magica six row-precision pneumatic seeder (75,000 seeds/ha) with dual pipe deflector. The seeds (Hybrid employed PR32G44; Pioneer Hi-Bred) were supplied in 2010 by Italian Seed Association and the quantity of dust abrasion resulted under 2g/q. Contaminated dust was mixed with an appropriate quantity of talc (used as a dispersing agent) in order to reach the desired concentration. We chose talc as dispersing agent because it is a common mineral material, not toxic to bees and it is usually added to seed boxes to reduce friction and stickiness and ensure smooth flow of seeds during planting. In a recent study it was shown that waste talc expelled during and after sowing represents another route of pesticide exposure for bees (Krupke *et al.*, 2012).

Laboratory study

The indirect contact toxicity of dust contaminated by the clothianidin-based product Poncho® was compared, in laboratory conditions, to that of spray formulation of the same active substance (Dantop ®) and at the same dose. In both treatments, forager bees (10 bees per cage) were exposed to clothianidin by walking for 3 h on treated apple leaves, placed on the bottom of plexiglass hoarding cage (13 x 6 x 11 cm). Bees were kept in darkness at 25 °C during the test. For the liquid formulation, the leaves were sprayed with 200 µl of test solution (water only in the control) and for the dust treatment, 0.01 g of Poncho® dust mixed with talc was applied (talc only in the control). During the trial, bees were fed with 50% (w/w) sugar solution. Five groups of 10 bees were used for each treatment. Mortality data was corrected for control mortality with Schneider-Orelli's formula and the effects of dust and liquid formulation were compared using Student t-test for each assessment hour. Before processing the mortality rate was arcsine transformed to normalize the data.

Semi-field study

In June 2010, a semi-field cage test was conducted following the EPPO 1/170 (4) guidelines (OEPP/EPPO, 2010) adapted to seed treatment. The study was carried out in an oilseed rape (*Brassica napus*) field of 2000 m² in the Experimental Farm of the University of Bologna. Six cages (three for each treatment) of 40 m² each covered with white anti-aphid net were set up before oilseed rape blooming. On May, 31st, with 50% blooming, in each cage, one nuke containing a healthy queen dated 2009 and bees arranged in three frames (about 5000 adult bees, two frames containing all brood stages and one with 20-25% of nectar and pollen stores) was introduced. All nukes were prepared at the same time with sister queens to guarantee uniform bee colonies. A trap for dead bee collection (type “underbasket”) was placed in front each nuke.

The treatment was applied on June, 7th at noon, when the crop was in full flowering and the bees were actively foraging. In each cage, 200 g of talc (pure in the control cages and containing 204.77 µg of clothianidin in the treated cages) was distributed uniformly with a mechanic pulverizer (Cifarelli® M3; Dusts-out: 0-6 Kg/min; Speed air: 125 m/sec; Volume air: 20 m³/min). The dose of clothianidin was calculated in order to assure the same concentration (per mq of soil) as that applied in the laboratory study. Later, four samples of the talc-Poncho® mixture used for the treatments, were analysed to assess the real concentration of active ingredient.

During the semi-field test, the following parameters were assessed:

1. Daily mortality: the daily number of dead bees in “underbasket” traps;
2. Strength of the colony: the number of adult bees and the brood extension assessed with the Liebefeld method (Imdorf *et al.*, 1987);
3. Flight activity: the number of bees exiting the nuke in 30”;
4. Foraging activity: the instant number of bees in three fixed plots of 0.25 m² each.
5. Foraging behaviour: the abnormal behaviour of the bees in each plot was recorded using a standardised approach by Giffard and Mamet (2009). The abnormal foraging behaviour was classified in three groups related to increasing levels of intoxication: a) motionless bees on plants, b) bees in cleaning activity, c) hanging-knocked out bees;
6. Bee behaviour in front of the nuke;
7. Socio-physiological status of the colony: a) thermoregulation capacity - temperature inside the nuke (between the two brood frames) was recorded by data logger iButton DS1923; b) Comb construction capacity – an empty frame was introduced in the nuke the day of treatment and the percentage of frame surface covered by built comb was subsequently measured. Both the thermoregulation and the comb construction capacities are considered two important physiological parameters to assess the vitality of a colony (Tautz, 2008).

Mortality and behavioural assessments were conducted before and at several moments after treatment: on days -3, -1, 0, 1, 2, 3, 5 and 7. Foraging and flight activities were assessed every two hours in the middle part of the daytime (10.00-12.00-14.00-16.00) except for day -3 when the data was collected only in the afternoon. The strength of the colonies was assessed once before (on day -4) and 7 and 15 days after the treatment.

The comb construction capacity was recorded 7 and 15 days after treatment. The internal temperature was recorded constantly from the day -3 till the day 5.

After 8 days from the treatment, the screening net was removed in order to allow the free foraging activity of bees. On 30 June, the colonies were moved to another site, about 6 Km away from the experimental field. On 15 July, other two frames were added in each nuke and in mid summer (August) and before wintering, anti-varroa treatments were applied, respectively with ApiVar® (a.i. Amitraz) stripes and with oxalic acid. The colony strength was assessed every two months until wintering and once after wintering (February 2011) in order to assess potential delayed effects.

We used repeated-measures ANOVA to analyse differences in daily bee mortality and colony strength between treatments and among the different observation days. To address normality and homoscedasticity, the daily mortality values were $\log(x+1)$ -transformed. The percentage (arcsine-transformed) of built comb in the two treatments was compared using *t*-test. The differences between treatments and days of the mean daily in-hive temperature were analysed with repeated-measures ANOVA. The flight activity and foraging activity were compared between the two treatments, separately before and after application, with Wilcoxon test. Since the current guideline (OEPP/EPPO, 2010) gives no particular indication about the method of elaboration and interpretation of semi-field and field data, in order to evaluate the level of bee mortality, we used the index proposed by Schmidt *et al.* (2003). This index is based on the ratio of daily bee mortality between and after treatment calculated for the treated colonies and divided by the same ratio calculated for the control colonies. Thus we obtain the deviation of the mortality in the treated-tunnels from the control-tunnels.

2.1.3 Results

Laboratory study

The analysis of the four samples of talc mixed with contaminated dust showed a.i. concentrations 10.0 ± 4.7 % lower than the estimated values.

Despite the real active ingredient concentration in dust was slightly lower than the expected one, no significant differences were found in the indirect toxicity test between the liquid and the dust

formulation. Our laboratory results showed that, up to 24th hour, mortality induced by the two products was comparable and below 15%. During the subsequent hours, the number of dead bees increased similarly in both treatments (fig. 2.1.1).

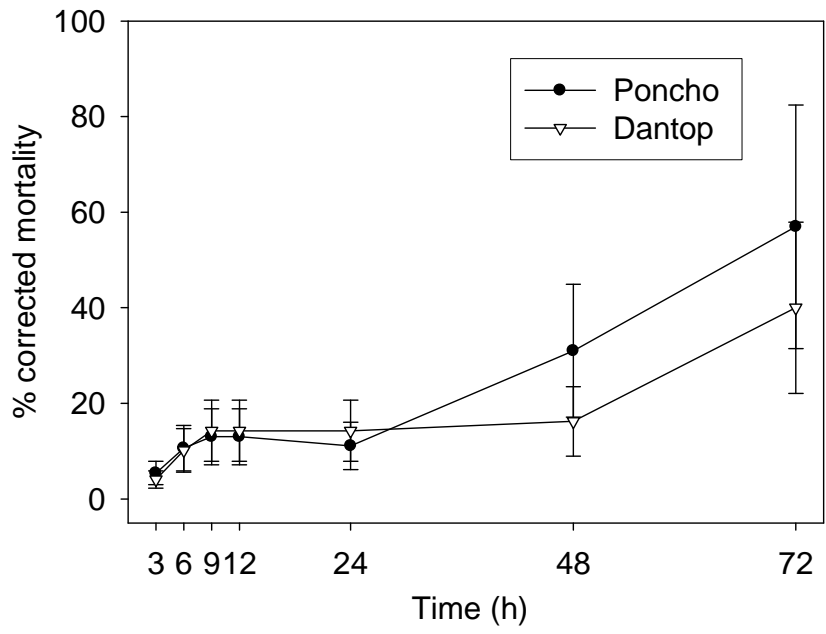


Figure 2.1.1. Corrected bee mortality (\pm SE) in dust (Poncho®) and liquid (Dantop®) formulation treatments (No statistical differences ($p=0.05$) were observed between treatments).

Semi-field study

We found no significant differences in bee mortality between treated- and control-cages ($F = 0.95$; $df = 1, 4$; $p = 0.38$) and among the days of the trial ($F = 1.99$; $df = 4, 24$; $p = 0.11$). However, we found a significant interaction between the two factors ($F = 4.10$; $df = 4, 24$; $p = 0.006$). In the treated-cages, the daily bee mortality increased in the first 2-3 days after the dust application, whereas it was stable in the controls. The bee mortality in the treated-cages was significantly higher than control in the first two days after treatment. The trend remained similar also during the third day but this difference wasn't confirmed statistically (fig. 2.1.2). The index proposed by Schmidt et al. (2003) was calculated basing on mortality data collected between day -3 and day 5. The relative

bee mortality was then ~10 times higher in treated than in control cages (tab. 2.1.1).

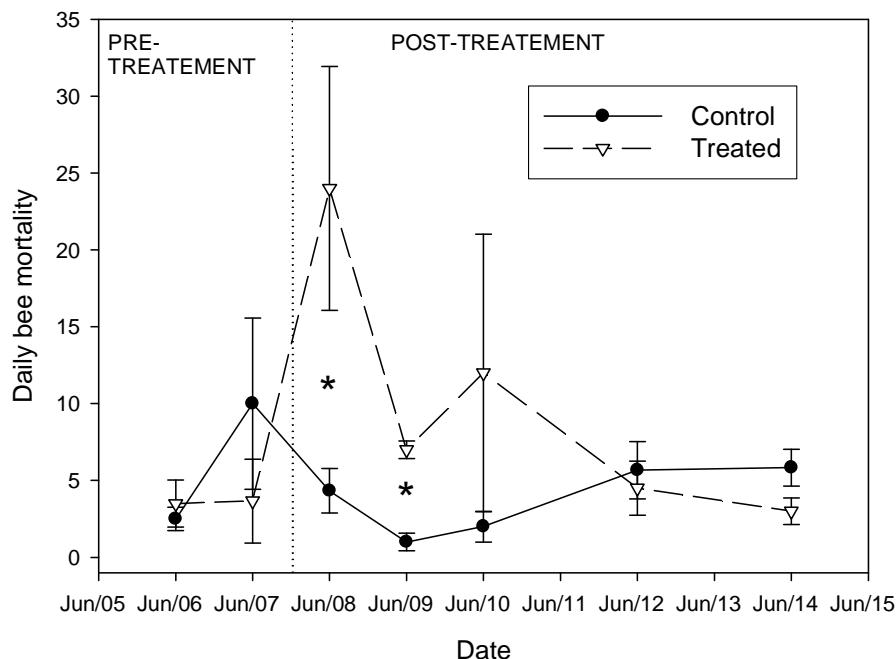


Figure 2.1.2. Mean \pm SE daily bee mortality in control and treated cages.

* Statistically significant differences between control and treated within the same day ($p=0.05$)

The colony strength (number of adult bees and brood) significantly changed during the trial but with similar trend in both treatments (tab. 2.1.2). The number of adult bees and brood cells decreased after 7 days from treatment due to the confined condition, but then rapidly increased during summer. Later, at the beginning of wintering the brood decreased as the mean environmental temperatures dropped to 10 °C. In February 2011, treated and control colonies showed adequate number of adult bees and brood to assure good colony growth during spring (fig. 2.1.3). In April, all the colonies were transferred from the nukes to the 10-frames hives.

The comb constructions started in all colonies after 7 days from the treatment and after 15 days the percentage of comb constructed was similar between treatments (control: $20.6 \pm 2.4\%$; treated: $22.2 \pm 14.7\%$) ($t = 0.29$; $p = 0.78$).

The in-hive temperature was in mean 35.3 ± 0.1 and 35.0 ± 0.1 °C in control and treated-cages respectively, thus we conclude that the thermoregulation capacity was not affected by treatment ($F = 0.69$; $df = 1, 4$; $p = 0.45$). In both treatments, the temperature decreased and showed large fluctuations during the confinement period whereas it became stable after the removing of the screening net ($F = 6.20$; $df = 17, 68$; $p < 0.001$). The treatment-days interaction was not significant ($F = 0.85$; $df = 17, 68$; $p = 0.63$).

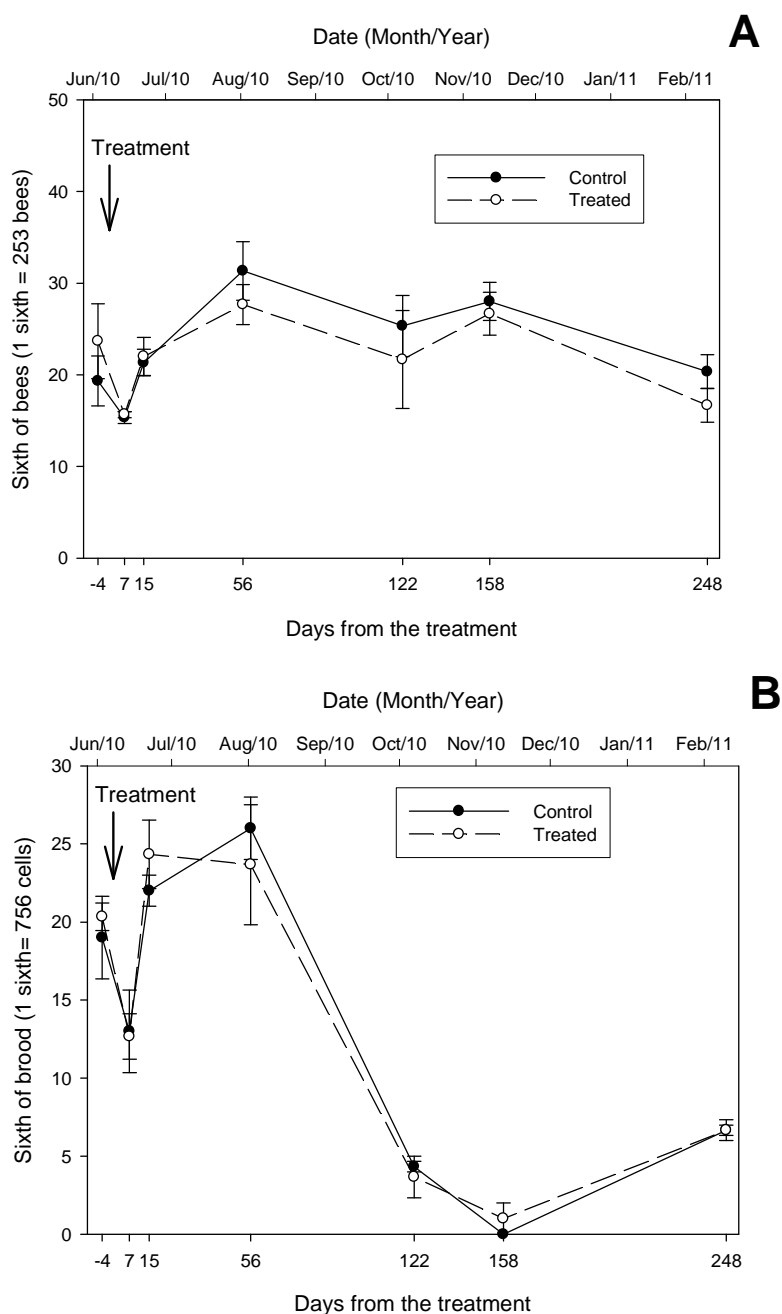


Figure 2.1.3. Mean \pm SE of number of sixths of adult bees (A) and brood (B) in control and treated cages.

We found no significant differences in flight activity between treatments before application (control: 8.1 bees; treated: 9.5 bees; $p = 0.26$). But after application, the flight activity in treated cages was significantly higher than in control ones (8.7 bees and 7.3 bees respectively; $p < 0.01$). The foraging activity (the total number of bees in the three plots) was similar between treatments, both before (control: 23.0 bees; treated: 22.6 bees; $p = 0.57$) and after application (control: 16.9 bees; treated: 16.2 bees; $p = 0.50$). In addition, the foraging behaviour observed on the plots showed no obvious symptoms of poisoning. This was demonstrated by the low frequency of abnormal

behaviours observed in both groups (tab. 2.1.3). However, in the treated cages, the day after the dust application, we noted many agitated bees and some bees (~10 per cage), showing abnormal behaviours (cleaning behaviour and uncoordinated body movements) in the entrance of the hive and.

Table 2.1.1. Index proposed by Schmidt *et al.* (2003) for the comparison of daily bee mortality in treated and control colonies.

# Colony	Treatment	Mean daily bee mortality before treatment (3 days)	Mean daily bee mortality after treatment (5 days)	Ratio post-treatment/pre-treatment
1	Control	9.00	4.80	0.93
3	Control	3.33	1.80	
5	Control	2.67	4.60	
2	Treated	0.33	7.80	9.67
4	Treated	6.67	6.40	
6	Treated	3.67	17.00	
Index treated/control				10.67

Table 2.1.2. Repeated measures ANOVA test for colony strength.

*Statistically significant differences ($p < 0.05$).

Effect	Adult bees			Brood		
	F	df	p	F	df	P
Treatment	0.23	1, 4	0.66	0.09	1, 4	0.78
Days	7.85	6, 24	< 0.01 *	107.26	6, 24	< 0.01 *
Interactions	0.76	6, 24	0.61	1.12	6, 24	0.35

Table 2.1.3. Total number of bees observed on oilseed rape plots exhibiting abnormal behaviour. Values between parentheses refer to pre-treatment.

N - absolute number of bees observed in the plots.

	Bees immobile on leavers or flowers	Bees engaging in cleaning activity	Hanging-knocked out bees
Control (N = 1669)	3 (0)	0 (0)	0 (0)
Treated (N = 1614)	10 (0)	2 (4)	0 (0)

2.1.4. Discussion and conclusions

The laboratory indirect toxicity test showed that bee mortality caused by the dust contaminated with clothianidin-based product Poncho® was not significantly different from that caused by liquid formulation (Dantop®), even if in our study the test concentration of the former was slightly lower than in the latter. Both application ways caused significant mortality rates, even if delayed in time.

This demonstrates that bees can get intoxicated after exposure to quantities of active ingredient dispersed during sowing of treated maize seeds and deposited on wild vegetation. In previous studies (ApeNet, 2010) sub-lethal effects were also observed in several bees exposed to the dust at the concentration found at 5 meters from edge of the sowing field.

Various studies have reported the sub-lethal and lethal effects of neonicotinoids on individual bees (Bortolotti *et al.*, 2003; Medrzycki *et al.*, 2003; Decourtye *et al.*, 2004a; Yang *et al.*, 2008), however, information on the potential effects of pesticides on colony level are scarce (but see Henry *et al.*, 2012). Semi-field and field studies are suitable to study the effects on colonies, including assessment of behaviour, bee mortality and the interaction among bees, exposed to the compound under realistic conditions. Compared to field studies, semi-field studies are easier to control and allow higher numbers of replicates which facilitates statistical evaluations. However, until now the available standardized test methods (EPPO 170/4) don't consider the possibility to study bee exposure to dust and don't give any particular indication in order to study long period effects and specific behaviours.

In this study we propose a new method to test in laboratory and in semi-field (cage) the effects of the dust dispersed during sowing operations on honey bees, knowing the exact exposure concentration of the active ingredient.

Only few methods have been proposed to assess in standardized way the impact of dust from coated seeds on bees. In a laboratory study, Giffard and Dupont (2009) test mortality of bees on *Tibouchina* foliage following the methodology based on EPA guideline relative to residues on foliage (EPA, 1996). The foliage of *Tibouchina* planted in the edge of the field, was exposed to dust dispersed during sowing of treated seeds. Assessments were conducted under controlled conditions and bees were introduced in containers with foliage collected 2 and 24 hours after sowing. Similarly, (Georgiadis *et al.*, 2011) proposed to assess the impact of dust to bees in semi-field studies simulating the sowing process carried out in a maize field surrounded by areas with flowering oilseed rape. In both studies, bees are exposed to the dust, simulating the field scenario but it is not possible to know the pesticide exposure concentration *a priori*. In our laboratory and semi-field method it is possible to apply the desired concentration estimated with specific sowing studies. In the present study we used the mean a.i. concentration deposited on the ground at 5 meters distance from the field's edge, during sowing with a drilling machine equipped with dual pipe deflector. Our results showed that this concentration is toxic to bees despite the deflector pipe modification reduced the quantity of dispersed active ingredients by an average of 50% compared with the unmodified seed drill (ApeNet, 2010).

After dust application, the mortality level observed in the semi-field study increased about 10-11 times compared to the control. The mortality was significantly higher than in control during the first 2 days and was still ecologically relevant during the 3rd day. Similar results were observed in a field study with thiametoxam. In this study, the bee mortality increased on the day of sowing and the number of foraging bees decreased on the day after sowing (Tremolada *et al.*, 2010).

In our semi-field test, sub-lethal effects (cleaning behaviours and agitation) were observed only in few bees in front of the treated hives and no effect was evident during foraging activity. Despite the peak of mortality observed after dust application, no significant differences emerged with regard to colony strength. Colony development decreased during confined period but increased from day 7 to day 15, i.e. after removal of the net that covered the cage. In fact, confined conditions resulted in a natural reduction of egg laying in control and in treated cages.

Comparing the treated cages with the control ones, the lethal effects on individual bees didn't affect the colony development, the socio-physiological parameters (thermoregulation and comb construction capacity) and didn't show long-term effects. Probably the homeostatic capacity of the colony avoided the colony decline despite the high bee mortality rate for 2-3 days.

It was estimated from the Khoury's model (Khoury *et al.*, 2011) that the colonies are able to survive with a mortality rate 3 times higher than control for few days but we can expect a colony decline if this large forager mortality rate lasts for longer than 10 days (Khoury *et al.*, 2011; Henry *et al.*, 2012). Such a situation is possible if the sowing period lasts several days as in the Po Valley, where the landscape is characterized by extended maize cultivation. However, even if the mortality peak didn't affect the colony development and survival, the forager loss may result in a decline of pollination service. This is particularly important in spring, in coincidence with maize sowing operations, when many crops and wild plants are in bloom.

In conclusion, the low active ingredient concentration dispersed at the edge of the field from the pneumatic seeder equipped with deflector, used as mitigation action, cannot be considered sufficiently safe for bees.

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2.2 Effects of indirect contact with neonicotinoids and fipronil contaminated dusts – laboratory study

In this study, the toxicity of the seed dressing formulations Poncho® (clothianidin), Gaucho® (imidacloprid), Cruiser® (thiamethoxam) and Regent® (fipronil) was assessed on forager bees. In order to do that, we considered the concentration of those active ingredients measured by CRA-ING and CRA-PAV in the experimentations carried out in 2010 (ApeNet, 2010, 2011). The a.i. concentrations found at a distance of 5 meters from the edge of the experimental field sowed with treated maize seeds, was chosen for our trials. Starting from this concentrations, we considered also treatments with 10, 100 and 1000 fold concentrated a.i..

2.2.1 Materials and methods

Adult forager bees were collected from a queen right colony in the farm of the University of Bologna and, after a slight anaesthesia with cold temperatures; bees were grouped by 10 in laboratory hoarding cages (13 x 6 x 11 cm). Bees were provided with sugar syrup (50% w/v sucrose) through the insertion of a no-needle syringe on the top of the cage. The experimental cages were divided into 4 treatment groups for each active ingredient, in order to test the toxicity of the pesticide concentration founded at 5 meters and further treatments 10, 100, and 1000 times more concentrated (tab. 2.2.1); an untreated control was also planned. Each treatment group consisted in three repetitions.

The contaminated dusts were obtained by a Heubach cylinder through the abrasion of treated seeds similar to those sold on the market. The active ingredient concentration was quantified by chemical analysis performed by CRA-API chemical laboratory. Afterwards, geometric dilutions in talc were performed in order to prepare different treatment concentrations that were conserved at 4°C and in darkness.

With respect to tested concentrations, clothianidin was employed at 6.25 µg/m² instead of 5.12 µg/m² as it has been done in the previous experimentation (chapter 2.1.2), as a consequence of further trials performed by CRA-ING to assess the quantity and the concentration of a.i. during the experimental maize sowing (ApeNet, 2010).

Following the same treatment protocol as explained in chapter 2.1.2, a paper covered with apple leaves coming from an organic apple orchard. Treatments were administered by spreading the leaves with 0.01g of previously prepared contaminated dusts. After 3 hours from the beginning of the experience, the treatments were removed and bees were left at controlled temperature conditions of 25°C± 2°C and darkness until the end of the experimentation.

The number of dead bees was registered at 3, 6, 9, 12, 24, 48 and 72 hours from the beginning of the treatment. Mortality data were statistically analyzed through ANOVA test.

Table 2.2.1. Utilised products and concentrations. Concentrations of active ingredients found at 5 m from the edge of an experimental field sowed with seed-treated maize and concentrations used for the experimental trials.

Commercial product	Active ingredient (insecticide)	$\mu\text{g}/\text{m}^2$ (5m)	$\mu\text{g}/\text{cage}$
Gaucho [®]	Imidacloprid	3.66	0.0209
Cruiser [®]	Thiamethoxam	2.77	0.0158
Poncho [®]	Clothianidin	6.25	0.036
Regent [®]	Fipronil	0.28	0.0016

2.2.2 Results and discussion

The lowest tested concentration (tab. 2.2.1) resulted not significantly different from control for all products, even though, for clothianidin, the $6.25 \mu\text{g}/\text{m}^2$ concentration determined a higher mortality at the beginning of the experience. Conversely, 100 and 1000 fold concentrated treatments were significantly different from control for the tested products (fig. 2.2.1 a, b, c, d). The lowest mortality percentages were attained with fipronil exposure.

The tested protocol demonstrated the toxic effect of high concentration of contaminated dust, thus confirming the suitability of such an experimental procedure to assess the effects of indirect contact with dusts. These results show that the measured field concentrations of all tested products are capable to entail a toxic effect on adult honey bees, when they are eventually walking on a contaminated surface. Even though the lowest concentration is not significantly different from control for all tested product, we can observe a slight toxic tendency at 72 hours from the beginning of the treatment.

The different experimental trials conducted by CRA-ING with the aim to establish reliable data on dust dispersal, highlighted an interesting trend related to the surface of the sowed field. In particular, the sowing experimentations conducted in 2009 was performed on a field surface of 1600 m^2 , whereas in 2010 the used surface was of 3 ha. Consistently, the concentrations of active ingredients found were higher for all the tested products (ApeNet, 2009, 2010). For those reasons, it could be hypothesized that the contemporary sowing of the widely extended maize area in the north of Italy, could result in a more relevant contamination than estimated. Consequently, the toxicity expressed by higher tested concentrations in this preliminary trial, could be considered as eventually possible.

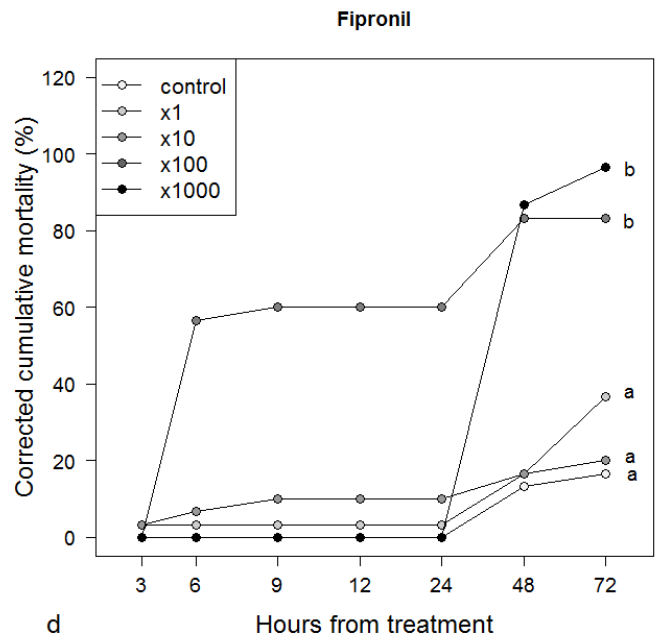
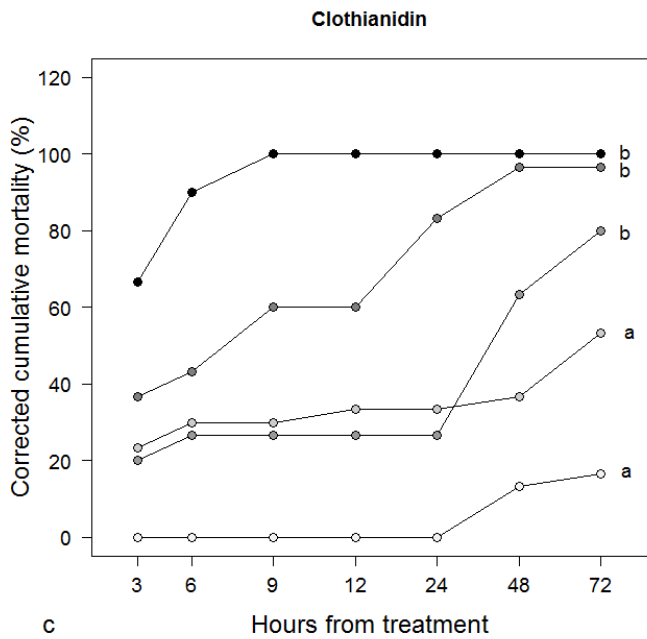
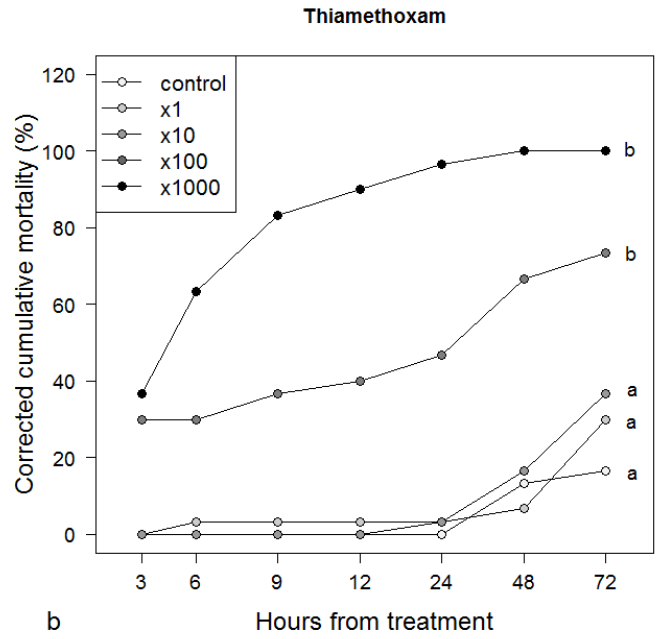
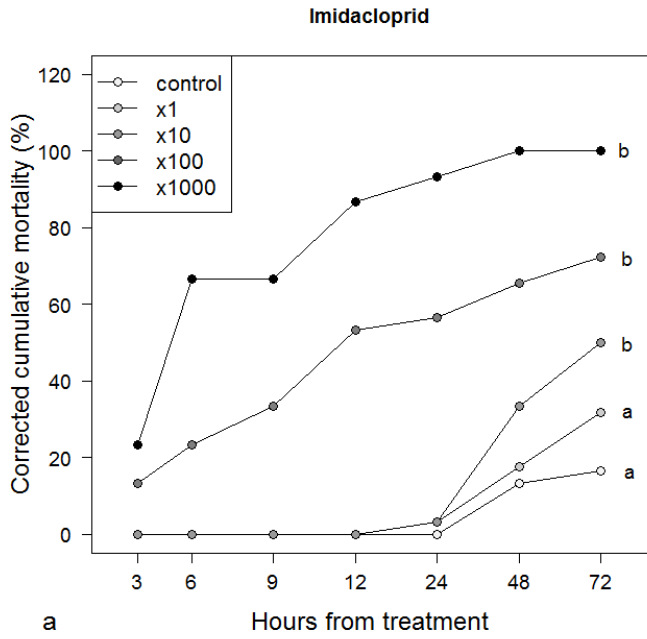


Figure 2.2.1. Effect of dusts contaminated with four active ingredients on honey bee mortality. The adult honey bee mortality was assessed for dusts experimentally contaminated with imidacloprid (a), thiamethoxam (b), clothianidin (c) and fipronil (d). Lines represent corrected cumulative mortality for the concentrations found at 5 m from the edge of a sown field (tab. 2.2.1) and 10, 100 and 1000 fold concentrated quantities.

2.3 Effect of indirect contact with clothianidin contaminated dusts on orientation –field study

The sublethal effects of neonicotinoids and fipronil have been largely investigated in the last years. Since these active molecules express their toxic action at a neuronal level, the most important effects are evidenced in cerebral functions. In laboratory conditions, a detrimental action in learning and memorization capacities has been evidenced, particularly for imidacloprid (Decourtye *et al.*, 2004a) and fipronil (Aliouane Y. *et al.*, 2009). In semi-field and field conditions, several studies have examined the effect of fipronil (Decourtye *et al.*, 2011) and clothianidin (Schneider *et al.*, 2012) in reducing honey bee foraging activity. The effects on orientation are mainly investigated with the evaluation of foragers homing ability, that is the capacity to find the way back to the hive. To this extent, the first product to be assessed has been imidacloprid. Three sublethal concentrations (100, 500 and 1000 µg/L), administered *via* ingestion, have caused a delay in the honey bee flights between the experimental hive and an artificial feeder (Bortolotti *et al.*, 2003), demonstrating a neonicotinoid detrimental effect on the orientation ability. More recently, the RFID technique has allowed to perform more extended studies, in which the effect of small doses of thiamethoxam on the homing flight ability has been demonstrated, as well (Henry *et al.*, 2012).

Here, the effect of clothianidin contaminated dusts, administered *via* indirect contact to forager bees, was assessed.

2.3.1 Materials and methods

After choosing an area with no flowering crops and few attractive vegetations spots within a 2 km range, a 10-frames healthy colony was placed. An artificial feeder was then located at 330 m from the hive, on the other side of a maize field. The feeder was provided with a high concentrated sucrose solution and a plastic device to make honey bees nourish.

After a one-day habituation period, honey bees went regularly foraging at the feeder. Foragers were then captured, marked with a coloured spot on the thorax and divided in two treatment groups (n=30). Honey bees were then placed in experimental cages, whose bottom was covered with leaves and treated with clothianidin contaminated talc (or pure talc for the control group), as described in the previous trials (see chapter 2.1.2). The cages were modified in order to reduce the height of the cage to 3 cm, this forcing honey bees to walk on the bottom of the cage. Clothianidin tested dose corresponded to the concentration found in maize sowing experimentations, as explained in chapter 2.1. The concentration measured in the field (6.25 µg/m²) was therefore adjusted to the cage bottom surface, so the final employed concentration was 0.044 µg/cage. Contaminated talc was kept in darkness and at 4°C until the utilisation. Honey bees were kept in the cages for 1 hour and 30 minutes (in darkness) and then released.

In 5 hours following the release, the honey bee presence at the feeder and at the entrance of the hive was visually assessed, in order to assess: (i) the percentage of bees that returned to the hive and to the feeder, (ii) the interval between two visits at the feeder and at the hive.

Collected data were converted into percentages and then were normalized by arcsine-root transformation. One way ANOVA was performed in order to assess differences in presences at the feeder and at the hive between treatments, whereas repeated measures ANOVA was used to compare flights duration.

2.3.2 Results and discussion

More than 80% of the bees came back to the hive in the observation period after the release. No significant difference was found between treated and control in the number of honey bees that came back to the hive in the 5 hours following the release ($p=0.69$). Similarly, a comparable number of bees visited the artificial feeder after having returned to the hive ($p= 0.53$) (Fig. 2.3.1). For both treated and control bees the number of individuals flying between the hive and the feeder diminished through time.

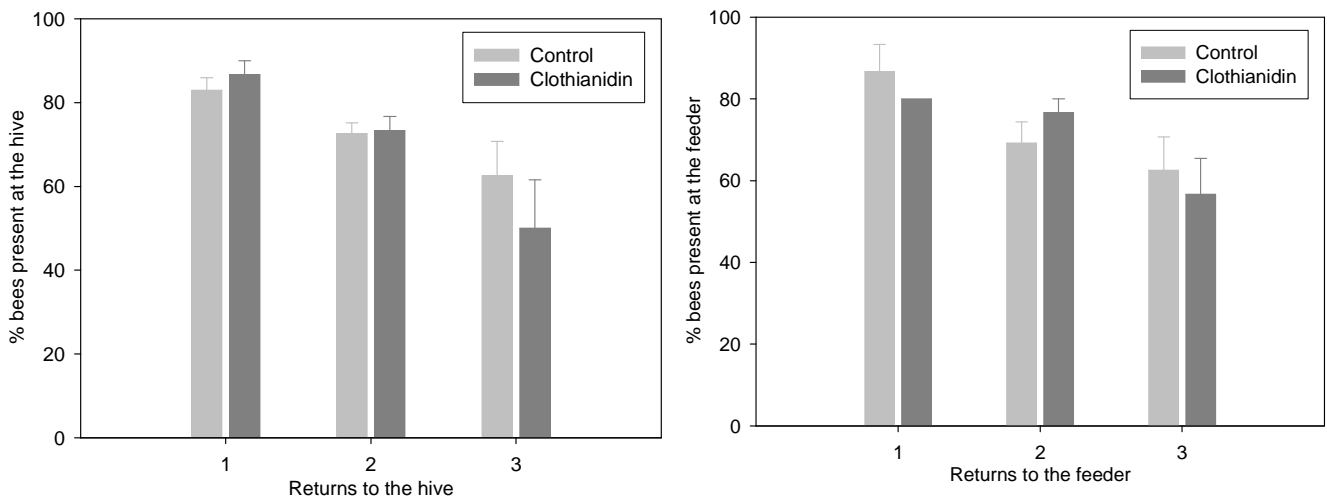


Figure 2.3.1. Percentage of honey bees presences at the hive (a) and at the artificial feeder (b) after the release and in following foraging flights. Error bars represent SE.

The duration of foraging flights was evaluated considering the time passed between two subsequent arrivals of the same bee at the hive. This data was similar between treated and control bees ($p=0.55$). Moreover, no differences was found between the duration of the first three foraging flights ($p=0.30$). On the other hand, the time spent in the hive was evaluated considering the time

interval between two visits at the artificial feeder. In this case, also, the observed behaviour was not different between treatment groups ($p=0.69$) (fig. 2.3.2).

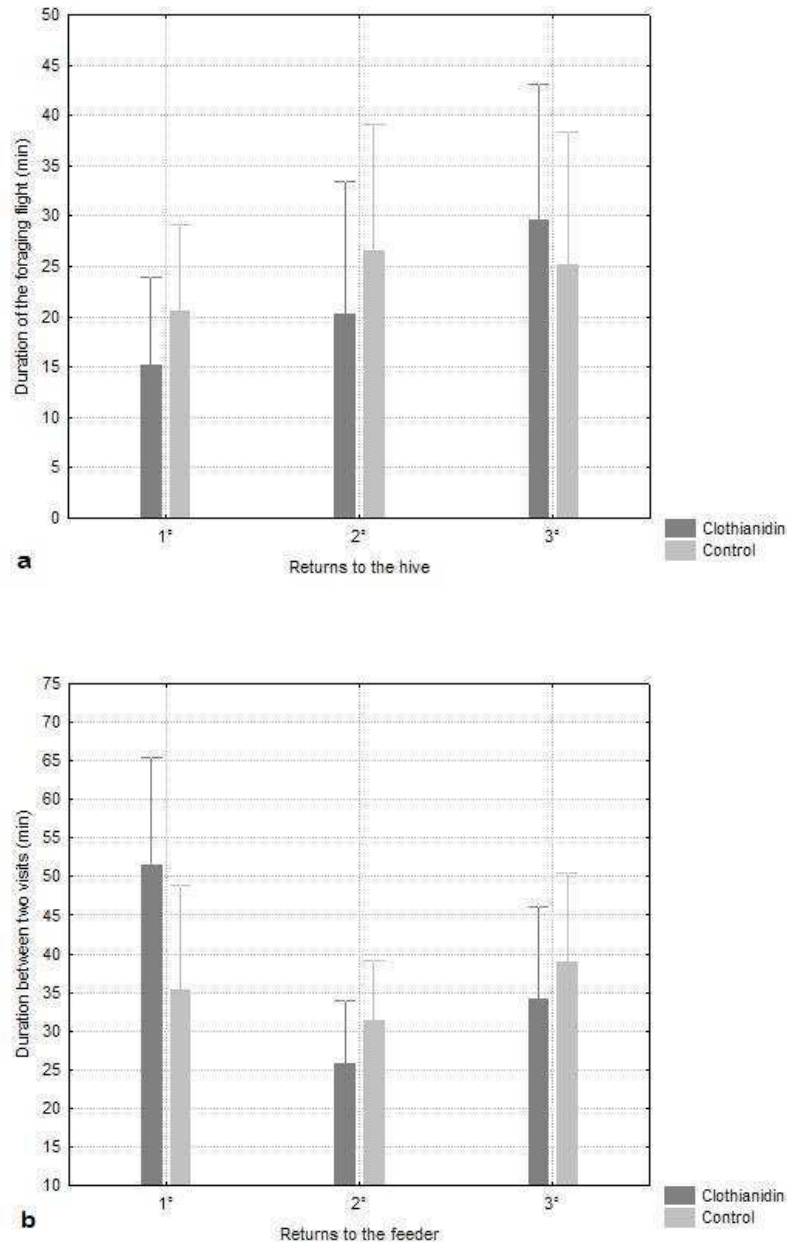


Figure 2.3.2. Time passed between two subsequent bee recordings at the hive (a) and at the feeder (b). Error bars show the 95% confidence interval.

The homing flight ability is one of the important sublethal endpoint to assess pesticide influence on behaviour and cognitive processes. The ingestion of contaminated solutions of imidacloprid, clothianidin and fipronil provoked a detrimental effect on honey bee capacity to flight back to the hive, when foraging (Bortolotti *et al.*, 2003; Decourtye *et al.*, 2011; Schneider *et al.*, 2012), but the effect of dust contamination on this behavioural trait has been never assessed.

Here, we developed a reliable method of bee intoxication to carry out field experimentations on indirect contact exposure to contaminated dusts. Concerning the pesticide effect, the number of bees returned at the hive and at the feeder in the first three foraging flights, was not different (fig. 2.3.1 a, b). However, for the first flight, treated honey bees were observed to come back to the feeder with a slightly higher delay than control bees (fig. 2.3.2 b). Even though not statistically significant, this observation might be explained as a tendency of treated bees to remain in the hive for a longer time.

In this experience, statistical analysis does not allow pointing out any difference between control and clothianidin treated bees, concerning homing ability, so that we conclude that the clothianidin concentration founded at 5 m from a maize sowed field, does not impair orientation capacities. However, we point out some principal comments on the experimental procedure that could be relevant for further research on this topic. The artificial feeder was placed at a distance of 330 m that might be not enough to evidence a slight but significant effect in disorienting bees. For instance, Henry *et al.* (2012) tested the homing flight capacity on 1 km distance. The employment of new techniques, as RIFD, would consent the management of a higher number of individuals and a longer observation period. The possibility of a continuous monitoring for at least 24 hours seems particularly useful since neonicotinoid and fipronil toxic action might be delayed in time and result in perceivable symptoms at long term.

However, other researches highlighted the significant role of neonicotinoids and fipronil contaminated dusts in olfactory learning and memory (ApeNet, 2010). This discrepancy indicates that it is not possible to exclude that contaminated dusts can be involved in cognitive processes impairment.

3 STUDY OF THE EFFECTS OF DIFFERENT PESTICIDES ON BIOCHEMICAL-PHYSIOLOGICAL CHANGES

Preface

The study of the effects of pesticides includes the assessment of the influence of such molecules at a subcellular level, on enzymes and metabolic pathways. This investigation might be pursued both with a genomic/proteomic approach and with a series of biochemical assays. The first method permits to evaluate the eventual differences in protein expression, thus considering enzymes concentration. The second approach is more focussed on the variations in enzymatic activity as a response to the exposure to several contaminants and stressors in general. A so-called multimarker approach is suitable to be employed for pesticide effects assessment. Therefore, the measure of the activity of a set of key-role metabolic enzymes provides an overview of intracellular pesticide induced modifications.

To this extent, different enzymes have been employed, particularly belonging to detoxification and oxidative stress response pathways. The use of this kind of method has been first developed in aquatic ecotoxicology, for biomonitoring purposes. In this approach, the sampling of individuals living in a polluted environment aims to individuate the traces of the exposition in significant variations of enzymes activity. This subject has been less investigated in terrestrial arthropods and pollinator insects, even though a recent studies have evidenced interesting results in honey bees (Badiou *et al.*, 2008; Badiou-Bénéteau *et al.*, 2012).

Here, we considered this issue with a different approach from biomonitoring. We in fact assessed, in laboratory conditions, the variation in enzymatic activities as a sublethal effect of a specific pesticide or a combination of products. Since sublethal effects are represented by the alterations of the organism physiology that don't involve death but that, in particular conditions, may lead to a weakening of individuals and colony, the changes at an enzymatic level could constitute a valuable tool to interpret pesticide impact on honey bee organism.

We therefore chose to test different pesticides, with different modalities of treatment. We carried out the experimentations with the following scheme:

- combined exposure to sublethal doses of *Bacillus thuringiensis* spores and fipronil;
- combined exposure to sublethal doses of *Bacillus thuringiensis* spores, followed by a contact treatment with deltamethrin;
- combined exposure to sublethal doses of difenoconazole, followed by a contact treatment with deltamethrin;

3.1 Chronic effect of three Cry toxins and combined effect of *Bt* and fipronil on adult honey bees: toxicity and physiological changes

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Keywords: honey bees; *Bacillus thuringiensis*; Cry toxins; biomarkers

In preparation

3.1.1 Introduction

The domestic honey bee (*Apis mellifera* L.) accounts for the most important pollination service of agricultural monocultures (Watanabe, 1994), contributing to the pollination of more than 66 percent of the world's 1500 crop species (Roubik, 2005) and to 35% of food crops (Klein *et al.*, 2007). During its intense foraging activity, a single honey bee can visit flower at distance higher than 6 km from the hive (Beekman and Ratnieks, 2000), searching for food sources, which are mainly represented by nectar and pollen, the latter being the first protein source for honey bees (Crailsheim *et al.*, 1992; Babendreier *et al.*, 2004). Predictably, that intense interaction with the environment exposes not only foragers but also hive bees and the brood to residues of pollutants and pesticides eventually present in pollen and nectar (Krupke *et al.*, 2012).

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium that has been known since the beginning of the century for its insecticidal properties, mainly against lepidopterans. Once ingested and activated in the intestinal tract of the target insect, the crystal toxins (Cry toxins) produced by the bacterium, cause cell lysis and ultimately lead to insect death (Gill *et al.*, 1992; Bravo *et al.*, 2007; Vachon *et al.*, 2012). As its potential in pest control was recognized, *Bt* spores were used in field treatments, with *Bt* var. *kurstaki* (B.t.k.) being the most widely used strain. Since 1996, biotechnologies permitted an extensively use of *Bt* in genetically modified crops (de Maagd *et al.*, 1999), providing Cry toxin expression in all the plant tissues and thus ensuring a more powerful protection against insect pests. Counterparty, this stimulated the research on possible side effects on non-target arthropods, particularly pollen consuming ones, as honey bees.

Amounts of *Bt* toxins are highly variable in pollen, mostly depending on whether the promoter sequence is constitutive or tissue-specific (Koziel *et al.*, 1993; Malone and Burgess, 2009). The genetically modified corn hybrid Event176, which contained a green-tissue and pollen specific promoter (Dutton *et al.*, 2003), expressed from 500 to 11000 µg/kg of Cry1Ab toxin, according to Fearing *et al.* (1997). Conversely, constitutive promoters accounts for lower amounts of toxin in pollen (Liu *et al.*, 2009) as in *Bt* maize MON810 containing approximately 2 µg/kg of Cry1Ab (Wraight *et al.*, 2000). A more recently released GM maize event, MON863, has been proved to express 77.000 µg/kg of Cry3Bb1 toxin in pollen (Duan *et al.*, 2002; Li *et al.*, 2008).

Thus, pollen-consuming organisms may be potentially exposed to high quantities of Cry toxins. Adult honey bees could therefore be exposed to *Bacillus thuringiensis* by foraging an area treated with *Bt* sprayed formulations, while the exposure by ingestion of pollen expressing Cry toxins may occur to both adult bees and brood (Malone *et al.*, 1999; Babendreier *et al.*, 2004).

Cry toxins have been generally proved not to affect adult honey bee survival. No lethal effect of Cry1Ab toxin has been demonstrated at doses up to 1000 µg/L administered via ingestion (Malone

and Minh-Hà, 2001; Ramirez-Romero *et al.*, 2005; Hendriksma *et al.*, 2011; Hendriksma *et al.*, 2012). Similar results have been found for other toxins, in particular Cry1Ah, conferring resistance to Lepidoptera in cotton (Dai *et al.*, 2012). On the other hand, learning capacities, food behaviour and foraging activity can be adversely affected by an oral exposure to Cry1Ab (Ramirez-Romero *et al.*, 2005; Ramirez-Romero *et al.*, 2008). When analyzing the effects on bees of *Bt* spore formulations, the *kurstaki* strain does not appear lethal to bees and bumblebees (Malone *et al.*, 1999; Mommaerts *et al.*, 2010). Conversely, a slight effect on mortality of foraging honey bees has been observed after an acute oral treatment with *kurstaki* strain spores (Brighenti *et al.*, 2007).

Thus, considering a lack of agreement regarding *Bt* impact on honey bees, a further knowledge on chronic effects of Cry toxins and *Bt* spores on adult honey bees seems to be needed. Moreover, investigating the combination of *Bt* with other plant protection products is relevant to the assessment of the risk for bees (Duan *et al.*, 2008). To date, no study have been focused on the joint effect on bees of a *Bt* treatment with another stressor, like a chemical pesticide, as it has been studied for other insects (Wu *et al.*, 2001; Morales-Rodriguez and Peck, 2009).

Fipronil is a phenylpyrazole insecticide and miticide that exhibits an antagonist action on the insect GABA and GluCl receptors, leading to an over activation of neurones and finally the death of insects (Ikeda *et al.*, 2003; Narahashi *et al.*, 2010). Its detrimental effects on learning and memory processes of bees have been extensively reported (El Hassani *et al.*, 2005; Aliouane Y. *et al.*, 2009; Bernadou *et al.*, 2009; Decourtye *et al.*, 2009; Decourtye *et al.*, 2011).

In this study, we have investigated the effects of *Bt* toxins and spores, in combination with Fipronil, on the honey bee. The presence of Fipronil in pollen and bee bread has been well documented (Chauzat *et al.*, 2006; Mullin *et al.*, 2010), consenting the hypothesis of a realistic combined exposure to both Fipronil and *Bt* residues. In addition, a secondary toxicity mechanism of Fipronil towards intestinal cells has been demonstrated (Vidau *et al.*, 2009; Cruz *et al.*, 2010). This shows that Fipronil can be active on the same target tissue as *Bacillus thuringiensis*.

In addition to the classical toxicological endpoints, like mortality and food consumption, different physiological functions have been investigated here and improved by the use of an approach involving biochemical biomarkers. We assessed the activity of Glutathione-S-Transferase (GST), Alkaline phosphatase (ALP), Glucose-6-phosphate dehydrogenase (G6PDH) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), following the administration of *Bt* alone or in combination with fipronil. The observable changes of enzymatic activity may, in fact, be considered as indicators of exposure to a chemical or a xenobiotic of a bioindicator organism (Lagadic *et al.*, 1994). Thus, measuring the variation of some physiological parameters, as

detoxification and metabolic enzymatic activity, can enhance the evaluation of toxic or perturbing effect of a pesticide.

Hence, the objectives of the present study were (i) to investigate the effects of three *Bt* toxins Cry1Ab-2, Cry1C-1, Cry3Aa when administered chronically for 10 days to adult honey bees, (ii) to evaluate the effects of a sublethal treatment with fipronil alone or in combination with a *B.t.k.* spore solution on honey bee survival and (iii) to study physiological changes induced by the different treatments.

3.1.2 Materials and methods

Tested products

Stock solutions of Cry toxins and *Bt* spores were prepared in distilled water. All the treatment solutions were prepared in distilled water and 0.1% dimethyl sulfoxide (DMSO). DMSO was used in order to enable a good fipronil solubilisation. Stock solutions were prepared and stored at -20°C and working solutions were freshly made by dilutions in sugar syrup (50% w/v sucrose) and renewed daily.

Honey bees

All bees were taken from queen right *Apis mellifera* honey bee colonies carefully monitored to check their health status. The 10-day mortality tests were carried out with adult bees collected from the hive supers. Bees were slightly anaesthetized with CO₂ and placed into plastic cages (6 x 8,5 x 10 cm), adapted from Pain type, in groups of 30 individuals and provided with candy (Apifonda; commercial sucrose feed paste) and water *ad libitum*. The 25-day survival test, which required a longer lifespan of individuals, was carried out with emergent honey bees. Emergent bees were obtained by placing brood frames in an incubator at controlled conditions (34°C ± 2°C, 60% ± 10% relative humidity, darkness) for one day. Newly emerged bees were placed in cages, without anaesthesia, in groups of 40 individuals, with a source of queen pheromone blend (one third of commercial Beeboost[®] stick). Fresh multifloral pollen was provided for the first three days and then replaced with a protein commercial preparation, added to the treatment syrup (1% v/v). After one day of adaptation to experimental conditions, dead honey bees were removed and replaced with new ones. The cages were placed in incubators at controlled conditions (28°C ± 2°C for adult bees; 34°C ± 2°C for the emergent bees; 60% ± 10% relative humidity; darkness) until the end of the experiments.

Modality of treatment and treatment groups

All insecticide treatments were administered for 10 hours per day then the honey bees were provided with candy and water *ad libitum* for the remaining 14 hours. Mortality and syrup consumption were recorded daily.

For each tested *Bt* toxin (Cry1Ab-2, Cry1 C-1 and Cry3 Aa), four treatment groups and a control group were formed in order to assess the effect of 10 µg/L; 1 µg/L; 0.1 µg/L and 0.01 µg/L concentrations. For each tested *Bt* strain (4Q2 and 4D1) two treatment groups and a control were formed in order to assess the effect of 100 µg/L and 1000 µg/L concentrations. A single treatment group consisted of 9 rearing cages, each containing 30 individuals.

In order to investigate the effect of Fipronil joint or disjoint with *Bt* strains, 10 treatment groups and a control group were set up. In this case, each group consisted of 6 rearing cages, each containing 40 individuals (tab. 3.1.1).

Table 3.1.1. Treatments, tested doses and evaluated endpoints.

Treatment	Tested doses	Endpoints
Cry1Ab	0.01, 0.1, 1, 10 µg/L	Adult honey bees mortality
Cry1C-1	0.01, 0.1, 1, 10 µg/L	Adult honey bees mortality
Cry3Aa	0.01, 0.1, 1, 10 µg/L	Adult honey bees mortality
<i>Bt</i> 4Q2	100, 1000 µg/L	Adult honey bees mortality
<i>Bt</i> 4D1	100, 1000 µg/L	Adult honey bees mortality
<i>Bt</i> 4Q2	100, 1000 µg/L	Honey bee survival and biomarkers
<i>Bt</i> 4D1	100, 1000 µg/L	Honey bee survival and biomarkers
<i>Bt</i> 4Q2 / Fipronil	100, 1000 µg/L / 1 µg/L	Honey bee survival and biomarkers
<i>Bt</i> 4D1 / Fipronil	100, 1000 µg/L / 1 µg/L	Honey bee survival and biomarkers
Fipronil	1 µg/L	Honey bee survival and biomarkers

Enzymatic activity assays

We performed enzymatic activity measurement on honey bees treated with *Bt* spores combined or not with 1 µg/L Fipronil. Three sampling dates were chosen: at the beginning of the trial, at 10 days and 20 days from the beginning. Only alive bees were sampled; the head, mid gut and abdomen devoid of the intestine were dissected and separated in order to analyze enzymatic activity in different compartments; all the samples were then stored at a -80° C. Four repetition were performed for each treatment group and three honey bees were sacrificed for each repetition. The tissue extracts were obtained by homogenizing (TissuLyser™; Qiagen; 5610 s at 30 MHz) three heads (or midguts or voided abdomens) in the extraction buffer (40 mM L-S phosphate buffer at pH 7.4; 10 mM NaCl; 1% Triton; protease inhibitors). The homogenates were then centrifuged at

15,000 g for 20 minutes at 4°C and the resulting supernatants kept in ice-cooled tubes. The extracts were then employed for enzymatic assays, performing three replicates for each repetition.

Glutathione-S-Transferase (GST) activity was spectrophotometrically assessed measuring the conjugation of reduced glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) using a method adapted from Habig et al. (1974). GST activity was measured in heads and midguts by adding 10 µl of enzymatic extract to the reaction mixture containing 1 mM ethylenediaminetetracetic acid (EDTA), 2.5 mM GSH, 1 mM CDNB and 100 mM Na/K-phosphate buffer at pH 7.4. GST activity was quantified by recording the appearance of conjugated product at 340 nm during 5 min.

Alkaline phosphatase (ALP) activity was assayed in a reaction medium containing 10 µl of enzymatic midgut extract, 20 mM MgCl₂, 2 mM p-nitrophenyl phosphate and 100 mM Tris-HCl buffer at pH 8.5. The enzymatic activity was measured by monitoring at 410 nm for 5 minutes through spectrophotometry the transformation of p-nitrophenyl phosphate to p-nitrophenol (Bounias et al., 1996).

Glucose-6-phosphate dehydrogenase (G6PDH) activity was assessed by measuring the transformation of glucose-6-phosphate to 6-phospho-gluconate through the reduction of β-Nicotinamide Adenine Dinucleotide Phosphate Hydrate (β-NADP). A medium containing 100 mM Trizma base buffer at pH 7.4, 1 mM D-Glucose 6-phosphate disodium (G6P Na₂), 0.5 mM β-NADP, 10 mM MgCl₂ was monitored for 5 minutes at 340 nm.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was assessed measuring at 340 nm the formation of glyceraldehyde-3-phosphate from glycerate-1,3-diphosphate, the latter formed by the conjugation of 3-phosphoglyceric acid (3-PGA) and 3-phosphoglyceric phosphokinase (3-PGK). The reaction medium was constituted by 80 mM triethanolamine buffer at pH 7.6, 7 mM 3-PGA, 4 mM L-Cysteine HCL neutralized with sodium bicarbonate, 2 mM magnesium sulfate (MgSO₄), 120 µM reduced β-Nicotinamide Adenine dinucleotide (β-NADH), 1.2 mM ATP, 1 mM EDTA, 5 U 3-PGK.

Statistical analysis

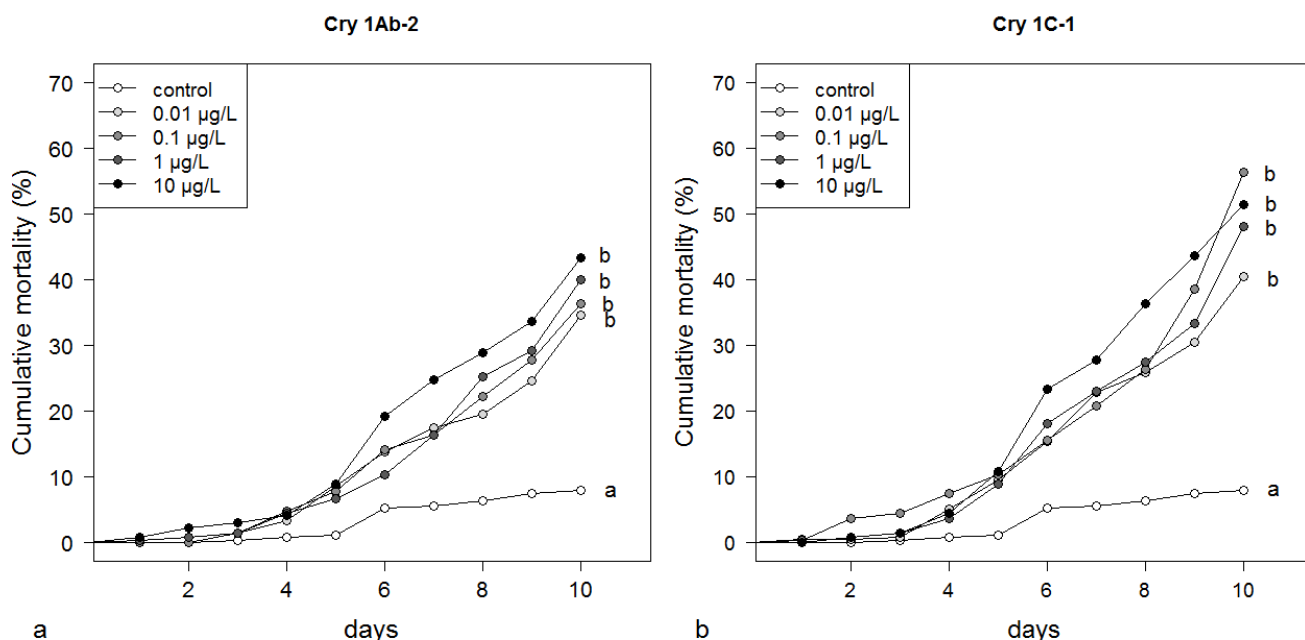
Mortality data were log-transformed and compared using a general linear model and an analysis of variance (ANOVA). Syrup consumption data were processed by a repeated measures ANOVA, followed by Tukey HSD post-hoc analysis. Enzymatic assay data were analyzed through a Mann-Whitney U test in order to obtain a between treatment comparison; ANOVA analysis was also used to define general tendencies. All comparisons described by p values inferior to 0.05 were considered as significantly different. All analysis were performed with R software (version 2.14.1).

3.1.3 Results

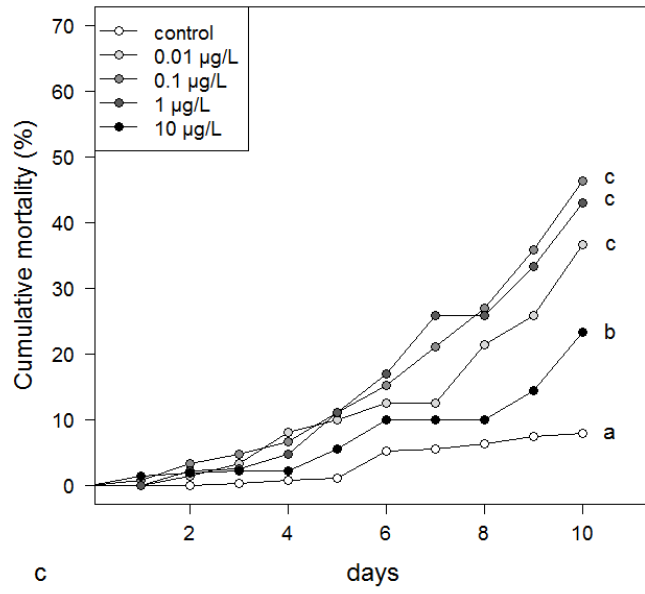
Effect of three *Bt* toxins on honey bee mortality and feeding behaviour

The effect of *Bt* toxins Cry1Ab-2, Cry1C-1 and Cry3Aa on adult bees was assessed through a chronic 10-day administration. Cry1Ab and Cry1C toxins are mostly used against Lepidopteran pests in maize and rice (Hofte and Whiteley, 1989; Martinez et al., 2004), whereas Cry3Aa is mainly used against Coleoptera in potato (Hussein *et al.*, 2006). The tested concentrations were chosen to be consistent with environmental realistic exposure. Thus, 0.01, 0.1, 1 and 10 $\mu\text{g}/\text{kg}$ of purified toxins were employed as tested concentration. The cumulative mortality of the control remained under 10% and was significantly lower than all other treatment groups ($p < 0.001$). For the Cry1Ab toxin, 10 $\mu\text{g}/\text{L}$ dose caused the highest mortality (43.3%) even though no significant differences were shown between treatments (fig. 3.1.1 a). The Cry1C-1 toxin expressed the most important effect, as honey bees died up to 56% as a consequence of the 0.1 $\mu\text{g}/\text{L}$ treatment, though statistical analysis showed no significant difference between doses (fig. 3.1.1 b). Honey bees treated with Cry3Aa toxin died at a higher rate than the control ($p = 0.016$), and the highest dose (10 $\mu\text{g}/\text{L}$) caused the lowest mortality among treatments ($p = 0.001$) (fig. 3.1.1 c).

No influence on syrup consumption can be attributed to treatment or dose for every tested toxin (fig. 3.1.1 d, e, f).

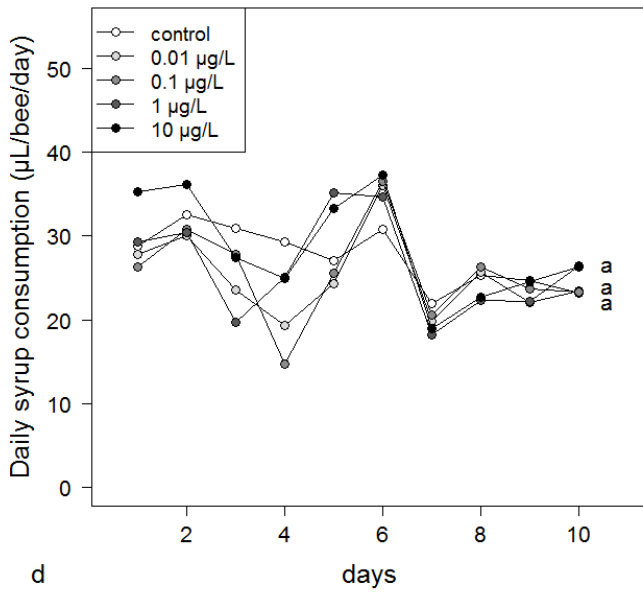


Cry 3Aa



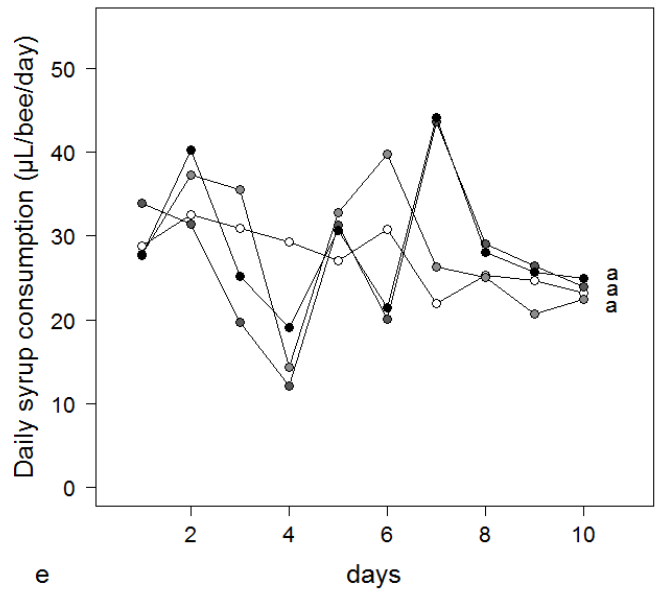
c

Cry 1Ab-2



d

Cry 1C-1



e

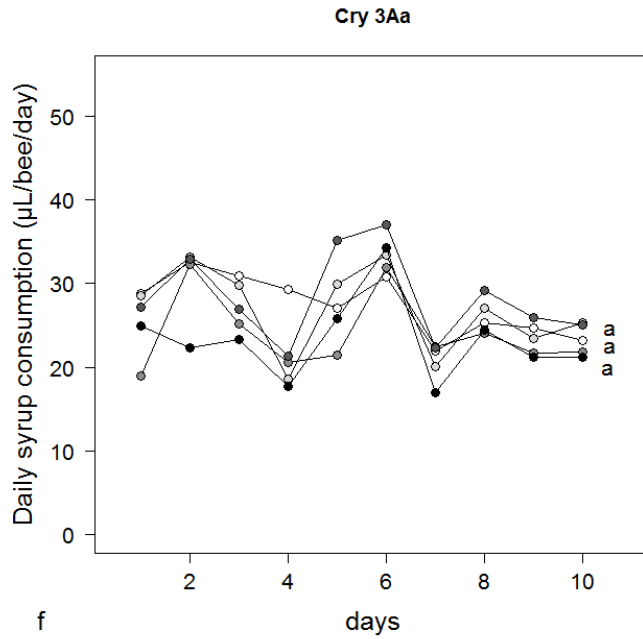
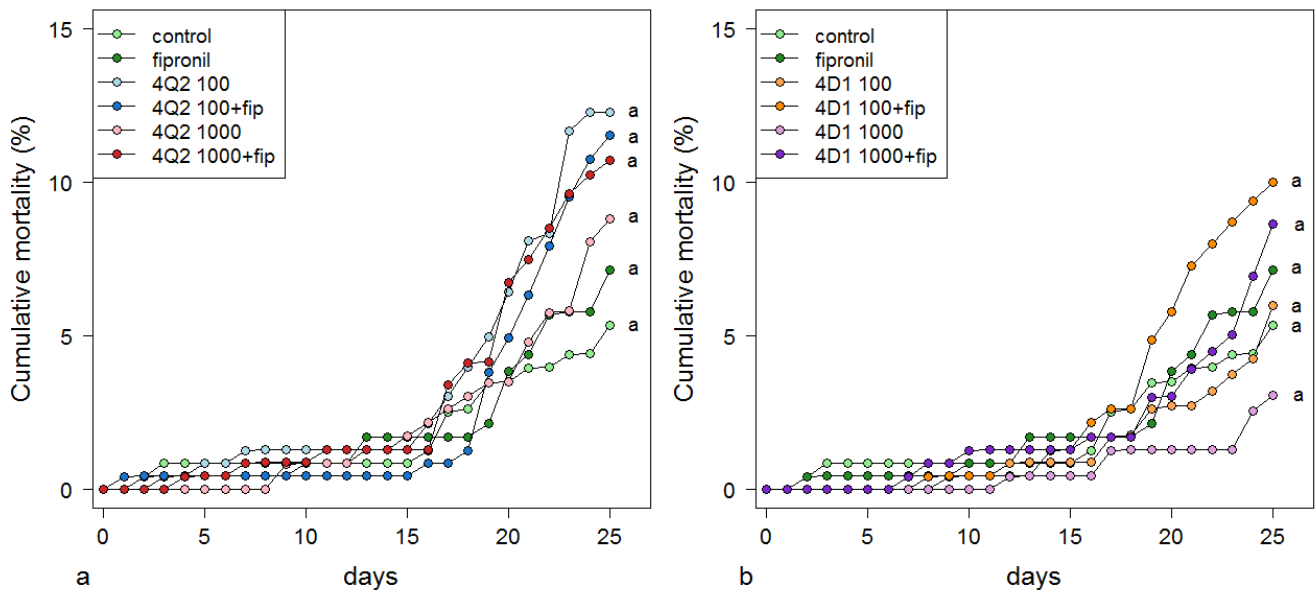


Figure 3.1.1. Effect of three Cry toxins on honey bee mortality and feeding behaviour. Data are represented as percent cumulative mortality for 10-day chronic exposure of adult honey bees to Cry1Ab-2 toxin (a), Cry1C-1 (b) and Cry3Aa (c) and daily average syrup consumption per bee when exposed to Cry1Ab-2 toxin (d), Cry1C-1 (e) and Cry3Aa (f). Each toxin has been tested with 4 different doses. Lines represents the mean of 9 repetitions (cages), each rearing cage containing 30 honey bees (n=270). Different letters indicate significant differences between treatments.

Combined effect of *Bt* spores and Fipronil on honey bee mortality and feeding behaviour

As *Bt* strains, *Bt* 4Q2 and *Bt* 4D1 were tested, the first being a modified strain that does not express any Cry toxin and the second representing a kurstaki strain and expressing Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B toxins. The tested concentrations were 100 and 1000 $\mu\text{g}/\text{kg}$. Fipronil was tested at 1 $\mu\text{g}/\text{L}$. This concentration is lower than the average residue amount found in pollen samples (Mullin *et al.*, 2010) and therefore consistent with environmental levels.

The joint effect of 4Q2/4D1 spores and fipronil on survival of emerging honey bees was investigated performing a 10-day treatment followed by a 15-day mortality assessment. The cumulated mortality remained under 2% for all the treatment groups at 15 days and did not exceed 15% at 25 days (fig. 3.1.2 a, b). No significant differences between *Bt* treated groups and control can be found at 25 days ($p=0.566$). The ANOVA analysis on all data revealed an effect of the treatments on feeding behaviour ($p=0.012$), as the combined administration of *Bt* 4Q2 and Fipronil resulted in a lower syrup consumption ($p=0.03$). Daily syrup intake was also influenced by time, thus by honey bee age ($p < 1 \times 10^{-16}$) (fig. 3.1.2 c, d).



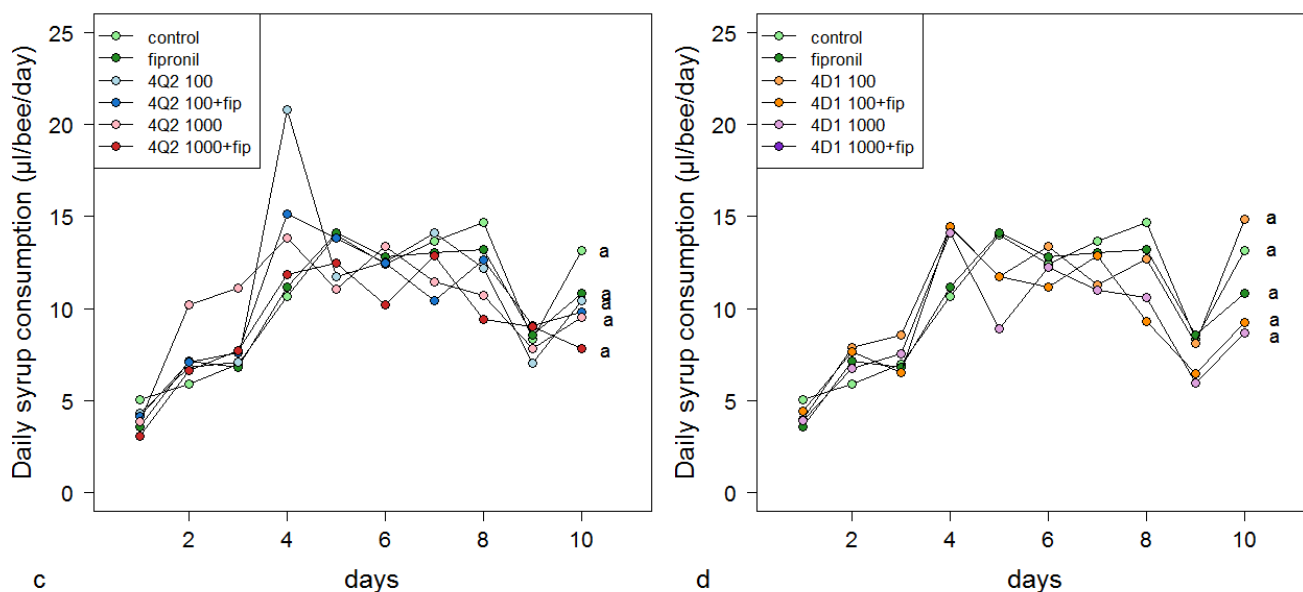


Figure 3.1.2. Effect of *Bt* 4Q2 and 4D1 strains joint or disjoint with Fipronil on honey bee survival and feeding behaviour. Cumulative mortality percentage for 10-day chronic exposure to *Bt* 4Q2 (a) and *Bt* 4D1 (b) strain joint and disjoint with fipronil, followed by a 15-day observation period. Daily average syrup consumption for a 10-day chronic treatment with *Bt*4Q2 (c) and *Bt*4D1 (d) joint and disjoint with fipronil. Lines represents the mean of 6 repetitions (cages), each rearing cage containing 40 honey bees (n=240). Different letters indicate significant differences between treatment groups (p<0.05).

Combined effect of *Bt* strain and fipronil on GST, ALP, G6PDH and GAPDH activity.

Enzymatic activities of Glutathione-S-transferase (GST), alkaline phosphatase (ALP), Glucose-6-phosphate dehydrogenase (G6PDH) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were evaluated at the end of the chronic treatment (day 10) and ten days after (day 20). Activity measures were statistically processed by a two-way ANOVA performed on all the data and by a Mann-Whitney U test in order to perform pairwise comparisons. Mann-Whitney results are shown in the boxplots, while ANOVA results are integrated in table 3.1.2.

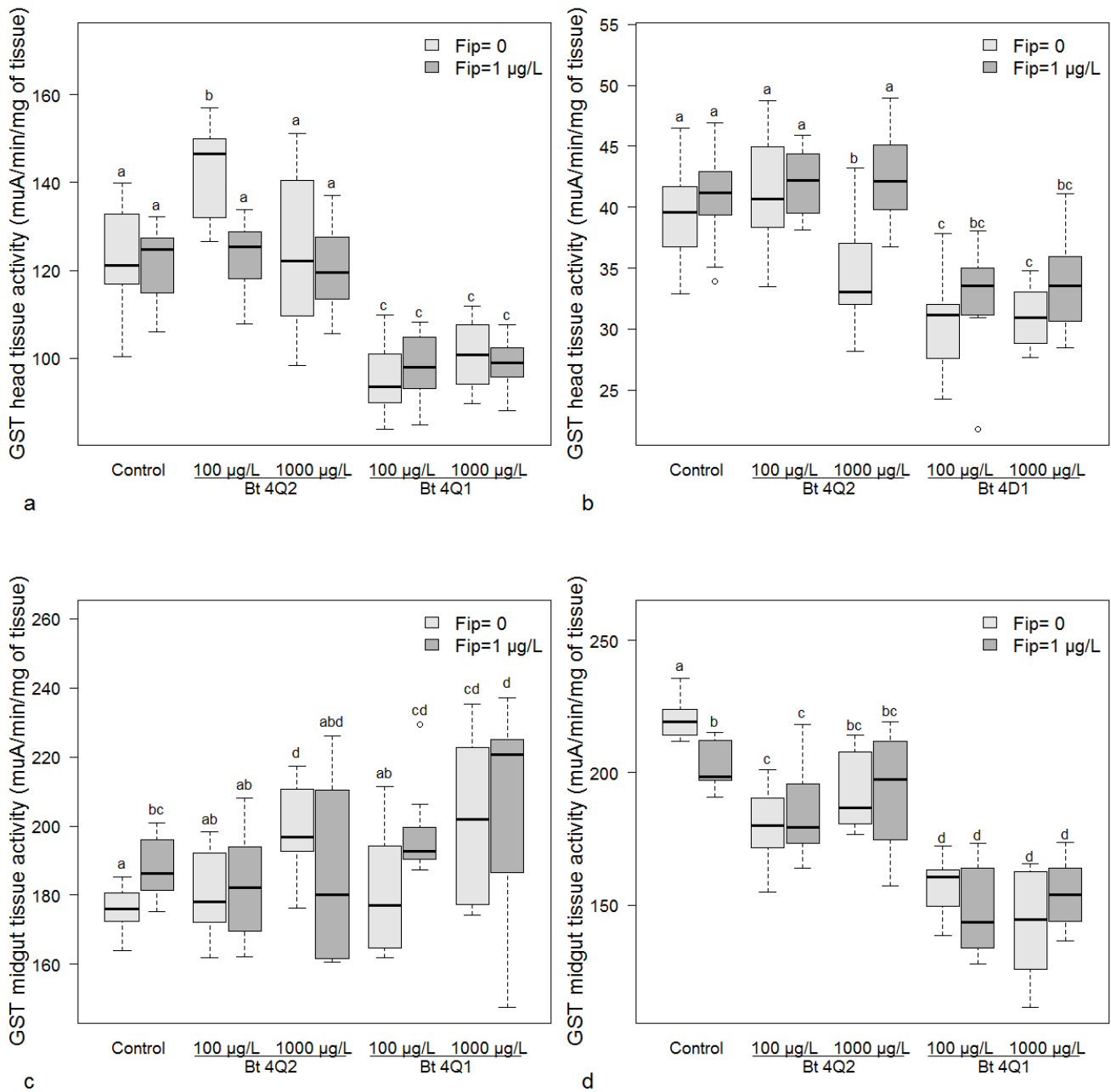


Figure 3.1.3. Effect of *Bt* 4Q2 and 4D1 strains joint or disjoint with Fipronil on GST activity. Glutathione-S-transferase (GST) activity was assessed in heads at day 10 (a) and day 20 (b) and in midguts (c, d) on the same sampling dates, respectively. Each treatment group is constituted by 4 repetitions performed in triplicate (n=12), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups (p<0.05). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

GST tissue activity was assessed in heads and midguts dissected from honey bees at 10 days and 20 days from the beginning of the experience. As shown in fig. 3.1.3 a and b, honey bees treated with *Bt kurstaki* at any dose expressed a significantly lower GST head activity at day 10 ($p < 1 \times 10^{-15}$), while no differences were found between the 4Q2 *Bt* treatment and the control. Similarly, the

kurstaki strain induced a decrease in GST activity in the midgut at day 20 ($p < 1 \times 10^{-15}$) (fig. 3.1.3 d), but the same effect was not found at day 10 sampling. As highlighted by ANOVA, the joint treatment with fipronil at day 20 increased GST head activity in all the treatment groups ($p = 0.000$).

ALP activity was assessed in midguts; at day 10 from the beginning of the experience, honey bees that had been treated with a *Bt* contaminated diet, showed a higher activity than the control ($p < 1 \times 10^{-05}$) and among them, ANOVA analysis evidenced a higher response for the *kurstaki* treated honey bees ($p = 0.008$). At day 10, fipronil caused a higher activity ($p < 1 \times 10^{-7}$) for all the treatment groups; in particular, Mann-Whitney U test showed a significant inducing effect for the 100 $\mu\text{g/L}$ dose ($p < 0.05$). At day 20, a lower ALP activity was found for the *Bt* treated bees ($p < 1 \times 10^{-12}$), with no significant differences between strains. Fipronil treated bees did not exhibit a different enzymatic activity, compared to fipronil non-treated bees (fig. 3.1.4 a, b).

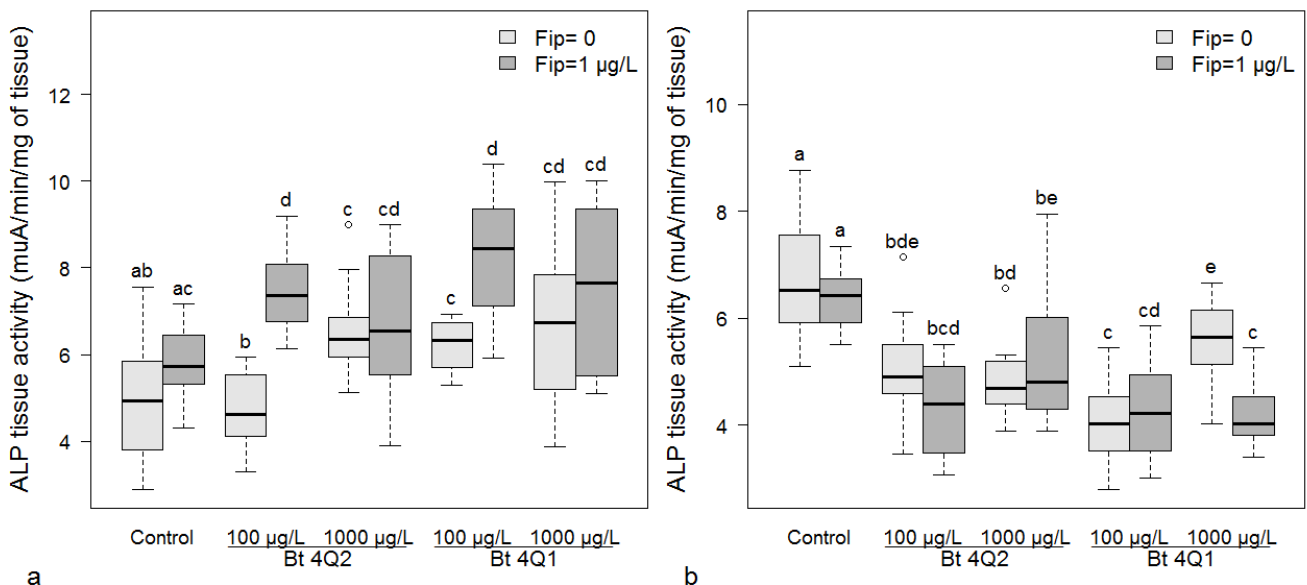


Figure 3.1.4. Effect of *Bt* 4Q2 and 4D1 strains joint or disjoint with Fipronil on ALP activity. Alkaline phosphatase (ALP) activity was assessed in midguts at day 10 (a) and day 20 (b). Each treatment group is constituted by 4 repetitions performed in triplicate ($n = 12$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

GAPDH activity was measured in the abdomens voided of the gut and the honey sac, at days 10 and 20. ANOVA analysis performed on all data showed an effect of fipronil in increasing enzyme activity on both sampling dates ($p = 0.007$ and $p < 1 \times 10^{-7}$, respectively); Mann-Whitney U test confirmed this result indicating a significant difference between fipronil treated and non treated bees for four treatment groups at day 10 and six treatment groups at day 20. However a tendency

can be identified in all the groups. No differences were found in function of *Bt* treatment or dose (fig. 3.1.5 a, b).

G6PDH activity was measured in the abdomens voided of the gut and honey sac. ANOVA analysis indicated no clear tendency of enzyme activity in function of *Bt* treatment. The 1µg/L treatment with fipronil determined a higher activity at day 10 in two treatment groups, *Bt* 4Q2 (1000 µg/L) and *Bt* 4D1 (100 µg/L), while at day 20 this effect can be found in the control, 4Q2 (1000 µg/L) and 4D1 (1000 µg/L). At day 20 fipronil inducing effect is confirmed also by ANOVA analysis performed on all data (p=0,003) (fig. 3.1.6 a, b).

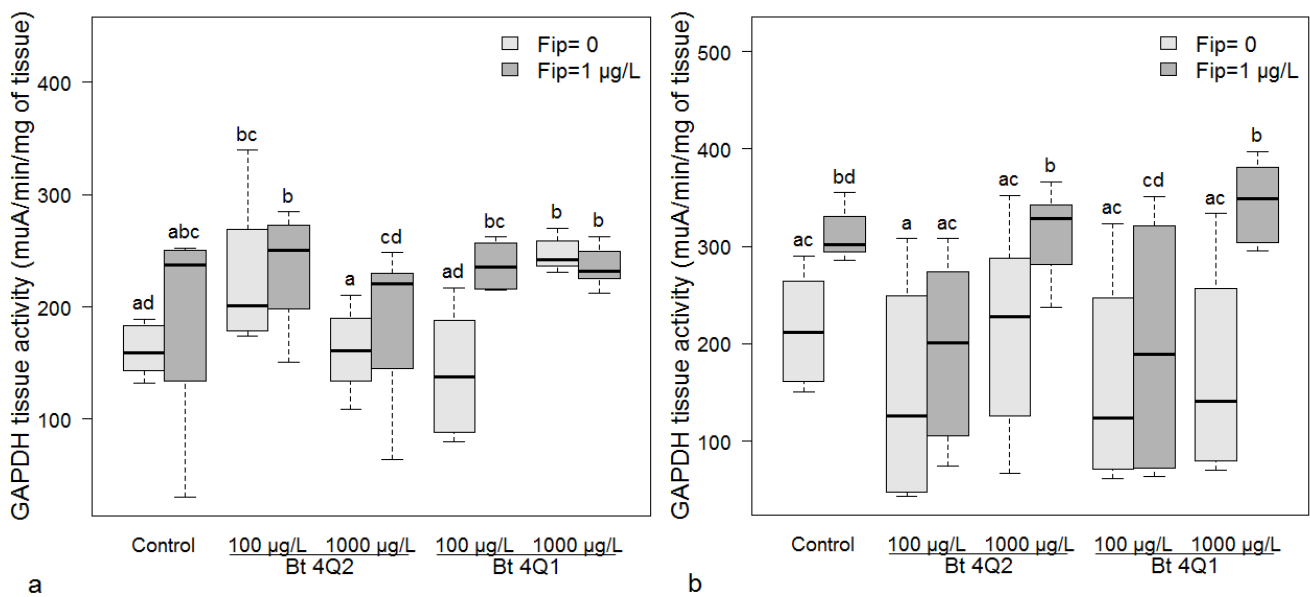


Figure 3.1.5. Effect of *Bt* 4Q2 and 4D1 strains joint or disjoint with Fipronil on GAPDH activity. GAPDH activity was assessed in abdomens at day 10 (a) and day 20 (b). Each treatment group is constituted by 4 repetitions performed in triplicate (n=12), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups (p<0.05). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

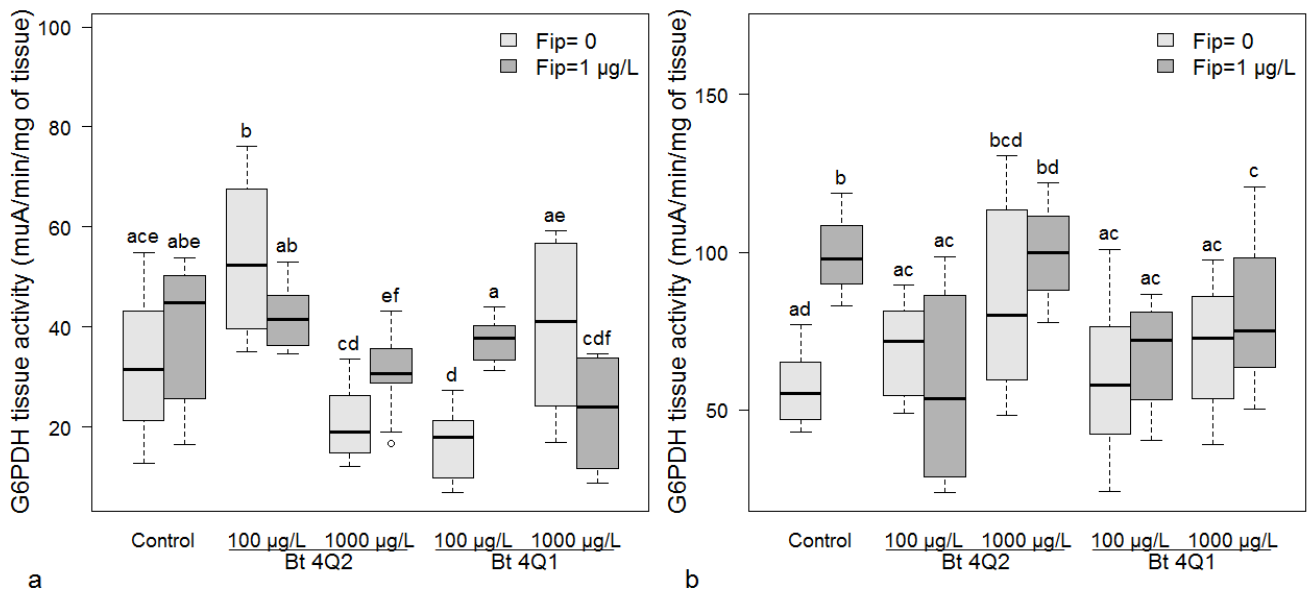


Figure 3.1.6. Effect of *Bt* 4Q2 and 4D1 strains joint or disjoint with Fipronil on G6PDH activity. Enzymatic activity was assessed in abdomens at day 10 (a) and day 20 (b). Each treatment group is constituted by 4 repetitions performed in triplicate (n=12), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups (p<0.05). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

3.1.4 Discussion and conclusions

Effect of three Cry toxins on adult honey bee mortality

As side-effects on pollinators represent a general concern about GM plants, many studies aimed to assess the potential effect of purified Cry toxins on honey bees. Here, we demonstrate that Cry1Ab, Cry1C and Cry3Aa purified toxins, may have a detrimental effect on adult honey bees when ingested, even at very low doses that can be found in the environment. To date, there is no study reporting an effect of a purified Cry toxin nor a *Bt* pollen-based diet on mortality of adult honey bees in laboratory conditions. A chronic administration (7 to 21 days) of various purified Cry toxins did not affect honey bee survival for Cry1Ba (Malone *et al.*, 2001), Cry1Ac (Han Peng *et al.*, 2010b), Cry1Ab (Ramirez-Romero *et al.*, 2008) and Cry1Ah (Dai *et al.*, 2012). Though it has been proposed that experiments using *Bt* pollen instead of contaminated syrup might be more conservative as a higher toxin availability is likely in syrup (Ramirez-Romero *et al.*, 2008), we showed significant effect on mortality at 10 days for doses lower than residues found in plant tissues and pollen (Liu *et al.*, 2009).

The experiments were performed with adult bees instead of emergent bees with the aim of considering a more veritable scenario with an heterogeneous age-structured population that could be found in the hive. As the average lifespan for a forager bee is estimated to be 7 days (Visscher and

Dukas, 1997), and the percentage of foraging bees in the experiment was unknown, we can consider that a certain percentage of individuals died for natural causes in such kind of experience. Nevertheless, we point out that all treatments were significantly different from control, thus showing a clear effect of toxin contamination.

Effect of a *Bt kurstaki* strain joint or disjoint with fipronil on adult honey bee mortality

As *Bt* formulation products represent an important way of pest control, to which honey bee could be exposed, we aimed to investigate the effects of a widely used *Bt* strain, *Bt var kurstaki* (4D1) on honey bee mortality. Non pathogenic bacteria and also bacterial coat component, when ingested, are reported to have an effect on honey bee physiology, in particular on immunity (Evans and Lopez, 2004). Therefore, a *Bt* strain (4Q2) deprived of the toxin encoding gene was used as a reliable negative control in order to account for eventual effects attributable to the bacterial spore components.

As opposite to the effect caused by purified toxins, we showed that *Bt* 4D1 spores at both 100 µg/L and 1000 µg/L dose have no effect on adult honey bee mortality. This result is in agreement with those obtained when treating bees with two *Bt* commercial formulation containing the same *kurstaki* strain (Malone *et al.*, 1999); similarly, Mommaerts (2010) concluded that no effect on mortality of bumblebees could be attributed to an oral *Bt kurstaki* treatment.

Consequently, with these results, we chose to test the effects of the same products when adopting a longer post-treatment monitoring, with the purpose of assessing honey bee survival. *Bt* 4D1 strain was confirmed to pose no hazards to honey bees on 25 days, therefore showing that it doesn't exhibit a delayed effect on mortality.

Moreover, we assessed the effect of a joint treatment with 1 µg/L Fipronil; several studies pointed out the enhanced toxicity of *Bacillus thuringiensis* when combined with chemical insecticides with respect to lepidopteran and coleopteran pests (Sudhakar and Dhingra, 2002; Singh *et al.*, 2007; Morales-Rodriguez and Peck, 2009). Thus, we tested the hypothesis of synergism between *Bt* and Fipronil insecticide. Assuming that the feeding behaviour of honey bees during the experiment was not influenced by treatments, we consider that they consumed an average of 0.01 ng Fipronil/bee/day (± 0.003), thus corresponding to approximately 1/470 of the oral LD50 (Decourtye *et al.*, 2005). In accordance with Vidau (2011) and Aufauvre (2012) this dose is confirmed to provoke no significant mortality when administered chronically; moreover the joint treatment did not enhance *Bt* toxicity.

Subsequently, we can confirm that *Bt kurstaki*, at doses consistent to recommended usage concentration and environmental residue levels, doesn't impair the survival of honey bees, and the

combination with a chemical insecticide as Fipronil doesn't show a synergistic nor an additive action.

Effects of a *Bt kurstaki* strain joint or disjoint with Fipronil on enzymatic biomarkers.

GST is a family of enzymes that plays a major role in detoxification of xenobiotics, also in invertebrates, (Siegfried and Young, 1993), conjugating them with reduced glutathione (GSH) (Baars and Breimer, 1980). Several studies highlighted other mechanisms of cell-defence operated by GST, focussing on its anti-oxidant role in removal of hydrogen peroxide and inhibition of lipid peroxidation (Felton and Summers, 1995; Barbehenn, 2002). For those reasons, GST has been widely employed as a valuable tool to study pesticide environmental contamination.

Here, we assessed GST activity both in heads and in midguts, the latter compartment being the most relevant for GST expression in honey bees (Diao *et al.*, 2006). We showed that *Bt* ingestion is linked to a strong decrease in GST activity, whereas no clear effect of the Fipronil treatment can be highlighted (fig. 4).

GST activity has been positively correlated to evolution of resistance towards chemical pesticides (Ottea and Plapp, 1984; Wei *et al.*, 2001; Enayati *et al.*, 2005; Boyer *et al.*, 2012) and more recently, to *Bacillus thuringiensis* modified crops (Guo *et al.*, 2012).

Conversely, the regulation of GST activity as consequence of a pesticide exposure in non-resistant populations is still discussed and not completely clarified. An increase in GST activity has often been related to insecticide exposure as consequence of an induction of the detoxification response (Baars and Breimer, 1980) or an improvement of anti-oxidant defences (Dubovskiy *et al.*, 2008; Printes *et al.*, 2011). Nonetheless, GST activity has also been demonstrated to be suppressed following a pesticide contamination (Baturu and Lagadic, 1996; Damasio *et al.*, 2010; Carvalho *et al.*, 2012). This evidence can be related to the fact that, subsequent to chemical contamination, GSH can undergo spontaneous oxidation, as it has been seen with paraquat in *Daphnia magna* (Barata *et al.*, 2005); consequently, a low substrate concentration results in a decrease in GST activity (James *et al.*, 2012).

Hence, we suggest that a *Bt* prolonged exposure may influence the GSH/GSSG balance leading to a reduced GST activity; however, a GSH assessment would be required to confirm this assumption.

Table 3.1.2. Activity of GST, ALP, G6PDH and GAPDH as function of Fipronil treatment, *Bt* treatment, *Bt* treatment doses and *Bt* strains. Statistical analysis were performed with ANOVA; differences were considered significant when p value was inferior than 0.05. Significance is indicated with ‘***’ when $p < 0.001$, ‘**’ when $p < 0.01$ and ‘*’ when $p < 0.05$.

	GST head		GST midgut		ALP midgut	
	day 10	day 20	day 10	day 20	day 10	day 20
Fipronil	0.021*	0.0001***	0.260	0.472	$<1 \times 10^{-7}$ ***	0.173
<i>Bt</i>	$<1 \times 10^{-16}$ ***	$<1 \times 10^{-16}$ ***	0.005**	$<1 \times 10^{-16}$ ***	$<1 \times 10^{-5}$ ***	$<1 \times 10^{-11}$ ***
Fipronil* <i>Bt</i>	0.027*	0.345	0.07	0.027*	0.676	0.573
4Q2 vs 4D1	$<1 \times 10^{-16}$ ***	$<1 \times 10^{-15}$ ***	0.029*	$<1 \times 10^{-16}$ ***	0.008**	0.096

	G6PDH abd.		GAPDH abd.	
	day 10	day 20	day 10	day 20
Fipronil	0.524	0.004**	0.007**	$<1 \times 10^{-6}$ ***
<i>Bt</i>	0.035*	0.165	0.063	0.126
Fipronil* <i>Bt</i>	0.653	0.002**	0.594	0.668
4Q2 vs 4D1	0.014*	0.011	0.265	0.955

ALP is a digestive enzyme involved in adsorption of molecules through the intestinal epithelium via their phosphorylation (Aufauvre *et al.*, 2012); this function has been proven in insects (Vlahovic *et al.*, 2009) and more recently a major role in mediating the toxicity of *Bacillus thuringiensis* has been described (Upadhyay and Singh, 2011).

Here, we find no influence of *Bt* treatment on ALP intestinal activity, but, interestingly, ALP activity was enhanced in *Bt* treated bees at day 10, whereas the opposite trend can be found at day 20. In *Helicoverpa armigera* larvae a major affinity of ALP to *Bt* CryAc toxin in the earlier stages and a lower involvement in the late larval development has been recently demonstrated (Upadhyay and Singh, 2011). Though we have no evident symptoms that demonstrate a *Bt* toxicity, this time-dependent enzyme response might us suggest a different involvement of ALP as a Cry receptor through time. Nevertheless, contrasting results between sampling dates preclude a clear interpretation of the relationship between *Bt* treatment and ALP levels in midguts.

Conversely, at the end of the treatment period, ALP activity resulted significantly higher in fipronil-treated bees, confirming the results found for a thiamethoxam sublethal intoxication (Badiou-Bénéteau *et al.*, 2012) and thus confirming ALP as a valuable marker for these two pesticides in bees.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Glucose-6-phosphate dehydrogenase (G6PDH) are two key-enzymes of the carbohydrate metabolism, the first mainly involved in the glycolysis pathway and, ultimately, in ATP production, the second catalyzing NAPDH formation via the pentose phosphate shunt.

Though it had been described as an “housekeeping” gene (Barber *et al.*, 2005), thus expressing quite constant protein levels, several studies highlighted that variations in GAPDH concentration play a role in transcriptional gene regulation, apoptosis induction (Sirover, 2005; Ortiz-Ortiz *et al.*, 2010) and response to oxidative stress conditions (Nicholls *et al.*, 2012). In particular, GAPDH could be reversibly inhibited by reactive oxygen species (ROS) and therefore redirecting the glucose flux towards the pentose phosphate pathway (Chuang *et al.*, 2005).

In this study, the *Bt* treatment did not influence the abdomen levels of GAPDH, whereas the Fipronil administration was linked to a higher enzymatic activity in both the sampling dates, thus suggesting an enhanced glucose metabolism and energy production.

As seen for GAPDH, G6PDH is strictly involved in oxidative stress remediation, as well (Grant, 2008); in fact, the energetic switch to the production of NADPH, due to the glycolysis inhibition, provides a reducing potential to avoid further molecules oxidation (Kletzien *et al.*, 1994; Verma *et al.*, 2007). Consistently to these evidences, the global G6PDH activity, in our results, augmented in response to fipronil at day 20; however the same general effect cannot be confirmed for day 10 sampling, even though four treatment groups of ten exhibited the same tendency.

Interestingly, GAPDH and G6PDH activities were both increased as consequence to fipronil ingestion, hence highlighting a different metabolic explication, that may involve a response to oxidative stress with regards to G6PDH, while might be correlated to other cellular modifications induced by Fipronil, with respect to GAPDH increased levels. We thus point out the interest of developing and improving the GAPDH/G6PDH biomarker tool for other pesticides in honey bees.

We conclude that Cry1Ab, Cry1C and Cry3Aa toxins express a chronic toxicity on adult honey bees, while no adverse effects can be attributed to sporal *Bt* formulation both with adult bees and emergent bees, even if in combination with a sublethal concentration of Fipronil. Different enzymatic biomarkers were improved, and in some cases, as in GST, our study permit to validate the use of that kind of bioindication method in honey bees; nevertheless a supplementary research involving semi-field and field experimentation would allow to confirm these results.

We consider these results relevant to evaluation of GM crops side effects even if a semi field and a field confirmation of such a phenomenon are needed. We also conclude that, to our experience, no relevant risk can be associated with *Bt kurstaki* treatments, confirming that the use of such products can be considered as safe.

3.2 Honey bees combined exposure to *Bt* spores and deltamethrin: toxicity and physiological changes

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In preparation

3.2.1 Introduction

Honey bees are exposed to a wide variety of environmental contaminants, mainly pesticides, due to their intense foraging activity and the large amount of potentially contaminated nectar and pollen that they collect and store in the hive. The simultaneous presence of several active ingredients in the stored pollen and bee bread has been well-documented (Chauzat *et al.*, 2006; Mullin *et al.*, 2010). Similarly, the exposure to multiple pesticide in the field is likely to occur when bees go foraging an area interested by successive treatments or when the field treatments are performed using a mixture of different active ingredients, both chemical and biological.

Even though a multiple pesticide exposure seems to be the most representative and realistic scenario of honey bees exposure in the field, little is known about the effect of multiple pesticides exposure. At present, a few studies focussed on binary mixture of pesticides. The combination of azoles fungicides and pyrethroid insecticides, via direct contact, has been proven to exhibit synergistic characteristics, both on mortality and olfactory learning performances (Vandame *et al.*, 1995; Vandame and Belzunces, 1998). Neonicotinoids insecticides and in particular those belonging to cyano-substituted group (i.e. thiacloprid and acetamiprid) have been also described as potentially synergic with azoles fungicides, since they share the same detoxification metabolic path as pyrethroids (Iwasa *et al.*, 2004).

Another important feature in combined pesticide toxicity is the sensitivity to the administered product. It is accepted that pesticide sensitivity in honey bees depends on many factors as season, (Meled *et al.*, 1998; Decourtye *et al.*, 2003) age (Guez *et al.*, 2001), brood rearing temperature (Medrzycki *et al.*, 2010), and presence of other stressors as pathogens (Alaux *et al.*, 2010; Aufauvre *et al.*, 2012), but few studies investigated the pesticide exposure as a sensitization factor.

Here, we consider the combined exposure of honey bees to a biological insecticide, *Bacillus thuringiensis*, and a chemical product, deltamethrin, when administered successively, trough time. In order to test this hypothesis on honey bees, we combined also two way of exposure, administering *B.thuringiensis* via ingestion, followed by an acute treatment with deltamethrin via direct contact. We considered, in fact, that in a field exposure scenario, honey bees might be exposed to *Bt* while foraging via nectar and pollen, followed by a direct exposure to an aerial spray insecticidal treatment with deltamethrin.

Bacillus thuringiensis is a gram-positive soil bacterium expressing insecticidal properties, as its spores contain toxic crystal proteins, named Cry toxins. The inactivated form of the toxin becomes active in insect gut where, following a receptor-mediated anchorage to the membrane of gut cells, it causes cell lysis and ultimately leads to the insect death. (Gill *et al.*, 1992; Bravo *et al.*, 2007;

Vachon *et al.*, 2012). Sporal formulation of *B.thuringiensis* var *kurstaki* are widely employed in order to control pests, especially Lepidopteran, on a vast number of crops.

Cry toxins genes are also employed for genetic transformation, in order to confer a long lasting insect resistance in all plant tissues. This technique is now diffused especially in maize, cotton, soybean and oil seed rape crops. The presence of Cry toxins in pollen has been proven for different toxins and in several cultures as maize and cotton (Fearing *et al.*, 1997; Han Peng *et al.*, 2010a), representing an exposure risk to beneficial arthropods and pollinators (Malone and Burgess, 2009).

At present, both *Bt* toxins and spores haven't been found to cause significant mortality neither to adult honey bees nor to larvae (Liu *et al.*, 2009; Han Peng *et al.*, 2010a; Dai *et al.*, 2012; Hendriksma *et al.*, 2012). Their influence on sublethal effect has been studied, as well: no effects of a *Bt* toxin, Cry1Ba, has been seen on morphological development (Malone *et al.*, 2004), whereas feeding behaviour and learning performances have been perturbed by a chronic Cry1Ab treatment (Ramirez-Romero *et al.*, 2008). Here, we investigated the effect of a sub chronic ingestion treatment of a *Bt kurstaki* strain expressing Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B toxins (hereafter referred to as *Bt* 4D1). As a reference, we also tested a modified strain not expressing any Cry toxin (hereafter referred to as *Bt* 4Q2).

Deltamethrin is a synthetic type II pyrethroid insecticide that express a high toxicity to insects, causing hyper excitation of the nervous system by impairing sodium channel action (Soderlund and Bloomquist, 1989). Its generalist mode of action determines a lack of selectivity towards other invertebrates as beneficial arthropods and pollinators, so exposing also honey bees to a relevant risk. Acute and chronic toxicity tests have proven the detrimental effect of deltamethrin on survival and learning performances of adult honey bees (Faucon *et al.*, 1985; Vandame *et al.*, 1995; Meled *et al.*, 1998; Pham-Delegue *et al.*, 2002).

In this study, we considered as relevant toxicity endpoints other than mortality rate, the response to treatments of enzymatic markers as glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), alkaline phosphatase (ALP), glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) tissue activity.

The objective of the present study was therefore (i) to study the combined toxicity to honey bees of *Bt* spores ingestion for 5 days followed by an acute contact treatment with deltamethrin field dose rate (ii) to evaluate the sensitization effect of *Bt* towards deltamethrin (iii) to study the physiological variations caused to both treatments, by evaluating the enzymatic activity of six stress marker enzymes.

3.2.2 Materials and methods

Honey bees

In order to obtain emergent bees, two brood frames were collected from a queen right *Apis mellifera* colony previously controlled for its health status. We used bees from the same colony in order to minimize the colony effect that was highlighted in other experimental studies. The brood frames were kept in an incubator at controlled temperature and humidity ($34^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $60\% \pm 10\%$ relative humidity, darkness) and emergent bees were collected after one day incubation. Therefore, at the beginning of the trial, bees were emerged since a minimum of one hour up to one day. Honey bees were then grouped by 30 in plastic rearing cages (6 x 8,5 x 10 cm), adapted from Pain type, with a source of queen pheromone blend (one third of commercial Beeboost stick) and provided with multifloral pollen, candy (Commercial Apifonda; honey and sugar) and water; cages were kept at controlled temperature and humidity ($34^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $60\% \pm 10\%$ relative humidity, darkness) for all the duration of the trial. After one day of adaptation to rearing conditions, dead bees were removed and the sub-chronic treatment was administered. Six experimental cages were used for each treatment group.

Experimental procedure

Bt spores dilutions were prepared in distilled water: stock solutions at 1 mg/L and 10 mg/L were prepared and conserved at -20°C . In order to obtain the treatment final concentrations (100 and 1000 $\mu\text{g/L}$), working solutions were freshly made by dilutions in sugar syrup (50% w/v sucrose) and renewed daily. *Bt* solutions were administered to bees 10 hours per day. During the treatment, the candy feeder and the water tube was removed, and then replaced each day at the end of the treatment. Syrup consumption was assessed by weighting each day the feeders before and after the treatment.

After 5 days of exposure to *Bt* spores, an acute contact treatment with deltamethrin was performed through a pulverizing tower modified from Potter type. A commercial deltamethrin liquid formulation (Decis Protech®) was used for the contact acute treatment through a pulveriser modified from Potter tower type; the concentration of a.i. deltamethrin was established in the field recommended dosage (7,5 g/ha) (fig 3.2.1). The concentration of a.i. in the working solution was calculated considering the surface of the disc used for the contact contamination and the volume of employed solution fallen down on the disc after the pulverisation. Final concentration solutions were prepared in distilled water the day before the intoxication and conserved in dark-glass bottles. Honey bees were anaesthetized with a slight CO_2 flux and then placed on the disc under the Potter

tower. After the acute treatment, bees were reintroduced in the rearing cages and in the incubator. Mortality was registered at 2, 4, 6, 24, 48 and 72 hours after treatment.

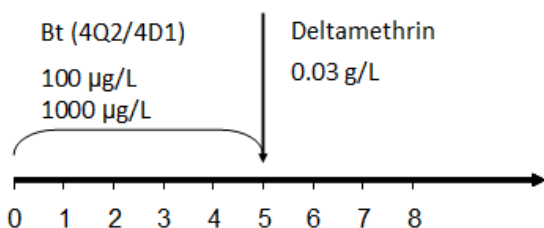


Figure 3.2.1. Scheme of the experimentation, tested products and doses.

Enzymatic assays

In order to perform enzymatic biomarker assays, alive honey bees were sampled at the end of the *Bt* treatment (day 5) and 72 hours after the acute treatment (day 8). Bees were dissected and heads, midguts and abdomens voided of the midgut and the honey sac, were conserved at -80°C . Four repetitions were performed for each treatment group and three honey bees were sacrificed for each repetition. The tissue extracts were obtained by homogenizing (TissuLyserTM; Qiagen; 5610 s at 30 MHz) three heads (or midguts or voided abdomens) in the extraction buffer (40 mM L-S phosphate buffer at pH 7.4; 10 mM NaCl; 1% Triton; protease inhibitors). The homogenates were centrifuged at 15,000 g for 20 minutes at 4°C and the resulting supernatants kept in ice-cooled tubes. The extracts were employed for enzymatic assays, performing three replicates for each repetition.

Glutathione-S-Transferase (GST) activity was spectrophotometrically assessed measuring the conjugation of reduced glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) using a method adapted from Habig et al. (1974). GST activity was measured in heads and midguts by adding 10 μl of enzymatic extract to the reaction mixture containing 1 mM ethylenediaminetetracetic acid (EDTA), 2.5 mM GSH, 1 mM CDNB and 100 mM Na/K-phosphate buffer at pH 7.4. GST activity was quantified by recording the appearance of conjugated product at 340 nm during 5 min.

Superoxide dismutase (SOD) activity was indirectly assessed by measuring at 560 nm the reduction of nitrobleu tetrazolium (*NBT*) by O_2^- generated by the xanthine/xanthine oxydase reaction. SOD activity is negative correlated to *NBT* reduction, competing for the same substrate (O_2^-). The assay was performed adding 10 μl of head or midgut enzymatic extract to 190 μl of reaction mixture containing 50 mM sodium carbonate/ disodium phosphate buffer at pH 7.8, 0.1 mM EDTA, 0.1 Xanthine, 0.025 mM *NBT*, 0.083 U/mL Xanthine oxydase.

Catalase (CAT) was assessed by measuring decrease of absorbance due to H₂O₂ extinction, at 240 nm for 10 minutes. 190 µl of 30 mM H₂O₂ and 100 mM NaH₂PO₄ buffer at pH 7.30 were added to 10 µl tissue extract (Beers and Sizer, 1952).

Alkaline phosphatase (ALP) activity was assayed in a reaction medium containing 10 µl of enzymatic midgut extract, 20 mM MgCl₂, 2 mM p-nitrophenyl phosphate and 100 mM Tris-HCl buffer at pH 8.5. The enzymatic activity was measured by monitoring at 410 nm for 5 minutes through spectrophotometry the transformation of p-nitrophenyl phosphate to p-nitrophenol (Bounias et al., 1996).

Glucose-6-phosphate dehydrogenase (G6PDH) activity was assessed by measuring the transformation of glucose-6-phosphate to 6-phospho-gluconate through the reduction of β-Nicotinamide Adenine Dinucleotide Phosphate Hydrate (β-NADP). A medium containing 100 mM Trizma base buffer at pH 7.4, 1 mM D-Glucose 6-phosphate disodium (G6P Na₂), 0.5 mM β-NADP, 10 mM MgCl₂ was monitored for 5 minutes at 340 nm.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was assessed measuring at 340 nm the formation of glyceraldehyde-3-phosphate from glycerate-1,3-diphosphate, the latter formed by the conjugation of 3-phosphoglyceric acid (3-PGA) and 3-phosphoglyceric phosphokinase (3-PGK). The reaction medium was constituted by 80 mM triethanolamine buffer at pH 7.6, 7 mM 3-PGA, 4 mM L-Cysteine HCL neutralized with sodium bicarbonate, 2 mM magnesium sulfate (MgSO₄), 120 µM reduced β-Nicotinamide Adenine dinucleotide (β-NADH), 1.2 mM ATP, 1 mM EDTA, 5 U 3-PGK.

Statistical analysis

Mortality data were log-transformed and compared using a general linear model and an analysis of variance (ANOVA). Enzymatic assay data were analyzed through a two-way ANOVA to define general tendencies and with Mann-Whitney U test in order to obtain a pair wise comparison between treatments. All comparisons described by p values inferior to 0.05 were considered as significantly different. All analysis was performed with R software (version 2.14.1).

3.2.3 Results

Mortality and feeding behaviour

The mortality registered during the sub-chronic exposure to *Bt* strains 4Q2 and 4D1 was not different from control; indeed all the treatment groups remained under 5% of mortality at day 5. After the acute contact exposure, a significant higher mortality than control was seen for all deltamethrin treated groups ($p < 1 \times 10^{-16}$) without differences between them (fig. 3.2.2 a).

The feeding behaviour was evaluated for the oral treatment with *Bt* spores, in the first 5 days of the trial. The syrup consumption was estimated as mean daily data adjusted with mortality. Honey bees consumed more syrup on the first day ($p < 0.01$) and the 4D1 treatment at the lowest dose (100 $\mu\text{g/L}$) was related to a higher consumption. In particular, on day 3 honey bees belonging to this treatment group consumed more syrup than the other ones (fig. 3.2.2 b).

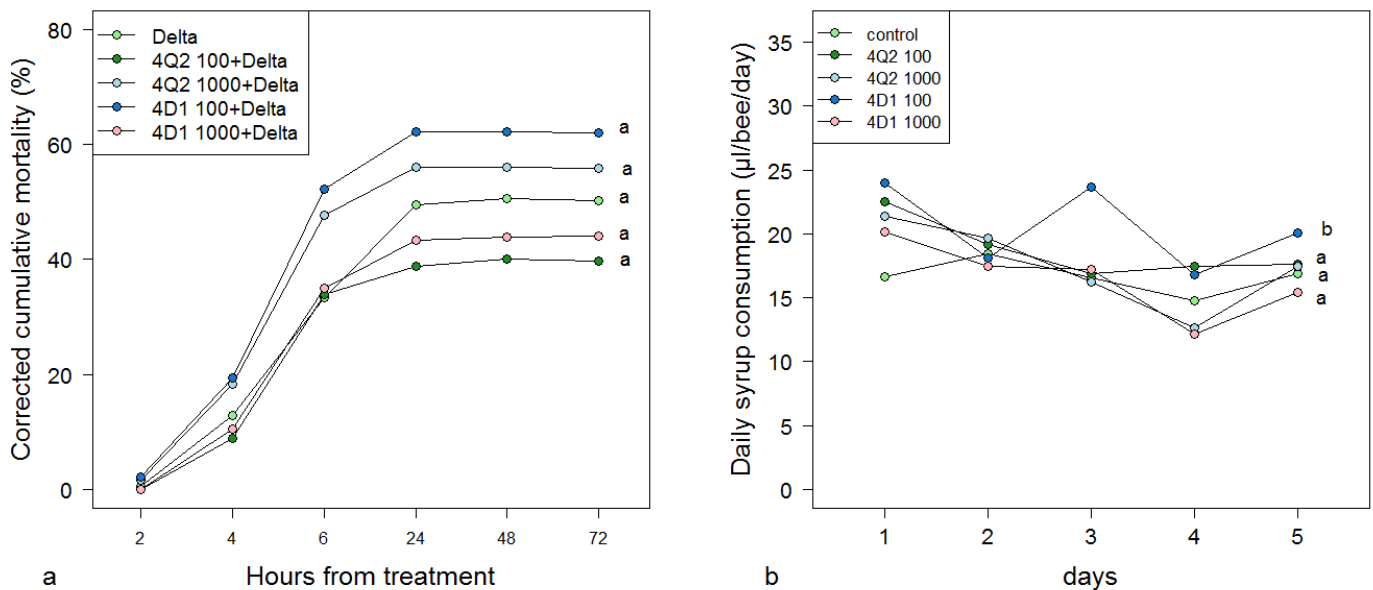


Figure 3.2.2. Effect of *Bt* spores combined with deltamethrin on mortality and syrup consumption. Data represents percent corrected cumulative mortality after the combined exposure to different doses of *Bt* spores from two *Bt* strains and deltamethrin (a) and mean daily syrup consumption ($\mu\text{l}/\text{bee}/\text{day}$) in dependence of *Bt* spores treatments (b). The deltamethrin untreated group had a mortality inferior to 5% and similar to control. Different letters indicate that groups are significantly different from control.

Enzymatic activity

GST activity

GST activity was measured in the heads and in the midguts. At day 5, a slight decreasing effect related to *Bt* exposure was evidenced in heads ($p = 0.006$) and midguts ($p = 0.05$), for both strains. At day 8, ANOVA performed on overall data showed that control bees had a significantly higher GST activity than *Bt* treated bees, both in heads ($p = 0.03$) and in midguts (0.0001). While in the heads both strains contributed to the decreasing effect, in the midguts, the lower GST level is mainly explicated by 4Q2 strain. Conversely, deltamethrin had no effect on GST activity (fig. 3.2.3; tab. 3.2.1).

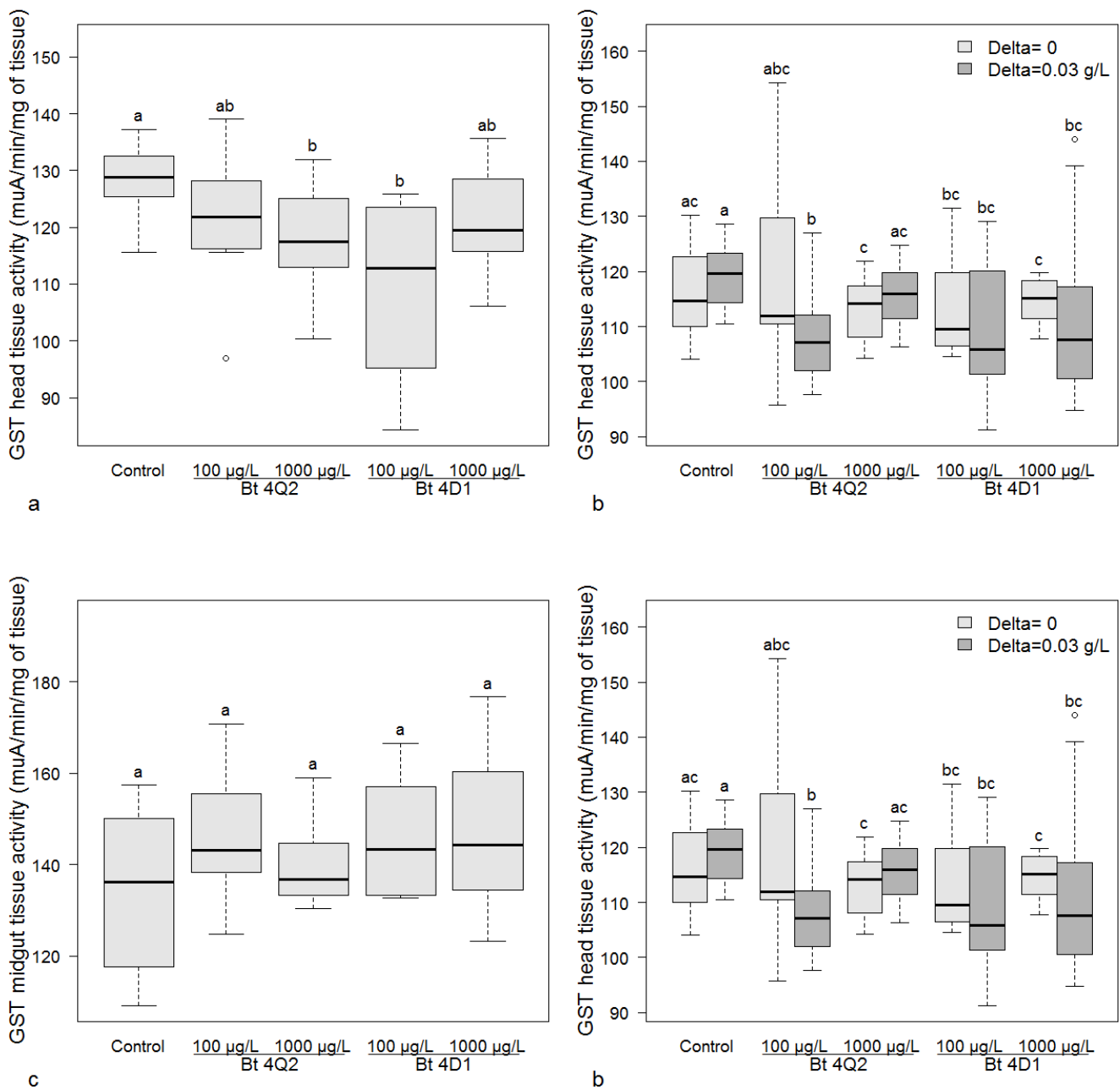


Figure 3.2.3. Effect of *Bt* combined or not with deltamethrin on GST activity. Glutathione-S-transferase (GST) activity was assessed in heads at day 5 (a) and day 8 (b) and in midguts (c, d) on the same sampling dates, respectively. Each treatment group is constituted by 4 repetitions performed in triplicate (n=12), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups (p<0.05). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

SOD activity

SOD activity was assessed in both heads and midguts. At day 5, control and treated bees expressed a similar SOD level in heads and in midguts (fig. 3.2.4 a, c). At day 8, ANOVA analysis highlighted a significant effect of *Bt* strains in increasing enzymatic activity, both in heads

($p=0.0001$), and midguts ($p<1\times 10^{-10}$); a between-strains comparison showed that 4D1 treatment was significantly higher than 4Q2, in both compartments (fig. 3.2.4 b, d). Though deltamethrin treated bees appeared no different from control ones, a significant interaction between treatments was evidenced by ANOVA analysis (tab. 3.2.1).

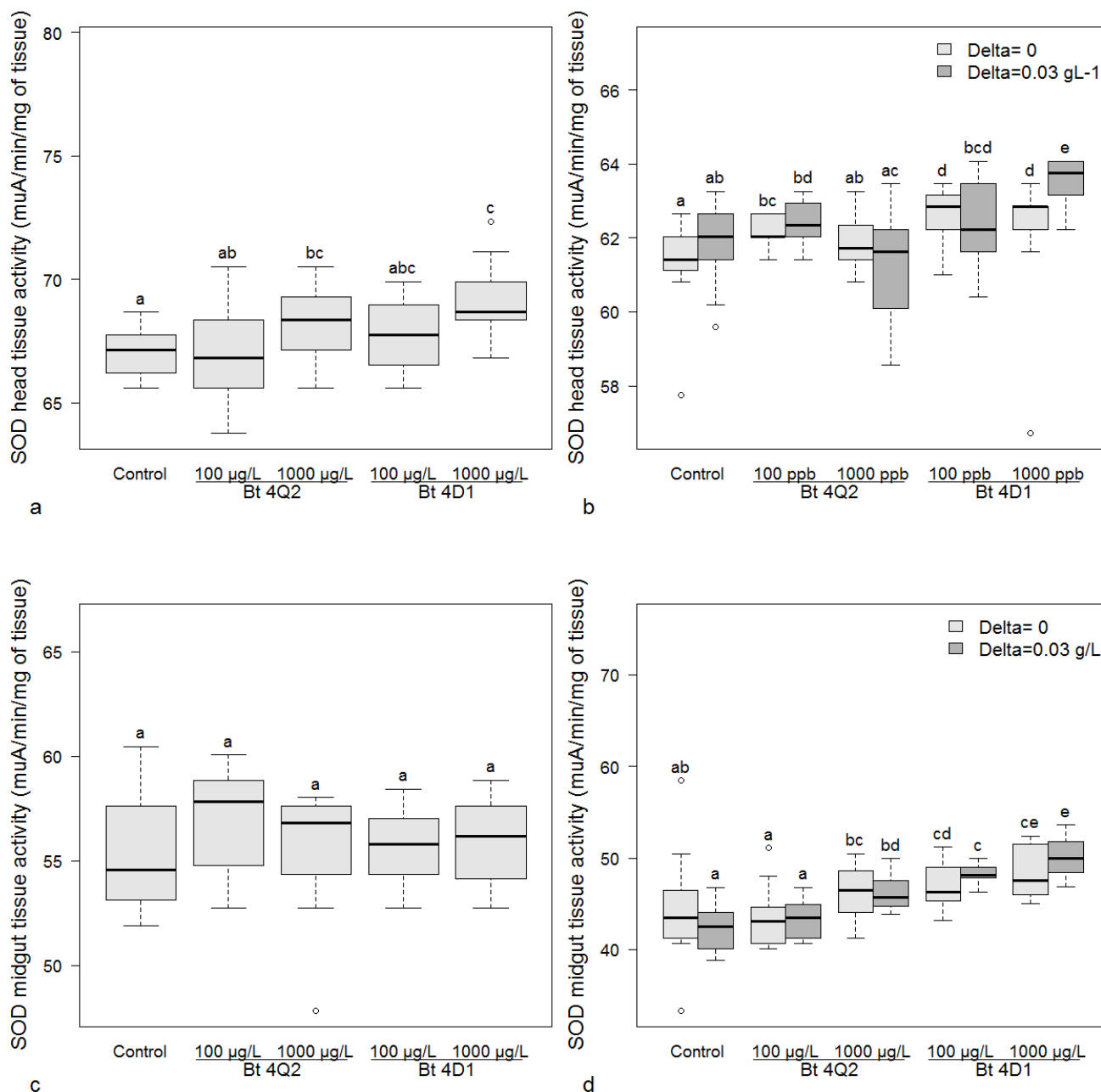


Figure 3.2.4. Effect of *Bt* combined or not with deltamethrin on SOD activity. Superoxide Dismutase (SOD) activity was assessed in heads at day 5 (a) and day 8 (b) and in midguts (c, d) on the same sampling dates, respectively. Each treatment group is constituted by 4 repetitions performed in triplicate ($n=12$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p<0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

CAT activity

The activity of catalase was measured in heads; *Bt* treatment resulted in a significant decrease of enzymatic activity at day 5 ($p= 0.004$), with 4D1 strain determining a lower activity than 4Q2 ($p=0.021$) (fig. 3.2.5 a). At day 8, the same reduction in enzyme activity was assessed ($p<1\times 10^{-6}$), with no differences between the strains (fig. 3.2.5 b; tab. 3.2.1). Since data showed no coherent variations of CAT activity as consequence of deltamethrin treatment, no clear effects of this product can be described.

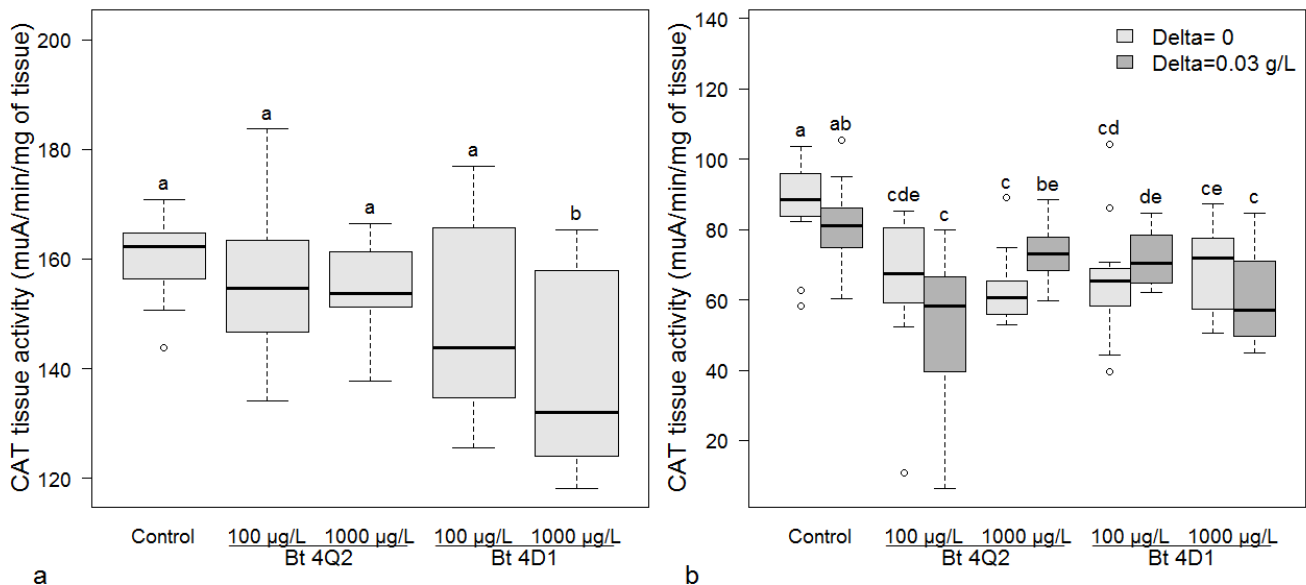


Figure 3.2.5. Effect of *Bt* combined or not with deltamethrin on CAT activity. Catalase (CAT) activity was assessed in heads at day 5 (a) and day 8 (b). Each treatment group is constituted by 4 repetitions performed in triplicate ($n=12$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p<0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

ALP activity

Alkaline phosphatase activity was measured in midguts. At day 5, ALP was found to be lower in *Bt* treated bees (0.004), in particular in 4D1 treatment group ($p=0.001$) (fig. 3.2.6 a). Three days later (day 8) a decrease of activity was recorded in *Bt* fed bees ($p<1\times 10^{-6}$); this effect cannot be related to a specific strain, as no significant difference was found between 4Q2 and 4D1 enzymatic levels. Moreover, ANOVA analysis performed on all data show an increasing effect of ALP activity due to deltamethrin exposure ($p=0.0002$; tab. 3.2.1), and the interaction between *Bt* and Deltamethrin treatment was significant ($p=0.025$) (fig. 3.2.6 b).

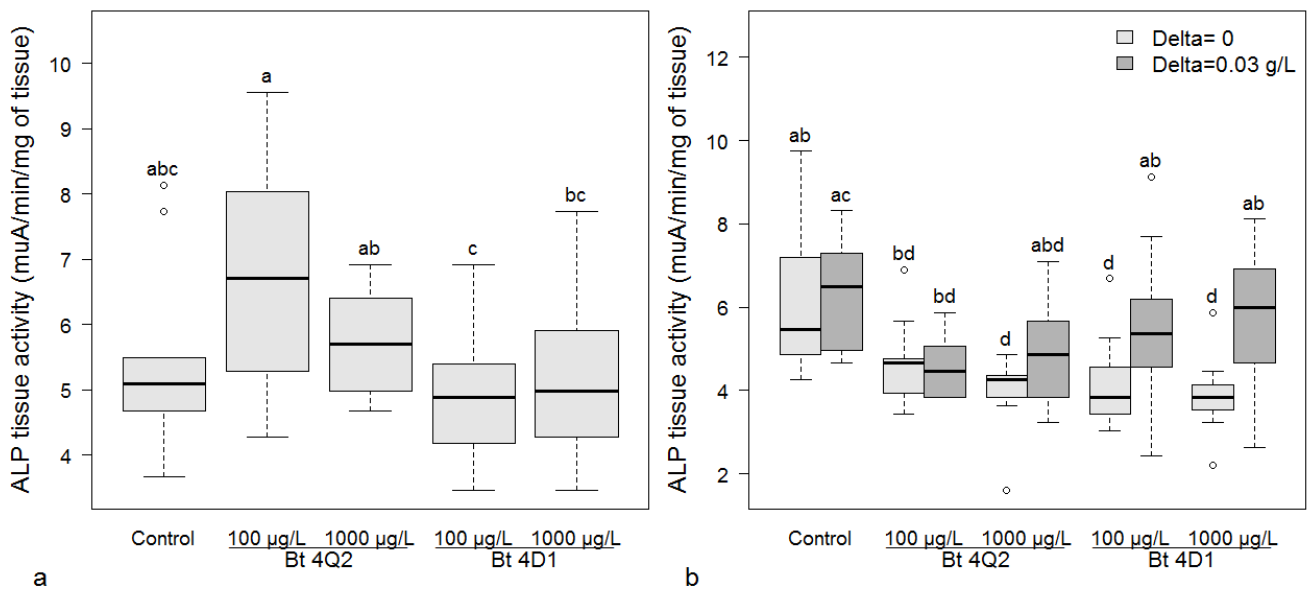


Figure 3.2.6. Effect of *Bt* combined or not with deltamethrin on ALP activity. Alkaline phosphatase (ALP) activity was assessed in midguts at day 5 (a) and day 8 (b). Each treatment group is constituted by 4 repetitions performed in triplicate (n=12), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups (p<0.05). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

GAPDH activity

The activity of this enzyme was measured in abdomens devoided of the gut and the honey sac. At the end of the subchronic treatment with *Bt* spores (day 5) a higher activity was found for the lowest dose of 4Q2 strain and the higher dose of 4D1 strain (Mann-Whitney U-test), even though no global effect can be attributed to *Bt*, as suggested by ANOVA performed on the overall dataset. Conversely, a significant difference between *Bt* treatment and control was showed at day 8 (p=0.020). A deltamethrin effect was highlighted, as well; in fact, enzymatic levels correspondent to deltamethrin treated bees, were lower than control (p=0.0005) and the combination between *Bt* and deltamethrin determined a stronger decreasing effect (p=0.004) (fig. 3.2.7 b; tab. 3.2.1).

G6PDH activity

G6PDH activity was measured in abdomens devoided of the gut and the honey sac. Comparable levels of this enzyme were found in control and treated bees at day 5 (fig. 3.2.8 a); Mann-Whitney U-test comparisons highlighted a significant lower level for the higher dose of 4D1 strain, but no overall effect of *Bt* can be confirmed by ANOVA analysis. At day 8, a decreasing effect of *Bt* spores ingestion was found, if compared with the control (p=0.0005) and 4D1 strain induced a stronger decrease in enzymatic activity than 4Q2 (p=0.048). Deltamethrin treated bees exhibited a lower G6PDH level than non treated ones (p<1×10⁻⁷) (fig. 3.2.8 b; tab. 3.2.1).

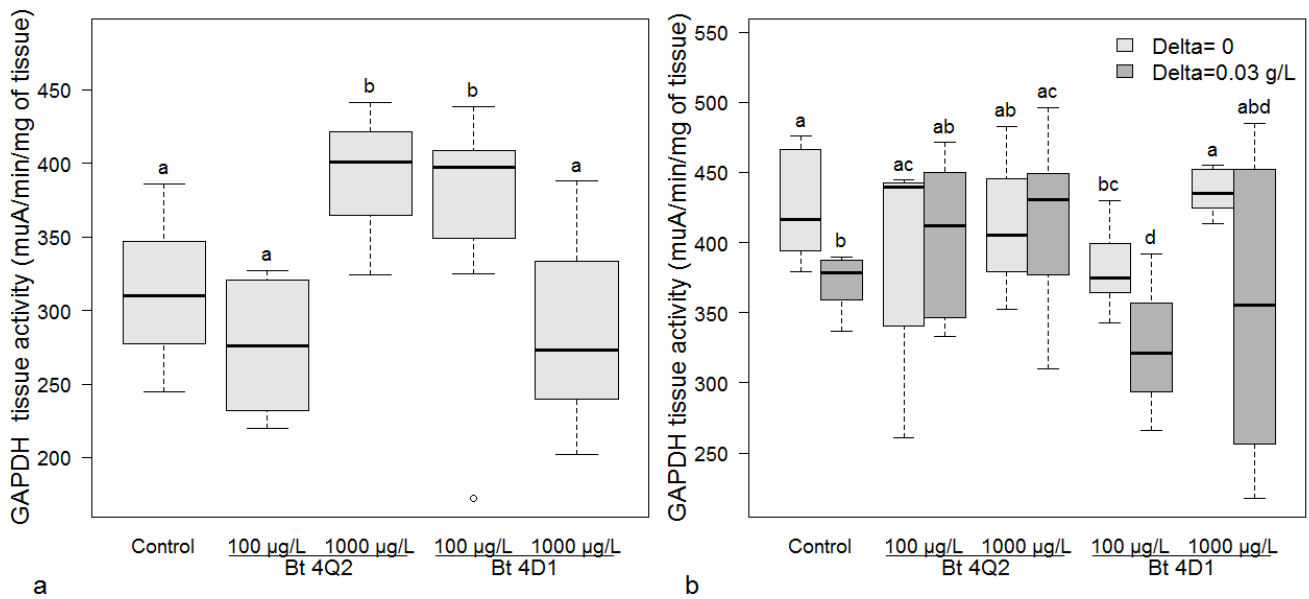


Figure 3.2.7. Effect of *Bt* combined or not with deltamethrin on GAPDH activity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was assessed in abdomens at day 5 (a) and day 8 (b). Each treatment group is constituted by 4 repetitions performed in triplicate ($n=12$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

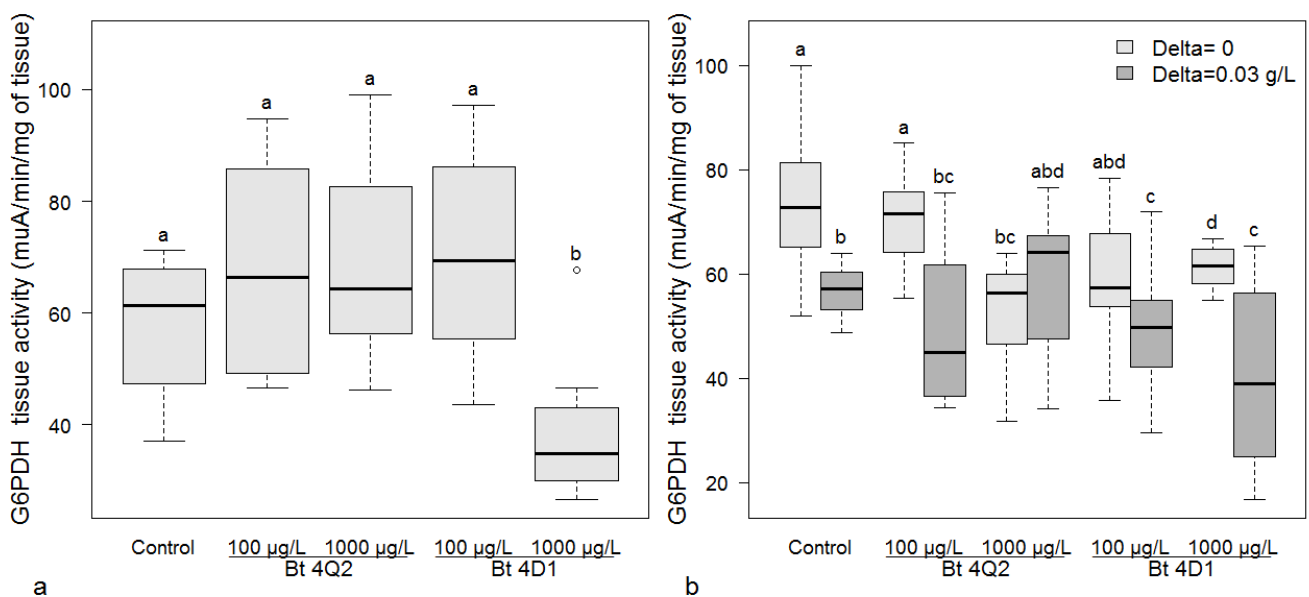


Figure 3.2.8. Effect of *Bt* combined or not with deltamethrin on G6PDH activity. Glucose-6-phosphate dehydrogenase (G6PDH) activity was assessed in abdomens at day 5 (a) and day 8 (b). Each treatment group is constituted by 4 repetitions performed in triplicate ($n=12$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the

measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

Table 3.2.1. AOV analysis of enzymatic activity data (p values). Activity of GST, CAT, SOD, ALP, GAPDH and G6PDH was analyzed as function of deltamethrin treatment, *Bt* treatment, *Bt* strains, and interaction between *Bt* and deltamethrin. Statistical analysis were performed with ANOVA. Differences were considered significant when p value was inferior than 0.05. Significance is indicated with ‘***’ when $p < 0.001$, ‘**’ when $p < 0.01$ and ‘*’ when $p < 0.05$.

	GST (head)		GST (midgut)		SOD (head)		SOD (midgut)	
	day 5	Day 8	day 5	day 8	day 5	day 8	day 5	day 8
Deltamet.		0.441		0.128		0.223		0.882
<i>Bt</i>	0.006 **	0.030 *	0.05 *	0.0001 ***	0.101	0.0001 ***	0.498	$< 1 \times 10^{-10}$ ***
Delta* <i>Bt</i>		0.409		0.184		0.246		0.121
<i>Bt</i> 4Q2/4D1	0.189	0.947	0.634	0.0001 ***	0.105	0.001**	0.498	$< 1 \times 10^{-9}$ ***
	CAT (head)		ALP (midgut)		GAPDH (abdomen)		G6PDH (abdomen)	
	day 5	Day 8	day 5	day 8	day 5	day 8	day 5	day 8
Deltamet.		0.483		0.0002 ***		0.0005***		$< 1 \times 10^{-7}$ ***
<i>Bt</i>	0.004 **	$< 1 \times 10^{-6}$ ***	0.004 **	$< 1 \times 10^{-6}$ ***	0.971	0.020 *	0.569	0.0005 ***
Delta* <i>Bt</i>		0.825		0.025 *		0.004 **		0.199
<i>Bt</i> 4Q2/4D1	0.021 *	0.305	0.001**	0.228	0.933	0.012*	0.313	0.048 *

3.2.4 Discussion and conclusions

Mortality

Consistently to toxicity data about *Bt* sporal formulations (Mommaerts *et al.*, 2010) and our previous experiences, no significant mortality effect was recorded during the sub chronic exposure. Deltamethrin provoked a significantly higher mortality at field recommended dose, even though honey bees previously treated with *Bt* spores didn't show a higher sensitivity to deltamethrin exposure. Thus, at a mortality level, we could not evidence a combined effect of these products nor a sensitization effect.

Enzymatic activity

As physiological parameters of pesticide effect, we evaluate the variation in enzymatic activities of three oxidative stress linked enzymes: Glutathion-S-Transferase (GST), Superoxyde dismutase (SOD) and Catalase (CAT). Furthermore, we considered the response of three metabolic enzymes as Alkaline phosphatase (ALP), Glucose-6-phosphate dehydrogenase (G6PDH) and Glyceraldeyde-3-phosphate dehydrogenase (GAPDH).

Oxidative stress enzymes

GST is a group of ubiquitous enzymes that plays a major role in the organism reaction to contaminants and cellular stress in general. The first described function of GST is its detoxification activity, due to the conjugation of xenobiotic molecules with reduced glutathione (GSH) (Baars and

Breimer, 1980). More recently, its involvement in cellular oxidative stress has been proposed. In fact, GST can operate in reducing damage of oxidizing conditions as lipid peroxidation and hydrogen peroxide production (Felton and Summers, 1995; Barbehenn, 2002).

Here, we show a reduction in GST tissue activity in heads after 5 days of sub chronic *Bt* treatment, compared to control levels. A slight decreasing trend, was also found 3 days after the end of the *Bt* exposure (day 8), both in heads and in midguts. As we previously verified, an exposure of 10 days to the same *Bt* strains at the same concentrations, determined a reduction in GST activity, even though the percentage of reduction compared to the control was higher in that experience, probably suggesting an effect of the exposure time. Moreover, both bacterial strains contributed to this result, so that we cannot ascribe the toxic strain to have some effect on GST activity. Consistently, a hypothesis about the involvement of the bacterial components in producing an enzyme variation might be proposed.

Superoxide dismutase and Catalase, can be considered as the most important cell defences against oxidative stress damages caused by reactive oxygen species (ROS) (Livingstone *et al.*, 1992). These two enzymes can operate in succession to remove oxygen intermediates: SOD is the principal scavenger for superoxide anions that are transformed into hydrogen peroxide. CAT can subsequently neutralize H₂O₂, converting it into H₂O and O₂, thus preventing peroxidation of lipids and other molecules. SOD variations are mainly regulated by substrate concentration and the enzyme activity increases are generally related to oxidative stress conditions in the cell. Here, SOD levels increases both in heads and in midguts in response to *Bt* treatment. Even if the augmentation is not so relevant after 5 days of exposure, at day 8 we register a clear increasing trend in function of *Bt* strain and dose. Consequently, we could expect an enhanced CAT level, as H₂O₂ concentration is augmented by SOD activity. However, at both sampling date, the catalase level in midgut decreases in dependence of *Bt* treatment. Indeed, as described by other studies (Kono and Fridovich, 1982; Gultekin *et al.*, 2000), CAT can be inhibited in condition of oxidative stress and high concentration of ROS. This findings are consistent with variations occurred in *Galleria mellonella* larvae after a *B. thuringiensis* exposure (Dubovskiy *et al.*, 2008). We can therefore hypothesize that *Bt* treatment lead to an oxidative stress status in honey bees, even though the percentage of enzymatic variations compared to control may allow to assume a low oxidative stress condition.

Chemical pesticides, as organophosphates and carbamates have also been related to SOD/CAT variations in response to oxygen reactive species accumulation (Ferrari *et al.*, 2011). Conversely, no effect of deltamethrin on oxidative stress enzymes has been evidenced in treated bees.

Intestinal metabolism

We evaluated the tissue activity of alkaline phosphatase, a digestive enzyme acting through phosphorylation of substrates to allow their adsorption through the intestinal epithelium. Its physiological variations have been related to contaminants exposure in insects (Badiou-Bénéteau *et al.*, 2012), even though an ALP-based biomarker tool is still not extensively employed.

Besides its main function in digestion, an important role of ALP in mediating the action of *B. thuringiensis* has been discovered; ALP might in fact represent an intestinal receptor of *Bt* involved in the development of bacterial toxicity through time (Upadhyay and Singh, 2011). Moreover, *Bt* resistant insects have been found to express less ALP in the intestinal membrane and counterparty higher ALP concentration in the gut lumen: the interaction between ALP and Cry toxins in the lumen resulted in a reduced anchorage to the epithelium membrane, thus leading to a reduced effect of *Bt* treatments (Caccia *et al.* 2012). Here, we find a *Bt* related reduction of ALP activity. However, having not measured the specific membrane enzyme activity, we cannot assume that this reduction is due to an interaction with Cry elements in the midgut.

ALP activity was proved to be stimulated by chemical pesticides as thiamethoxam (Badiou-Bénéteau *et al.*, 2012), even though the biochemical mechanism relating pesticide presence to ALP increased activity isn't well known yet. In superior organisms, like fishes, ALP increase in response to a synthetic pyrethroid, cypermethrin, is explained as a symptom of tissue damage, with particular reference to liver necrosis (Firat *et al.*, 2011). Here, we point out the deltamethrin linked increase in ALP enzymatic activity, consistently with those findings. Furthermore, a positive interaction between *Bt* and deltamethrin treatment is evidenced, since the combination of both treatments leads to a higher ALP level compared to *Bt*-treated bees, for both *Bt* strains.

Energetic metabolism enzymes

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Glucose-6-phosphate dehydrogenase (G6PDH) take part to the carbohydrate and energetic metabolism, being the key-enzymes of glycolysis and pentose phosphate pathway, respectively. These two enzymes are still scarcely employed as biochemical markers of pesticide exposure in insects and other organisms, even though some studies investigated this topic. Besides its main role in glucose catabolism, it has been shown that GAPDH is involved in cell death associated with oxidative stress, generated by a pesticide exposure (i.e. paraquat) (Ortiz-Ortiz *et al.*, 2010). G6PDH is involved in NADPH production, that is necessary to provide the reductive potential to face oxidative stress conditions, also maintaining reduced glutathione (GSH). The exposure to chemical pesticides as organophosphates and herbicides determines a decrease in G6PDH activity in human erythrocytes, thus reducing the organism capacity to react to oxidative damage (Aliciguzel *et al.*, 2001).

Here, deltamethrin has a decreasing effect both on GAPDH and G6PDH. This result suggests a reduced glucose metabolism that would determine, at the same time, a reduced energetic supply by reducing glycolysis, and a lower capacity to face oxidative stress conditions with a lower G6PDH activity.

In conclusion, *Bt* treatment with spores have been confirmed not harmful to honey bees, since no significant mortality is expressed. The recorded variations in enzymatic levels suggest a physiological effect of such a treatment, sometimes not related to the expression of Cry toxins, as in the case of GST. This result raises interest on effects of bacterial components as cell wall molecules on honey bee physiology. The pyrethroid deltamethrin caused some important modifications, especially to ALP and GAPDH/G6PDH levels. For those reasons, we point out the interest of future research on the development of such biomarkers for chemical pesticides exposure.

3.3 Physiological changes induced by a combined treatment with difenoconazole and deltamethrin

In this third experience, we tested the effect of a subchronic treatment with the fungicide difenoconazole followed by a contact acute treatment with deltamethrin. Difenoconazole is an azole fungicide, whose interaction with pyrethroids insecticides, and deltamethrin in particular, has been assessed in previous studies (Meled *et al.*, 1998; Vandame and Belzunces, 1998). Azoles fungicides can succeed in inhibiting the development of the fungal cell wall by interfering with P450 monooxygenases. This monooxygenase is also involved in detoxification pathways for several pesticides, and particularly pyrethroids (Vandame and Belzunces, 1998). Hence, it has been proved that the exposure to azoles fungicides, enhances the honey bee sensitivity to a following or contemporary pyrethroid administration. Synergistic effects can be evidenced in a significant augmentation of bee mortality, that occurs for contact exposure with doses higher than 25 g/ha (Meled *et al.*, 1998), or in the impairment of the termoregulation capacities, starting from 850 ng/bee (Vandame and Belzunces, 1998). Here, we chose to test extremely low doses of difenoconazole, administered orally. In fact, the presence of residues of such substance in pollen has been demonstrated, with amounts going from 10 µg/Kg in fresh pollen loads (Skerl *et al.*, 2009) to 130 µg/Kg in bee bread (Mullin *et al.*, 2010). Conversely, deltamethrin was administered via direct contact, with a simulation of a pulverization treatment. Also in this case, we chose to test low doses of deltamethrin, corresponding to 1/5, 1/50 and 1/250 of the field recommended dose (7.5 g/ha) (fig. 3.3.1).

The objective of this study was therefore to evaluate the toxicity and the effect on various enzymes of a subchronic treatment with the selected fungicide, followed by three different doses of deltamethrin.

3.3.1 Material and methods

Honey bees

Emergent honey bees were obtained by incubating a brood frame from a healthy queen -right colony at controlled conditions (34°C ± 2°C; 60%± 10% RH; darkness). After emergence, bees were grouped by 30 and kept in experimental cages, establishing 6 petition for each treatment (n=180). The rearing conditions were controlled and a constant temperature of 34°C ± 2°C with 60%± 10% of relative humidity was assured. All the cages were provided with candy, water, a multifloral pollen supply and a source of queen pheromone blend (one third of commercial Beeboost). The subchronic treatment was administered 10 hours per day through a contaminated syrup (50% sucrose in tap water) containing 5 µg/L of difenoconazole. In order to allow a good solubility of this product, 0,1% DMSO was added to all solutions. A stock solution was prepared in distilled water and then stored at -20°C, whereas working solutions were prepared and renewed each day in syrup. The treatment consumption and the mortality were registered daily.

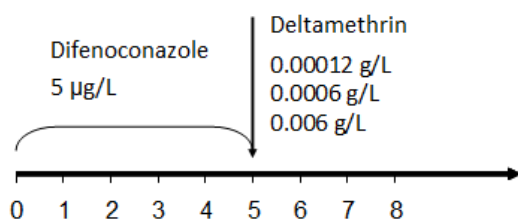


Figure 3.3.1. Scheme of the experimentation, tested products and doses.

Sampling for enzymatic assays were performed the last day, keeping only alive bees, and conserving them at -80°C . After dissection, the heads and the midguts were separated and analyzed. Three repetitions were established for each treatment group, with 3 honey bees used for each extraction (repetition). Each repetition was then measured in triplicate.

Biochemical assays

In each treatment group, we measured the activity of GST in the heads and in the midguts, the activity of CAT in the heads and the ALP activity in the midguts. Experimental procedure was the same as used in the other experimentations. Detailed protocols are described in the Annex (experimental procedures).

Statistical analysis

Biochemical assays data were comprehensively analyzed by a one way ANOVA, considering the enzymatic activity as function of the deltamethirn, difenoconazole treatment and treatments interaction. Mann-Whitney U-test was then used in order to evidence pair wise comparisons between treatment groups. Statistical analysis of pairwise comparisons is described in the figures, while a summary of AOV performed on whole datasets is shown in table 3.3.1.

3.3.2 Results and discussion

Mortality

Mortality data were always under 5%, for all treatment groups, with no differences between control and any other treatment. Therefore, we considered all the tested products and doses as sublethal and no toxic for honey bees.

Enzymatic assays

GST activity was assessed in heads and midguts at the end of the experimentation. In the heads, the difenoconazole treatment resulted in a significant increase of GST activity ($p < 1 \times 10^{-9}$), while deltamethrin determined a lower enzymatic level ($p = 0.026$). In the midguts, deltamethrin induced a reduction in activity ($p = 0.000$), whereas difenoconazole had no relevant influence (fig. 3.3.2). As noticed in previous experience, GST does not provide a clear response following a chemical pesticide exposure.

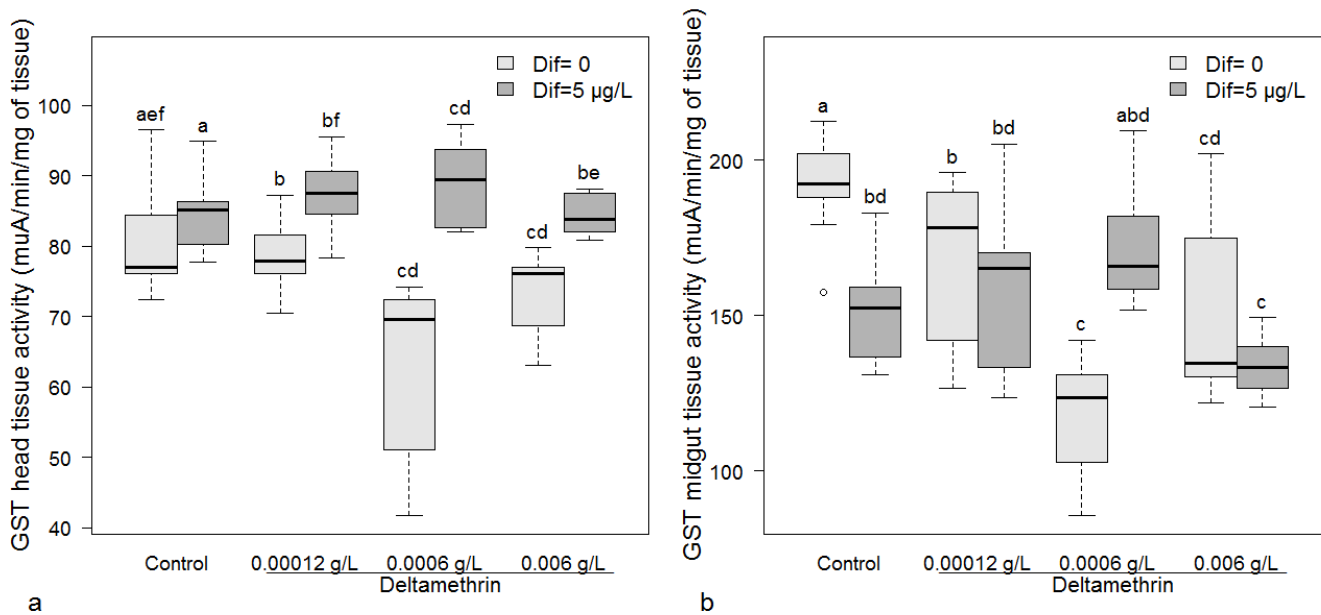


Figure 3.3.2. Effect of difenoconazole combined or not with deltamethrin on GST activity. GST activity was assessed in heads (a) and midguts (b) at day 8. Each treatment group is constituted by 3 repetitions performed in triplicate ($n=9$), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

The ALP activity, measured in midguts, was not influenced by any of the deltamethrin concentrations, while the difenoconazole treatment resulted in an enhanced activity ($p = 0.02$). This contaminant mediated augmentation of ALP was already demonstrated by two authors (Bounias *et al.*, 1996; Badiou-Bénéteau *et al.*, 2012), even though little is known about the mechanisms that relate the exposure to pesticide and an enhancement of this enzyme.

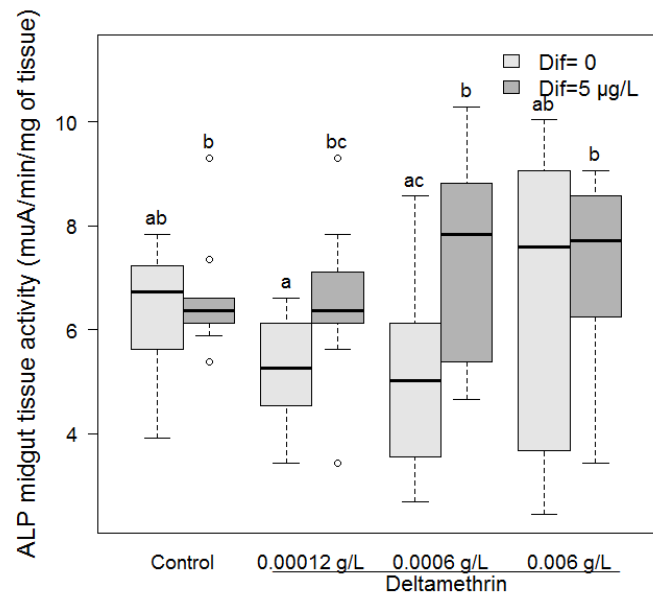


Figure 3.3.3. Effect of difenoconazole combined or not with deltamethrin on ALP activity. ALP activity was assessed in midguts at day 8. Each treatment group is constituted by 3 repetitions performed in triplicate (n=9), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

CAT level was assessed in the heads, where its activity was significantly inhibited by deltamethrin ($p < 1 \times 10^{-6}$). In particular, it is interesting to notice a concentration dependent effect, so that the higher concentration led to the lowest CAT level (fig. 3.3.4). Conversely, no influence could be attributed to difenoconazole exposure. However, a significant effect of the interaction between treatment was found ($p = 0.004$). The reduction of catalase activity as consequence of the exposure to a pesticide or a general stressor was highlighted by several authors (Kono and Fridovich, 1982; Gultekin *et al.*, 2000) This variation was interpreted as an inhibition caused by the accumulation of the enzyme substrate, H_2O_2 . Interestingly, the combination of difenoconazole and deltamethrin causes a weaker effect on catalase activity.

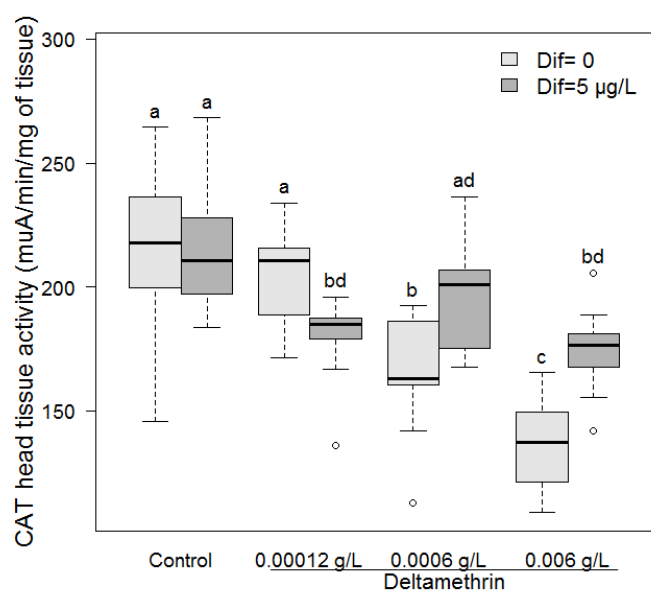


Figure 3.3.4. Effect of difenoconazole combined or not with deltamethrin on CAT activity. CAT activity was assessed in heads at day 8. Each treatment group is constituted by 3 repetitions performed in triplicate (n=9), each sample containing 3 individuals. Differences between treatment groups were estimated by Mann-Whitney U test: different letters indicate a significant difference between groups ($p < 0.05$). Data are represented as boxes corresponding to 50% of the measures; the line symbolizes the median, whiskers include 90% of the data and outliers are represented by circles.

Table 3.3.1. AOV analysis of enzymatic activity data (p values). Activity of GST, CAT and ALP was analyzed as function of deltamethrin and difenoconazole treatments, alone and in interaction. Statistical analysis were performed with ANOVA. Differences were considered significant when p value was inferior than 0.05. Significance is indicated with ‘***’ when $p < 0.001$, ‘**’ when $p < 0.01$ and ‘*’ when $p < 0.05$.

	GST (head) day 8	GST (midgut) day 8	ALP (midgut) day 8	CAT (head) day 8
Deltamethrin	0.026 *	0.000 ***	0.313	$< 1 \times 10^{-6}$ ***
Difenoconazole	$< 1 \times 10^{-9}$ ***	0.758	0.022 *	0.054
Delta*Difenoconazole	0.013 *	0.019 *	0.804	0.004 **

4 GENERAL DISCUSSION AND CONCLUSIONS

In this research, two main issues of pesticide risk assessment to honey bees were addressed: the evaluation of the risk of exposure and the assessment of the different effects caused by pesticides.

In the first part of this study, a specific way of exposure, first highlighted in consequence of numerous honey bee mortality cases, was investigated. As those important honey bee mortalities happened in the same period and in the same area as maize or sunflower sowing, several scientific researches in Europe related these accidents to the massive dust dispersal occurring during the sowing operations. The contaminated dusts were in fact supposed to contain residues of the pesticides used for seed dressing treatment (Comité Scientifique et Technique, 2003; Greatti *et al.*, 2003; Pistorius *et al.*, 2009). However, the entity of the contamination was never estimated with specific researches.

Here, in the framework of a wider project on the causes of honey bees mortalities in Italy (Apenet project), the quantification of the actual dust contamination was carried out and the real possibility for forager bees to be exposed to contaminated dusts, was further demonstrated. The numerous bee losses accidents in 2008, together with the results of the whole project ultimately contributed to important regulatory actions in Italy, with the suspension of the use of maize seed treatment with neonicotinoids and fipronil, up to this day.

Within this project, we proposed an experimental methodology to assess the risk associated with dust exposure, with respect to different active ingredients employed in seed dressing formulation (imidacloprid, thiamethoxam, clothianidin and fipronil).

In laboratory conditions, all tested products were demonstrated to be toxic to honey bees. The acute exposure *via* indirect contact to contaminated dusts caused a higher mortality than control, even though, at the lowest concentration, it didn't entail a significantly different mortality. Nevertheless, it has been observed as the surface of the sowed field has a positive relationship with the amount of the dust deposit (ApeNet, 2010). Considering this observation, it could be hypothesized that the actual entity of the environmental contamination caused by the pesticide seed treatment could be higher than measured in the experimental trials. That being so, the significant toxicity provoked by 10 to 1000 fold concentrated treatment might be relevant to risk assessment purpose.

In laboratory conditions, we demonstrated that the acute toxicity of clothianidin contaminated dusts, is comparable to that of the liquid formulation of the same active ingredient. Therefore, since no standardized test methods were considered so far (OEPP/EPPO, 2010), we evidence the convenience of a specific risk assessment for dusts toxicity to honey bees.

In semi-field conditions, an effective protocol for testing dust effects on mortality and several sublethal traits was developed, and the detrimental effect on small colonies was evidenced, showing a higher mortality soon after the treatment. This results are consistent to those obtained in another field experimentation, where thiamethoxam dust toxicity on honey bee mortality was highlighted (Tremolada *et al.*, 2010).

The honey bees orientation capacity was then evaluated in a field experience, after the treatment with clothianidin contaminated dusts. Neonicotinoids provoke negative effects on homing flight and foraging activity, as demonstrated in field conditions for imidacloprid (Bortolotti *et al.*, 2003), thiamethoxam (Henry *et al.*, 2012) and clothianidin (Schneider *et al.*, 2012). The influence of neonicotinoids residues in dusts was also proven to adversely influence honey bee olfactory and visual learning (ApeNet, 2010). However, the homing ability and the duration of foraging flights were not impaired by the exposure to clothianidin dusts, in our experimentation. Unfortunately, a limited sample size and the impossibility to carry out a field test repetition, didn't allow a more accurate investigation.

In the second part of this research, the assessment of pesticides effects has been taken into account, investigating some of the physiological changes that can be detected at a biochemical level in response to a pesticide exposure. Even though the majority of sublethal effects is generally represented by behavioural traits, we consider that a deeper knowledge of pesticide-induced changes at a subcellular level could be relevant to risk assessment extent. We therefore evaluated various kind of pesticides and different modalities of exposure as potential stressors to honey bees. To this aim, three experimentations were carried out, testing the combination of *Bacillus thuringiensis* and fipronil, of *Bacillus thuringiensis* and deltamethrin and of difenoconazole fungicide and deltamethrin. As endpoints, the activity of different stress-linked enzymes was evaluated. More in particular, we assessed the activity of GST, CAT, SOD, ALP, GAPDH and G6PDH. These enzymes are involved in different cellular tasks, from detoxification (GST), prevention of oxidative damage (CAT/SOD and GST) and glucose metabolism (GAPDH and G6PDH). Nonetheless, they are strictly related to each other, as shown in figure 1.

We observed some significant variations of GST activity in function of the exposure to different substances and the analyzed organ. The most relevant changes were related to *Bt* treatment: in the first experience a strong decrease in GST levels were registered following the *Bt kurstaki* (4D1) chronic exposure at both sampling date, until 20 days after the beginning of the trial (tab. 4.1). This response was confirmed in the midgut at day 20, as well. Similarly, in the second experience, we measured GST levels after 5 days of treatment, and we observed a *Bt* related decrease (tab. 4.2). For

those reasons, we can hypothesize a time-dependent GST variation, in response to *Bt* so that a 10-day exposure determines a more evident effect than a 5-day administration. At the same time we cannot totally attribute the decreasing effect to the toxins expressed by *Bt*, since in the second experience, the non toxic strain (4Q2) had a major role in diminishing GST levels. As proposed in chapter 2.1, the lower GST activity in correspondence with *Bt* treatment might be related to the spontaneous oxidation of GSH that occurs in oxidative stress conditions (Barata *et al.*, 2005). Since GSH represents the main substrate for GST, the lack of this compound may determine a fall of the enzyme activity. On the other hand, neither deltamethrin nor fipronil showed a clear effect on this enzyme. On the contrary, the third experimentation evidence as deltamethrin determined a decrease in midgut GST level. Therefore, we cannot associate the deltamethrin exposure with a clear trend in GST variations,

Table 4.1. Physiological changes induced by *Bt* spores and deltamethrin

10 days				
	4Q2 100/1000 µg/L	4D1 100/1000 µg/L	4Q2 + fipronil 100/1000 µg/L	4D1 + fipronil 100/1000 µg/L
GST Head	↑ / -	↓ / ↓	- / -	↓ / ↓
GST Midgut	- / ↑	- / ↑	- / -	↑ / ↑
ALP Midgut	- / ↑	↑ / ↑	↑ / ↑	↑ / ↑
GADPH Abdomen	↑ / -	- / ↑	↑ / -	↑ / ↑
G6PDH Abdomen	↑ / -	↓ / -	- / -	- / -
20 days				
	4Q2 100/1000 µg/L	4D1 100/1000 µg/L	4Q2 + fipronil 100/1000 µg/L	4D1 + fipronil 100/1000 µg/L
GST Head	- / ↓	↓ / ↓	- / -	↓ / ↓
GST Midgut	↓ / ↓	↓ / ↓	↓ / ↓	↓ / ↓
ALP Midgut	↓ / ↓	↓ / ↓	↓ / ↓	↓ / ↓
GADPH Abdomen	- / -	- / -	- / ↑	- / ↑
G6PDH Abdomen	- / -	- / -	- / -	- / ↑

Table 4.2. Physiological changes induced by *Bt* spores and deltamethrin

8 days				
	4Q2 100/1000 µg/L	4D1 100/1000 µg/L	4Q2 + Delta 100/1000 µg/L	4D1 + Delta 100/1000 µg/L
GST Head	- / -	- / -	↓ / -	- / -
GST Midgut	↓ / ↓	- / -	↓ / -	- / -
SOD Head	↓ / -	↑ / ↑	- / -	↑ / ↑
SOD Midgut	- / -	↑ / ↑	- / -	↑ / ↑
CAT Head	↓ / ↓	↓ / ↓	↓ / ↓	↓ / ↓
ALP Midgut	- / ↓	↓ / ↓	- / -	- / -

GADPH Abdomen	- / -	↓ / -	- / -	↓ / -
G6PDH Abdomen	- / ↓	- / ↓	↓ / -	↓ / ↓

Table 4.3. Physiological changes induced by difenoconazole and deltamethrin

	8 days		
	Difenoc. 5 µg/L	Deltamethrin 0.00012 / 0.0006 / 0.006 g/L	Difenoc. + Deltamethrin 0.00012 / 0.0006 / 0.006 g/L
GST Head	-	↓ / ↓ / ↓	- / ↑ / -
GST Midgut	↓	↓ / ↓ / ↓	↓ / - / ↓
CAT Head	-	- / ↓ / ↓	↓ / - / ↓
ALP Midgut	-	- / - / -	- / - / -

SOD/CAT have been examined as the most important enzymes involved in prevention of cell damage provoked by ROS excess (Livingstone *et al.*, 1992). These two enzymes, in the heads, show an opposite trend, with SOD increasing and CAT diminishing following the exposure to a stressor. We verified this occurrence with the exposure to *Bt* (tab. 4.1, 4.2) and, regarding CAT, with the combined exposure to difenoconazole and deltamethrin (tab. 4.3). As suggested by Kono *et al.* and Gultekin *et al.* (1982; 2000), a stress condition that determines a high concentration of superoxide anions might, at the same time, enhance SOD activity as a O₂⁻ scavenger and inhibit CAT activity due to an accumulation of hydrogen peroxide. In a more general contest, this interpretation is consistent to the considerations proposed for GST variations. Since hydrogen peroxide accumulates, in fact, it might be involved also in GSH oxidation, thus leading to GST activity reduction (fig. 4.1).

Alkaline phosphatase (ALP) showed an inconstant behaviour in response to *Bt* that doesn't allow to affirm a solid interpretation. Counterparty, it seems interesting to analyze its variations in dependence on the chemical pesticides that were considered, fipronil, deltamethrin and difenoconazole. Fipronil effect was assessed at the end of a chronic 10-day treatment and after 10 more days from that sampling. In the first date fipronil was linked to a significant augmentation in ALP activity compared to non fipronil treated groups. After 10 days from the end of the treatment, this effect was no longer observed. Similarly, deltamethrin at 0.03 g/L concentration, induced a significant increase in ALP levels (tab. 4.2). Interestingly, deltamethrin at very low concentrations, corresponding to 1/5, 1/50 and 1/250 of the field recommended dose did not influence ALP levels. This suggests a concentration-dependent response of ALP to deltamethrin. Though this enzyme hasn't been extensively studied in relationship with pesticide exposure in insects, our results are consistent with those obtained employing thiamethoxam as a chemical pesticide (Badiou-Bénéteau *et al.*, 2012). Further investigations, also with other toxic compound are necessary to determine

which aspect of these chemical insecticides involves intestinal enzyme activities and ALP modifications. Eventual findings might be relevant to better understand their principal of secondary mode of action.

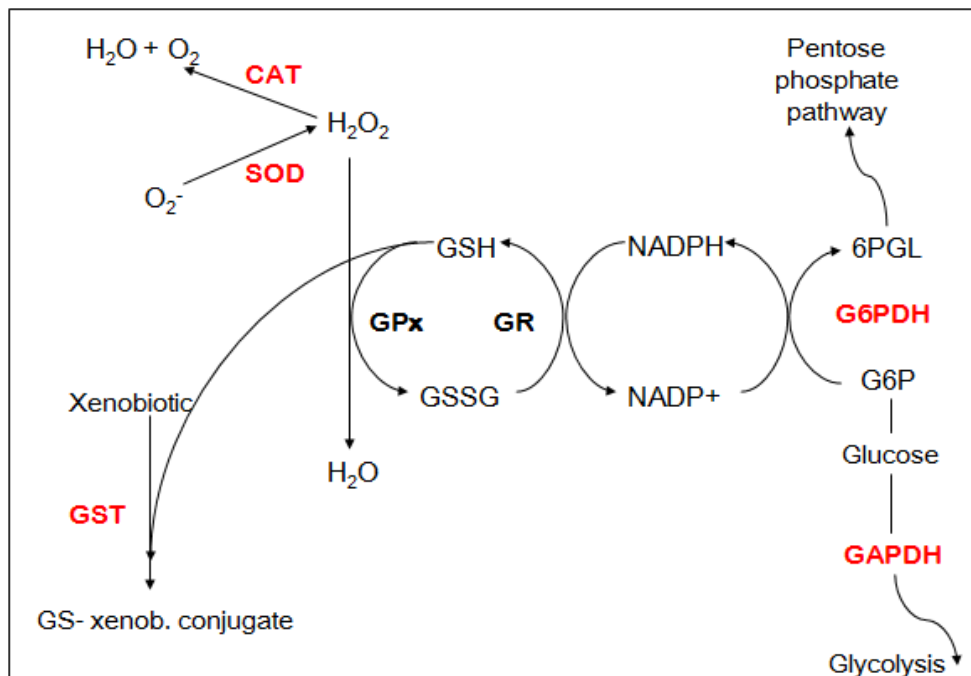


Figure 4.1. Overview of metabolic connection of enzymes employed in this research

GAPDH and G6PDH were analyzed as key role enzymes for glucose metabolism, even though their involvement in other metabolic processes and in oxidative stress damage prevention has been evidenced (Ortiz-Ortiz *et al.*, 2010). Here, we measured the variations of these enzymes in the abdomen, following the combined treatment of *Bt* and fipronil or deltamethrin. Our results seem particularly interesting with regards to chemical pesticides effects. Fipronil (1µg/L) chronically administered for 10 days, provoked a significant augmentation in both enzymes activity, whereas deltamethrin caused the opposite effect, determining a decrease in tissue activity.

The studies on the stress-induced reaction of these two enzymes were rarely carried out on insects. Their combined role in pesticide-induced stress is not well known and the interpretation of their changes in response to a contaminant exposure is contrasting. For example, some authors suggest that a decrease in GAPDH and an increase in G6PDH might follow a pesticide exposure. In this interpretation, GAPDH might be inhibited by a high concentration of ROS, thus slowing down glycolysis. The higher availability of glucose-6-phosphate is then redirected to the pentose phosphate pathway, to enhance the production of NADPH (Kletzien *et al.*, 1994; Verma *et al.*, 2007). On the other hand, some other studies propose that a pesticide-dependent decrease of

G6PDH represent a sign of the insufficient capacity of an organism to react to oxidative stress conditions (Aliciguzel *et al.*, 2001). For those reasons, we cannot suggest a clear interpretation of the founded results for these two enzymes.

In conclusion, with this research work we proposed an effective protocol to assess the toxicity of pesticide contaminated dusts to honey bees, in laboratory, semi field and field conditions.

The *a priori* determination of the environmental contamination with neonicotinoid and fipronil residues in sowing dusts, could allow further studies on lethal and sublethal effects on honey bee, with the aim of describing an accurate dose-effect relationship. Moreover, it would permit useful comparisons between the toxicity expressed by dusts and other formulations, in order to perform a complete risk assessment.

The study of the effect of different pesticides on important metabolic enzymes revealed the consistent response to some of them to pesticides-induced stress. In particular, we put the attention on GST response to *Bt* treatment, the coupled variation of SOD/CAT and the changes in ALP levels following a chemical pesticide contamination. Moreover, this study shows that very low doses or concentrations of different kind of pesticides, are able to elicit significant changes in physiological conditions. For those reasons, we stress the attention on the potential usefulness of such biochemical endpoints to improve the investigation of pesticide effects, considering different biological organisation levels.

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6 ANNEX - EXPERIMENTAL PROCEDURES

6.1 Protein extraction

Preparation of the extraction buffer

- Prepare a 2X LS-phosphate buffer: dilute 80 mM NaH_2PO_4 and 20 mM NaCl; adjust pH to 7.4;
- Prepare a 10X Triton 100x: dilute 10% w/v of Triton 100x in distilled water;
- Prepare 200X proteases inhibitors (pepstatin A, Leupeptin, Aprotin, Trypsin, Antipain).

Preparations of the tissues (4°C)

- Label and weight tubes and place 3-5 heads/midguts/abdomens in each tube;
- Weight tubes;
- Place a steel ball in every tube;
- Add the extraction buffer in function of the sample mass (10% w/v):
- Homogenize samples with a TissueLyser during 10 seconds for 5 times with 30 seconds of pause between every time. Repeat this sequence twice with a pause of 30 minutes, placing the samples at 4°C;
- Separate solid tissues from protein extract through centrifugation: centrifuge the tubes at 15000 rcf for 20 minutes (at 4 °C) in ;
- Withdraw the supernatant liquid with a micropipette.

6.2 Glutathione-S-Transferase (GST) activity assay

Solutions and buffer preparation

- Prepare a phosphate buffer (5X): add KH_2PO_4 (500 mM) to Na_2HPO_4 (500 mM) to get to pH=7.4;
- Prepare 100 mM EDTA solution in distilled water (100X);
- Prepare 25 mM GSH solution in distilled water (10X);
- Prepare 100 mM CDNB solution in acetonitrile (100X);

Preparation of the reaction mixture

- Introduce in a reservoir:
 - 4400 μL Tampon phosphate (5X)
 - 220 μL EDTA 100X
 - 2200 μL GSH 10X

220 μL CDNB 100X
13860 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 10 μL of extraction buffer in blank wells;
- Introduce 10 μL of tissue extract in sample wells;
- Introduce 190 μL of reaction mixture in all wells;

Enzymatic assay

- Read the plate in the spectrophotometer at 340 nm wavelength
- Read kinetic data

6.3 Catalase (CAT) activity assay

Solutions and buffer preparation

- Prepare NaH_2PO_4 buffer (5X) and get pH to 7;
- Prepare 300 mM H_2O_2 in distilled water (10X).

Preparation of the reaction mixture

- Introduce in a reservoir:
4400 μL Tampon phosphate 5X
2200 μL H_2O_2 10X
14300 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 10 μL of extraction buffer in blank wells;
- Introduce 10 μL of tissue extract in sample wells;
- Introduce 190 μL of reaction mixture in all wells.

Enzymatic assay

- Read the plate in the spectrophotometer at 240 nm wavelength;
- Read kinetic data.

6.4 Superoxyde dismutase (SOD) activity assay

Solutions and buffer preparation

- Prepare phosphate/carbonate buffer: add KH_2PO_4 (500 mM) to Na_2HPO_4 (500 mM) to get to pH=7.8;
- Prepare 1 mM H_2O_2 in distilled water (10X);
- Prepare 0.5 mM xanthine in distilled water (5X);
- Prepare 0.25 mM NBT in phosphate/carbonate buffer (10X);
- Prepare a xanthine oxydase solution of 0.833 U/mL (10X).

Preparation of the reaction mixture

- Introduce in a reservoir:
 - 2200 μL Tampon phosphate/carbonate 10X
 - 2200 μL EDTA 10X
 - 4400 μL Xanthine 5X
 - 2200 μL NBT 10X
 - 7700 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 10 μL of extraction buffer in blank wells;
- Introduce 10 μL of extraction buffer in refence wells;
- Introduce 10 μL of tissue extract in sample wells;
- Introduce 20 μL of xanthine oxydase solution reference and sample wells;
- Introduce 170 μL of reaction mixture in all wells.

Enzymatic assay

- Read the plate in the spectrophotometer at 560 nm wavelength;
- Read kinetic data.

6.5 Alkaline phosphatase (ALP) activity assay

Solutions and buffer preparation

- Prepare Tris-HCl buffer: mix 500 mM Tris-HCl and 100 μM MgCl_2 and get to pH 8.5;
- Prepare 20 mM p -NPP in distilled water (10X).

Preparation of the reaction mixture

- Introduce in a reservoir:
 - 4400 μL Tampon Tris-HCl 5X
 - 2200 μL ρ -NPP 10X
 - 14300 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 10 μL of extraction buffer in blank wells;
- Introduce 10 μL of tissue extract in sample wells;
- Introduce 190 μL of reaction mixture in all wells.

Enzymatic assay

- Read the plate in the spectrophotometer at 410 nm wavelength;
- Read kinetic data.

6.6 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity assay

Solutions and buffer preparation

- Prepare 400 mM Triethanolamine buffer and get pH to 7.6 (5X);
- Prepare 70 mM 3-PGA in distilled water (10X);
- Prepare 40 mM L-Cysteine HCl neutralized with sodium bicarbonate, in distilled water (10X);
- Prepare 20 mM MgSO_4 in distilled water (10X);
- Prepare 12 mM β -NADH in distilled water (100X);
- Prepare 12 mM ATP in distilled water (10X);
- Prepare 100 mM EDTA in distilled water (100X);
- Prepare 3-PGK at 2500 U/mL in distilled water (100X);

Preparation of the reaction mixture

- Introduce in a reservoir:
 - 4400 μL Triethanolamine buffer 5X
 - 2200 μL 3-PGA 10X
 - 2200 μL L-Cysteine HCL 10X
 - 2200 μL MgSO_4 10X
 - 220 μL β -NADH 100X
 - 220 μL EDTA 100X
 - 220 μL 3-PGK 100X
 - 7590 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 5 μL of extraction buffer in blank wells;
- Introduce 5 μL of tissue extract in reference wells;
- Introduce 5 μL of tissue extract in sample wells;
- Introduce 20 μL of ATP in blank and sample wells and 20 μL of distilled water in reference well;
- Introduce 175 μL of reaction mixture in all wells.

Enzymatic assay

- Read the plate in the spectrophotometer at 340 nm wavelength;
- Read kinetic data.

6.7 Glucose-6-Phosphate dehydrogenase (G6PDH) activity assay

Solutions and buffer preparation

- Prepare 500 mM Trizma base buffer and get pH to 7.4 (5X);
- Prepare 10 mM G6P in distilled water (10X);
- Prepare 100 mM MgCl_2 in distilled water (10X);
- Prepare 5 mM NADP in distilled water (10X).

Preparation of the reaction mixture

- Introduce in a reservoir:
 - 4400 μL Trizma base buffer 5X
 - 2200 μL G6P 10X
 - 2200 μL MgCl_2 10X
 - 2200 μL NADP 10X
 - 9900 μL distilled water

Microplate preparation (96-well microplate, U-bottom)

- Introduce 10 μL of extraction buffer in blank wells;
- Introduce 10 μL of tissue extract in sample wells;
- Introduce 190 μL of reaction mixture in all wells.

Enzymatic assay

- Read the plate in the spectrophotometer at 340 nm wavelength;

- Read kinetic data.

6.8 List of abbreviations

GSH: reduced glutathione

EDTA: ethylenediaminetetraacetic acid

CDNB: 1-Chloro-2,4-dinitrobenzene

NBT: nitroblue tetrazolium

ρ -NPP: ρ -nitrophenyl phosphate

3 PGA: 3-Phosphoglyceric acid

β -NADH: β -nicotinamide adenine dinucleotide reduced

ATP: adenosine-5'-triphosphate

3-PGK: 3-phosphoglycerate kinase

G6P: Glucose 6-phosphate

NADP: Nicotinamide adenine dinucleotide phosphate