HONEYBEE *Apis mellifera* L., 1758 (Hymenoptera: Apidae) ENZYMES AS POSSIBLE BIOMARKERS FOR THE ASSESSMENT OF ENVIRONMENTAL CONTAMINATION WITH PESTICIDE

STEPHAN MALFITANO CARVALHO

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Orientador

Prof. Dr. Geraldo Andrade Carvalho

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APROVADA em 26 de fevereiro de 2010

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LAVRAS

MINAS GERAIS – BRASIL

A ***Deus,*** pelo dom da vida!!!

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SUMMARY

RESUMO

CARVALHO, Stephan Malfitano. **Enzimas da abelha *Apis mellifera* L., 1758 (Hymenoptera: Apidae) como possíveis biomarcadoras em avaliação da contaminação ambiental por pesticidas.** 2010. 105 p. Tese (Doutorado em Entomologia) - Universidade Federal de Lavras, Lavras.*

As abelhas do gênero *Apis*, principalmente *Apis mellifera* L., 1758, têm grande destaque, mundialmente, tanto pelos seus produtos quanto pelo importante serviço de polinização. No ambito científico, diversas pesquisas são realizadas objetivando aprimorar conhecimentos sobre sua organização social, biologia e comportamento, além de proteção dessa espécie contra fatores exógenos, tais como os xenobióticos. Dentre as técnicas moleculares que podem ser empregadas, estudos bioquímicos podem ser utilizados para avaliar (a) o sistema de detoxificação por meio das enzimas carboxylesterases (CaE) e glutathione S-transferase (GST) que, dentre as inúmeras funções que possuem, são responsáveis pela resistência de insetos a pesticidas; (b) enzimas do estresse oxidativo, como catalase (CAT) e GST, as quais têm como funções eliminar peróxidos e radicais livres em espécies reativas ao oxigênio; (c) a alkaline phosphatase (ALP) que está ligada principalmente ao processo digestivo e transporte de metabólitos; (d) biomarker de exposição a xenobióticos, visando determinar a qualidade ambiental e (e) o efeito sobre a acetylcholinesterase (AChE), que tem como função mediar a transmissão do impulso nervoso em sinápses colinérgicas.

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ABSTRACT

CARVALHO, Stephan Malfitano. **Honeybee *Apis mellifera* L., 1758 (Hymenoptera: Apidae) enzymes as possible biomarkers for the assessment of environmental contamination with pesticide.** 2010. 105 p. Thesis (Doctorat in Entomology) - Universidade de Lavras, Lavras.*

The honeybees of the genus *Apis*, mainly *Apis mellifera* L., 1758, stand out greatly in the worldwide context both for their products and the important pollinating activities. In the field of science, much research is carried out to improve knowledge on their social organization, biology, behavior in addition to the protection of this species from exogenous agents, such as xenobiotics. Out of the molecular techniques which can be used, biochemical studies can be utilized to evaluate (a) detoxification system by means of the enzymes carboxylesterases (CaE) and glutathione S-transferase (GST), which out of the several functions are responsible for insect resistance to insecticides; (b) oxidative stress enzymes, such as catalase (CAT) and GST, which has functions to eliminate peroxides and free radicals in oxygen-reactive species; (c) alkaline phosphatase (ALP), bound mainly to the digestive process and metabolite transport; (d) biomarker of exposition to xenobiotics, aiming to determine environmental quality and (e) the effect on acetylcholinesterase (AChE), which has a function to mediate nervous impulse transmition at cholinergic synapses.

* Advisory committee: Dr. Geraldo Andrade Carvalho – UFLA, Lavras/MG, Brazil / Dr. Luc Philippe Belzunces – INRA, Avignon, France.

CHAPTER 1

ENZYMATIC STUDIES ON *Apis* spp. HONEYBEES: A REVIEW

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1 INTRODUCTION*

Among the several social honeybees found across the world, the ones of the genus *Apis* are the most studied due to their wide geographic distribution, their hive products and being responsible for pollination of several plant species, both native and cultivated (Delaplane & Mayer, 2005). With the outcome of agricultural techniques, mainly the large scale use of pesticides to control insect pests and diseases in crops, a negative impact on honeybees was detected, probably due to the lack of detoxification system (Atkins et al., 1981). This compounds, known as xenobiotics can affect honeybees in two ways, the lethal (death) and the sublethal. The lethal effects are relatively easy to be characterized; however, the sublethal effects may not show clear symptoms of intoxication, though they may damage aspects related to biology, such as survival and colony population (Devillers, 2002; Desneux et al., 2007).

Like all animal species, honeybees have a complex enzymatic system responsible for several vital activities for their survival, mainly the oxidative stress, detoxification, digestive metabolism and transport (Rockstein, 1978; Chapman, 1998), thus several studies were carried out to detect susceptibility of *A. mellifera* to insecticides. Gilbert & Wilkinson (1974) studied microsomal oxidases and Yu et al. (1984) evaluate these oxidases, as well as GST, esterases, epoxide hydrolase and DDT-dehydrochlorinase, and suggested that honeybee susceptibility is probably linked to the absence of microsomal oxidase activity in its stomach or to the lack of satisfactory amounts of fat body, important in xenobiotic detoxification processes.

* Enzyme abbreviations: AChE = acetylcholinesterase (EC 3.1.1.7); CAT = catalase (EC 1.11.1.6); ALP = alkaline phosphatase (EC 3.1.3.1); GST = glutathione S-transferase (EC 2.5.1.18); CaE-1,CaE-2 and CaE-3 = carboxylesterases (EC 3.1.1.1)

Claudianos et al. (2006) conducted comparative studies in order to identify the possible cause of honeybee susceptibility to insecticides. It was detected that *A. mellifera* have fewer gene codifiers of total proteins (±11000) when compared to *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) (±13500) and *Anopheles gambie* Giles, 1926 (Diptera: Culicidae) (±14000). The absence of these genes occurs in three enzymes responsible for detoxification, GST, cytochrome P450 and CaE, which are responsible for the main detoxification processes. Generally speaking, honeybees have only 29% of all GST present in *D. melanogaster* and *A. gambie*; 48.5% of P450 and 46.5% of CaE.

Another important enzymatic system is the one responsible for the oxidative stress in reactive oxygen species (ROS), mediated by enzymes such as CAT, superoxide dismutase (SOD), GST, glutathione peroxidase and glutathione reductase. These enzymes are able to eliminate peroxides and free radicals (i.e. O_{2^-}; OH•; NO• and H_2O_2) which are produced by aerobic cellular metabolism, or induced by an external factor such as environmental stress caused by xenobiotics and feeding (Barreiros et al., 2006). Accumulation of peroxides and free radicals may cause problems suh as cytotoxicity and damage of cellular structures inducing programmed cell death (PCD), inhibition of enzymatic activity in addition to protein, RNA and DNA oxidation (Korsloot et al., 2004). For example in the *A. mellifera* queen, in which the oxidative stress mechanism is fundamental in the spermatozoon conservation in the spermatheca for long periods (Weirich et al., 2002).

The enzyme ALP is of great importance to digestive processes and transport of metabolites. This group of isozymes have catalytic function linked to the participation of metallic ions and is frequently used in human biochemical studies as an indicator of diseases in the liver and bone (Posen, 1967). One of its characteristics is the capacity of hydrolyzing several phosphate esters, such as

substrates oxyphosphate monoesters S-phosphorothioates, phosphoramidates, thiophosphate and phosphate (Coleman, 1992). Akai (1969) and Moldenke (1976) reported that ALP is directly linked to digestive processes and active transport of metabolites in insects; in addition, Suresh et al. (1993) also demonstrated increase of ALP synthesis and activity of an invertebrate, when in the presence of copper ions.

This review reports studies carried out with the enzymes AChE, ALP, CAT, CaE and GST in *Apis* honeybees, considering their potential use in environmental quality monitoring programs, due to the importance of detoxification, oxidative stress, digestive processes and transport of metabolites for honeybee survival.

2 ACETYLCHOLINESTERASE

2.1 General aspects

Acetylcholinesterase (AChE - EC 3.1.1.7) is found at cholinergic synapses to hydrolyze acetylcholine in all insects, mediating neural transmition. Two class of insecticides widely used in agriculture, organophosphates and carbamates, have AChE as a target, inhibiting its catalytic effect causing honeybees' death. Under normal conditions, the AChE reaction takes place predominantly with acetylcholine degradation; however, it is a reversible reaction (O`Brien, 1976):

$$\underset{\text{(acetylcholine)}}{C_7H_{16}NO_2} + H_2O \xrightarrow{\text{AChE}} \underset{\text{(acetate)}}{C_2H_3O_2^-} + \underset{\text{(choline)}}{C_5H_{14}ON^+}$$

AChE belongs to the group of hydrolases, which act as a catalyst in reactions between a substrate and a water molecule, and are classified into three

groups: (A) the enzyme that hydrolyze paraoxon or other triesters, present in mammalian serum and absent in bird serum; (B) serine hydrolases, inhibited by organophosphates such as paraoxon, including acetylcholinesterase and carboxylesterase, and (C) esterases not interating with organophosphates (Aldridge, 1953; Thompson, 1999). Among the hydrolases, CaE (3.1.1.1) will be discussed later.

Belzunces et al. (1988b) studied some biochemical and physico-chemical characteristics of honeybee AChE, and observed that this enzyme is presente predominantly in the brain of these insects; in the hydrophilic form (soluble) with 3% to 6% and amphiphilic form (membranary) with 94% to 97% of the total. The amphiphilic form is bound to the cellular membrane by a glycophosphatidylinositol anchor, and can be changed to phosphatidylinositol-specific phospholipase on hydrophilic form. The two forms are in distinct conformations, are similar in eletrophoretic mobilities, molecular mass and sensitivity to eserine and BW284C51 (1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one-dibromide). As AChE is predominantly in a membranary form, the use of non-ionic detergent is needed for the extraction to take place, such as Lubrol-PX and Triton X-100, which have slight differences in the extracted AChE, but both efficient in the enzyme solubilization. In enzymatic characterization tests, this was considered as a true esterase or acetylcholinesterase, inhibited by BW284C51 and not affected by iso-OMPA (tetra-isopropyl pyrophosphortetramide), which is a specific inhibitor of pseudocholinesterase (butyrylcholinesterase) (Belzunces et al., 1988a) (Table 1). Badiou et al. (2007) confirmed the existence of two amphiphilic forms of AChE extracted from *A. mellifera* heads, distinguished by their ionic properties.

Zhang et al. (2005) made comparative studies of AChE between *Apis cerana cerana* Fabricius, 1793 and *Apis mellifera ligustica* Spinola, 1806. They found no differences between those enzymes extracted from the head and

abdomen, however, their activity was higher than the one from the thorax in *A. cerana*. No difference was observed between AChE affinity in the evaluated substrates (acetylthiocholine iodide, acetyl-β-(methyl)thiocholine iodide, s-propionylthiocholine and butyrylthiocholine); nevertheless, the AChE of *A. mellifera* hydrolyzed more rapidly the substrates than in *A. cerana*. In assays with inhibitors, it was observed that the AChE of *A. mellifera* was more sensitive as compared to *A. cerana*.

According to O`Brien (1976), the AChE of vertebrates is found in erythrocytes, nerves and muscles. Nevertheless, in insects, butyrylcholinesterase (BuChE) is not found in this case and AChE is abundant and may act on the substrate butyrylcholine, showing a BuChE activity. During development of honeybee pupae and queens, Polyzou et al. (1997) observed that AChE was inhibited by BW284C51 or eserine, and this enzyme showed 14% of BuChE activity.

2.2 Pesticide inhibition

Early studies carried out with AChE of *A. mellifera* intended to know the mode of action of insecticides. Metcalf & March (1949) evaluated the toxicity of parathion and its metabolites using *A. mellifera* and *Musca domestica* L., 1758 (Diptera: Muscidae) as insect models. They detected different inhibitor effects of insecticides on AChE of both species, with di-isopropyl *p*-nitrophenyl thiophosphate showing a slight inhibition of honeybee AChE and strong inhibition in the case of the fly, whereas parathion and paraoxon affected the action of this enzyme in both species. These results raised the hypothesis of structural differences of AChE found in *A. mellifera* and *M. domestica*. Later, Metcalf & March (1950), when comparing the effects of organophosphates, carbamates and theirs metabolites on AChE of *A. mellifera*, *M. domestica* and white mouse, discovered that the AChE of these species differs in aspects related

to the structure of the molecule, affecting the physicochemical characteristics, and showing a distinct behavior when exposed to some of these compounds. This hypothesis was also raised by Rahman et al. (1989), after comparing the inhibitor effect of some biphenyl derivates on AChE of honeybees, rice weevil, fish, pigeon and rat. Evidence was found that depending on its origin; important differences may be present in molecule toxicity and the degree of inhibition of the enzyme.

Barker et al. (1978) showed no differences of AChE activity in newly-emerged honeybees up to 36 days, suggesting that the activity of this enzyme is constant, and in cases where the inhibition of its activity is higher than half of the normal activity, this may be related to intoxication by insecticides. However, Nazer et al. (1974) observed that newly-emerged honeybees, that develop internal tasks, have two times as much activity of AChE as compared to the ones with food collecting responsibilities in the field. It was demonstrated that older honeybees have higher sensitivity to malaoxon. Shapira et al. (2001) suggested that the laborial division among the workers can occur along with the AChE alterations, being that in forager honeybees, lower catalytic ability was observed. Possible explanation may be related to the decreased levels of AChE encoded in the mRNA, making the learning ability easier in honeybees.

Kasturi Bai & Reddy (1977), after application of several organophosphates and carbamates in *Apis cerana indica* Fabricius, 1793, observed that owing to the different degrees of AChE inhibition, 39%; 63% and 96%, honeybees presented symptom hyperactivity, *knock down* and paralysis, respectively. In this study, the first symptoms of intoxication were found only when AChE inhibition reached 35%, with death of honeybees at 96%. The authors suggested that studies of ACHE inhibition should be used as an evaluation criterion in cases of honeybee intoxications by insecticides.

The effects of some insecticides, pyrethroids, carbamates, organophosphates and organochlorines, besides AChE storage conditions, were evaluated by Westlake et al. (1985). After extracted and maintained at 25^{o}C, it was not possible to quantify the enzymatic activity after one week due to desnaturation; nevertheless, no loss in activity was observed when stored between -20^{o} and 4^{o}C. On the other hand, in honeybees treated by contact with organophosphates and carbamates, significant differences in AChE inhibition was found; however, nothing was observed with pyrethroids and organochlorines. Fewer changes in AChE activity was observed when they were intoxicated orally by carbamates, suggesting a rapid form of metabolization/decomposition via enzymatic system of the digestive tract. These authors also stressed the use of AChE monitoring routines for the assessment of the contamination with pesticides mainly in cases of sublethal doses.

2.3 Biomarker studies

Studies with honeybees AChE have been oriented towards a new context, especially for use as terrestrial/aerial biomarkers in environmental monitoring programs. Stefanidou et al. (1996) reported this potential; however, with the used biochemical techniques, it was impossible to distinguish the insecticides with anticholinesterase action. Bendahou et al. (1999) investgated the sublethal effect of cypermethrin and fenitrothion on carbohydrate levels and some enzymes of *A. mellifera*. As for pyrethroid, no alteration in AChE was detected; however, inhibition higher than 60% of this enzyme by fenitrothion, even at lower doses was observed. Stefanidou et al. (1998) suggested the use of non-specific cholinesterase, BuChE, may also indicate likely cause of honeybee intoxication with insecticides with anticholinesterase action, this being a method both sensitive and of high fidelity.

Recently, Badiou et al. (2008) report that not only organophosphates and carbamates can be responsible for inhibition of AChE; after evidence found that there was a reduction in specific activity of AChE in honeybees killed by 25 ng of deltamethrin, as compared to control, suggesting that this was owing to the complex and indirect mode of action of this group of insecticides. Evaluating this enzyme in living honeybees, there was a significant increase in AChE activity. These authors demonstrated that AChE could be used as a biomarker in honeybees exposed to deltamethrin.

Further evaluation studies on the use of enzymes as biomarkers were carried out by Rabea et al. (2010), by exposing the honeybees to insect growth regulator and spinosad, and evaluating the AChE from the head, thorax and abdomen. The results reveal that spinosad, chlorfluazuron and oxymatrine inhibited AChE activity, regardless of its origin from the insect body. There was an increase in the degree of inhibition of the enzyme related to the doses of the insecticides used, reinforcing the use of AChE from honeybees for use in environmental quality assessment.

3 CARBOXYLESTERASE

3.1 General aspects

Carboxilesterase (CaE - EC 3.1.1.1), like AChE, are type B esterases, which catalyze the hydrolysis of either aliphatic or aromatic esters, except choline ester (Aldridge, 1953; Thompson, 1999). These enzymes, considered as of Phase 1, are responsible for hydrolysis of xenobiotic molecules such as insecticides, resulting into higher solubility in water, facilitate its excretion and consequently causing little toxicity (Dauterman & Hodgson, 1978):

$$\text{carboxylic ester} + H_2O \xrightleftharpoons{\text{CaE}} \text{alcohol} + \text{carboxylate}$$

In addition to its detoxification function and as found in the whole body, CaE can also be associated to digestive processes, embriogenic diapause and larval development, in the composition of the venom of some Hymenoptera and used as a polimorfism enzyme for the identification of honeybee species (Rockstein, 1978). According to Benton (1967), CaEs are highly active in honeybee venom, mainly with the substrates α and β naphthyl acetate and β naphthyl stearate, and can be possibly associated with the predigesting of preys and not in toxicological processes. In others cases, CaE may be used as polimorfic isozymes to distinguish the different species of the genus *Apis* (Nunamaker et al., 1984).

Tanabe et al. (1970) investigated the esterasic pattern in some hymenoptera species and observed that the eletrophoretic migration of enzymes in *A. mellifera* is faster than in *Apis indica japonica* (Radoszkowski, 1877), and that there are similarities among enzymatic patterns in *A. mellifera*, *Polistes chinensis antennalis* Perez, 1905 (Hymenoptera: Vespidae) and *Xylocopa appendiculata circumvolans* Smith, 1873 (Hymenoptera: Apidae). The lower esterasic activity was detected in *A. mellifera* males (haploids) when compared to females (diploids), possibly due to the partogenetic origin. By means of inhibition assays, it was found that diisopropyl fluorophosphate and eserine have high inhibitory capacity of CaE either in *A. mellifera* or in *A. indica japonica*.

According to Tripathi & Dixon (1968) the amounts of esterases, among all enzymes present in *A. mellifera* haemolymph, show a reduction during larval development, its induction and activity being related to the diet and presence of hormones. Gilliam & Jackson (1972a, 1972b) detected the presence of several enzymes in *A. mellifera* haemolymph, along with CaE. In evaluating the enzymatic pattern during the honeybee's developmental phases, it was found that CaE have activity alterations among the phases of egg, larva, pupa and

newly-emerged adult, and that the patterns remained constant during adult life, even in honeybees with different activities in the hive.

The factors affecting honeybee development/biology led Bitondi & Mestriner (1983) to study the presence CaE in Africanized honeybees. They found six CaE isozymes, which differ not only by electrophoretic mobility, but also by substrate specificity, inhibitors, caste and development phases. From the six isozymes found, those named 1, 2 and 4 had no changes during *A. mellifera* ontogenic development, but variations were found in CaE 3, 5 and 6, with 3 highly active in the larval phase, probably related to genetic and/or owing to feeding. Isozymes 5 and 6 were more active in pupa and adult phases, enforcing the hypothesis that there are highly specialized enzymatic mechanisms which are activated at determined time and that can be suspended/deactivated after their expression. Another CaE, called esterase-1a, was identified by Ruvolo-Takasusuki et al. (1997) in the abdomen of Africanized drones, queens and egg-laying workers, suggesting that this enzyme may be associated to sexual maturation of these insects.

3.2 Pesticide effects

Among the several studies with the CaE, those related to the effects on insect resistance are more frequent. Yu et al. (1984) showed that homogenates from the midgut of *A. mellifera* has high activity of CaE; and after feeding them contaminated food with malathion at sublethal dose, the enzyme activity was reduced. *In vivo* and *in vitro* assays with CaE from *A. mellifera* were carried out by Belzunces et al. (1994), who observed the toxic effect of the transgenic products, wheat germ agglutinin (WGA) and bowman birk soybean trypsin inhibitor. Results indicated that these compounds slightly affected CaE activity in the *in vitro* assays using WGA; with no differences in enzymatic activity.

As CaE degrades the juvenile hormone, Figueiredo et al. (1996) studied the performance of these enzymes during the immature phases of Africanized honeybees (fifth instar larva, pre-pupa and pupa) after being exposed to dichlorvos. The electrophoresis studies showed that only one CaE was not inhibited by dichlorvos, and the other five were inhibited to different extents and phases of development of honeybees. The pre-pupas treated did not reach the subsequent phase, dying. These results show in some cases that insecticides have not only neurotoxic effect but can also act in enzymatic systems responsible for control in insect development.

3.3 Biomarkers studies

Hashimoto et al. (2003) studied the possibility of using CaEs as bioindicators when Africanized honeybees were exposed to several doses of thiamethoxam, either by topic application or ingestion of contaminated food. Regardless of the way of application, CaE 1, 2, 4 and 5 were inhibited, and the newly-emerged honeybees being the most sensitive to the insecticide, suggesting that new studies should be carried out to check the effect of this neonicotinoid on honeybees under field conditions.

Attencia et al. (2005) suggested the use of CaE 3 and 4 in environmental monitoring programs, when Africanized honeybees colonies were contaminated with methyl parathion and malathion. A reduction in the activity of these isoenzymes takes place soon after the early days of contamination, but later, an opposite effect was observed, with the increase in CaE enzymatic activities, probably in order to detoxify/degrade the molecule. After 21 days, regardless of the sublethal concentration used, the enzymatic activity returned to normal.

4 GLUTATHIONE S-TRANSFERASE

4.1 General aspects

Glutathione S-transferase (GST - EC 2.5.1.18) is a group of isoenzymes responsible mainly for the detoxification of several xenobiotics in insects. They are classified as of Phase II, can combine with a great variety of hydrophilic compounds (i.e. glutathione, glucose and amino acids), to xenobiotics or metabolites. Among catalysis functions, GST participates in the excretion processes of poor nutritional compounds produced by cellular metabolism, and in the transport of important lipophilic compounds (Fournier et al., 1992; Tu & Akgül, 2005). In animals, GST may still be classified as cytosolic isozymes or microssomal isozymes, and can be present in great amounts, representing over 10% of all proteins extracted from rat liver (Jakoby, 1985). GST using different pathways can eliminate the toxic oxygen produced in cells with aerobic metabolism (Barreiros et al., 2006).

Due to great losses of honeybee colonies, Papadopoulos et al. (2004a, 2004b) studied the physiological characteristics, sensitivity of inhibitors, substrate specificity and kinetic parameters of GST from *Apis mellifera macedonica* Ruttner, 1988. GST was obtained from cytosolic fractions of the whole body of adults (without sting and venal gland), larvae and pupae. The results showed the high affinity of GST to CDNB (1-chloro-2,4-dinitrobenzene), independent development phases when compared to other substrate, such as DCNB (1,2-dichloro-4 nitrobenzene) and TPBO (*trans*-4-phenyl-3buten-2-one). Only hematin inhibited GST activity in larva, pupa and adult phases, although the different inhibitors tested affected GST of adults. By chromatofocusing analysis with the pH range from 4 to 7, the honeybee crude extract of cytosolic fraction showed two GST isozymes, one alkaline (pI of 7.5) related to stress and another acid (pI of 4.58); however the acid form had high activity in opposition

to the alkaline in the phases of larva and pupa. In adults, the opposite was observed, with predomination of high activity of the alkaline isozyme. In stress assays caused by low temperatures, hunger and intoxication by insecticides, the isoenzymes react in an independent form, and most of time the alkaline form presented high activity since it is directly involved to stress protection.

Comparative studies by Diao et al. (2006) demonstrated that GST showed more activity in all the studied fractions (head, thorax, abdomen without midgut and midgut) of *A. mellifera* and *A. cerana*, preferentially in cytosol. The major activities in both species were obtained in crude extracts, being that GST activity in *A. mellifera* was higher than in the Asiatic species in all parts studied (head, thorax, abdomen without midgut and midgut). Also, distinct differences were observed during the developmental phases of these two species, and again the high activity was in *A. mellifera*. Comparing five different species, *A. mellifera carnica, A. mellifera ligustica, A. mellifera anatolica*, *A. mellifera caucasica* and *A. cerana cerana*, several differences were observed in parameters V_{max} and K_m, the major being by the affinity of CDNB to the Asiatic specie, reaching 4.85-fold in addition to the averages of the other species; however, the specific activity was 1.9-fold lower than the average of the remaining ones.

4.2 Detoxification studies

In preliminary studies with GST and aryl hydrocarbon hydroxylase of *A. mellifera* and *Varroa jacobsoni* (Oudermans) (Acari: Mesostigmata), Baars & Driessen (1984), found that both enzymes were active, with similar GST values for both species. Yu et al. (1984) also evaluated the activity of several enzymes in *A. mellifera* responsible for insecticide detoxification, and the GST extracted from midgut showed a high activity, mainly when the honeybees were contaminated with sublethal doses of permethrin.

An important characteristic of honeybees is their social organization, where each worker, is assigned to a specific function in a set time, known as laborial division. Based on this, Smirle & Winston (1988) studied the behavior of two detoxification enzymes, GST and mixed-function oxidase (MFO), from *A. mellifera* workers emergence until the beginning of foraging activities. The observations revealed that there is an indirect relation between age/activity and the amount of protein, so newly-emerged has higher amounts of protein and lower specific activity, in opposition to the old honeybees, with small amounts of protein but high specific activity. It is believed that this condition may be a strategic adaptation for honeybee's exploration of contaminated environment with pesticides. Diao et al. (2006) observed a similar pattern of GST activity in *A. mellifera* during 20 days after emergence, but on the 21st day, when foraging activities start for many honeybees, GST activity was almost three times higher.

Smirle & Robinson (1989) studied the hypothesis that GST activity is related to honeybee's age and behavior using an experimental hive with 2000 young workers, queen and frame. Under this condition, nurse and forager honeybees may have similar task. A group of honeybees at 7, 14 and 21 days old were later used as a GST source. Although with the same age, and even with different tasks, no difference was detected in GST activity among them in the period between 7 and 21 days; however, forager honeybees had higher activity of this enzyme when at 21 days, suggesting that the increase is related to search for food and not to age.

According to Smirle (1990), both GST and MFO are directly related to honeybee tolerance to insecticides. From the relation between doses of some insecticides and the enzymes responsible for the detoxification, it follows that by increasing the dose of diazinon and propoxur, for instance, there is an increase of these enzymes. However, an inverse situation was found with aldrin, when

both enzymes were inhibited with increasing dose. For carbaryl, no linear behavior was detected, but a difference among colonies was observed.

Smirle (1993) studied the activity of GST and MFO, taking into consideration the size of colony population and the larva/adult ratio. Studies on sister colonies demonstrated an inverse ratio between population and enzymatic activity, where the increase in population results into decrease of GST and MFO activity. As the ratio of the amount of larva/adult increased, high levels of enzymatic activity of both enzymes were observed; suggesting that in higher larva/adult ratio there is an adaptative process to protect the colony against possible xenobiotics. Nielsen et al. (2000) studied the effect of flumethrin in GST enzymatic activity and glutathione peroxidase in several developmental phases of *A. mellifera.* The presence of this insecticide/miticide used in the control of *Varroa* induced GST enzymatic activity in the phases of larva, pupa and adult, as compared to the control.

4.3 Oxidative stress

The majority of studies with GST in *Apis* is directly related to the ability of this enzyme to promote the detoxification. However, some researchers observed the activity of this enzyme is associated to honeybee reproduction, mainly due to the long sperm storage periods in the spermatheca. In this context, Weirich et al. (2002) evaluated the activity of some enzymes of oxidative stress, GST, CAT and SOD, extracted from several structures of *A. mellifera* body (Table 1). Results indicate higher GST activity in the ventriculi of all castes of *A. mellifera*, being 2-fold higher in workers that in virgin or mated queens. High activity was detected in thoracic muscles and spermatheca of mated queens, poorer activity was observed in haemolymph, regardless of the caste.

Collins et al. (2004) evaluated sperm storage and the antioxidative activity of enzymes in *A. mellifera*. In mated queens, the organs related to sperm

storage show from ten to twenty-fold GST transcripts as compared to virgin queens. In drones, tissues have high levels of GST of encoding transcripts.

5 CATALASE

5.1 General aspects

Catalase (CAT - EC 1.11.1.6) is a hydroperoxidase found mainly in the peroxissomes. The catalytic effect is made by converting hydrogen peroxide into water + oxygen:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Although insects have short life cycle when compared to vertebrates, they are deeply affected by the oxidative stress during the phases of development, reproduction and longevity (Korsloot et al., 2004). CAT is the major antioxidant defense found in peroxisomes, but it can also be related to the biosynthesis of compounds in insects used in their protection (Blum, 1978). During the acclimation processes, increased CAT activities were observed in *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) submitted to stress caused by reduction in temperature (Hoffmann, 1985).

According to Felton & Summers (1995), antioxidant enzymes like CAT are directly related to the detoxification by ROS produced by xenobiotics such as paraquat and several allelochemicals. In addition to this, with an overexpression of CAT and SOD, lifetime expectation of *D. melanogaster* can increase by 30%, reducing losses and physiological performances as a result of age.

Few studies are available with CAT from honeybees (Table 1). Jimenez & Gilliam (1996) quantified CAT and acyl-CoA oxidase in *A. mellifera* adults

with known age. They detected CAT in peroxisomes and microperoxisomes of homogenates from the midgut, directly related to adult's development and behavior. CAT specific activity increased with age and it was higher in foragers; as observed by Smirle & Winston (1988) for GST and MFO; on the other hand, midgut-extracted protein was low. A probable explanation to this phenomenum is that flying activities and age might have induced oxidative stress, increasing CAT, GST, SOD levels protecting cells from toxic products of aerobic metabolism associated to foraging and senescence. Felton & Summers (1995) reported that when high amounts of peroxides and free radicals are formed in tissues during insect flight, CAT, GST and SOD, high activities take place or in specialized mechanisms, like bioluminescence. They also found higher activity of these enzymes on winged adults as compared to their respective larval phases.

5.2 Oxidative stress

Weirich et al. (2002) evaluated the effect of CAT, GST and SOD in several honeybee tissues in order to check the action of these enzymes in sperm conservation processes during long periods in *A. mellifera* queens. They concluded that mated queens had high CAT and GST activity in spermatheca, as compared to virgin ones. However, higher CAT activity was found in semen and also in midguts of mated queens, workers and unmated queens. It was suggested that higher CAT activity in the midgut may be associated to a diet with high levels of H_2O_2, and in reproduction-related organs, the presence of CAT may be involved in semen protection against oxidative stress along queen's lifespan.

Using RT-PCR techniques, Collins et al. (2004) confirmed that mated queen's spermatheca have from 10 to 20 times as many CAT encoding-transcripts as compared to virgin ones, with higher levels with age; old queens reached up to 10 times more in virgin queens; also increasing these levels with age, with 10 times as much as in young queens. In drones, reproduction-related

tissues have high levels of CAT encoding-transcripts, suggesting that the presence of this enzyme is directly related to protectition/conservatition of semen during storage.

6 ALKALINE PHOSPHATASE

6.1 General aspects

Alkaline phosphatases (ALP - EC 3.1.3.1) are enzymes of the hydrolase group, responsible for the removal of the phosphate group from several types of substrates (nucleotides, proteins and alkaloids) in a process called dephosphorylation. ALPs are more active in alkaline pH, above eight, and are involved mainly in digestive processes, transport of metabolites and antioxidants, with their catalytic function involved with the participation of metallic ions (Coleman, 1992). In medicine, ALP activity is widely used as a marker in diagnostics of diseases in liver, bones and cancer (Posen, 1967; Lange et al., 1982).

The early studies with honeybee ALP were reported when these enzymes were extracted from *A. mellifera* venom (Benton, 1967) (Table 1), evidencing the presence of two forms of phosphatase, the alkaline and the acid, their presence probably not being related to toxicological functions.

With increasing interest in ALP functions, either in energetic metabolism or as infection and disease marker, Bounias (1978) conducted a detailed study of the physico-chemical characteristics of this enzyme extracted from the midgut of *A. mellifera*. It was observed that this enzyme was present in a main fraction and that their properties are quite stable from one colony to another of the same subspecies. According to Delage-Darchen et al. (1982), ALP is present in several honeybee species and in *A. mellifera* may be found in several glands, such as mandibular, hypopharyngeal, cephalic labial, and

thoracic labial; nevertheless its major expression is in midgut. Jimenez & Gilliam (1990) demonstrated that in the midgut of *A. mellifera*, ALP showed its activity in elongated microvilli of the striated border, and inside of microbodies.

6.2 Xenobiotics effects

A study related to the effect of a pirethroid in ALP was carried out by Bounias et al. (1985). After the intrathoracic application of 0.1pmol deltamethrin per honeybee, they observed that ALP enzymatic activity varied considerably, after an initial period of inhibition, followed by higher activity stimulation after 2 hours after application and, finally, a return to normal conditions of activity after 3 hours. Bounias et al. (1996) studied the toxic effect of cupric salts used to control *Varroa* in *A. mellifera* over ALP activity, observing a varied behavior in function of intoxication. In the case of cupric gluconate salts, ALP activity had biphasic effect, with maximum activity in the concentration of 1mM, but a reduction above this concentration. On the other hand, cupric sulfate salts induced only ALP increase in activity, with a maximum of its enzymatic expression at the concentration of 5mM. In cases of increase in enzymatic activity of ALP, it was suggested that colonies may be benefited in cases of contamination, probably by protection against xenobiotics. According to these authors, the mechanism involved in this response is unknow, probably being that ALP can modulate signaling routes mediated by anti-stress proteins.

7 PERSPECTIVES

The use of biochemical tools to evaluate the environmental impact of xenobiotics, mainly enzymatic studies, gains more space each day in the scientific context and international regulatory agencies, due to the easiness of implementation of the techniques, and can preview and minimize risks for man,

ecosystem and beneficial species like honeybees. Honeybees are exposed to several polluting agents, mainly pesticides in agricultural areas, turning them excellent bioindicator organisms of environmental quality. Additionally, the evaluation of these biomarkers may improve the management of honeybees in agroecosystems, increasing the agricultural and hive production and preserve this important pollinator.

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TABLE 1 Summary of the main studies of AChE, CaE, CAT, GST and ALP on *Apis* spp. honeybees.

Enzyme	Species	Source	Reference
AchE	*A. mellifera*	Head	Metcalf & March (1949)
AchE	*A. mellifera*	Head	Metcalf & March (1950)
AchE	*A. mellifera*	Head	Rockstein (1950)
AchE	*A. mellifera*	Head	Casida (1955)
AchE	*A. mellifera ligustica*	Head	Nazer et al. (1974)
AchE	*A. cerana indica*	Thorax	Kasturi Bai & Reddy (1977)
AchE	*A. mellifera*	Head	Barker et al. (1978)
AchE	*A. mellifera*	Head	Westlake et al. (1985)
AchE	*A. mellifera*	Head	Belzunces et al. (1988a)
AchE	*A. mellifera*	Head	Belzunces et al. (1988b)
AchE	*Apis* spp.	Head	Rahman et al. (1989)
AchE	*A. mellifera*	Head	Belzunces et al. (1990)
AchE	*A. mellifera*	Head	Belzunces & Colin (1991)
AchE	*A. mellifera*	Head	Belzunces et al. (1992)
AchE	*A. mellifera*	Head	Gauthier et al. (1992)
AchE	*A. mellifera*	Head	Stefanidou et al. (1996)
AchE	*A. mellifera*	Head	Belzunces & Debras (1997)
AchE	*A. mellifera*	Head	Polyzou et al. (1997)
BuChE	*A. mellifera*	Head	Stefanidou et al. (1998)
AChE and adenosine triphosphate	*A. mellifera*	Head	Bendahou et al. (1999)
AchE	*A. mellifera ligustica* *A. cerana cerana*	Head, thorax and abdomen	Zhang et al. (2005)
AchE	*A. mellifera*	Head	Badiou et al. (2007)

TABLE 1 Continued…

Enzyme	Species	Source	Reference
AchE	*A. mellifera*	Head	Badiou et al. (2008a)
AchE	*A. mellifera*	Head	Badiou et al. (2008b)
AchE	*A. mellifera*	Head	Badiou & Belzunces (2008)
AChE and adenosine triphosphate	*A. mellifera*	Head, thorax and abdomen	Rabea et al. (2010)
CaE and alkaline/acid phosphatase	*A. mellifera*	Venom	Benton (1967)
AChE, ALP, CaE, CAT and GST	*A. mellifera mellifera*	Head and midgut	Carvalho et al. (2010)
AChE, ALP, CaE, CAT and GST	*A. mellifera mellifera*	Head and midgut	Carvalho et al. (2010)
CaE	*A. mellifera*	-	Tripathi & Dixon (1968)
CaE	*A. mellifera* *A. indica japonica*	Whole body	Tanabe et al. (1970)
CaE, malate dehydrogenase, lactate dehydrogenase and α-glycerophosphate dehydrogenase	*A. mellifera*	Hemolymph	Gilliam & Jackson (1972a, 1972b)
CaE and malate dehydrogenase	*A. mellifera*	eggs	Nunamaker and Wilson (1981)
CaE	Africanized honeybee	Whole body	Bitondi & Mestriner (1983)
CaE and malate dehydrogenase	*Apis florea* *Apis dorsata* *Apis cerana*	-	Nunamaker et al. (1984)

TABLE 1 Continued…

Enzyme	Species	Source	Reference
CaE and trypsin-like protease	*A. mellifera*	Midgut	Belzunces et al. (1994)
CaE	Africanized honeybee	Whole larval body	Figueiredo et al. (1996)
CaE	Africanized honeybee	Several sources	Ruvolo-Takasusuki et al. (1997)
CaE	Africanized honeybee	Whole body	Hashimoto et al. (2003)
CaE	Africanized honeybee	Whole body	Attencia et al. (2005)
GST and aryl hydrocarbon hydrosylase	*A. mellifera*	Midgut	Baars et al. (1984)
Microsomal oxidades, GST, CaE, epoxide hydrolase and DDT-dehydrochlorinase	*A. mellifera*	Head and midgut	Yu et al. (1984)
GST and mixed-function oxidase	*A. mellifera*	Midgut	Smirle & Winston (1988)
GST	*A. mellifera*	Midgut	Smirle & Robinson (1989)
GST and mixed-function oxidase	*A. mellifera*	Midgut	Smirle (1990)
GST and mixed-function oxidase	*A. mellifera*	Midgut	Smirle (1993)
GST and gluthathione peroxidase	*A. mellifera*	Whole body	Nielsen et al. (2000)
GST	*A. mellifera macedonica*	Whole body	Papadopoulos et al. (2004a)
GST	*A. mellifera macedonica*	Whole body	Papadopoulos et al. (2004b)

TABLE 1 Continued…

Enzyme	Species	Source	Reference
GST	*A. mellifera anatolica* *A. mellifera carnica* *A. mellifera caucasica* *A. mellifera ligustica* *A. cerana cerana*	Head, thorax and abdomen	Diao et al. (2006)
ALP	*A. mellifera*	Midgut	Bounias (1978)
ALP and others	*A. mellifera*	Glands and midgut	Delage-Darchen et al. (1982)
ALP	*A. mellifera*	Midgut	Bounias et al. (1985)
Alkaline and acid phosphatase	*A. mellifera*	Midgut	Bounias et al. (1996)
CAT and acyl-CoA oxidase	*A. mellifera*	Midgut	Jimenez & Gilliam (1996)
CAT, GST and SOD	*A. mellifera*	Several sources	Weirich et al. (2002)
CAT, GST and SOD	*A. mellifera ligustica*	Several sources	Collins et al. (2004)

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CHAPTER 2

DIFFERENTIAL PROFILE OF SOME ENZYMES FROM *Apis mellifera mellifera* L., 1758 FOR THEIR FUTURE USE IN ENVIRONMENTAL QUALITY ASSESSMENTS

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ABSTRACT

Characteristics such as extraction, activity, variability and stability during storage of enzymes from crude extracts of *Apis mellifera mellifera* L., 1758 head (acetyl cholinesterase - AChE and carboxylesterases - CaE-1, CaE-2 and CaE-3) and midgut (glutathione *S*-transferase - GST, alkaline phosphatase - ALP and catalase - CAT) were studied. This information will be useful for future use of these biochemical tools in environmental quality assessment programs. High yield of extraction was obtained in only one step of extraction, with results of 65.04, 82.77, 83.47, 76.55, 80.22, 99.88 and 88.57% of total activity, for AChE, CaE-1, CaE-2, CaE-3, CAT, GST and ALP, respectively. The specific activity (SA) and tissue activity (TA) of enzymes showed the same patterns, whereas the high activity found may be due to enzymes synthesized or stored in the tissue (head or midgut) used as sources. These enzymes showed different patterns in SA after the storage at -80^{o}C. AChE, CaE-2 and CaE-3 were show to be stable after 60 days' storage, although small variations were found over the time, without important losses in their activity. The SA of CaE-1 varied during the storage period, but it was possible to find activity at the last assessment (day 60). ALP showed slight and gradual loss in SA during the storage period. Interestingly, CAT and GST had different patterns, whereas CAT decreased the SA from the fresh extract as compared with that stored for 3 days, at the rate of 36.87%, and GST increased the SA in the same period at 90.36%. Both CAT and GST had the same behavior and stability from assessment from the third to the last day of storage.

Keywords: Honeybee; biomarker; acetylcholinesterase; carboxyl esterase; glutathione S-transferase; catalase; alkaline phosphatase.

1 INTRODUCTION*

The presence of a xenobiotic in the environment may be a risk. A complementary approach to detecting exposure to xenobiotics and their potential impact on living organisms is monitoring by using the honeybees as bioindicators and their enzymes as biomarkers. The use of honeybees *Apis mellifera mellifera* L., 1758 (Hymenoptera: Apidae) as model insects for the development of biomarkers on terrestrial environments is interesting, since they have intense foraging activity, which brings them into contact with a variety of pollutants (Cluzeau, 2002; Leita et al., 2004). The activity of honeybees benefits both economy and the environment for their highly valued products (honey, wax, royal jelly, pollen and propolis). For the maintenance of biodiversity, since they are responsible for 80% of the pollination of all botanical species and for the increase of plant production (Klein et al., 2007). The worldwide value of pollination reaches €153 billion at agronomic level, which in 2005, accounted for 9.5% of the total value of world's agricultural production used for human food (Gallai et al., 2009).

The most recent definition of biomarker is "an observable and/or measurable change at the molecular, biochemical, cellular, physiological or behavioral level, which reveals the present or past exposure of an individual to, at least, one chemical substance of polluting character" (Lagadic et al., 1997). According to Depledge & Fossi (1994), ecotoxicology studies should provide information related to toxicity of xenobiotics on the biota, besides preventing the origins/destination and concentration of a particular molecule in the environment. Thus, biomarkers have high relevance for international regulatory agencies to register exposure/effect of environmental pollutants and

* Enzymes abbreviations: AChE = acetyl cholinesterase (EC 3.1.1.7); CAT = catalase (EC 1.11.1.6); ALP = alkaline phosphatase (3.1.3.1); GST = glutathione S-transferase (EC 2.5.1.18); CaE-1, CaE-2 and CaE-3 = carboxylesterases (3.1.1.1)

invertebrates like honeybees, important study models since they represent organisms that explore several trophic levels.

The use of honeybees in environmental assessment represents a engagement between the requirements of scientific research and its feasibility. According to Celli & Maccagnani (2003), honeybees act on the detection of environmental pollution in two ways: (i) by their death resulting from the effect of toxic molecules, and (ii) through residues of chemical compounds found in colony products or in their bodies. Thus, honeybee becomes an excellent model of evaluation of environmental quality, indirectly contributing towards studies of xenobiotics in the environment. The first report of using honeybees as bioindicators goes back to 1935, in Czechoslovakia, where Svoboda used this species to evaluate environmental quality in areas of high population density and industrial activities (Porrini et al., 2002). Some studies have dealt with biomarkers in the honeybee (Stefanidou et al., 1996; Hashimoto et al., 2003; Badiou et al., 2008), because they can translate information on environmental health in relation to the agricultural practice used.

Betwwen analytical and molecular techniques to evaluate the effects of residues of xenobiotics in *A. mellifera*, both biochemical and enzymatic are fundamentals. The study of enzyme patterns considered as of exposition (i.e. GST, AChE and CaE) can indicate the presence of some kind of insecticide in honeybee bodies (Hyne & Maher, 2003). From those enzymes, the esterase group, including AChE and CaE, is used as a pattern in studies of environmental contamination by xenobiotics, mainly by organophosphates and carbamates (Thompson, 1999). Others, like morphological and immune-histochemical techniques, are used to evaluate the effect of insecticides on honeybees' silk glands, Malpighian tubules and guts, using markers as heat shock protein and cell death (Malaspina & Silva-Zacarin, 2006).

Gilbert & Wilkinson (1974) and Yu et al. (1984) evidenced the presence and activity of several enzymes responsible by the detoxification system, like epoxidase, hydrolase, *O*-demethylase, GST and CaE. After honeybee genome sequencing, Claudianos et al. (2006) suggested that the lack in detoxification enzymes may be related to susceptibility to insecticides, when compared to *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) and *Anopheles gambie* Giles, 1926 (Diptera: Culicidae) which have higher amounts of enzymes of the GST, P450 and CaE families.

A series of enzymes from *A. mellifera* were tested in this study to investigate their potential use as biomarkers in the future. AChE is an enzyme of the nervous system which is responsible for hydrolysis of acetylcholine in cholinergic synapses and is used as a biomarker when it is inhibited/stimulated by several insecticides (Stefanidou et al., 1996; Badiou et al., 2008). GST and CaE can play an active role in detoxification of endogenous/exogenous compounds, inducible by different chemicals. CaE are phase I enzyme, which reacts with non-polar compounds through hydrolysis and protect organisms against pesticides. Moreover, GSTs also are important in protecting the tissues from oxidative stress acting in diferents routes (Bitondi & Mestriner, 1983; Maxwell, 1992; Hyne & Maher, 2003; Papadopoulos et al., 2004a). CAT is a peroxisomal hydroperoxidase that catalyses the conversion of hydrogen peroxide into oxygen and water, responsible for protection of insect tissue against the oxidative process which occurs in oxygen reactive species (Felton & Summers, 1995; Jimenez & Gilliam, 1996). ALP is a group of isozymes responsible for hydrolysis in digestive processes, transport of metabolites, being that their catalytic function involves the participation of metallic ions (Bounias et al., 1996; Coleman, 1992).

Due the importance of *A. mellifera* in the socio-economic context and the potential use in their enzymatic systems in environmental quality

assessment, this work studied the characteristics of extraction, activity, variability and storage of the enzymes AChE and CaE obtained from heads of honeybees, and CAT, GST and ALP from midguts.

2 MATERIAL AND METHODS

2.1 Chemicals

Antipain; aprotinin; leupeptin; pepstatin A; trypsin inhibitor; sodium phosphate monobasic; sodium chloride (NaCl); Triton® X-100; acetylthiocholine iodide (AcSCH.I); 5,5'dithio-bis(2,nitrobenzoic acid) (DTNB); sodium bicarbonate; α and β naphthyl acetate (α-NA or β-NA); *p*-nytrophenyl acetate (*p*-NPA); 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51); Fast Garnet GBC; sodium dodecyl sulfate (SDS); hydrogen peroxide (H_2O_2); sodium phosphate dibasic; potassium phosphate monobasic; ethylenediaminetetra-acetic acid (EDTA); 1-chloro-2,4-dinitrobenzene (CDNB); L-glutathione reduced (GSH); acetonitril; acetone; Trizma® base (Tris); hydrochloride acid (HCl); magnesium chloride ($MgCl_2$); bovine serum albumin (BSA) and *p*-nitrophenyl phosphate (*p*-NPP) were obtained from Sigma Aldrich (France).

2.2 Honeybees

Forager honeybees *Apis mellifera mellifera* L., 1758 were collected in June/July, 2008 directly from a single colony in UMR406 Abeilles et Environnement, Avignon, France. The maintenance in laboratory was in an experimental cage (Pain, 1969), feeding *ad libitum* with Candy paste at 28±2^{o}C, 60±10% RH, and 12 hours' photophase.

2.3 Samples preparation

2.3.1 Heads

Honeybees were previously anesthetized at -5°C and fresh heads obtained by cutting them off with a scalpel. To isolate the AChE and CaE, the weight of three heads was obtained in previously weighted microtube and the correspondent volume of 10% (w/v) extraction solution added. The extraction solution medium was phosphate buffer 40 mM pH 7.4; 10 mM NaCl; 1% Triton® X-100 and 2 µL/mL of a mixture of the protease inhibitors antipain, aprotinin, leupeptin, pepstatin A, and trypsin inhibitor in equal parts (Belzunces et al., 1988a). The homogenization of the heads was made by using the high speed shaker TissueLyser II (Qiagen®) for 90 seconds, and afterwards, the samples were centrifuged at 13.000 *g* for 20 minutes. The supernatant was recovered for use right before enzymatic essays. All procedures were carried out at 4°C.

2.3.2 Midguts

Midguts were obtained by pulling stings off from anesthetized honeybees at -5°C, and likewise for heads, weights determined in microtubes and a volume of 10% (w/v) extracting solution was added. This solution contained 40 mM phosphate buffer pH 7.4; 10 mM NaCl and 2 µL/mL of a mixture (equal parts) of the protease inhibitor antipain; aprotinin; leupeptin; pepstatin A and trypsin inhibitor. After homogenization of the samples with the high speed shaker TissueLyser for 90 seconds, microtubes were centrifuged at 13.000 *g* for 20 minutes and the supernatant recovered for biochemical analysis. All procedures were carried out at 4°C.

2.3.3 Protein determination

Protein concentrations were determined following the method of Bradford (1976) with a Bio-Rad Protein Assays kit (Bio-Rad Laboratories, France), and BSA as a standard.

2.4 Extraction, specific/tissue activity and variability

Fresh extracts from 45 honeybees were prepared to determine the extraction efficiency. Inside each microtube (total of 15), three heads or midguts were placed as reported in section 2.3. After the first stage of extraction, the supernatant obtained was recovered and a new homogenization performed, all supernatants being stored individualy. Only two stages of extraction were needed to homogenize the midgut, due its being a very soft tissue, when compared with the head, a very sclerotized structure, hard to be homogenized three steps being needed to promote the total homogenization. Extracts were analyzed singly and in triplicate, with the total activity (100%) calculated by the sum of each activity from the same microtube. Enzyme activity was expressed in percentage of each extraction step.

The specific and tissue activity for each enzyme was determined along of this essay, using the first extraction from the fresh extract of head and midgut, respectively. Also, the variability among each microtube from the first extraction was determined.

2.5 Storage stability essay

The enzymatic stability after storage was evaluated during the period from fresh to 60 days, in both head and midgut extracts from honeybees. Two pools, one of each extract, was prepared from 72 heads or midguts as in section 2.3, with the total volume divided and distributed in equal parts in 32 microtubes. Four microtubes were used in the dosage assay with fresh extract

(right after preparation of extract), and the other 28 stored at -80°C. Different times, fresh extracts, 3, 7, 14, 21, 30, 45 and 60 days were used to determine de stability of theses enzymes under storage conditions at -80°C. Four microtubes were used for each dosage, both in triplicate readings. Results were expressed in specific activity for each enzyme.

2.6 Enzymatic procedures

Analytical procedures were performed using Varian® Cary 1E UV-Vis spectrophotometer at 25±0.5°C. In the visible spectrum, disposable semi-micro cuvettes in polyfoam magazine (Ratiolab®, Germany), and for the UV spectrum, semi-micro UV-Disposable cuvette (Plastibrand®, Germany) were used. All enzyme samples were assayed in 1mL of final volume and read in triplicate. Specific activity was expressed in nanomoles of hydrolyzed substrate per milligram of protein/minute (except for CAT).

2.6.1 Acetylcholinesterase

AChE activity was determinated according to the method of Ellman et al. (1961) at 412 nm. The reaction was carried out in 100 mM of phosphate buffer at pH 7.0; 1.5 mM of DTNB; 0,3 mM of AcSChI and 20µL of the extract from the heads.

2.6.2 Inhibition of acetylcholinesterase by BW284C51

To prevent competition between AChE and CaE for the same substrate, the true inhibitor of AChE, BW284C51 (Austin & Berry, 1953) was used. The ideal concentration of this inhibitor to use in CaE measurement was determined on incubated AChE in the assay medium in a range of 10^{-10} to 10^{-4} mM of BW284C51 for 20 minutes at 25±0.5°C in darkness. The AChE activity was

determined as in section 2.6.1 and data expressed as percentage of control activity.

2.6.3 Carboxylesterase

Three CaE isozymes were evaluated, each with their substrate specificity: α-NA (CaE-1), β-NA (CaE-2) and *p*-NPA (CaE-3). All procedures were carried out according to Gomori (1953) and Asperen (1962). The inhibition of AChE from head extract (5μL for α-NA/ β-NA and 10μL for *p*-NPA) by 10^{-4} mM BW284C511 were carried out in phosphate buffer 100 mM pH 7.0; 0.005% Triton X-100 and distilled water for 20 minutes at 25±0.5°C in darkness (see section 2.4.2) After this time, 0.4 mM of one of each substrate in alcohol solution was added and the reaction developed during 3 minutes, its being interrupted when 1.5 % of SDS + 0.4 mg/mL of Fast Garnet GBC were added (except for *p*-NPA substrate, because the reaction was stopped only with 1.5 % of SDS) and the absorbance read immediately at 568 nm for α-NA; 515 nm for β-NA and 410 nm for *p*-NPA.

2.6.4 Alkaline phosphatase

ALP activities were performed by following the methods of Bessey et al. (1946) and incorporated for honeybees by Bounias et al. (1996). The reaction took place 100 mM Tris-HCl buffer pH 8.5; 2 mM of *p*-NPP; 20 μM $MgCl_2$ and 25 μL of extract from midgut, read at 410 nm.

2.6.5 Catalase

The breakdown of H_2O_2 by CAT was carried out according to Beers & Sizer (1951), with the medium assay made with 100 mM phosphate buffer at pH 7.0, 10 mM of H_2O_2 and 25 μL of extract from midgut. The reaction at 240 nm

was expressed as the rate of decomposition of H_2O_2 (m*k*/sec/mg protein), following the techniques of Cohen (1970) and Aebi (1984).

2.6.6 Glutathione S-transferase

GST reaction was made according to Habig et al. (1974), and the reaction at 340 nm was: 100 mM of sodium/potassium phosphate buffer at pH 7.4; 1 mM of EDTA, 2.5 mM of GSH, 1 mM of CDNB (diluted in acetonitrile: water, 3:7) and 25 µL of crude extract from midgut.

2.7 Data analysis

All procedures were performed using the statistical software R® (2009), calculating the mean and the standard error (S.E.) for sum of efficiency extraction and specific activity for each enzyme. Data from inhibition of AChE by BW284C51 was fitted with the logistic model from the "drc" package (Analysis of dose-response curves) (Ritz & Streibig, 2005). One-way analysis of variance (ANOVA) was performed with data from enzyme variability, and mean confidence limits at 95% calculated.

3 RESULTS

3.1 Inhibition of acetylcholinesterase by BW284C51

It was found through the AChE essay with the inhibitor BW284C51 at 10^{-10} M of concentration minimally affected enzymatic activity and at 10^{-04} M inhibited almost all AChE present in head extract of *A. mellifera*. The value of the inhibitor concentration of 50% (IC_{50}) was $7.30e^{-7}$ M with confidence limits (95%) from $6.02e^{-7}$ to $8.57e^{-7}$ M (Figure 1).

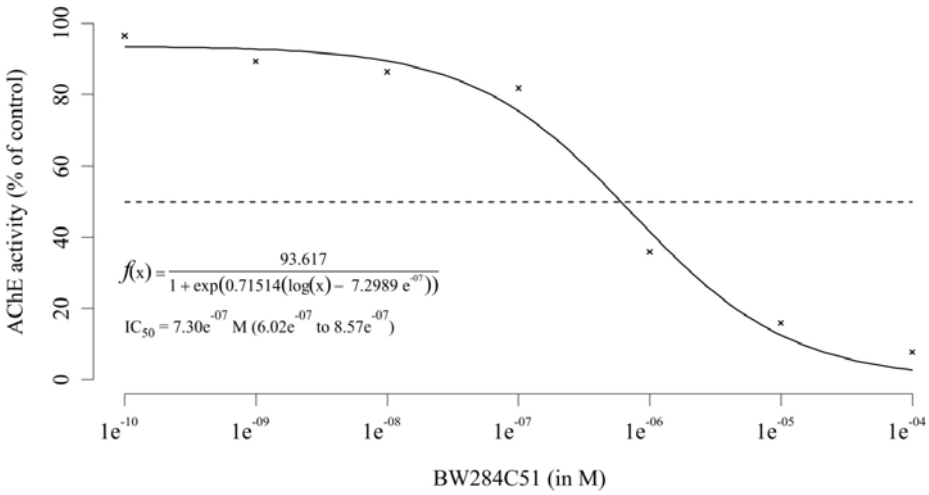

FIGURE 1 Percentage of honeybee AChE activity after incubation with several concentrations of the true acetylcholinesterase inhibitor BW284C51.

3.2 Extraction, specific/tissue activity and variability

A third extraction step was needed to remove all enzymes from the head, but only two for enzymes in the midgut. Of all enzymes evaluated, AChE showed the lowest values in extraction at the first step with average of 65.04%. At the second and third steps, a remaining activity for AChE of 25.20 and 9.76%, respectively was observed. As for CaE-1, CaE-2 and CaE-3, high extraction efficiency was obtained immediately at the first step, with averages of 82.77, 83.47 and 76.55%, respectively. At the remaining second and third steps, the averages were: CaE-1, of 14.75 and 2.49%; CaE-2, 15.98 and 0.55% and for CaE-3, 17.88 and 5.56%, respectively. Among cytosolic enzymes, higher extraction efficiency was observed for GST, with 99.88% extraction at the first step, and the remaining 0.12% for the second step. High efficiency was also found in ALP, with average activities of 88.57 and 11.43% at the first and second steps, respectively. As for CAT, medium activity was of 80.22% at the first and 19.78% for the second step (Table 1 and Figure 2).

TABLE 1 Percentage of enzymes extracted after two (midgut) or three (head) steps of homogenization (means ± S.E.; n=45).

Enzyme	Tissue	Sum of percentual extraction		
		First	Second	Third
AChE	Head	65.04 ± 0.26	25.20 ± 0.30	9.76 ± 0.04
CaE-1		82.77 ± 0.23	14.75 ± 0.16	2.49 ± 0.12
CaE-2		83.47 ± 0.34	15.98 ± 0.31	0.55 ± 0.16
CaE-3		76.55 ± 0.46	17.88 ± 0.36	5.56 ± 0.21
CAT	Midgut	80.22 ± 0.89	19.78 ± 0.89	not performed
GST		99.88 ± 0.08	0.12 ± 0.08	
ALP		88.57 ± 0.81	11.43 ± 0.81	

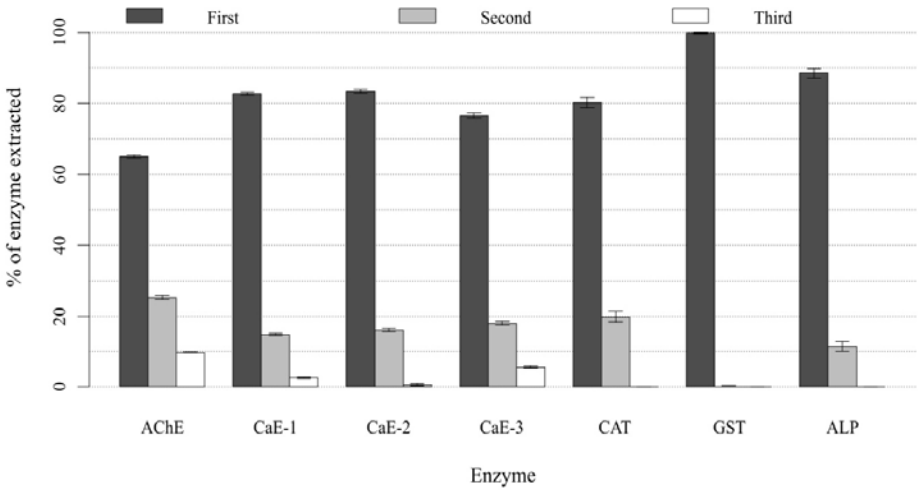

FIGURE 2 Percentage of enzyme extracted after three (head – AChE and CaE) or two (midgut – CAT, GST and ALP) sequential steps (mean ± S.E.; n=45).

Increased enzymatic activities were found in the specific (SA) when compared to tissue (TA) activity (Table 2). For the enzymes from the head of *A. mellifera*, SA of AChE was 22.20 nmol/min/mg of protein and TA of 6.54 nmol/min/mg of tissue, and 3.39 being the ratio between the two. For CaE, higher activities were found for CaE-2, followed by CaE-3 and CaE-1, with SA averages of 61.09, 42.40 and 39.69 nmol/min/mg and TA of 18.12, 12.58 and

11.78 nmol/min/mg of tissue of protein, respectively. For midgut enzymes, GST exhibited SA of 321.68 nmol/min/mg of protein and TA of 20.80 nmol/min/mg of tissue. In ALP, the SA was 7.69 nmol/min/mg of protein and TA of 0.50 nmol/min/mg of tissue. The CAT SA was 49.87 m*k*/sec/mg of protein and TA of 2.97 m*k*/sec/mg of tissue (Table 2).

TABLE 2 Specific/tissue activity and the ratio between enzyme activities (mean ± S.E.; n=45).

Enzyme	Substrate	Specific activity (SA) nmol/min/mg of protein	Tissue activity (TA) nmol/min/mg of tissue	Relation SA/TA
AChE	AcSCH.I	22.20 ± 0.55	6.54 ± 0.25	3.39
CaE-1	α-NA	39.69 ± 0.80	11.78 ± 0.41	3.37
CaE-2	β-NA	61.09 ± 1.06	18.12 ± 0.72	3.37
CaE-3	*p*-NPA	42.40 ± 0.98	12.58 ± 0.83	3.37
GST	CDNB	321.68 ± 10.10	20.80 ± 1.04	15.47
ALP	*p*-NPP	7.69 ± 0.21	0.50 ± 0.04	15.38
		m*k*/sec/mg of protein	m*k*/sec/mg of tissue	
CAT	H_2O_2	49.87 ± 1.65	2.97 ± 0.25	16.79

There was a pattern in SA and TA ratio of enzymes from head and midgut of *A. mellifera*. The results showed greater quantities of protein in midgut when compared with head and the higher SA found for the different enzymes in the two tissues studied suggests that each enzyme exists predominantly in the tissue from which it is derived or showed high functionality (Table 2).

By the analysis of Figures 3 and 4, one can see that there were variations among the different samples analyzed; each one prepared using three honeybees, amounting to 45. SA of AChE ranged from 17.34 to 31.89 nmol/min/mg, with average of 22.20 nmol/min/mg of protein, with different averages among samples ($P<2.20e^{-16}$). As for CaE, SA varied from 31.16 to 53.99 nmol/min/mg for CaE-1, average of 39.69 nmol/min/mg ($P<7.35e^{-15}$); 48.44 to 81.03

nmol/min/mg and average of 61.09 nmol/min/mg for CaE-2 ($P<4.81e^{-11}$), and from 29.70 a 61.56 nmol/min/mg and average of 42.40 nmol/min/mg of protein for CaE-3 ($P<4.15e^{-9}$) (Table 2). For midgut enzymes, SA varied from 29.36 to 71.44 m*k*/sec/mg and average of 49.87 m*k*/sec/mg of protein ($P<6.90e^{-10}$) for CAT. The average SA of GST was 321.68 nmol/min/mg, with values varying from 238.85 to 439.67 nmol/min/mg of protein ($P<6.27e^{-15}$). Yet for ALP, the lowest SA was 5.54 nmol/min/mg and the highest 12.08 nmol/min/mg with average of 7.69 nmol/min/mg of protein ($P<1.43e^{-7}$) (Table 2).

3.3 Storage stability

Figures 5 and 6 showed stability of enzyme fresh extracts up to 60 days, during storage at -80°C. Among the enzymes from head, the greatest variation was found in CaE-1, but even at 60 days, it was possible to find activity at the end of the period. Variation in AChE is lowest when compared to CaE-1, and may be considered stable (Figures 5A and 5B). For CaE-2 and CaE-3, slight variation was found however the SA of these enzymes was stable until day 60 (Figures 5C and 5D).

When enzymes from midgut were analyzed, ALP showed a slight decrease in SA over the time, the activity varying from 6.10 nmol/min/mg in fresh extract to 5.27 nmol/min/mg of protein at the last evaluation (60 days) (Figure 6A). For CAT and GST, different patterns were observed, and highest losses were detected as compared to ALP. With CAT, SA losses of 36.87% from initial fresh extract (87.57 m*k*/sec/mg of protein) at the third day of storage (55.28 m*k*/sec/mg of protein); however, SA remained stable up to 60 days (Figure 6B). Contrary to CAT, GST activity increased in comparison to the fresh and frozen extracts. After 3 days of storage at -80°C, SA was 344.36 nmol/min/mg as compared to 180.90 nmol/min/mg of protein for the fresh

extract, with an increase of 90.36%. The SA of GST was stable after the third day in other assessments at the end of the experiment (Figure 6C).

4 DISCUSSION

4.1 Inhibition of acetylcholinesterase

Results demonstrated that these enzymes, present in the *A. mellifera mellifera* head, are true cholinesterases or acetylcholinesterases, acting in rapid hydrolysis of acetylcholine at the cholinergic synapses. According to Austin & Berry (1953), the use of cholinesterase inhibitors is important when studies of this group of enzymes are carried out, BW284C51 being a reversible inhibitor, selective and inhibiting only AChE. The use of this inhibitor was recommended by Dary et al. (1990) to prevent competition between AChE and CaE for the same substrate in studies for determination of the general activity of CaE in insects. The btained results agree with those reported by Belzunces et al. (1988a), who classified *A. mellifera* AChE as true. Studying *A. mellifera* queens, Polyzou et al. (1997) verified that AChE (membrane and soluble) obtained from several pupal stages are true cholinesterases, inhibited with 10^{-5} M of BW284C51. Similar results were reported by Badiou et al. (2007), where both honeybee AChE membrane forms were completely inhibited by using BW284C51 at 10^{-5} M; even though not affected by iso-OMPA (tetra-isopropyl pyrophosphortetramide), a specific inhibitor of pseudo cholinesterase (butyrylcholinesterase).

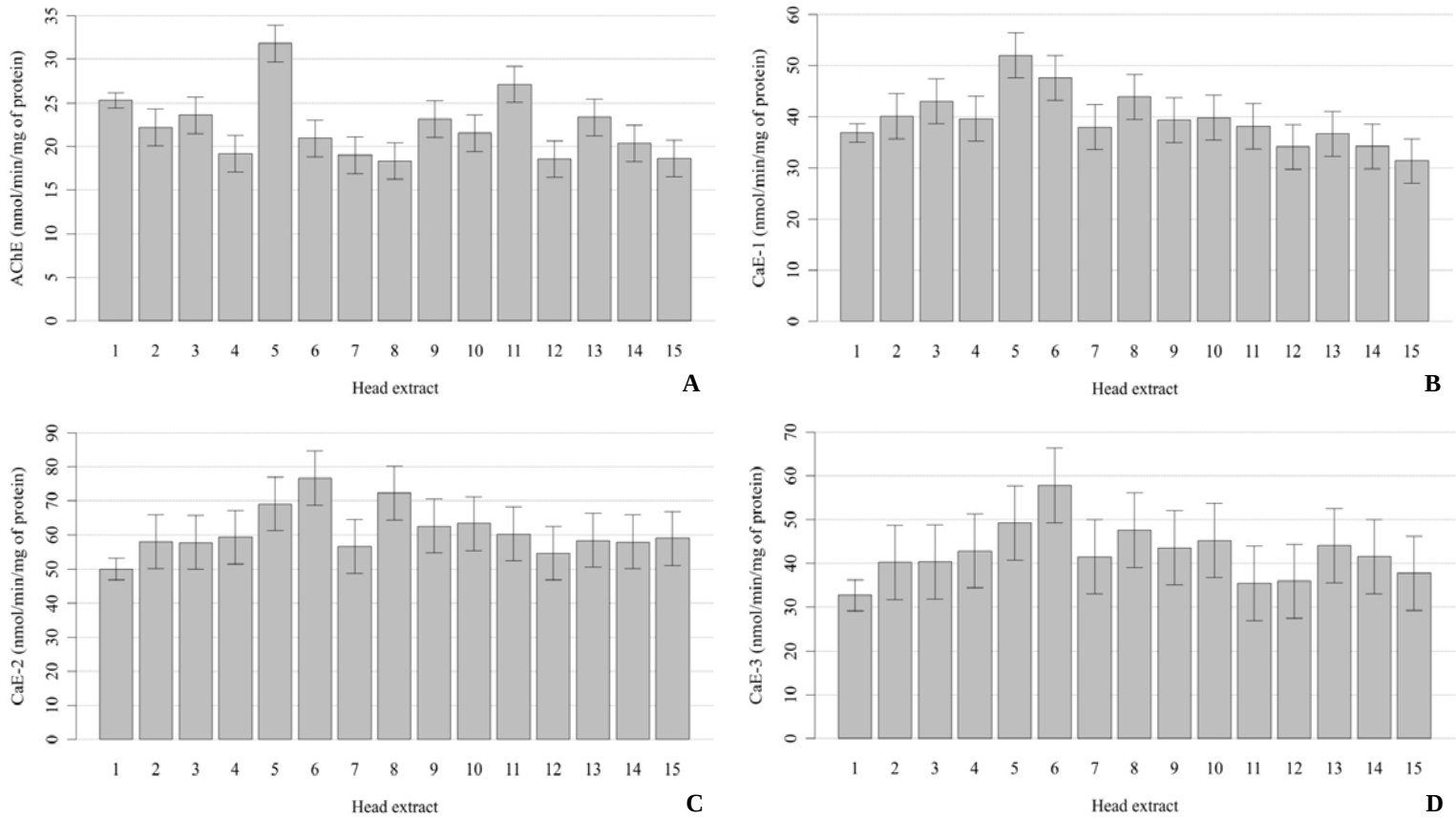

FIGURE 3 Variability of acetylcholinesterase and three carboxyl esterase among each sample (n=45; three heads per sample; ± $CL_{95\%}$).

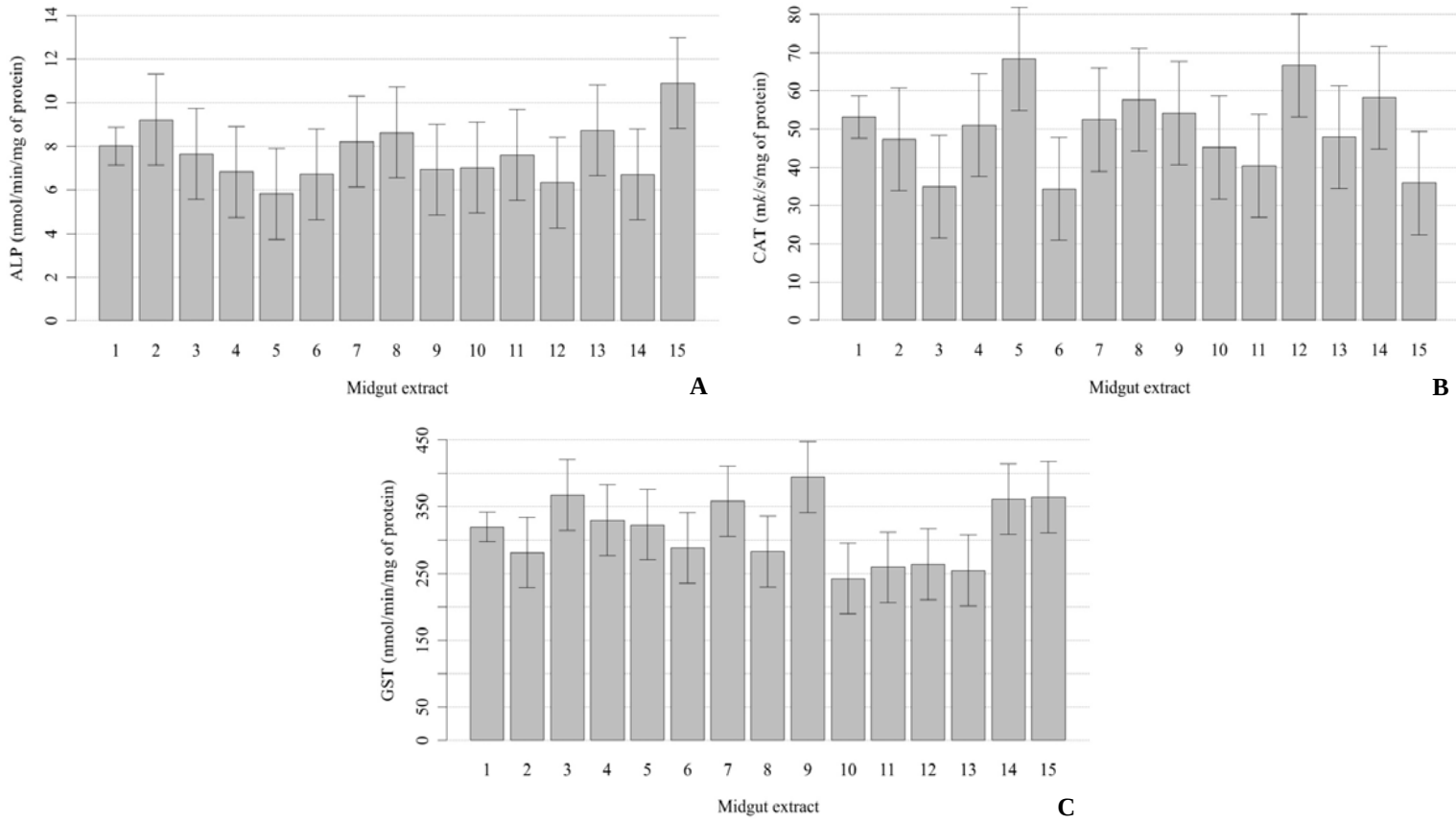

FIGURE 4 Variability of catalase, glutathione S-transferase and alkaline phosphatase among each sample (n=45; three midguts per sample; ± $CL_{95\%}$).

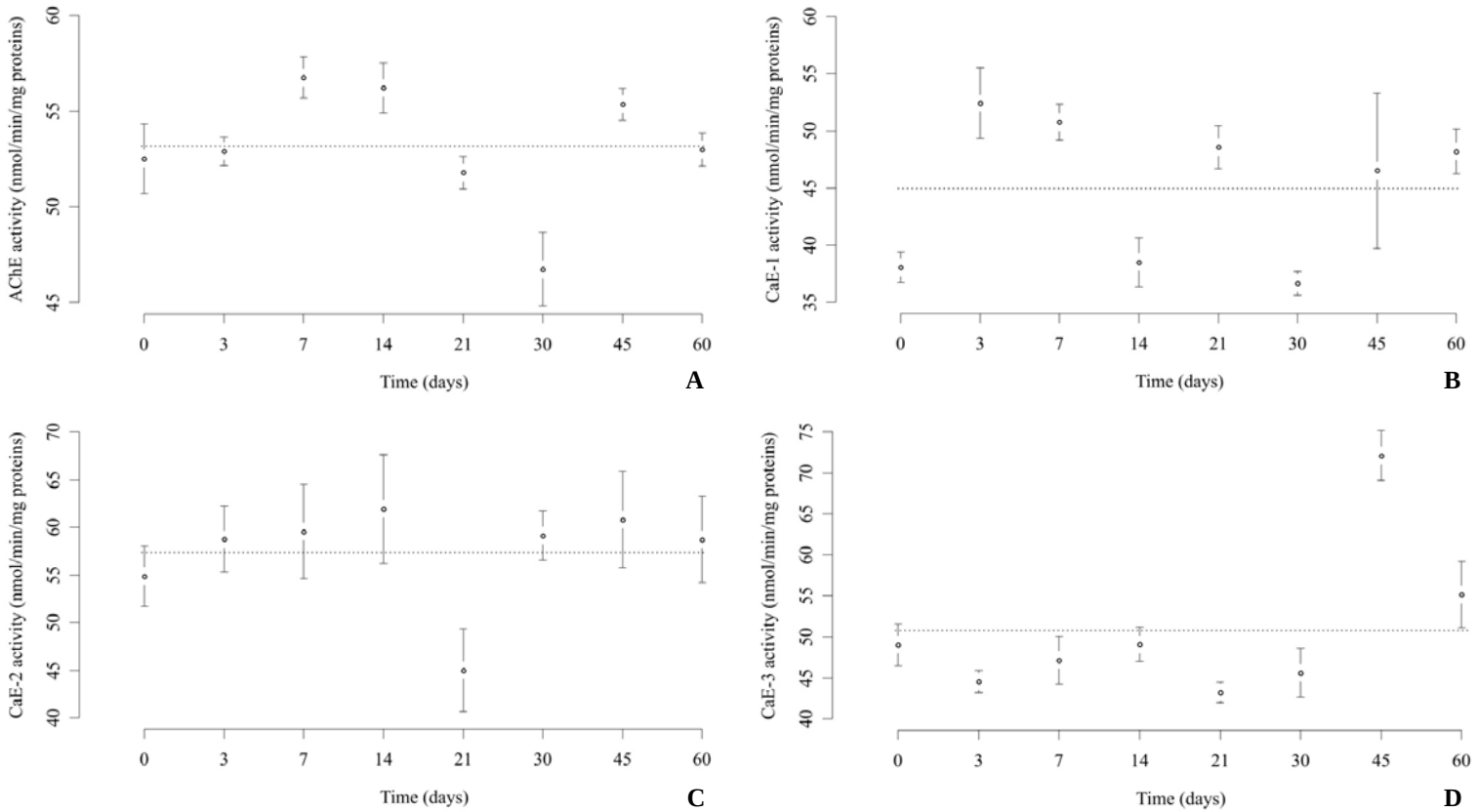

FIGURE 5 Enzyme patterns of AChE and CaE (head extract) during the storage period of fresh extract up to 60 days at -80^{o}C.

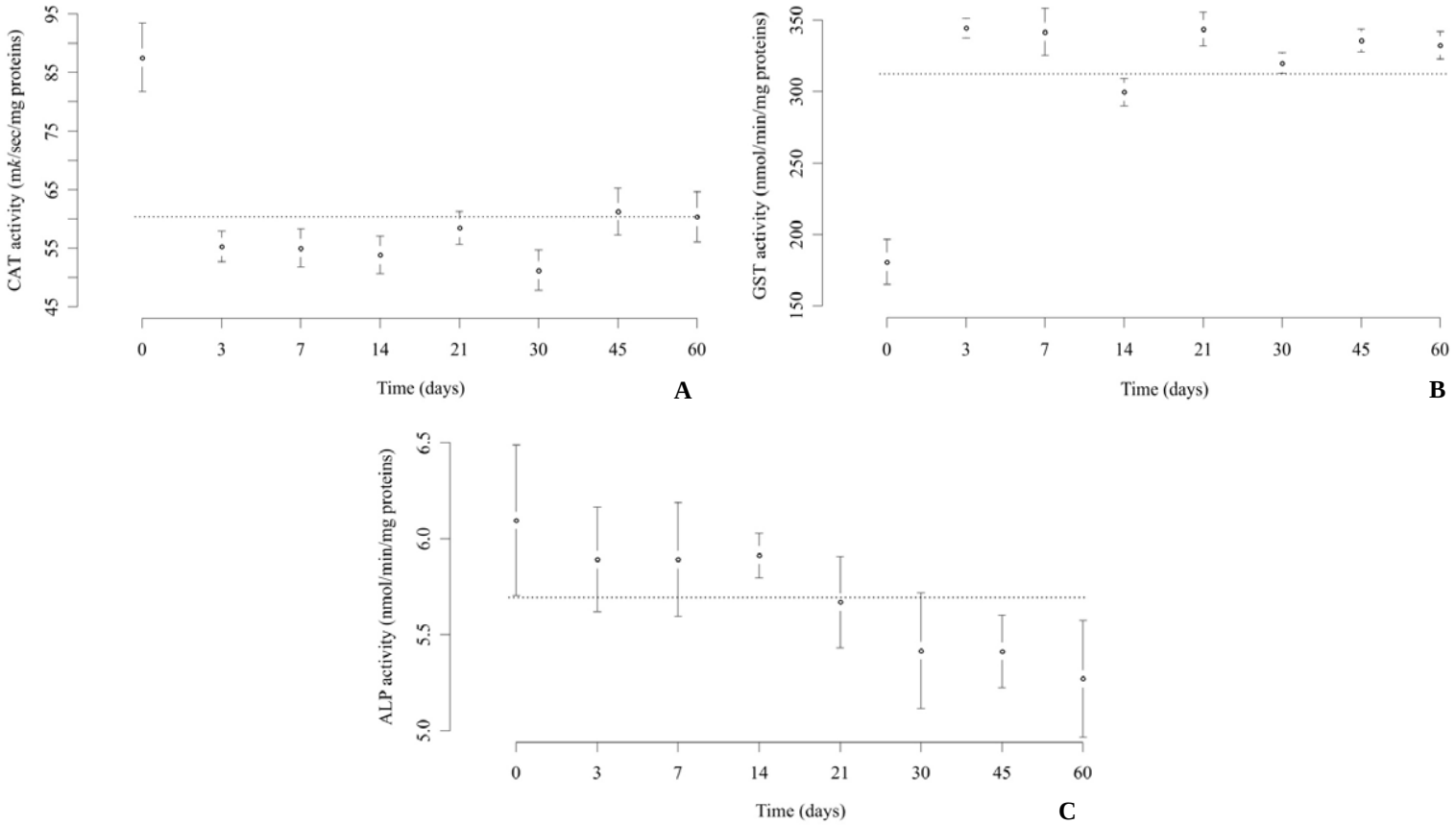

FIGURE 6 Enzyme patterns of CAT, GST and ALP (midgut extract) during the storage period of fresh extract up to 60 days at -80^{o}C.

4.2 Extraction, specific/tissue activity and variability

High yield was obtained with the first stage alone during the extraction procedure, with values varying from 65.04 to 99.88%, average of 82.36%. Extraction results were representative and reproducible and may be considered satisfactory, and only one stage was used to extract all enzymes from the head and midgut of *A. mellifera*.

According to Papadopoulos et al. (2004a), GST is present in all *Apis mellifera macedonica* Ruttner, 1988 stages, with higher SA when CDNB was used in adults. These authors observed that kinetic characteristics change with insect development, with a predominant isozyme with acid pH in the larval stage and another alkaline in adults. Another work by Papadopoulos et al. (2004b) suggested that both isozymes (acid and alkaline) are independently induced by factors like insecticide and environmental conditions (low temperature, starvation and xenobiotic exposure). Diao et al. (2006) informed high activity of GST in midgut of some species of honeybees, preferentially present in the cytosol fraction, in comparison to other tissues, such as head, thorax and abdomen without midgut. High activity of GST was found in the adult stage of *A. mellifera* in all assays. Thus, these authors suggested that the high activity of GST in the midgut is associated with the digestive system, to protect it in the case of ingestion of chemical- contaminated food.

The use of honeybee head extract as a source of AChE in this work is based on several reports in the literature. Belzunces et al. (1988a, 1988b) observed that AChE is mainly synthesized, stored and transported in the brain of honeybees, stressing the presence of two forms, one hydrophilic (3 to 6%) and the other amphiphilic, representing 94 to 97% of total AChE, which is attached to the cellular membrane by a glycophosphatidylinositol anchor, with the need to use a non-denaturant detergent, like Triton X-100 to perform the extraction. The constancy activity of AChE in *A. mellifera* was found by Barker et al. (1978),

reporting that no significant differences were detected among the different stages of honeybees. According to Zhang et al. (2005), there is no difference between AChE obtained from head, thorax and abdomen of *A. mellifera* and *Apis cerana* Fabricius, 1793, which may be related to the existence of only one site of synthetization, the brain. When evaluated the distribution of this enzyme in the body, it was found 65.70% of AChE in *A. mellifera* head, 18.70% in the thorax, and 15.60% in the abdomen. Polyzou et al. (1997) studied the behavior of AChE during the development of *A. mellifera* queens and concluded that 98% of the enzyme found in all developmental stages are in the amphiphilic form, and only 2% is of hydrophilic type. Nevertheless, out of the total AChE activity, 14% was ascribed to butyrylcholinesterase activity, accounting for the use of the true inhibitor BW284C51 in CaE studies.

In this work, CaEs were assayed using extracts obtained from heads of honeybees, with high activity and constancy. Yu et al. (1984) also demonstrated that CaEs are very active in the midgut of *A. mellifera*, which may be used as another source of CaE. However, these authors used no detergents to extract the enzyme. Acoording to Dary et al. (1990), the use of Triton X-100 increased the extraction of CaE in some cases, such as in mosquito-resistant strains. Delage-Darchen et al. (1982) also reported the activity of CaE in glands found in the head and midgut of honeybees, and extract of head with Triton X-100 was prepared for the AChE assay and also in CaE measurements. In the studies by Belzunces et al. (1994), CaE from honeybee was extracted in a medium containing the non-ionic detergent Lubrol-PX, which does not disrupt membrane. The review by Thompson (1999) suggested the use of Triton-X100 in the assay with CaE using α-NA as a substrate. The classification of CaEs may be based mainly on their specificity to the substrate, whereas Bitondi & Mestriner (1983) marked six *A. mellifera* CaE, each with independent

characteristics. High CaE activity from honeybee was found with α-NA and β-NA and in less intensity for *p*-NPA.

ALP shows several functions in honeybees, whereas binding with the metabolism and the transport of metabolites, usually in the presence of metallic ions (Coleman, 1992). Their presence was found in different body structure/secretions of honeybees, such as venom (Benton, 1967), low quantities in mandibular, hypopharingeal, cephalic and thoracic labial glands and with high activity in the midgut (Bounias, 1978; Delage-Darchen et al., 1982). Studies carried out in this work with enzymes are important to reinforce their use as biomarkers since this group of isozymes was highlighted by studies standing out their activity after contamination by xenobiotics such as deltamethrin and cupric salts, to be considered an important toxicological marker (Bounias et al., 1985, 1996).

Jimenez & Gilliam (1996) reported high activity of CAT found in peroximes on the honeybee's midgut, the same as observed in this work. These authors demonstrated the age-dependent activities, which increased with the age of workers, probably as a protection to contaminated forage fields. In addition to this, Weirich et al. (2002) reported other several tissues in different castes of honeybees where CAT is active, such as in the spermatheca and midgut. In semen or other structure of reproduction of honeybees, the role of these enzymes are to increase sperm longevity by the antioxidative system (Collins et al., 2004), or in the midgut due to the high exposure to food with different concentrations of H_2O_2 or xenobiotics (Weirich et al., 2002).

Variability is a serious problem in analytical procedures and can impair the use of certain biomarkers in the environmental assessment. It may be reduced to a minimum rate, when different endogenous/exogenous factors, genetic differences, sex, gender and species are known (Almar et al., 1987). As an example, it has been suggested that female beetles are more susceptible to

physiological stress than males (Stone et al., 2002). Moreover, most enzymes may be changed in the course of insect development, giving significant variability (Polyzou et al., 1997). Thompson (1999) described some factors that affect the variability of esterases, such as species, interindividual factors, age, diurnal changes and sex. It is widely accepted that the effects of pollutants can differ according to the pattern of enzyme expression or activity. The variability observed could be attributed to: (i) the existence of half sibs in the colony due to the fecundation of the queen by different males, (ii) the age of foraging honeybees which could vary from 18 to 38 days in summer until 140 days in winter and (iii) food availability, race and activities performed (Winston, 1987). In the specific case of *A. mellifera*, some works were developed to know the different patterns of enzymes. Smirle & Winston (1988) and Diao et al. (2006) observed that difference in enzymatic activity of GST and mixed-function oxidases between newly emerged and foraging honeybees was due to the period of adaptation to explore contaminated ecosystems, compensating for protein loss by increasing activity. In other study, Smirle (1993) verified that these enzymes are affected by factors as colony population and the brood/adult workers ratio, the mechanism to protect larvae from xenobiotics. Different responses of these enzymes are also reported in several colonies of honeybees (Smirle, 1990).

In environmental assessments, differential responses of biomarkers must be attributable to the effect of pollutants and not to other factors. Thus, the choice of a specific group, age, and health of *A. mellifera* can increase the realibility on the used procedures. Moreover, it is more convenient to use foraging honeybees, which are easily captured, age-calibrated by identifying paint marks and the first ones in contact to xenobiotics (Depledge & Fossi, 1994).

4.3 Storage stability

The results showed that enzymes were stable after deep freezing, but in some cases, presented different behavior. The storage temperature of enzymes can vary, whereas -80°C is a routine in some works (Escartin & Porte, 1997; Weirich et al., 2002). Westlake et al. (1985) found no losses in AChE activity when stored between -20°C and 4°C for 26 days, with only some variations, but significant losses at room temperature. Thompson (1999) advises the storage of brain in ice to minimize loss in activity during transport and analysis; however, the tissues should be stored at low temperatures during extended periods of time, when AChE is stable for 2 weeks. With the purpose of storing various sheep enzymes for further analysis, Jones (1985) verified that AChE was stable and at -20°C its stability was 224 days. However, ALP losses occur with a period of stability of 14 days after deep freezing at -20°C. When stored at the same temperature during four months, no losses were found in AChE, and ALP, with 10% activity losses in plasma and serum, respectively. Even with gradual decrease of ALP activity, it can be found after 60 days of storage, but Benton (1967) reported that honeybee venom stored at 4°C for one year may cause substantial losses until total denaturation of these enzymes.

According to Belzunces et al. (1994), the crude extract from the midgut of *A. mellifera* may be stored for 2 weeks without any loss of CaE activity using *p*-NA as a substrate. No losses were observed in CaE from ryegrass (*Lolium perenne* L., 1753), assayed with α-NA, β-NA after two years of storage at -20 °C (Wouters & Booy, 2000). GST and CAT displayed specific behavior with both increase and decrease in their activity, respectively, between 0 and 3 days of deep freezing, implying that measurements should not be made during this period. The process of CAT storage before their analysis is employed because no loss was verified at -85°C (Yumoto et al., 2000). Delcros et al. (1998) verified that CAT activity didn't differ in fresh or frozen wheat doughs after

three months of storage at -20°C. Unlike the results reported in this research, GSTs do not increase their activity, but decrease at rates which ranges owing to the species from vertebrate: monkey, dog, human, mouse and rat, when submitted to cryopreservation (Diener et al., 1993, 1994; Steinberg et al., 1999). The difference between the results reported and those in the literature can be related to *A. mellifera*, but future research should be carried out for this species.

5 CONCLUSIONS

The honeybee enzymes AChE, CaE, CAT, ALP and GST showed a good pattern for use in environmental quality monitoring programs:

- All enzymes were extracted with high yield, varying between 65.04 at 99.88% of total activity for AChE and GST, respectively;

- The high specific activity found indicates the good choice of *A. mellifera* tissue (head or midgut) as a source of these enzymes;

- Enzymes may be frozen to be used later in analysis even with some variation during storage;

- Variations within each enzyme group may be minimized using a correct experimental design, knowing characteristics such as age, health and origin of the honeybee and the use of a negative control.

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CHAPTER 3

SUBLETHAL EXPOSURE OF *Apis mellifera mellifera* L., 1758 TO PESTICIDES: BIOCHEMICAL STUDIES FOR ASSESSMENT OF THE ENVIRONMENTAL QUALITY

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ABSTRACT

The development of biomarkers to assess the impact of pesticides in the environment is important, so that work the acute toxicity of deltamethrin, fipronil, spinosad and thiamethoxam to *Apis mellifera mellifera* L., 1758 was determined in this work and the biochemical effect of these compounds on the enzymes acetylcholinesterase (AChE), carboxylesterases (CaE-1; CaE-2 e CaE-3), glutathione S-transferase (GST), catalase (CAT) and alkaline phosphatase (ALP) was also studied. The acute toxicity bioassays showed topic LD_{50} values of 50.65, 5.83, 47.11 and 51.16 ng/honeybee for deltamethrin, fipronil, spinosad and thiamethoxam, respectively. Other honeybees were then intoxicated by means of topic application with sublethal doses of the insecticides ($^{1}/_{10}$ and $^{1}/_{20}$ of LD_{50}), and 24h afterwards, the head and midgut were obtained to prepare the enzymatic extracts. Results indicated that all insecticides, except deltamethrin, affect the enzymatic activity of AChE. For CaE-1, deltamethrin and thiamethoxam induce inhibition and spinosad stimulus. On the other hand, CaE-2 was inhibited by spinosad and fipronil and stimulated by deltamethrin and thiamethoxam; CaE-3 was inhibited by deltamethrin and thiamethoxam. Among midgut enzymes, only ALP was inhibited by fipronil, and enzymes ALP, CAT e GST were stimulated by the remaining insecticides, resulting into increase in their specific activity. These results showed the potential use of these enzymes as biomarkers of exposition; nevertheless, other studies should be conducted to evaluate the enzymatic behavior in relation to the residual effect of these insecticides, the different pathways of exposition and the time of exposition.

Keywords: Honeybee; biomarker; acetylcholinesterase; carboxylesterase; glutathione S-transferase; catalase; alkaline phosphatase; deltamethrin; fipronil; spinosad; thiamethoxam.

1 INTRODUCTION*

The honeybee *Apis mellifera* L., 1758 (Hymenoptera: Apidae) has a great agronomic, environmental and socioeconomic interest, allowing substantial yields in honey, royal jelly, polen and propolis, besides pollinating several plants with resulting increase in production and quality of food (Delaplane & Mayer, 2005; Tautz, 2008; Gallai et al., 2009). The honeybee stands out due to easiness of handling, wide geographical distribution, known biology, short lifespan and high reproductive potential (Devillers, 2002).

The advance in agricultural techniques, with the intense use of pesticides, expansion of monocultures and even the interaction with pathogens responsible for honeybee diseases, are important factors related to vanishing of honeybees and significantly worsening food production (Stokstad, 2007). During foraging period, honeybees go an average 1.5 to 3.0 km around for collecting nectar, pollen, resins and water, that may be contaminated by some source of pollutants, mainly pesticides which in general are able to cause honeybees' death and/or affect their development (Cluzeau, 2002; Chauzat et al., 2009). Among the differents insecticides employed in fields, we can stand out the pyrethroid deltamethrin, acting in sodium/potassium gates causing a rapid membrane depolarization of neuron; the phenylpyrazole fipronil that act as an antagonist to γ-aminobutyric acid (GABA), the spinosad, belonging to a new class naturalyte, which have a complex action mechanism in the nervous system, acting on nicotinic receptors and GABA; and thiamethoxam, a second generation of neoniconinoid, being an agonist to the acetylcholine receptor (Elliott, 1980; Durham et al., 2001; Mayes et al., 2003; Elbert et al., 2008).

* Enzyme abbreviations: AChE = acetylcholinesterase (EC 3.1.1.7); CAT = catalase (EC 1.11.1.6); ALP = alkaline phosphatase (EC 3.1.3.1); GST = glutathione S-transferase (EC 2.5.1.18); CaE-1,CaE-2 and CaE-3 = carboxylesterases (EC 3.1.1.1)

According to Celli & Maccagnani (2003), the honeybees can be used to detect environmental quality, or their mortality, residues in the hive products or honeybee bodies, being that it is thought to be possible to anticipate the impact caused by xenobiotics with use of biochemical tools. However, studies are needed to obtain information on the response of enzymes used as biomarkers, thus validating their use for complementary studies of environmental quality (Vasseur & Cossu-Leguille, 2003). The honeybee's detoxification systems have been studied due to the importance of the impact of insecticides on honeybees. Gilbert & Wilkinson (1974) reported that drone and workers have the enzyme microsomal oxidase, responsible for the primary metabolism of pesticides and other xenobiotics. Yu et al. (1984), revealed the presence of enzymes of detoxification microssomal oxidase, GST, CaE, hydrolase and DDT-dehydrochlorinase in the midgut of honeybees. Concerning an insect of social organization, Smirle & Winston (1987, 1988) and Smirle (1990, 1993) reported that the effect of enzymes of detoxification can also be influenced by differences among colonies, age of workers, population, amount and intensity of brood feeding. Nielsen et al. (2000) observed stimuli of GST activity and glutathione peroxidase in several developmental stages of honeybees after treatment with flumethrin.

In the first time, the inhibition/induction of enzymes from the detoxification system cannot be used as a measure of insecticide potentialization, but it can be used as as biomarkers of exposition (Walker, 1998). According to Hyne & Maher (2003) and Vasseur & Cossu-Leguille (2003), many enzymes have potential for use in environmental monitoring programs, either as of exposition (AChE, CaE; GST; metallothioneim and P450) or oxidative systems (CAT, glutathione peroxidase/reductase and superoxide dismutase). Stefanidou et al. (1996, 1998) called the attention to the use of honeybee AChE and butyrylcholinesterase in programs of environmental

monitoring. Thompson (1999) reported several factors, advantages and methods that can be employed using CaEs as biomarkers of environmental quality. Attencia et al. (2005) verified the possibility of use of CaE as biomarkers, after inhibition of some isozymes extracted from honeybees exposed to organophosphates. Hashimoto et al. (2003) suggested the use of these CaE isozymes as indicators of exposition to honeybees exposed to different concentrations of thiamethoxam.

Badiou et al. (2008) detected AChE inhibition in dead honeybees after treatment with deltamethrin or in association to pirimicarb, and increase in specific enzymatic and tissue factor in surviving honeybees, indicating that AChE can be used in monitoring programs when honeybees are exposed to deltamethrin. Rabea et al. (2009) detected the possibility of using honeybee enzymatic system as a biomarker. After exposed to chlorfluazuron, oxymatrine and spinosad, inhibition of AChE and adenosine triphosphatase was detected, and the use of honeybees in the environmental monitoring assessment was suggested. Stefanidou et al. (1996) reported the potential use of honeybee AChE as a biomarker of exposition to organophosphates and carbamates.

Due to the importance of honeybees in social, economic and ecological aspects, the increase in knowledge about biomarkers for evaluation of environmental impact of pesticides in agricultural areas is needed, so, this work was conducted to determine the acute toxicity of deltamethrin, fipronil, spinosad and thiamethoxam to *A. mellifera*, and study the *in vitro* biochemical effects of subletal exposition to these compounds on the enzymatic activities of AChE, CaE, GST, CAT and ALP.

2 MATERIALS AND METHODS

2.1 Chemicals

The pesticides deltamethrin (99.8%), fipronil (97.6%), and thiamethoxam (99.7%) were purchased from Pestanal®, Sigma-Aldrich (France). Spinosad (96%) was obtained from Cluzeau Info Labo (France). For all assays, antipain; aprotinin; leupeptin; pepstatin A; trypsin inhibitor; sodium phosphate monobasic; sodium chloride (NaCl); Triton® X-100; acetylthiocholine iodide (AcSCH.I); 5,5'dithio-bis(2,nitrobenzoic acid) (DTNB); sodium bicarbonate; α and β naphthyl acetate (α-NA or β-NA); *p*-nytrophenyl acetate (*p*-NPA); 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51) were used; Fast Garnet GBC; sodium dodecyl sulfate (SDS); hydrogen peroxide (H_2O_2); sodium phosphate dibasic; potassium phosphate monobasic; ethylenediaminetetra-acetic acid (EDTA); 1-chloro-2,4-dinitrobenzene (CDNB); L-glutathione reduced (GSH); acetonitril; acetone; Trizma® base (Tris); hydrochloride acid (HCl); magnesium chloride ($MgCl_2$); *p*-nitrophenyl phosphate (*p*-NPP) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (France).

2.2 Honeybees

Foraging honeybees *A. mellifera mellifera* were collected from July to August, 2008 in frames for the comb in a single colony of honeybees at UMR406 Abeilles et Environnement, INRA/Avignon, France. They were maintained in the laboratory in experimental cages (10 x 10 x 8 cm), feeding *ad libitum* with Candy paste at 28±2°C, 60±10% RH, and 12 hours' photophase.

2.3 Acute toxicity assay

Dose-response experiments were carried out to determine the lethal dose (LD_{50}) for each pesticide, according to the French directive CEB nº 230 (AFPP, 2003). Several dilutions in acetone of each pesticide were made and 1 µL of each solution was applied on the thorax of honeybees anaesthetized with CO_2 for 1 minute, using a 50 µL Hamilton syringe with a repeating dispenser. The experiment was run in a completely randomized design with 75 honeybees (three cages with 25 honeybees) for each pesticide concentration. The control received only applications of acetone. Laboratory conditions for maintenance of the honeybees were described in section 2.2.

2.4 Intoxication assay

After determination of LD_{50} of each pesticide, experiments were conducted to intoxicate honeybees with sublethal doses equivalent to $^1/_{10}$ and $^1/_{20}$ of LD_{50} ($LD_{50/10}$ and $LD_{50/20}$), to be used in the enzymatic assays and in the control assay (acetone only). All procedures, dilutions, laboratory conditions, honeybees, and chemicals were reported in sections 2.2 and 2.3.

Honeybees were anaesthetized with CO_2 (1 minute) and 1µL of each concentration/pesticide was applied on the dorsal thorax. After contamination, 75 honeybees per treatment (three cages with 25 honeybees each) were kept in the experimental cage and placed in a climatic room. On the following day (24 hours after intoxication), each cage with honeybees were placed into an ultra-freezer at -80°C to kill the honeybees and for storage, until preparation of samples to be used in enzyme assays.

The assays were undertakent in two steps, each one with two pesticides and Control due to the difficulty carrying through all assays and prepare the samples at the same time.

2.5 Sample preparation

2.5.1 Heads

Heads of honeybees, stored at -80°C, were obtained by cutting them off from the body using a scalpel. Nine heads per cage were placed in three microtubes (2 mL), and weighed. Phosphate buffer 40 mM pH 7.4, containing 10 mM NaCl, 1% Triton® X-100 and 2 µL/mL of a mixture (equal parts) of the protease inhibitors antipain, aprotinin, leupeptin, pepstatin was added, obtaining a head extract at final concentration of 10% (w/v). The homogenates of head were obtained using a high speed shaker (TissueLyser II, Qiagen®) with stainless steel to disrupt the tissue during 90 seconds, and so the samples were centrifuged at 13.000 *g* for 20 minutes and the supernatant stored in microtubes at -80°C to the analysis. All extraction procedures were carried out at 4° C.

2.5.2 Midgut

The midguts were obtained by pulling the sting out of the honeybees that were stored at -80°C; nine midguts (per cage) were equally divided in three microtubes and weighted. 40 mM phosphate buffer pH 7.4 with 10 mM NaCl and 2 µL/mL of a mixture (equal parts) of the proteases inhibitor (antipain, aprotinin, leupeptin, pepstatin A and trypsin inhibitor) at the final rate of 10% (w/v) was added. The tissues were processed as above (item 2.5.1), by high-speed shaking with stainless steel for 90 seconds and the homogenates centrifuged at 13.000 *g* for 20 minutes. The supernatant was stored in microtubes at -80°C until the analysis; all extraction procedures were conducted at 4°C.

2.5.3 Protein determination

To determine the specific activity of each enzyme, proteins were quantified according to the method proposed by Bradford (1976), using the Bio-Rad Protein Assays kit (Bio-Rad Laboratories, France) and BSA as a standard.

2.6 Enzyme assays

All enzymatic assays were performed with Variant® Cary 1E UV-Vis spectrophotometer at 25°C and final volume of 1 mL, disposable semi-micro cuvette (Ratiolab®, Germany) for the essays in the visible spectrum and the semi-micro UV-Disposable cuvette (Plastibrand®, Germany) for UV spectrum. The activity of the sample was determined in triplicate for all enzymes and the activity expressed in nanomoles of product formed per milligram of protein/minute (except for CAT, see item 2.6.3).

2.6.1 Acetylcholinesterase

AChE activity was determined following the method described by Ellman et al. (1961) at 412 nm. The final concentration of the reaction was 0.3 mM of AcSCH.I, 1.5 mM of DTNB, 100 mM sodium phosphate buffer at pH 7.0, 0.02% of Triton X100 and the 20 µL of crude extract obtained from the heads of the honeybees.

2.6.2 Carboxylesterase

Activities of three CaEs were determined using three different substrates: α-NA; β-NA and *p*-NPA. The technique was described by Gomori (1953) and incorporated in entomological studies by Asperen (1962). The true inhibitor for AChE, BW284C51 (Austin & Berry, 1953) was used to avoid that AChE competed with CaE for the same substrate. The first step of reaction carried out was to inhibit AChE by the addition of crude extract of heads from

honeybees (5µL for α-NA/ β-NA and 10µL for *p*-NPA) in the presence of phosphate buffer 100 mM pH 7.0; 10^{-4} mM BW284C51, 0.005% Triton X-100 and distilled water for 20 minutes at 25°C in darkness. After incubation, 0.4 mM of a substrate (α-NA; β-NA or *p*-NPA) was added and after 3 minutes, the reaction was interrupted with 1.5% of SDS + 0.4 mg/mL of Fast Garnet GBC and the azo compounds formed were monitored at 568 nm (α-NA); 515 (β-NA) and 410 (*p*-NPA).

2.6.3 Catalase

The CAT reaction employed was described by Beers & Sizer (1951). However, CAT have abnormal kinetics, we used the method suggested by Cohen et al. (1970) and Aebi (1984) to express the activity as the rate of decomposition of H_2O_2 (m*k*/sec/mg protein). The final concentrations were 100 mM phosphate buffer pH 7.0; 10 mM of H_2O_2 and 25 µL crude extracts from the midgut. The optical density was determined at 240 nm.

2.6.4 Glutathione S-transferase

The method proposed by Habig et al. (1974) with some modifications was used to evaluate GST activity. Conjugation of GSH with CDNB was measured at 340 nm in the presence of 100 mM of sodium/potassium phosphate buffer at pH 7.4; 1 mM EDTA; 2.5 mM GSH; 1 mM CDNB and 25 µL of crude extract from the midgut. Since CDNB is insoluble in water, it was prepared in acetonitril: water solution (3:7).

2.6.5 Alkaline phosphatase

ALP was assayed using the procedures of Bessey et al. (1946) and Bounias et al. (1996). The reaction occurs at 410 nm in the presence of 100 mM

Tris-HCl buffer pH 8.5; 2 mM *p*-NPP; 20 µM $MgCl_2$ and 25 µL of crude extract from the midgut.

2.7 Statistical analyses

Analyses were performed using the statistical software R® (2009). Mortality data from the dose-response assays, which were obtained 24 hours after the treatment of honeybees with the pesticides, were analyzed using a logistics model from the "drc" package (Analysis of dose-response curves) (Ritz & Streibig, 2005), and the respective lethal doses 50 (LD_{50}) and confidence limit at 95%. For enzyme assays, activity data were analyzed from one-way analysis of variance (ANOVA) and means of activity of each enzyme compared by contrast analysis, forming clusters with similar behavior (Kuhn et al., 2009).

3 RESULTS

3.1 Acute toxicity assay

The acute toxicity bioassays revealed that among the tested insecticides, fipronil was the most toxic to *A. mellifera* with LD_{50} of 5.83 ng/honeybee. The others insecticides, deltamethrin, spinosad and thiamethoxam showed LD_{50} value of 50.65; 47.26 and 51.16 ng/honeybee, respectively. From the respective LD_{50} values of each insecticide, the sublethal doses $LD_{50/10}$ and $LD_{50/20}$ were determinated, which were employed in the intoxication assay (Table 1, Figures 1a, 1b, 1c and 1d). The *Knock down* effect was observed only in the case of deltamethrin; however, honeybee recovered 1 to 2 hours after the application of lower doses. In the intoxication assay (see section 2.4), all honeybees survived after exposed to sublethal doses ($LD_{50/10}$ and $LD_{50/20}$) of the insecticides.

TABLE 1 Acute toxicity of pesticides to *A. mellifera.*

Insecticide	Purity[a]	LD_{50}[b]	$CL_{95\%}$[c]	χ^2[d]	D.F.[e]	$LD_{50/10}$[f]	$LD_{50/20}$[f]
Deltamethrin	99.8	50.65	43.33 – 57.97	21.88	15	5.07	2.53
Fipronil	97.6	5.83	5.05 – 6.60	25.98	21	0.58	0.29
Spinosad	96.0	47.11	44.22 – 49.99	24.74	18	4.11	2.36
Thiamethoxam	99.7	51.16	46.71 – 55.61	18.82	13	5.12	2.56

[a] in percentage [b] in ng/honeybee for 24h [c] Confidence limit at 95%
[d] Chi-square [e] Degrees of freedom [f] Determinated from the LD_{50} value

3.2 Enzymatic assay

Different patterns of enzyme activities were observed by the exposition of the honeybees to the insecticides (Tables 2, 3, 4 and 5). By contrast analysis, no differences were found in the amounts of protein among the different treatments with the insecticides and their respective control (data not show), the enzyme activities were expressed in specific activity. No differences were found for AChE after exposition of honeybees to sublethal doses of deltamethrin. For the CaE group, deltamethrin at $LD_{50/10}$ inhibited the specific activity of CaE-1 and CaE-3 as compared to Control with activities of 40.12 and 56.19 nmol/min/mg of protein in the insecticide treatment and in the control treatment 52.40 e 65.06 nmol/min/mg of protein, respectively. Stimulus was detected for CaE-2 in both sublethal doses of deltamethrin to honeybees, with an average of 82.51 and 67.16 nmol/min/mg of protein (51.99 nmol/min/mg protein in Control).

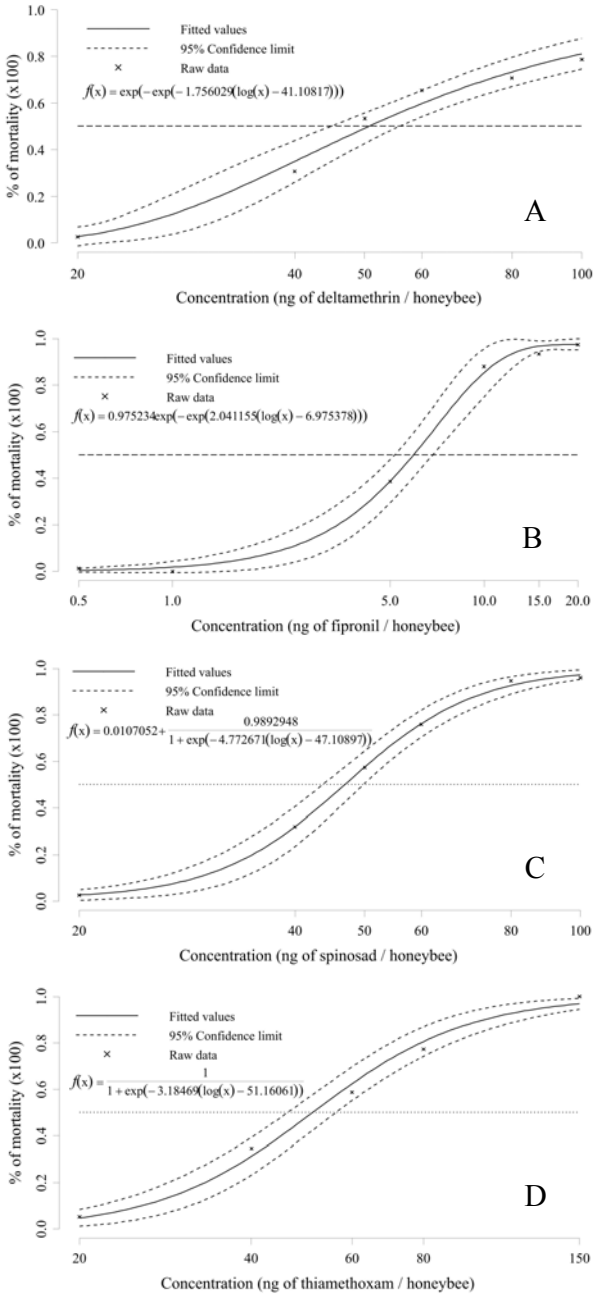

FIGURE 1 Dose-response assay after the exposure of *A. mellifera* to deltamethrin (A), fipronil (B), spinosad (C) and thiamethoxam (D).

In relation to the enzymes from honeybee midgut, ALP, CAT and GST, an increase in enzymatic activity after intoxication for both sublethal doses of deltamethrin was detected, indicating stimulus after contamination by this pyrethroid; for ALP, means for the two doses were 6.51 and 6.49 nmol/min/mg of protein (5.31 nmol/min/mg for Control); for CAT, 89.41 and 88.57 m*k*/sec/mg of protein and 65.99 m*k*/sec/mg for Control, and for GST, means after intoxication were 459.19 and 448.53 nmol/min/mg of protein, respectively and de 407.78 nmol/min/mg for Control (Table 2).

When honeybees were intoxicated by fipronil, no differences were detected in CaE-1 and CaE-3 esterases obtained from honeybee heads as compared to Control. However, CaE-2 was inhibited when honeybees were exposed to low dose of fipronil on $LD_{50/20}$, with average of 83.62 nmol/min/mg of protein and 93.39 nmol/min/mg for Control. Likewise, AChE was also inhibited by $LD_{50/20}$ of fipronil, with an average of 55.60 nmol/min/mg of protein as compared to Control (62.66 nmol/min/mg). Fipronil affected ALP, CAT and GST originated from honeybee midgut. Stimulus for enzyme activity was only observed in ALP in both doses evaluated, with 5.97 and 5.37 nmol/min/mg of protein, respectively and 6.40 nmol/min/mg for the Control. For CAT and GST, the $LD_{50/10}$ of fipronil stimulated these enzymes with averages of 162.77 m*k*/sec/mg of protein and 257.62 nmol/min/mg, respectively (Table 3).

It was detected that spinosad affected enzymes of *A. mellifera*, except CaE-3 (Table 4). In the group of enzymes extracted from honeybee heads, it was observed that the $LD_{50/10}$ of this insecticide stimulated CaE-1 activity when compared to the Control, with averages of 52.20 e 46.18 nmol/min/mg of protein, respectively. For CaE-2, the dose $LD_{50/10}$ of spinosad inhibited enzymatic activity, with average of 86.26 nmol/min/mg of protein, compared to 93.39 nmol/min/mg for the Control. For AChE, this naturalyte insecticide inhibits its activity in the two doses tested, $LD_{50/10}$ and $LD_{50/20}$, with average of

49.71 and 56.53 nmol/min/mg of protein, respectively, and 62.66 nmol/min/mg for the Control. Considering cytosolic enzymes obtained from the midgut, spinosad stimulated the specific activity of ALP, CAT and GST. For CAT and GST, both doses induced increase in specific activity as compared to the Control, averages of 157.11 and 142.31 m*k*/sec/mg of protein for CAT and 277.62 and 263.13 nmol/min/mg of protein for GST, respectively. Only the highest dose induced the stimulus of ALP activity, with average of 7.17 nmol/min/mg of protein and 6.40 nmol/min/mg for Control.

Among the CaE extracted from honeybee head, only CaE-2 activity was stimulated in both doses of thiamethoxam with averages of 71.28 and 69.37 nmol/min/mg and 51.99 nmol/min/mg of protein for the Control. The remaining CaE had their activities inhibited, with CaE-1 affected by both doses evaluated, with averages of 41.07 e 38.51 nmol/min/mg of protein, respectively. For CaE-3, only $LD_{50/20}$ affected enzymatic activity as compared to the Control, with averages of 51.63 nmol/min/mg of protein. Likewise to that observed with spinosad, all midgut enzymes were stimulated when honeybees were intoxicated with thiamethoxam. ALP and GST had their activities increased, with major enzymatic activities observed at lower thiamethoxam doses, with average of 5.93 e 6.45 and 441.06 and 470.10 nmol/min/mg of protein, respectively. For CAT, only the dose $LD_{50/20}$ stimulated enzymatic activity, average of 88.37 m*k*/sec/mg of protein (Table 5).

4 DISCUSSION

The acute toxicity assays with the populations of *A. mellifera* under study is important to determine the real LD_{50}, avoiding thus intercolonial variations, season of year, age, stress, development and population size (Smirle & Winston, 1987, 1988; Smirle, 1990, 1993; Harris & Woodring, 1992). All

insecticides were toxic, howver, fipronil was the insecticide most toxic to *A. mellifera*, with lower acute toxicity (LD_{50} at 24 hours) 5.83 ng/honeybee, and deltamethrin, spinosad and thiamethoxam has a similar acute toxicity varying from 47.11 to 51.16 ng/honeybee. The estimated LD_{50} of fipronil on this work is similar to the one obtained by Tingle et al. (2003) and Decourtye et al. (2005), who found averages from 4 to 6 ng/honeybee. However, Atkins et al. (1981) and Decourtye et al. (2005) reported a topic LD_{50} from 62 to 67 ng/honeybee for deltamethrin, respectively, higher than the one found in this study (50.65 ng/honeybee). For spinosad, it was observed that the value obtained in this study (47.11 ng/honeybee) agrees with the one described by Mayes et al. (2003) and Miles (2003), which can vary from 25 to 78 ng/honeybee. According to Iwasa et al. (2004), the LD_{50} of thiamethoxam is 30 ng/honeybee, value 30% times as low as the one obtained in this work; Hashimoto et al. (2003) however, found LD_{50} of this insecticide higher than 50 ng/honeybee for foragers of Africanized honeybees.

Inhibition and stimulation patterns of enzymatic activity were observed after exposition of honeybees to the insecticides (Tables 2, 3, 4 and 5), showing that despite the lack in some detoxification enzymes (i.e. CaE, GST and P450) compared with *Drosophila melanogaster* (Meigen, 1830) and *Anopheles gambie* Giles, 1926, honeybees have an active enzymatic system (Claudianos et al., 2006). No difference was detected in AChE enzymatic activity after honeybees' intoxication with sublethal doses of deltamethrin, in opposition to what was observed by Badiou et al. (2008). This difference was likely a result of the higher doses tested by those authors, it is five times as high as the ones used in this study. The lack of alteration in AChE activity by deltamethrin may be related to time after exposition to this pyrethroid along with detoxification enzymes, as reported by Vandame et al. (1995). These last authors found that only a small amount of non-metabolized deltamethrin was found in the

honeybees' bodies after three hours of exposition to sublethal doses (≤5 ng/honeybee), suggesting degradation by P450 enzymes.

TABLE 2 The effect of sublethal exposure with deltamethrin in enzymes from *A. mellifera*.

Tissue	Enzyme	Dose	Specific activity ($IC_{95\%}$)	Contrast group	Effect
			in nmol/min/mg of protein		
Head	AChE	Control	61.29 (57.75 – 64.98)	1	-
		$LD_{50/10}$	61.41 (52.74 – 70.22)	1	-
		$LD_{50/20}$	58.76 (50.15 – 67.51)	1	-
	CaE-1	Control	52.40 (49.12 – 55.81)	1	-
		$LD_{50/10}$	40.12 (32.39 – 47.97)	2	Inhibition
		$LD_{50/20}$	50.80 (42.83 – 58.91)	1	-
	CaE-2	Control	51.99 (48.73 – 55.39)	3	-
		$LD_{50/10}$	82.51 (73.91 – 91.29)	1	Stimulation
		$LD_{50/20}$	67.16 (58.86 – 75.61)	2	Stimulation
	CaE-3	Control	65.06 (61.49 – 68.62)	1	-
		$LD_{50/10}$	56.19 (47.59 – 64.79)	2	Inhibition
		$LD_{50/20}$	62.03 (53.44 – 70.63)	1	-
Midgut	GST	Control	407.78 (389.42 – 418.15)	2	-
		$LD_{50/10}$	459.19 (426.16 – 484.22)	1	Stimulation
		$LD_{50/20}$	448.53 (415.50 – 473.55)	1	Stimulation
	ALP	Control	5.31 (4.98 – 5.65)	2	-
		$LD_{50/10}$	6.51 (5.66 – 7.36)	1	Stimulation
		$LD_{50/20}$	6.49 (5.64 – 7.34)	1	Stimulation
			in m*k*/sec/mg of protein		
	CAT	Control	65.99 (52.99 – 80.96)	2	
		$LD_{50/10}$	89.41 (55.02 – 126.07)	1	Stimulation
		$LD_{50/20}$	88.57 (54.24 – 125.17)	1	Stimulation

Spinosad inhibited AChE in both doses studied. Rabea et al. (2009) reported similar results when spinosad was given in food for *A. mellifera*, it can inhibit AChE and adenosine triphosphatase at the medium rate of inhibition of 48.19%. Even not being an inhibitor of AChE, spinosad shows a mode of action characterized by excitation of the insect nervous system, inducing non-volunteer contractions followed by prostration and death. Nicotinic acetylcholine receptors

were also affected, altering GABA-gated chloride currents (Salgado, 1998; Salgado et al., 1998; Mayes et al., 2003).

TABLE 3 The effect of sublethal exposure with fipronil in enzymes from *A. mellifera*.

Tissue	Enzyme	Dose	Specific activity ($IC_{95\%}$)	Contrast group	Effect
			in nmol/min/mg of protein		
Head	AChE	Control	62.66 (59.07 – 66.39)	1	-
		$LD_{50/10}$	64.28 (55.49 – 73.22)	1	-
		$LD_{50/20}$	55.60 (46.98 – 64.35)	2	Inhibition
	CaE-1	Control	46.18 (41.95 – 50.40)	1	-
		$LD_{50/10}$	51.50 (41.30 – 61.70)	1	-
		$LD_{50/20}$	48.81 (38.60 – 59.01)	1	-
	CaE-2	Control	93.39 (88.99 – 97.92)	1	-
		$LD_{50/10}$	93.64 (82.93 – 104.50)	1	-
		$LD_{50/20}$	83.62 (73.08 – 94.30)	2	Inhibition
	CaE-3	Control	83.84 (79.68 – 88.14)	1	-
		$LD_{50/10}$	86.76 (76.56 – 97.10)	1	-
		$LD_{50/20}$	68.69 (58.82 – 78.69)	1	-
Midgut	GST	Control	242.21 (232.49 – 251.92)	2	-
		$LD_{50/10}$	257.62 (234.16 – 281.08)	1	Stimulation
		$LD_{50/20}$	242.13 (218.67 – 265.59)	2	-
	ALP	Control	6.40 (5.89 – 6.91)	1	-
		$LD_{50/10}$	5.97 (4.74 – 7.21)	2	Inhibition
		$LD_{50/20}$	5.37 (4.13 – 6.60)	2	Inhibition
			in m*k*/sec/mg of protein		
	CAT	Control	126.35 (112.38 – 141.43)	2	-
		$LD_{50/10}$	162.77 (126.88 – 199.91)	1	Stimulation
		$LD_{50/20}$	130.04 (95.37 – 165.84)	2	-

Thiamethoxam acts as an agonist to acetylcholine (nAChR) receptor (Elbert et al., 2008) and curiously inhibits the enzymatic activity of AChE in honeybees. Rodrigues et al. (2010) described similar results of AChE for rats contaminated with doses of thiamethoxam, the highest one inhibiting enzymatic activity from 2 hours until 7 days after intoxication. These authors suggested that the mechanism responsible for inhibition of AChE by thiamethoxam has not been completely clarified, and can be related to an indirect effect of the main

compound or its metabolites when at sublethal doses. These compounds acting on acetylcholine receptors may induce different patterns of expected enzyme activity, and compensatory mechanisms may be present until reestablishment of normal physiological conditions. According to Badiou et al. (2008), a likely explanation for inhibition of AChE by deltamethrin is related to a complex way of action when at sublethal doses. For Fipronil, inhibition of AChE at lower dose may be related to an indirect mode of action of the insecticide, or its metabolite fipronil-sulfone, which have identical toxicity and neurophysiologic effects. For these two compounds, the mode of action involves antagonism of the neurotransmitter γ-amino butyric acid (GABA) (Durham et al., 2001). As evidenced in this work, Cole et al. (1993) also found that the criket central nervous system when stimulated by ivermectin, can have up to 57% of its activity inhibited by application of fipronil.

Among the CaEs evaluated, a different pattern related to the four insecticides at their different concentrations was detected. Deltamethrin and thiamethoxam inhibited activity of CaE-1 and CaE-3 and stimulated CaE-2; spinosad stimulated CaE-1 and inhibited CaE-2. Activity inhibition of CaE-2 was detected only for fipronil. According to Thompson (1999), inhibition of esterases type B (i.e. carboxylesterases) in several animals by organophosphates and carbamates, may be used in environmental quality monitoring programs. Bitondi & Mestriner (1983) reported that *A. mellifera* have six esterase isozymes, and each can distinguished by eletrophoretic mobility, specificity to the substrate, in addition to inhibitory properties. Yu et al. (1984) reported that guts of *A. mellifera* have high activity of CaE and had reduction to specific activity of this enzyme after ingestion of contaminated food with sublethal doses of malathion. Figueiredo et al. (1996) found that diclorvos applied to honeybees on fifth instar, pre-pupa and pupa, only CaE-1 was not affected, the remaining CaE inhibited at different degrees and phases. With the same results, Hashimoto

et al. (2003) observed that thiamethoxam have inhibitory action on four CaEs in Africanized honeybees, and suggested the use of these enzymes as biomarkers of exposition. Attencia et al. (2005) also suggested the use of CaE to evaluate *A. mellifera* exposure to methyl-parathion and malathion, after detecting the inhibition of these enzymes even 14 days after intoxication by these organophosphates.

TABLE 4 The effect of sublethal exposure with spinosad in enzymes from *A. mellifera*.

Tissue	Enzyme	Dose	Specific activity ($IC_{95\%}$)	Contrast group	Effect
			in nmol/min/mg of protein		
Head	AChE	Control	62.66 (59.07 – 66.39)	1	-
		$LD_{50/10}$	49.71 (41.22 – 58.33)	3	Inhibition
		$LD_{50/20}$	56.53 (47.90 – 65.30)	2	Inhibition
	CaE-1	Control	46.18 (43.11 -49.39)	2	-
		$LD_{50/10}$	52.20 (44.55 – 60.00)	1	Stimulation
		$LD_{50/20}$	49.05 (41.47 – 56.77)	2	-
	CaE-2	Control	93.39 (88.99 – 97.92)	1	-
		$LD_{50/10}$	86.26 (75.67 – 96.99)	2	Inhibition
		$LD_{50/20}$	93.00 (82.30 – 103.85)	1	-
	CaE-3	Control	83.84 (80.42 – 87.27)	1	-
		$LD_{50/10}$	85.10 (76.83 – 93.37)	1	-
		$LD_{50/20}$	82.58 (74.31 – 90.84)	1	-
Midgut	GST	Control	242.21 (229.75 – 254.66)	2	-
		$LD_{50/10}$	277.62 (247.55 – 307.69)	1	Stimulation
		$LD_{50/20}$	263.13 (233.06 – 293.20)	1	Stimulation
	ALP	Control	6.40 (5.91 – 6.91)	2	-
		$LD_{50/10}$	7.17 (5.96 – 8.40)	1	Stimulation
		$LD_{50/20}$	6.19 (5.01 – 7.40)	2	-
			in m*k*/sec/mg of protein		
	CAT	Control	126.35 (121.22 – 131.61)	3	-
		$LD_{50/10}$	157.11 (144.21 – 170.16)	1	Stimulation
		$LD_{50/20}$	142.31 (129.61 – 155.15)	2	Stimulation

TABLE 5 The effect of sublethal exposure with thiamethoxam in enzymes from *A. mellifera*.

Tissue	Enzyme	Dose	Specific activity ($IC_{95\%}$)	Contrast group	Effect
			in nmol/min/mg of protein		
Head	AChE	Control	61.29 (58.77 – 63.88)	1	-
		$LD_{50/10}$	63.49 (57.32 – 69.73)	1	-
		$LD_{50/20}$	58.25 (52.15 – 64.41)	2	Inhibition
	CaE-1	Control	51.93 (48.67 – 55.33)	1	-
		$LD_{50/10}$	41.07 (33.84 – 48.91)	2	Inhibition
		$LD_{50/20}$	38.51 (30.85 – 46.30)	2	Inhibition
	CaE-2	Control	51.99 (48.72 – 55.39)	2	-
		$LD_{50/10}$	71.28 (62.90 – 79.83)	1	Stimulation
		$LD_{50/20}$	69.37 (61.02 – 77.87)	1	Stimulation
	CaE-3	Control	64.43 (60.79 – 68.21)	1	-
		$LD_{50/10}$	65.33 (56.43 – 74.37)	1	-
		$LD_{50/20}$	51.63 (43.01 – 60.38)	2	Inhibition
Midgut	GST	Control	407.78 (391.12 – 424.45)	3	-
		$LD_{50/10}$	441.06 (400.82 – 481.31)	2	Stimulation
		$LD_{50/20}$	470.10 (429.85 – 510.34)	1	Stimulation
	ALP	Control	5.26 (4.95 – 5.58)	3	-
		$LD_{50/10}$	5.93 (5.16 – 6.71)	2	Stimulation
		$LD_{50/20}$	6.45 (5.68 – 7.25)	1	Stimulation
			in m*k*/sec/mg of protein		
	CAT	Control	67.55 (59.52 – 75.59)	2	-
		$LD_{50/10}$	70.12 (50.72 – 89.52)	2	-
		$LD_{50/20}$	88.37 (68.97 – 107.77)	1	Stimulation

The CAT activity was stimulated for all insecticides evaluated, acting in oxidative stress from reactive oxygen species, deals with the elimination of toxic cellular oxygen produced by own aerobic cellular metabolism or any external factor like environmental stress caused by xenobiotics (Barreiros et al., 2006). Jimenez & Gilliam (1996) showing that CAT activity in honeybee guts is directly proportional to age, with higher levels found in foraging honeybees, suggesting that this enzyme can metabolize deleterious pro-oxidants generated by aerobic metabolism, making this enzyme potential for environmental quality monitoring programs. Weirich et al. (2002) and Collins et al. (2004) reported that high CAT activities are found in several *A. mellifera* in organs like midgut,

spermatheca and haemolymph, their being associated with the preservation of sperm in the queens' body, and as a protector of exogenous factors such as environmental poluents, impairing age process and cell death (Felton & Summers, 1995).

ALP is a group of isozymes responsible for hydrolysis in digestive processes and metabolite transport, with catalytic function, it is involved with metallic ions (Coleman, 1992). Delage-Darchen et al. (1982) found ALP in several glands of *A. mellifera*, but its major frequency and activity are related to the midgut. The increase in ALP activity after 24h of treatment with deltamethrin was detected in this work, but inhibition periods followed by induction were observed by Bounias et al. (1985) in *A. mellifera* intoxicated with sublethal doses of this same pyretroid. Borges et al. (2007) reported that sublethal doses of cypermethrin applied in silver catfish *Rhamdia quelen* (Quoy and Gaimard, 1824) (Siluriformes: Heptapteridae) induced the activity of ALP even 8 days after intoxication. Potential use of ALP in environmental quality monitoring programs here discussed was also target in some studies like the one by Suresh et al. (1993), verifying that ALP obtained from hemolymph of invertebrates can be used as an indicator of exposure to copper. Bounias et al. (1996) detected increase in ALP activity obtained from A. *mellifera* midguts when treated with cupric salts, suggesting that the increment of enzymatic activity may be related to honeybee's contact with several xenobiotics.

As here found**,** the stimulation of GST showing the activity of these enzyme in detoxification, transport of lipofilic compounds and tissue protection caused by xenobiotics. Much research showing the activity of GST, in most cases, this enzyme is responsible for insect resistance to insecticides (Fournier et al., 1992) or connected to insect development. Weirich et al. (2002) and Diao et al. (2006) verified that GST is present in all developmental phases of honeybees, with major activity in the midgut. Baars & Driessen (1984) reported GST

activity in *A. mellifera* and Yu et al. (1984) that the major activity is obtained when CDNB is used as a substrate. In essays with sublethal doses of pyretroids and organophosphates, only permethrin induced GST activity. Nevertheless, all evaluated insecticides in this work induced activity of this enzyme, but at different doses. Smirle & Winston (1988) demonstrated that GST showed different patterns of activity in function of age, due to labor division among workers. GST activity is inversely proportional to age, where the newly emerged has higher amounts of proteins, but lower specific activity. Yet, those with foraging activities have more specific activities and fewer amounts of protein, probably due to the adaptative capacity of honeybees to explore environments contaminated with insecticides. As observed in this study, similar results were obtained by Nielsen et al. (2000) and Papadopoulos et al. (2004), who detected that GST increases the activity when honeybees, independly of the phases of development, were exposed to deltamethrin and flumethrin.

5 CONCLUSIONS

- Deltamethrin, fipronil, spinosad and thiamethoxam were toxic to *A. mellifera*;

- The use of sublethal doses of these insecticides do not cause death of honeybees, however, their use at sublethal doses induces a different patterns of enzyme activity;

- Even the enzymes acetylcholinesterase, carboxylesterase; glutathione *S*-transferase; catalase and alkaline phosphatase exhibit distinct patterns when honeybees are exposed to pesticides, they can be used as biomarkers of exposition;

- It suggests that new experiments are performed to observe the behavior of these enzymes in relation to different forms of honeybee's exposition to insecticides, time of exposition and after intoxication periods.

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