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USE OF ACETYLCHOLINESTERASE IN APIS MELLIFERA AS A BIOMARKER OF PESTICIDES POLLUTION IN SOUSS MASSA REGION (SOUTH OF MOROCCO)

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ABSTRACT

The aim of this work is to study and test, as biomarker of pesticide contamination, the acetylcholinestersae activity (AChE) in the honeybee Apis mellifera. Kinetic parameters, organ distribution and the response of the AChE to field and laboratory exposure to pesticide were studied. The extraction of the enzyme must be carried out in Tris buffer or phosphate buffer. AChE activity was distributed mainly in the head compared to the thorax and the abdomen. The optimum assay temperature is 25 °C while the optimal pH is 9. The apparent Km value was 0.4 mM and the apparent Vmax was 6.8 nmoles/min/mg proteins. The study of the effect of exposure of Apis mellifera to deltamethrine and agricultural contamination were used as a preliminary test to demonstrate the reaction of AChE to a type of pesticide used in the Souss Massa region. Results showed an effect that depends on the exposure dose. The comparative study of AChE activity according to sites in the Souss Massa were conducted in Houara region (considered as a contaminated site) and Tassila (considered as a control site).The bee Apis mellifera shows a high AChE activity in animals from contaminated sites (Houara),compared to those recolted in the control site (Tassila).

Keywords: Acetylcholinesterase, Apis mellifera, Biomarker, Honeybee, Pesticides, Pollution, Souss Massa, Morocco

1. INTRODUCTION

The honeybees have important environmental and economic roles. These organisms are a vital part of agricultural ecosystems and intervene in the production of honey and royal jelly more their participation in the pollination and diversification of flora. They provide thus substantial benefits to the maintenance of the biodiversity and the productivity of both natural and agricultural ecosystems (Devillers, 2002).

These social insect are also interesting to use as sentinels species for ecotoxicological studies and as monitors of environmental health and quality and for detecting various pollutants or as test organisms for estimating the terrestrial ecotoxicity of xenobiotics (Bromenshenk, 1988; Cesco, 1994).

However and despite these advantages, these insects live critical conditions in some area resulting in a collapse and death of bee colonies. This degradation result from multiple factors such as pathogens, bad nutrition, natural habitat degradation, and pesticides. These latter are

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often incriminated and constitute one of many factors contributing to the decline of honeybees. These compounds, widely used in agricultural practice to protect crops from disease and pests, are known to cause problems to the apiculture industry by their widespread distribution (Barganska, 2014).

Their presence in or near areas beekeeping areas could can cause the collapse of bee colonies and their production in addition to reduce the quality and value of honey.

Pesticides constitute a global threat requiring the establishment of tool to monitor their environmental pollution and toxicity risk to organisms. Consequently, it is important to develop analytical technique able to detect theses xenobiotics.

In the last decade, an increasing interest has focused on the study and use biomarkers as ecotoxicological indicators of contamination. Such biological indicators are diagnostic and prognostic early warming tools and have been extensively used to reveal the exposure of organisms to various chemicals contaminants (Peakall, 1992). Such indicators, based on measurable perturbation of physiological or biochemical parameters after xenobiotic exposure, offer the potential of specificity, sensitivity and application to a wide range of organisms and for discriminating contamination over broad geographic regions (Walker *et al.* 2001).

These parameters, translating pesticides effects, can be seen and considered at all response levels: molecular, cellular, histological, individual, or even at higher ecological levels such as population, community, or ecosystem. In this context biochemical alterations as some enzymatic inhibition are often used as biomarkers of environmental health (Burgeot *et al.,* 1996). These parameters have had a great utility in environmental monitoring applications and offer a rapid and sensitive mean of following the impact of chemicals on living organisms, like pesticides on honeybees. Some have been extensively field validated and are already used in routine application in international programs (Burgeot *et al.,* 1996).

In this context, acetylcholinesterase enzymatic activity (AChE), allowing control and modulation of the neural transmission in animals, is one of the early validated biomarkers characterized in human environmental exposure which was used as biomarker of effect on nervous system following complex exposure to organophosphorus compound and carbamate insecticides (Payne *et al.* 1996; Bocquené *et al.,* 1997; Lafaurie, 1998; Lioneto *et al.* 2012).

The purpose of our work is to investigate the possibility of use of AChE activity as a biomarker of pesticides contamination in Souss Massa Region (South of Morocco). *Apis mellifera* was used as biological model in our study. This sentinel organism shows a high sensitivity towards the most widely used pesticides in agricultural ecosystems (Porrini et al., 2014).

In this article, we studied characteristics and organ distribution of AChE and the response of the enzyme to detlamethrine in the laboratory and to contamination in the field. Each of these parameters must be considered and optimized in order for a biomarker to be accurate and reproducible when using in biomonitoring approach.

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2. MATERIAL AND METHODS

Sampling sites

Foraging honeybees*(Apis mellifera)*were sampled in two sites: I*)* Site of Tassila located in Agadir city far from any human activity and considered as a control site and *ii)*Site of Houara (or Ouled Teima) located at 44 Km from Agadir and known for its intensive agricultural activity and exposed to various phytosanitary products. This last site was considered as contaminated site (Figure 1).

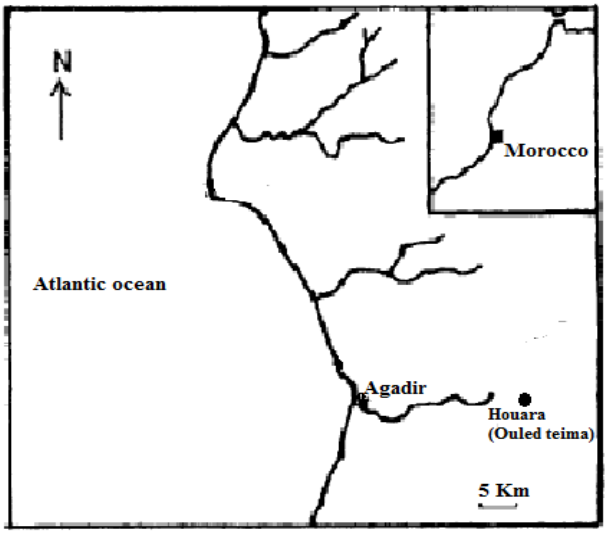

Figure 1: Sampling sites: Agadir (Tassila) and Houara

Studies of characteristics of AChE (assay conditions, Kinetic parameters, organ distribution and laboratory exposure to pesticide) and effect of laboratory exposure to deltamethrine were conducted on honeybees sampled from Tassila site and the response of the AChE to field exposure to pesticide were studied with animals from Ouleid Teima site.

Sample preparation

Foraging bees were collected from hives then introduced into an aerated plastic box until the laboratory where they are directly treated or stored at -30 °C until their use.

For the preparation on the extract, the tissues (whole animal or organs) were collected, thawed and washed with cold buffer (pH 7,4), weighed and homogenized in three volumes (w/v) of the same buffer using an Ultra turax homogenizer. Homogenates were centrifuged at 9000g for 30 min and the resulting supernatant (post-mitochondria1 fraction or S9) containing crude enzyme were directly used for assay or frozen (-30°C) until use.

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For organ distribution of ACHE activity, the extraction was conducted comparatively in the head, thorax and the abdomen.

For the study of the response of the AChE to field and laboratory exposure to pesticide (Delamethrine), extract was prepared as mentioned above.

Acetycholinestersase assay

The AChE activity in the extracts was measured by the method described by Ellmnan *et al.* (1961) using acetylthiocholine (ATC) as substrate.

The reaction mixture was prepared in buffer (*tris*-hydroxy-methyl-aminomethane or sodium phosphate buffer), containing DTNB at a final concentration of 0.08 Mand 50 µl of the tissue homogenate. 50µl of ATC at 0.045Mwas added to start the reaction. AChE activity was recorded at 412 nm for 2 min. Different temperature (10°C to 50°C) and pH (5 to 11) were tested before choice of optimal values (25°C and was 7.4 respectively). AChE activity was expressed in nmol of hydrolysed ATC/min/mg protein.

Proteins estimation

For quantitative determination of proteins, the method of Lowry *et al*. (1951) was used with bovine serum albumin as the standard.

Statistical analysis

The data were analyzed by ANOVA and the least significant difference (LSD) using STATISTICA.

3. RESULTS AND DISCUSSION

As mentioned above, biomarkers, like AChE, can provide information on the health of organisms and ecosystems. These measurable changes of cellular or biochemical functions were used widely as early warning signal for diagnostic or prognostic tools for assessing xenobiotic effects. To accomplish this goal, biomarkers must be studied and well known before their validation. These objectives include qualification, verification and assay optimization of the biomarkers analysis.

In this context, the characterization of biomarker is very important so that it could be properly applied in environmental monitoring. About that, the first step is the samples preparation, which showed to be a very important step in the measurement of biomarker. In our study, the use of AChE activity in a model organism *Apis mellifera* need to choose a buffer able to create an environment like cellular medium and ensuring a good expression of the enzymatic activity.

In our study, the main factors for assaying AChE activity (Temperature, pH, ionic strength (buffer) and the proper concentrations of substrates of AChE (Acetylthiocholine) were

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considered. Buffer of samples preparation, organ distribution, and response to laboratory and field exposure to pesticides were also studied.

Regarding the choice of buffer, Tris(*Tris*-hydroxy-methyl-aminomethane) and sodium phosphate buffer were tested for the extraction of AChE activity of *Apis mellifera*. Figure 2 shows that their use allowed to have a measurable activity, but the Tris buffer provides more activity than the Phosphate buffer, with 6,86 and 6,15 nmol/min/mg P respectively.

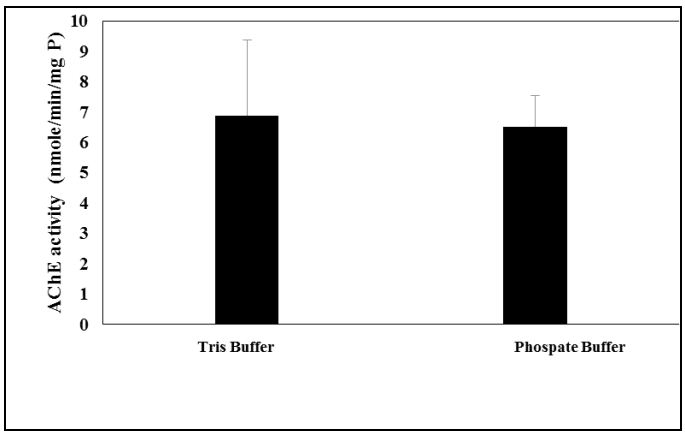

Figure 2: Effect of buffer of extraction of S9 on AChE activity of *Apis mellifera* sampled in Tassila (Agadir). ***Enzyme activity was determinate in S9 of whole animal at pH 7.4 for2 min. The values indicate mean ±SD, (n=6)***

These results confirm the effect of buffer, and particularly Tris and sodium phosphate buffers, on the properties of AChE (While *et al.,* 2011). The two solutions were widely considered as universal buffers used in many enzyme assays and other biological applications. They possess many properties allowing maintaining and optimizing protein stability during every stage of the procedure of extraction and assay.

These results are in agreement with those obtained for several organisms (Bocquené *et al*., 1997; Najimi *et al.,* 1997) and specially in *Apis mellifera (*Badiou and Belzunces, 2008).

In the rest of our study, Tris buffer was chosen because of its wide use for most biological experiments and its pKa approximately of 8,1 at 25°C making it effective buffer for adjusting and stabilizing pH between 7 and 9 with a high capacity and resistance to changes in pH comparatively to others buffers (Sambrook and Russell, 2001; Bisswanger, 2014). Otherwise, its very freely solubility and inertia in water in many enzymatic systems and its inexpensive cost contributed to its choice.

For the temperature, the effect on *Apis mellifera* AChE was between 10°C and 50°C (Figure 3). The weakest activities were observed for temperatures below 20°C and above 35°C and the highest activity were found at 25°C considered as optimal temperature. This value is in the range

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temperature (20°C to 30°C) described for AChE activities described in many other species (Suteau *et al.*, 1988) and particularly in *Apis mellifera*.

These results traduce the dependence of AChE activity on the temperature and the obligation to consider it in AChE use as biomarker. Indeed, two of the most important factors affecting AChE activity are the temperature of the environment from which the organism was sampled and the temperature of the assay (Hogan, 1970).

For assay temperature specific to our study, 25°C was chosen as optimum temperature for the following of our work.

This value is in the range temperature (20°C and 30°C) described for AChE assay in many organisms. This value is frequently preferred because of experimental reasons and easier manipulation.

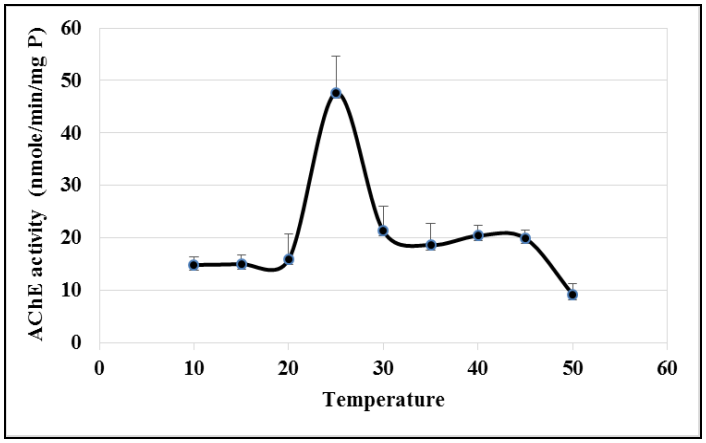

Figure 3: Effect of temperature incubation on AChE activity of *Apis mellifera* sampled in Tassila (Agadir). *Enzyme activity was determinated in S9 of whole animal at pH 7.4 for 2 min. The values indicate mean ±SD, (n=6)*

The relationships between *Apis mellifera* AChE activity and pH of the incubation mixture are shown in Figure 4.

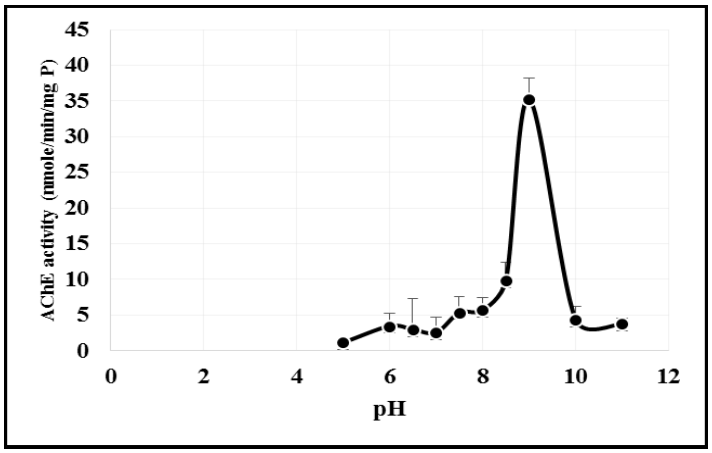

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Figure 4: Effect of pH incubation on AChE activity of *Apis mellifera* sampled inTassila (Agadir) .*Enzyme activity was determinated in S9 of whole animal at 25°C for 2 min. The values indicate mean ±SD, (n=6)*

As physiological functions, the activity of AChE seems to be very sensitive and depends to pH variation of assay buffer. The weakest values were observed for pH below 7 and above 10 and the maximal activity was obtained at pH 9.This evolution in bell-shaped allure is typical for most enzymes (Bisswanger, 2014) and traduces the dependence and the interval of the stability and activity of AChE on the pH. Indeed, each enzyme is stable and active within the range of its pH optimum but its activity is slow above or below this value because the enzyme may be denatured by extreme pH (whether high or low).

These results appeared to be similar to those obtained with other invertebrates like *Mytilus galloprovincialis* and *Perna perna* (Najimi *et al*., 1997*)* and *Mytlilus edulis* (Bocquené *et al.*, 1997) or vertebrates species like peacock bass (*Cichlao cellaris*) (Silva *et al.* 2013), *Colossoma macropomum* (Dia Assis *et al*, 2010), rat (Eränkö, 1973) and in human (Kaya *et al.* 2013).

Despite the maximum activity obtained in our study at pH 9, we have choosen pH 7.4 for assay. This value is in the range pH (7 to 8.8) described for AChE assay in many organisms, and specially *Apis mellifera*, and frequently preferred because of experimental reasons and easier manipulation and maintenance.

Results about organ distribution of AChE activity in *Apis mellifera* are presented in Figure 5. The highest activity is noticed in the head followed by the thorax and abdomen.

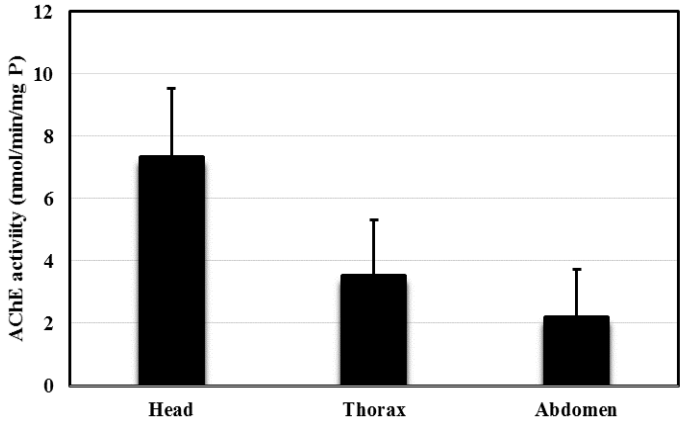

Figure 5: Organ distribution of AChE activity of *Apis mellifera* sampled in Tassila (Agadir) .*Enzyme activity was determinated in S9 tissue at25°CandpH 7.4 for 2 min. The values indicate mean ±SD, (n=6)*

The abundance of AChE activity in the head could be explained by the role of this organ in nervous transmission where this control of nerve transmission in the cholinergic synapses by hydrolysing the neurotransmitter acetylcholine. Similar results were obtained by Zhang *et al.* (2015) and Badiou *et al.* (2008) which have described that the honey bee presented two membrane-bound AChE (93-97% of total activity) and a soluble form (3-7% of total activity),

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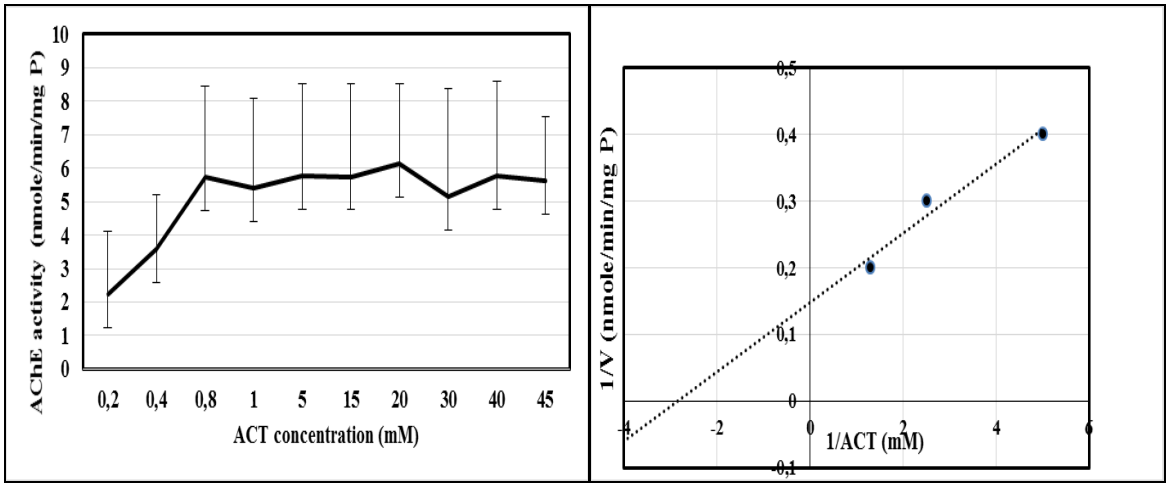
mainly localized in the head. This organ was often used to characterization of AChE activities in many insects (Gnagey *et al.*, 1987; Badiou and Belzunces, 2008; Badiou *et al.*, 2008; Zhang *et al.*, 2015). The same results was also described in *Drosophilia* and other insects in which most of the AChE activity is found in the central nervous system rather than in the periphery (Jan and Jan, 1976; Badiou *et al.*, 2008).

Michaelis-Menten representation of the kinetic of *Apis mellifera* AChE activity (Figure 6A) showed an increase according to the concentration of substrate before reaching its maximum value at the concentration of 0.8 mM acetylthiocholine. The maximum activity appeared at 0.8 mM of ACT in the incubation mixture.



A ***B***

Figure 6: Effect of acetythiocholine (ACT) concentration in the incubation mixture on AChE activity of *Apis mellifera* sampled in Tassila (Agadir) *(A) Michaelis-Menten representation; (B) LineweaverBurke representation. Enzyme activity was determinated in S9 of whole animal at 25°C and pH 7.4 for 2 min. The values indicate mean ±SD, (n=6)*

The linear regression of Lineweaver-Burke representation exhibited a significant correlation coefficient and the apparent biomolecular constant value (Km) was 0.4 mM and the apparent maximal velocity (Vmax) was 6.8 nmoles/min/mg proteins (Figure 6B). This Km values were comparable to those described for other types of bees such as *Apis cerana* (0.24 mM) (Badiou *et al.*, 2008; Zhang *et al.* 2005). The Km values observed in our study seem also to be close to those described in *Triatoma infestans* (Wood *et al.*, 1979) and some fish species, like *Oreochromis niloticus* (Rodriguez Fuentes and Gold-Bouchot 2004), *Pleuronectes vertulus* and *Pleuronychtis verticalis (*Rodriguez Fuentes *et al.*, 2008) and *Colossoma macropomum* Dia Assis *et al*, 2010.

The AChE activity seems to be inductible in *Apis mellifera* exposed in laboratory conditions to 0.5 an 1 ppm of delatmethrine or collected in Houara region which is known for its intensive agricultural activity and exposed to various phytosanitary products (Figures 7 and 8).

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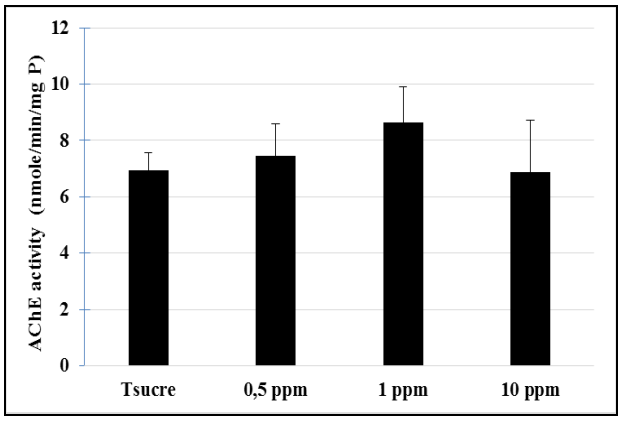

Figure 7: Response of AChE activity of *Apis mellifera* sampled in Tassila aflter laboratory exposure to Deltamethrine .*Enzyme activity was determinated in S9 of whole animal at 25°C and pH 7.4 for 2 min. The values indicate mean ±SD, (n=6)*

This kind of variation, which seems contradictory because anticholinesterase pesticides are considered as AChE inhibitors, have been described in many studies. Indeed, Boily *et al.* (2013) described that AChE activity decreases following exposure to herbicides atrazines and glyphosate, and a slight increase by exposure to neonicotinoids However, Badiou *et al.* (2008) showed an increase in AChE activity in *Apis mellifera* survivors following exposure to pyrethroids pesticides.

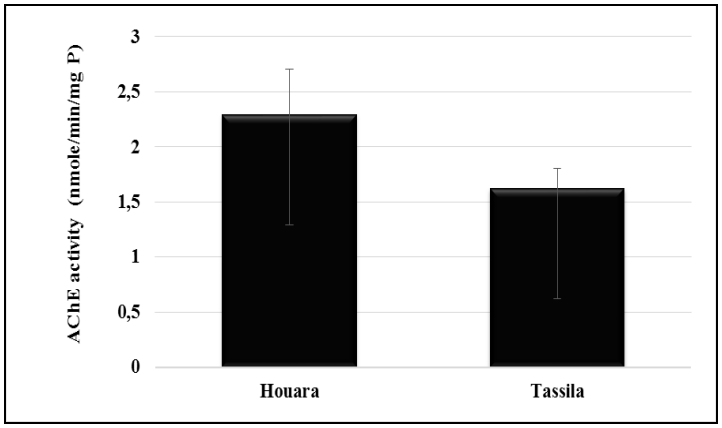

Figure 8: Comparative response of AChE activity of *Apis mellifera* sampled in Tassila (Control site) and Houara (contaminated site). ***Enzyme activity was determinated in S9 of whole animal at 25°C and pH 7.4 for 2 min. The values indicate mean ±SD, (n=6)***

These contaminants are known by their mimic action of acetylcholine (ACh) at the AChE nicotinic receptor (nAChR) Boily *et al.* (2013). Indeed, neonicotinoids are considered as ACh agonists that bind in the same way as ACh on the N-terminal subunit domain, mimicking the natural neurotransmitter by binding with high affinity, applying neuronal hyperexcitation and a

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membrane imbalance. The protracted action of neonicotinoids is mainly due to an absence of degradation by AChE (Vo *et al.*, 2010).

On a molecular level, Samson-Robert *et al.* (2015) described in neonicotinoid-exposed bees an increase in gene expression encoding AChE. The same mechanism was mentioned by Badiou and Belzunec (2008) for *Apis mellifera* bees exposed to delthametrine. According to these mechanisms, the increases in AChE of bees recorded during the exposure of animals to deltamethrine or bees harvested in the agricultural site (Howara) under our experimental conditions, would probably be an intracellular signaling that would have stimulated the expression of AChE gene, and consequently resulted in the increase of AchE activity, as has already been shown by Badiou and Belzunec (2008).

CONCLUSION

Our preliminary study gives a basic information on conditions assay of AChE activity of *Apis mellifera* in Souss Massa region (South of Morocco). It suggests that the enzyme is sensitive to field and laboratory exposure to pesticide (Delamethrine which is used in crop protection in Houara). It has a potent insecticidal activity with an appreciable safety margin.). Therefore, the activity of this enzyme may possibly be used as a biomarker of pollution by phytosanitary products

These results allow us to propose this biomarker for the monitoring of critical conditions incriminating pesticides in some area and resulting in collapse and death of bee colonies and known to cause problems to the apiculture industry.

Every program of research must consider the behavior of the enzyme in this species, as a result of different organ levels, and variations in AChE activity related to different pesticide exposure.

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