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Full Length Research Paper

Histological changes and biochemical parameters in the hepatopancreas of terrestrial gastropod *Helix aspersa* as biomarkers of neonicotinoid insecticide exposure

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In this study, adult snails, *Helix aspersa* were used to estimate the effect of aneonicotinoid insecticide (thiametoxam) on biochemical parameters and histological changes in the hepatopancreas of this gastropod after a treatment of six weeks. During this period, snails were exposed by ingestion and contact to fresh lettuce leaves which were soaked with an insecticide solution. The thiametoxam test solutions were 0, 25, 50, 100 and 200 mg/L. The results of the biochemical dosages (total carbohydrates, total proteins and total lipids) showed significant decreases at two concentrations (100 and 200 mg/L) of thiametoxam. However, the histological examination of the hepatopancreas of the treated snails showed alterations as a response to all the treatments, and revealed the degeneration of the digestive tubules and the breakdown of the basement membrane in a dose-dependent manner, leading to a severe deterioration of the tissues in the concentration of 200 mg/L thiametoxam. The dosage of carbohydrates, lipids and proteins supported by the study of histological changes on the hepatopancreas of *H. aspersa* can be considered as potential biomarkers of exposure to thiametoxam.

Key words: Insecticide, *Helix aspersa,* thiametoxam, hepatopancreas, biochemical study, histological study.

INTRODUCTION

Insecticides constitute a means of combating diseases and it is the most effective against the major diseases of the cultivated plants, and are necessary in the preservation and even in the increase of agricultural yields. However, most of the molecules of insecticides are highly toxic and are difficult to biodegrade. Their excessive use can engender fatal consequences for all the constituents of the environment.

In Algeria, the usage of insecticides and other phytosanitary products spreads more and more with the development of agriculture. So, the analyses of the residues of pesticides are not systematically made. Thus, in this context, we estimated with an experimental study, the effect of a neonicotinoid insecticide, the thiametoxam which is used as a commercial preparation (25 g of thiametoxam in 100 g of insecticide), on the terrestrial gastropod *Helix aspersa*. It is widely used in the Algerian North-East region against biting and sucking insects of cereals, fruit trees and vegetable crops. It is used in fields at doses ranging from 800 to 4000 mg/L of insecticide (commercial formulation), corresponding to 200 to 1000 mg/L of thiametoxam (active ingredient).

Even if most of the treatments are applied to the air

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Table 1. Dilutions of thiametoxam used and the corresponding pH.

Group	Treatment administered	pH
I	Control snails, untreated	5.80
II	Snails treated with 25 mg/L of thiametoxam	6.52
III	Snails treated with 50 mg/L of thiametoxam	6.12
IV	Snails treated with 100 mg/L of thiametoxam	5.81
V	Snails treated with 200 mg/L of thiametoxam	5.18

parts of the plants, a considerable part of the products always reach the soil, where bacteria, mushrooms, seaweeds, insects and terrestrial invertebrates live. Among these invertebrates, the snail *H. aspersa*, herbivore and detritivore, plays a major role in numerous ecosystems (Russell-Hunter, 1983). Due to its position in the trophic chain, it is the prey of numerous predators such as birds, mammals or invertebrates. Thus, it can be at the origin of transfers of contaminants (Laskowski and Hopkin, 1996b). Consequently, this terrestrial gastropod is more often used to estimate the impact of contaminations on different physiological and behavioral functions (Beeby and Richmond, 1988; Radwan and Salama, 1999; Coeurdassier et al., 2000, 2001; Gomot-de Vaufleury, 2000; Snyman et al., 2002, 2005; Salama et al., 2005; Regoli et al., 2006), either during laboratory tests or directly on sites.

Terrestrial snails such as *Helix aspersa* are well known for their capacities to accumulate different classes of chemicals in their tissues, particularly, the hepatopancreas (Gomot, 1997; Regoli et al., 2006). The possible use of cellular alterations on the gastropods' hepatopancreas as biomarkers for the exposure to xenobiotics have been investigated (Marigomez et al., 1998; Snyman et al., 2005; Radwan et al., 2008). However, little information is available in the literature concerning the study of the biochemical and the histological markers of mollusks which are exposed to insecticides (Hamed et al., 2007; Radwan et al., 2008) and especially, thiametoxam.

The aim of this study was to determine the effect of the insecticide, thiametoxam on the biochemical and histological parameters of a non-target organism, *H. aspersa*, which is one of the most abundant gastropod in north-east Algeria.

MATERIALS AND METHODS

Thiametoxam is a synthetic organic insecticide included in the neonicotinoids chemical family, class of thianicotynils. Its chemical formula is 3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]-oxadiazinan-4-ylidene-N-nitroamine). It is the most important new class of insecticides which was developed in the last three decades. Its advantage is that it fights against insects that are resistant to other pesticide classes. It also has a moderate toxicity to mammals (Bingham et al., 2008) birds and fish (Tomizawa and Casida, 2005), because they bind at the postsynaptic nicotinic acetylcholine receptor, more abundant in insects than in warm-blooded animals.

The thiametoxam was used as a commercial preparation. We chose four doses (25, 50, 100 and 200 mg/L) which are lower or equal to the concentrations that are applied in field. Indeed, the insecticide is applied in culture at concentrations ranging from 800 to 4000 mg/L, corresponding to 200 to 1000 mg/L of thiametoxam (active ingredient). Thus, the chosen concentrations are related to the concentrations that are lower than those encountered by snails in the field.

Experimental design

The snails used are the adults of *H. aspersa* collected in an untreated site by chemicals, situated in the north-east region of Algeria. Then, the snails were transferred to the laboratory, where they adapted to the controlled conditions described by Gomot (1994) (temperature 20 ± 2°C, photoperiod 18 hL/6hO, humidity 80 to 90%) during two weeks. However, they were exclusively fed with leaves of fresh lettuce. The 75 chosen individuals had a meanbody weight of 14.53 ± 0.2 g and a mean shell diameter of 36 ± 0.5 mm. They were divided into five groups of 15 animals each and were maintained in aerated plastic boxes (25 × 13.5 × 16.5 cm). The five groups of snails were fed with fresh lettuce (control snails), or fed with lettuce (during 30 s) soaked in an insecticide solution according as shown in Table 1. All the thiamethoxam dilutions were prepared with instilled water. The solutions of insecticides were renewed every week. The food was renewed thrice a week, when boxes were cleaned. The experiment was done for six weeks under the controlled laboratory conditions previously mentioned.

Biochemical dosages

At the end of the sixth week of exposure, snails were weighed. Then, they were starved for two days. Afterwards, 12 specimens chosen at random from each treated group were killed by decapitation and the hepatopancreas of each animal was quickly excised and weighed. Eight organs which were chosen from each experimental group were used for biochemical dosages. After the dissection, the extraction of the different constituents (total proteins, total carbohydrates and total lipids) was realized according to the process of Schibko et al. (1966) on a fragment (100 mg) of the hepatopancreas homogenized in 1 ml of trichloroacetic acid (TCA) in 20%. The dosage of total proteins was made according to the method of Bradford (1976), by using the bovine serum albumin as standard. The reading of absorbance was done at 595 nm. The dosage of total carbohydrates was determined according to Duchateau and Florkin (1959). This method uses glucose as standard, and the reading of absorbance was done at 620 nm. Total lipids are quantified according to the method of Goldsworthy et al. (1972), using the vanillin method. Absorbance was read after 30 min of darkness at 530 nm. The amount of metabolites was measured in an aliquot of 100 µl. Dosages were expressed as µg/mg of the analyzed tissue.

Histology

Four other glands were used for histological studies. These glands were fixed in the liquid of Bouin (Preece, 1972) for three days. Samples were prepared for analyses after several stages of rinses in demineralized water, dehydration in baths of alcohol in increasing degree and impregnation in a bath of paraffin wax (Odendaal, 2002). Then, the samples of hepatopancreas were included in the paraffin by means of molds (bars of Leuckart). Finally, samples were sectioned at a thickness of 6 µm by a Leitz microtome and stained using the hematoxylin and eosin method (Martoja and Martoja, 1967). Slides of four individuals of each exposure group (control and treated) were observed with the light microscope (Leica DM LB2).

Statistical analysis

For every concentration of insecticide, the results were expressed as mean ± standard deviation (SD), with significant level $p \leq 0.05$. The results obtained from treatments were compared with those obtained from the control using Student's test followed by the analysis of the variance (ANOVA) in one way of classification. All the calculations were made by means of the software MINITAB of analysis and data processing version 13.31.

RESULTS

Behavioral responses

Snails exposed to thiamethoxam showed a series of symptoms. The first symptom was the loss of chemoreception so that the snails were no longer attracted to food in terms of time in dose-related manner as compared to the control snails. At higher thiamethoxam concentration (200 mg/L), snails spent most of their time at the top of the boxes without showing any locomotion and feeding activities after the first week of exposure. At 100 mg/L of thiametoxam, almost all the snails refused to consume leaves of lettuce offered as food after three weeks of exposure. However, in lower concentrations (50 and 25 mg/L), most of the animals showed a normal activity during the experimental period.

Estimation of feeding rate

The mass of lettuce consumed by snails was not measured because we noticed that the lettuce leaves soaked in insecticide was rapidly losing its freshness as compared to that of the control snails (soaked in distilled water). Thus, it was impossible to quantify the mass of consumed leaves with precision. Consequently, it was impossible to estimate the feeding rate.

Biochemical parameters

The influence of pH of treated lettuce on the hepatopancreas was not significant. As pH values of tested solutions ranged between 5.18 and 6.52 (Table 1), it is likely that the effects observed on hepatopancreas were more due to the chemical than the low pH.

Effects of commercial thiametoxam insecticide formulation (0, 25, 50, 100 and 200 mg/L) on hepatopancreatic constituents (total carbohydrates, total lipids and total proteins) in *H. aspersa* were estimated after six weeks of treatment. All the previous constituents are shown in Figures 1A, B and C. Biochemical analysis revealed very significant differences in the rate of different metabolites in snails exposed to thiametoxam as compared to the control group. As shown in Figure 1, the content of total carbohydrates was not significantly reduced for 25 and 50 mg/L of thiamethoxam. Additionally, a very significant reduction in total carbohydrates was observed in snails exposed to 100 mg/L of thiamethoxam and highly significant in the concentration 200 mg/L as compared to the control value. On the other hand, the content of total proteins (Figure 1B) and total lipids (Figure 1C) was very significantly decreased for 100 and 200 mg/L of thiametoxam in tissues of treated snails as compared to the control.

Histopathology

A histological section of the hepatopancreas of the control group is illustrated in Figure 2a. It shows that the tissue of the digestive gland essentially consist of the juxtaposition of numerous digestive tubules which have various shapes and sizes and which are separated by inter tubular space, composed of hemolymphatic vessels and hemocytes. A simple epithelium of several cellular types lines the lumen of the tubules. These cells have various morphologies, but they have three main cellular types: digestive, calcium and excretory cells (Zaldibar et al., 2008). Tubules are maintained coherently by the intertubular connective tissue. Digestive cells constitute the major cellular component of the digestive gland tubule epithelium, and they are relatively polymorphic according to the stage of digestion (Heusser and Dupuy, 2011).

After 6 weeks of treatment, the histological examination of the hepatopancreas of the contaminated snails shows changes as response to all the treatments. In the concentration, 25 mg/L of thiametoxam (Figure 2b), a remarkable increase in the number of excretory vacuoles, a partial degeneration of some digestive cells and more and larger intertubular connective tissue were observed. So, in the concentration, 50 mg/L (Figure 2c), the same changes were observed, and they were accompanied by alterations in apical cell border, the release of fragments into the lumen, and breakdowns in the basement membrane of the digestive tubule with alterations of muscular fibers. The number of breakdowns increased in a dose-dependent manner. In the concentration, 100 mg/L (Figure 2d), the histological examination showed a very advanced degeneration of

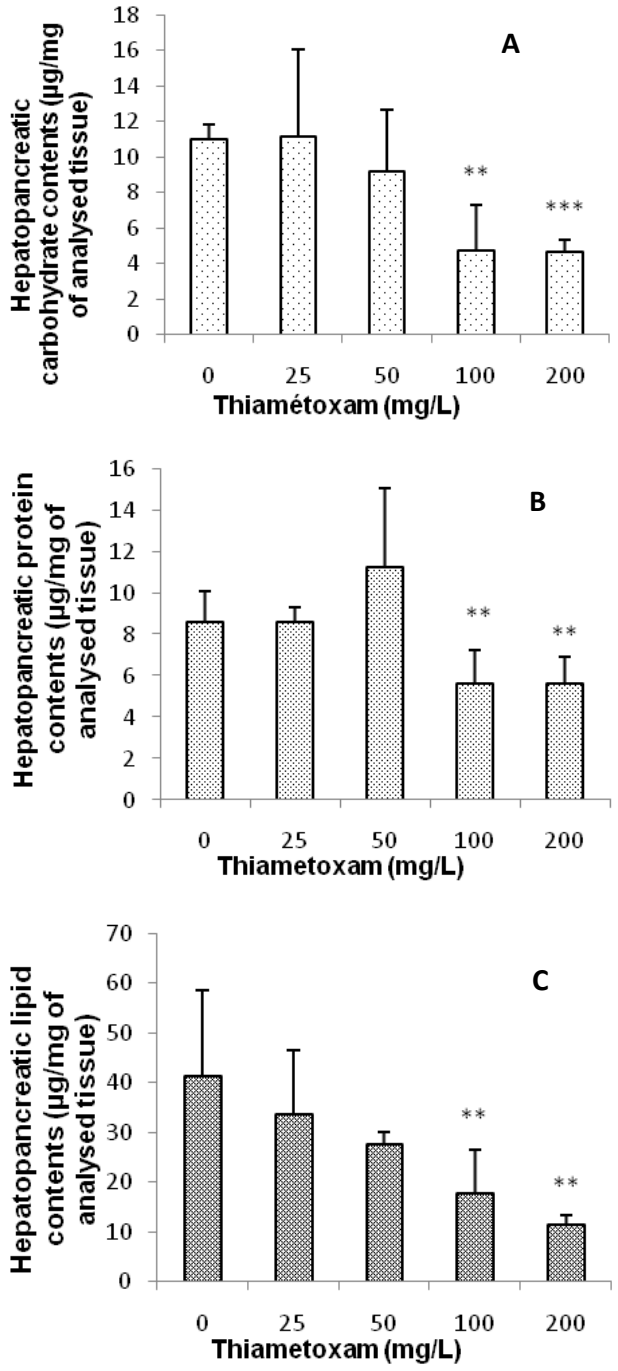

Figure 1. Rate of total contents (µg/mg of analyzed tissue) of carbohydrates (A), proteins (B) and lipids (C) in the hepatopancreas of *H. aspersa* after six weeks of exposure to 0, 25, 50, 100 and 200 mg/L of thiametoxam administered by ingestion. Values are expressed as mean ± standard deviations; n = 8; asterisks symbolizes significant difference between control and the treated groups; ** $P \leq 0.01$; *** $P \leq 0.001$.

the digestive cells, the calcium cells and the basal membrane of the digestive tubules. The tubules showed a reduced lumen with tissular fragments. The digestive tubules seem mingled in some places, because of the very important degeneration of the basement membrane. Finally, in the concentration, 200 mg/L (Figure 2e), the connective tissues, digestive tubules and their membranes were severely damaged.

DISCUSSION

Biochemical parameters in organisms exposed to toxic contaminants have been used as biomarkers and can constitute an important diagnostic tool to assess the exposure and effects of xenobiotics (Forbes et al., 1997; McLoughlin et al., 2000).

The evolution of the rate of total carbohydrates in the hepatopancreas of treated snails was dose-related. Indeed, carbohydrates constitute the primary and the immediate source of energy (Moussard, 1999). In stress conditions, carbohydrate reserves depleted to meet energy demands (Arasta and al., 1996). Our results are in accordance with those of El-Wakil and Radwan (1991). In addition, Padmaja and Rao (1994) suggested that the depletion of glycogen content in the tissue of the freshwater snail *Bellamya dissimilis* (Müller), exposed to endosulfan, methyl parathion, quinalphos and nuvanmay are due to direct utilization of glycogen for energy generation, to counter hypoxia caused by these pesticides. Additionally, a dose-dependent decrease of total proteins and total lipids from 100 mg/L of thiametoxam was noted in tissues of the treated snails when compared with the control.

Lipids constitute a restricted energy fuel which is proposed for tissues if necessary after carbohydrates, but proteins are mainly involved in the architecture of the cell. During chronic periods of stress, they are also a source of energy (Padmaja and Rao, 1994). Under stress conditions, the snails need more energy to detoxify the toxicant. Since snails have a limited amount of carbohydrates and lipids, the next alternative source of energy to meet the increased energy demand is proteins (Moussard, 1999). Also, these results are similar to those of Radwan et al. (2008). They showed the significant effect of both tested carbamates (methomyl and methiocarb) on the decrease of rates of total proteins and lipids in the tissues of *Eobania vermiculata* up to 7 days, involves the possibility of these chemicals to exert cytotoxic effects which are highly dependent on interference with lipoprotein levels and rate of biosynthesis. Moreover, decrease in total protein reserves of snail tissues may be partly due to the stress of the tested carbamates resulting from an imbalance between the rate of protein synthesis and degradation. Padmaja and Rao (1994) suggested that the decrease in tissue lipid and proteins of the freshwater snail *B. dissimilis*, under pesticides exposure could be due to several mechanisms like direct utilization by cells for energy needs. Eissa et al. (2002) reported that the harmful effect of chemical compounds could be attributed to the enhancement of energy utilization

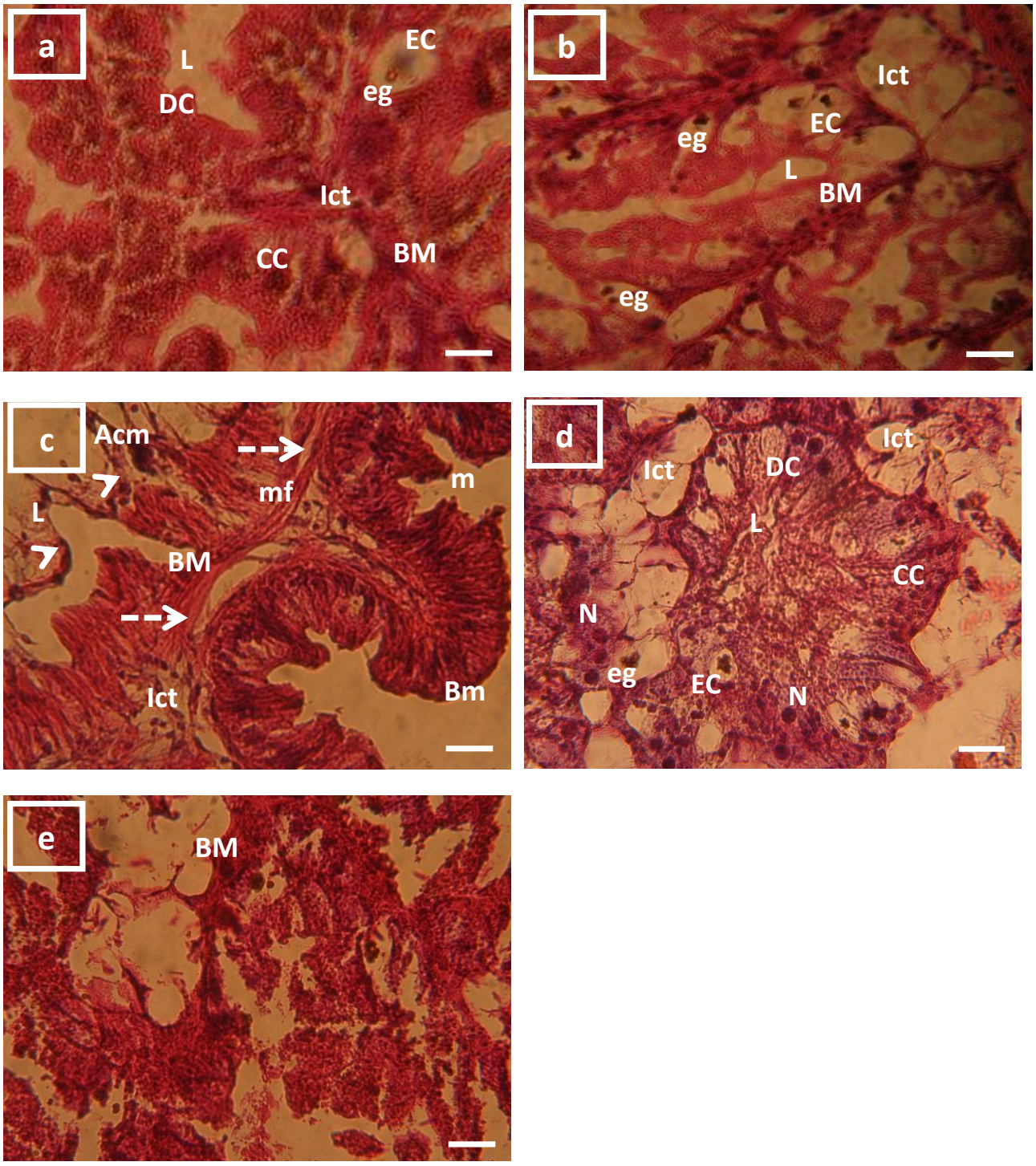

Figure 2. Histological sections of the hepatopancreas of snails *H. aspersa* in control and treated groups (after six weeks of treatment with thiametoxam) stained with hematoxilyn-eosin. L, Digestive tubule lumen; DC, digestive cells; CC, calcium cells; EC, excretory cells; lct, intertubular connective tissue; eg, excretory granules; BM, basement membrane; mf, muscular fibers; m, microvilli; Bm, border in brush of microvilli; N, nucleus; Acb, apical cell border. (a) Control snails, untreated showing the juxtaposition of numerous digestive tubules of various shapes and size. (b) snails treated with 25 mg/L of thiametoxam, showing a remarkable increase in the number of excretory vacuoles and partial degeneration of some digestive cells. (c) snails treated with 50 mg/L of thiametoxam showing alterations in apical cell border (arrow heads) and breakdown into the basement membrane (arrows) of digestive tubule with alterations of muscular fibers. (d) snails treated with 100 mg/L of thiametoxam showing reduced lumen and digestive tubules seems merged in some places, because of the very important degeneration of the basement membrane. (e) snails treated with 200 mg/L of thiametoxam showing tissues severely damaged. Scale bars: 50 µm (a, b, d and e); 20 µm (c).

and/or destructions of cells organelles of treated snails that may lead to protein synthesis.

The histological examination of the hepatopancreas showed that the thiametoxam provokes marked structural disruption from the lowest tested concentration. Indeed, the exposure to pesticides can cause important cytological changes in the hepatopancreas which plays a crucial role in the detoxification of pollutants (Frias-Espericueta et al., 2008). These observations are in agreement with those of Russell et al. (1981), Jonnalagadda and Rao (1996) and Chabicovsky et al. (2004). The histological study also revealed degeneration of the digestive tubules, fragmentation of the digestive cells and breakdown of basement membrane in a dose-dependent manner, leading to a severe deterioration of the tissues for the highest concentrations. These results corroborate with those of Radwan et al. (2008) and Hamed et al. (2007), according to whom the structural changes and the loss of digestive cells essentially and other cellular types seem to be a general response after an exposure to pesticides, in particular to carbamates for the land snail *E. vermiculata*. The deterioration of the digestive cells consequently induces the change of the global digestive process, provoked by the ingestion of different classes of chemical pollutants (Valavanidis et al., 2006). On the other hand, the structural changes observed at the higher concentrations could be due to starvation caused by the repulsion of contaminated food and thus, by induced estivation.

In this study, biochemical levels of hepatopancreatic constituents were largely reduced after thiametoxam intoxication at the highest tested concentrations of this insecticide, whereas the deterioration of hepatopancreatic tissues was observed in the lowest tested concentrations. So, these results indicate that histological alterations in the hepatopancreas reflect better, the toxic effects of the tested insecticide than the dosage of biochemical contents. This can be attributed to the fact that thiametoxam at low tested concentrations does not interfere with the energetic metabolism of hepatopancreatic cells, and more exactly, the biosynthesis and the biodegradation of energetic molecules (carbohydrates, lipids) or of proteins (Gil and al., 1989; Padmaja and Rao, 1994). Nevertheless, the reduction of the different constituents at the highest tested concentrations of thiametoxam could be caused by the alterations of tubular cell structures in general and it could especially be caused by the destruction of the basal and topical membranes. Thus, these alterations disturb the permeability of these membranes, slow down the food transport of nutrients, and also disturb all the digestive processes (Bourne et al., 1991). Similarly, Triebskorn and Florschuütz (1993) showed that the cloethocarb interferes with the passage of food in the digestive tract of the slug *Deroceras reticulatum*.

In this study, we exposed snails to different concentrations of thiametoxam for six weeks but in the natural environment, the snails may be exposed to pesticides in their lifetime and there is a need to test whether these responses are alike (Wang and Rainbow, 2005). After laboratory experiments, Wu et al. (2005) concluded that while some biomarkers (enzyme induction and lysosomal integrity) are clearly reversible after pollution reduction, other responses (cell damage, pathology) may be permanent and not reversible. In order to elucidate this issue (long term, re-adaptation), experiments will be performed in the future. It has been shown that the recovery time changes depending on the biomarkers, the pollutant and the species sensitivity (Wu et al., 2005). In addition, it would be interesting to quantify thiametoxam and its metabolites in snail's tissues to elucidate the effects of this insecticide on land snails.

The puzzle on the environmental consequences of pesticides requires the development of adapted methods for the analysis of their effects on the soil invertebrates, and particularly on the snail *H. aspersa*.

This occurrence presents contribution of research on the sub-chronic exposure of snails to the contamination by the thiametoxam under laboratory conditions. Results indicate that snails were sensitive to pollution with selected concentrations. This toxicological global approach revealed significant decreases of energetic constituents (rate of total carbohydrates, total proteins and total lipids), and remarkable changes of the hepatopancreatic tissue in the individuals of land snail *H. aspersa* exposed to the lettuce contaminated with the insecticide, likely to be used as biomarker of exposure, at first to thiametoxam then to other insecticides belonging to the same chemical family, currently present in the environment. Then, with regards to these results, this approach can be generalized in the other pesticides more recently used, and be the object of evaluation of the risks which they can undergo in the terrestrial ecosystems.

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