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Evaluation of acetylcholinesterase, glutathione S-transferase and catalase activities in the land snail *Helix aspersa* exposed to thiamethoxam

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Abstract
In the present study, adult snails *Helix aspersa* were used to estimate the effect of a neonicotinoid insecticide, thiamethoxam on the acetylcholinesterase (AChE), glutathione S-transferase (GST) and catalase (CAT) activities after a treatment of 6 weeks. During this period, snails were exposed by ingestion and contact to fresh lettuce leaves which were soaked with an insecticide solution. The thiamethoxam test solutions were 0, 25, 50, 100 and 200 mg/L, which are lower or equal to the concentrations that are applied in field. AChE activity was concentration-dependently inhibited by thiamethoxam. Also, GST and CAT activities were induced by neonicotinoïde insecticide with a dose-dependent manner. The responses of these enzymes are probably related to an increase in the release of reactive oxygen species in the presence of thiamethoxam. These results suggest the neurotoxic effect of thiamethoxam on snails exposed to the lettuce contaminated with this insecticide. In same time, this compound has effects on the digestive system, in addition to this neurotoxic effect in *Helix aspersa* (non-target organism).

Keywords: *Helix aspersa*, insecticide, thiamethoxam, AChE, GST, CAT

1. Introduction
The continuing need for novel and selective insecticides acting on pests has led to the development of new groups of compounds. The newest major group of insecticides is the neonicotinoids, which include the thiamethoxam [1]. This insecticide is neurotoxic compound that act on ion channels within the insect nervous system, acting mostly as agonist of insect nicotinic acetylcholine receptors [2]. The thiamethoxam is widely used in the North-East region of Algeria against biting and sucking insects of cereals, fruit trees and vegetable crops.
This insecticide is designed to induce high mortality in populations of target organisms. However, it can have collateral effects on non-target species by disturbing their physiology and by inducing illness in populations of living organisms [3]. Among these organisms, the snail *Helix aspersa aspersa* (syn. *Cantareus aspersus* or *Cornu aspersum* O.F. Müller, 1774) [4], herbivore and detritivore, plays a major role in numerous ecosystems [5]. 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 the contaminants transfers [6]. Consequently, this terrestrial gastropod is more often used to estimate the impact of contaminations on different physiological, biochemical and behavioral functions [7-12].
Terrestrial snails are well known for their capacities to accumulate different classes of chemicals in their tissues such as the brain, lungs, kidneys and particularly, the hepatopancreas [12, 13]. Only few data have been collected regarding the biochemical snail response to insecticides [10, 14, 15] and specially thiamethoxam. In invertebrates, the immune and detoxification systems respond quickly to chemical and biological stresses [16]. In this context, it seems interesting to try to characterize the disturbance of organisms by biochemical approaches through monitoring of biomarkers.
In this work the main aim was to investigate the effect of thiamethoxam on the activities of three selected enzymes in the snail *H. aspersa*, which is one of the most abundant gastropod in north-east Algeria.
We measured the following activities: acetylcholinesterase (AChE), glutathione S-transferase (GST), and catalase (CAT). We decided to test the following hypotheses:

(i) Thiamethoxam is neurotoxic compound, acting mostly as agonist of insect nicotinic acetylcholine receptors. The treatment with this insecticide will cause significant variations in AChE activity of non-target organism (snail).
(ii) Snails exposed to thiamethoxam stress will have significant variations in the activities of two hepatopancreatic enzymes: GST and CAT.

2. Materials and Methods

2.1 Chemicals

Thiamethoxam 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]-oxadia-zinan-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 has also a moderate toxicity to mammals [17, 18] birds and fish [1], because they bind at the postsynaptic nicotinic acetyl-choline receptor, more abundant in insects than in warm-blooded animals.

The thiamethoxam was used as a commercial preparation. We chose five doses (0, 25, 50, 100 and 200 mg/L) which are lower or equal to the concentrations that are applied in the 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 thiamethoxam (active ingredient). Thus, the chosen concentrations are related to the concentrations that are lower or equal than those encountered by snails in the field. The experiment was conducted from mid-October until late November 2012.

2.2 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) [19] (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 mean body weight of 14.53 ± 0.2 g. 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 to 25, 50, 100 and 200 mg/L respectively). 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.

2.3 Preparation of samples and biochemical dosages

At the end of the sixth week of exposure, snails were weighed. Then, 5 snails chosen at random from each treated group were killed by decapitation and the hepatopancreas of each animal was quickly excised and weighed. Five organs (head and hepatopancreas) which were chosen from each experimental group were used for biochemical dosages. The heads are used to determine AChE activity. At the same time, the hepatopancreas of the same specimens were used to measure the activities GST and CAT respectively.

Each head was homogenized in ice-cold 1 ml of detergent solution (10 mM Tris-HCl buffer, pH 7.2 with 160 mM sucrose) at 12 000 rpm for 2min, five repetitions are performed. The homogenates were centrifuged at 5000×g for 5min. The supernatant is recuperated to serve as the enzyme source.

AChE activity [EC 3.1.1.7] was assayed [20] using acetylthiocholine (AtCh) as a substrate. In the reaction catalyzed by AChE, AtCh is hydrolyzed to acetic acid and thiocholine. The thiocholine, in the presence of DTNB (5'-dithio-bis-2-nitrobenzoic acid) gives a yellow product 5-thio-2-nitrobenzoicb acid (TNB). TNB was measured spectrophotometrically at 412 nm. AChE activity was expressed as µmol of thiocholine released h^{-1} mg^{-1} of protein.

Hepatopancreatic samples were homogenized in ice-cold 0.05 M phosphate buffer (pH 7.4) in a Teflon-glass homogenizer. Homogenates were centrifuged at 15,000×g for 10 min. For each enzyme, five repetitions are performed.

GST activity [EC 2.5.1.18] was measured [21] using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. In the reaction catalyzed by GST, glutathione conjugates with CDNB. The concentration of the complexes was measured spectrophotometrically at a wavelength of 340 nm. The results were expressed as µmol of CDNB·min^{-1}·mg $protein^{-1}$.

CAT activity [EC 1.11.1.6] was measured following the method proposed by Regoli et Principato (1995) [22]. The rate of H_2O_2 reduction was measured spectrophotometrically at a wavelength of 240 nm. The results were expressed as µmol of reduced H_2O_2 min^{-1}·mg $protein^{-1}$.

Protein concentration was determined using the method described by Bradford (1976) [23]. Bovine serum albumin was used as the standard.

2.4 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.

3. Results and Discussion

Effects of commercial thiamethoxam insecticide formulation (0, 25, 50, 100 and 200mg/L) on enzymatic activities (AChE, GST and CAT) in *H. aspersa* were estimated after six weeks of treatment. All the previous activities are shown in Figures 1A, B and C.

The effects of thiamethoxam on AChE activity of snail's heads are showed in Figure 1A. A significant reduction ($p \leq 0.05$) in AChE activity was observed in snails exposed to 100 and 200 mg/L as compared to the control.

The oral treatment with 50 mg/L increased significantly ($p \leq 0.05$) GST activity compared to the control (Fig. 1B). Then, the concentrations 100 and 200 mg/L induced a highly significant increase ($p \leq 0.001$) of GST activity, with a dose-dependent effect.

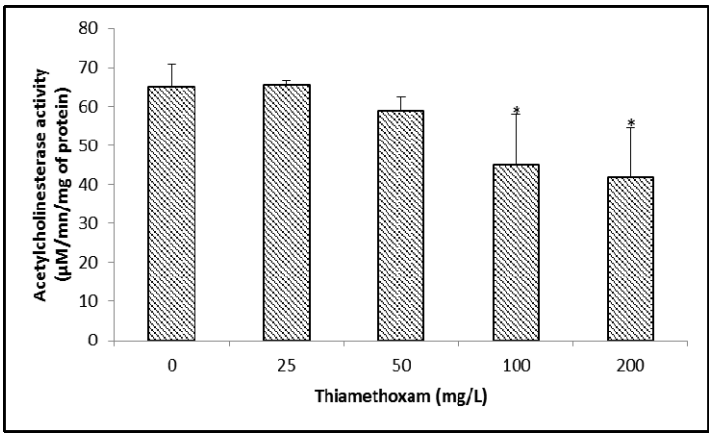
Effects of thiamethoxam on CAT activity of hepatopancreas are presented in Figure 1C. The treatments with 50, 100 and 200 mg/L induced a significant increase ($p \leq 0.05$) in the activity of the enzyme, with a dose-dependent effect.

The analysis of AChE, GST and CAT activities by one-way analysis of variance showed highly significant ($p \leq 0.001$) effects of treatment with thiamethoxam for the two biomarkers AChE and GST respectively, and significant ($p \leq 0.05$) effect for CAT activity.

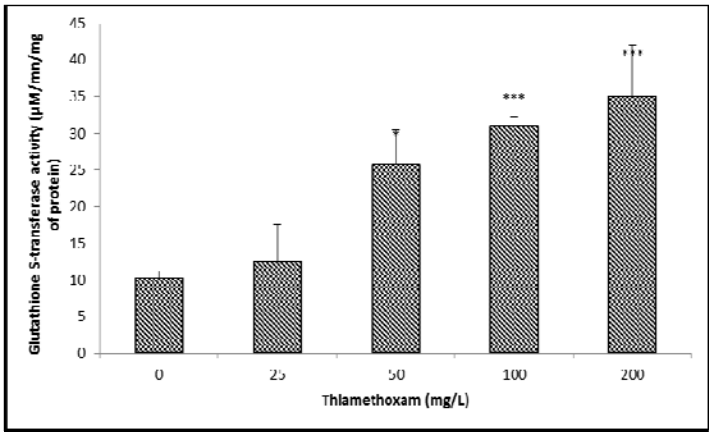
Biochemical parameters in organisms exposed to toxic contaminants have been used as biomarkers and can constitute an important diagnostic tool to assess the exposure and effects of xenobiotics [24, 25]. Biomarkers of environmental stress measured in this study are AChE, GST and CAT activities.

The insecticide enters in the body of the snails by various ways and crossing several barriers before reaching the hepatopancreas which is the organ of detoxification of xenobiotics [26-29]. They can be reversibly absorbed by the hepatopancreas then they are detoxified and finally excreted [30].

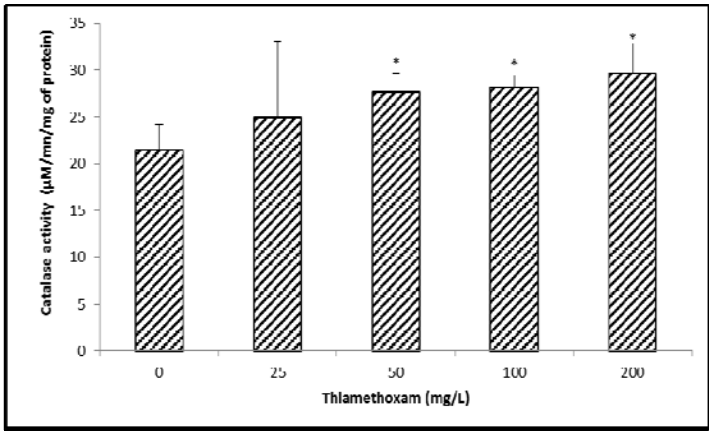
AChE activity plays no role in detoxification in vertebrates but it is involved in the mechanisms of transmission of nerve impulses throughout the body. The inhibition of the enzyme by many neurotoxic compounds leads to an accumulation of the chemical messenger, the acetylcholine in the synaptic space, which maintains a constant transmission of nerve impulses, leading to the death of the individual [31]. Among the neurotoxic compounds, organophosphorus insecticides and carbamates are considered the most potent and specific inhibitors of cholinesterase [32, 33].



(A)



(B)



(C)

Fig 1: Acetylcholinesterase activity in the heads of *H. aspersa* (A), Glutatione S-transferase activity (B), and Catalase activity (C) in the hepatopancreas of snails after six weeks of exposure to 0, 25, 50, 100 and 200 mg/L of thiamethoxam administered by ingestion. Values are expressed as mean ± standard deviations; n = 5; asterisks symbolizes significant difference between control and the treated groups; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

In invertebrates, the role of cholinesterase is less clear, although the existence of cholinergic neurons as well as specific acetylcholine receptors has been demonstrated in mollusks and gastropods [34]. Thus, AChE activity is used as a marker of exposure to inhibitors pesticides in mollusks.

Our results show that thiamethoxam inhibited significantly AChE activity in *H. aspersa*. Given the fact that AChE activity is a specific biomarker of exposure to organophosphate pesticides [35], brominated and carbamate pesticides [36], it is certain that the differences measured on this enzyme activity reflect less a specific effect that a total impact of the contamination.

Neurotoxic effects of non-molluscicides organic substances were evaluated by Rorke *et al* (1974) [37], who observed 90% inhibition of the cholinesterase activity in the hemolymph of *H. aspersa* induced *in vitro* by 10^{-4}g/L of fenitro-oxon (OPs) or physostigmine (carbamate). The fenitrothion and the diethylphénylphosphate (OPs) do not cause cholinesterase inhibition at the concentration of 10^{-3}g/L [38]. Inhibition of AChE activity in *H. aspersa*, has also been reported after topical treatment by the methomyl, carbofuran, chlorpyrifos and paraquat [11]. In *Pomacea patula*, Mora *et al* (2000) [39] show that the inhibition of AChE activity is correlated to the concentration of carbaryl in the same organ. In *H. aspersa*, Coeurdassier *et al* (2001) [8] have also shown that AChE activity was inhibited by 10% after 7 days of exposure to dimethoate at concentrations very near to those recommended for application in the field.

The GST are phase II biotransformation enzymes. Their function is to combine a large variety of substrates with a molecule of glutathione (GSH) to permit their elimination. These substrates may be endogenous molecules, as well as xenobiotics including pesticides [40-43].

Thus, the treatment by thiamethoxam applied orally induces a significant increase of GST activity in the hepatopancreas of *H. aspersa*. Since the GST enzymes are among the first antioxidant enzymes to respond to the presence of a xenobiotic, the induction of this enzyme's activity probably indicates a high rate of conjugation with GSH compound tested, and possible activation of antioxidant defenses. On the other hand, the direction and intensity of the changes depend on the species, gender, animal fitness, exposure time and concentration of the thiamethoxam, as well as on the presence of other toxic chemicals in the environment.

The induction of GST activity is in agreement with results reported by Gagné *et al* (2006) [44].

Demonstrating that the pollutants increase this enzyme's activity in the digestive gland of bivalve *Mya arenaria*. The increased GST activity observed may be due to the activation of the antioxidant system by natural pesticides. However, no significant change in GST activity was observed in mussels *Mytilus galloprovinvialis* collected from severely contaminated site by PCBs [45]. However, Cheung *et al* (2002) [46] observed an induction of GST activity, correlated positively with the amount of PCBs in mussel *Perna viridis* transplanted in many contaminated sites around Hong Kong. Finally, Torres and Mason (2002) [47] observed inhibition of GST activity (*in vitro*) in snail *H. aspersa* exposed to 10 µM of tributyltin.

The CATs are enzymes, which prevent peroxidation of the biological molecules induced by hydrogen peroxide. They are sensitive to numerous contaminants inducing an oxidative stress on cell membranes, such as PAHs, PCBs and some pesticides [48] and metals [49]. Results showed an increase [50] or a decrease in activity [49]. The hypothesis retained is that this enzymatic activity seems to be very sensitive to anthropic or natural environmental factors [51]. This hypothesis is supported by the results obtained by Pellerin-Massicotte *(*1997) [52], observing induction of catalase activity in an unpolluted site. This induction could be due to physiological stress as a repetition of lay. According to these authors, catalase may be sensitive to subtle changes in environmental conditions.

In *H. aspersa*, the induction of CAT activity is obtained after treatment with thiamethoxam. The effect was especially distinct from 50 mg/L (Fig. 1C) of this neonicotinoid. Thus, the thiamethoxam belong to the large group of oxidative stress-generating chemicals in the hepatopancreas of *H. aspersa*. Similar results were observed by Salama *et al* (2005) [11], in the same species of gastropod after exposure to methomyl and chlorpyrifos. However, the same authors found that this enzymatic activity decreased after exposure to carbofuran and paraquat. El-Gendy *et al* (2009) [53] reported an induction of hepatopancreatic CAT activity in *Theba pisana*, after treatment with pesticides containing copper.

H. aspersa appears sensitive to the treatment with the thiamethoxam in laboratory. But, in the wild, snails can be exposed to different mixtures of pesticides throughout their lives. Therefore, it is necessary to determine whether these responses are similar [54]. After laboratory experiments, Wu *et al* (2005) [55] concluded that while some biomarkers such as enzyme induction are clearly reversible after reduction of the pollution, other responses (cell damage) can be permanent and not reversible. To clarify this question, experiments must be performed in the future. It has been described that the adaptation duration changes with time of recovery based biomarkers, the pollutant and the sensitivity of species [55].

4. Conclusion

The number of molecules available in the market is considerable and not all are subject to further evaluation. Also, many data on the residual quantities actually present in the environment, their behavior, their fate in these compartments and their toxicity are missing.

The study of the response of some environmental stress biomarkers in the snail *H. aspersa* exposed to thiamethoxam revealed a neurotoxic action resulting in inhibition of AChE activity. These nerve disturbances may explain, in part, the observed decreases in the duration of food intake and the amount of food ingested. Additionally, the insecticide induces the detoxification system through an increase in GST and CAT activities. These changes are probably related to an increase in the release of reactive oxygen species in the presence of xenobiotics in general, and of thiamethoxam in this case.

Thus, the neonicotinoid would seem that in addition to its neurotoxicity in insects, it is also neurotoxic in terrestrial gastropods (non-target organisms).

In addition, it would be interesting to quantify thiamethoxam and its metabolites in snail's tissues and especially, head and hepatopancreas to elucidate the effects of this neonicotinoid on terrestrial gastropods including *H. aspersa.*

Our enzymatic exploration after exposure to thiamethoxam in *H. aspersa* remains to complete using enzymatic dosages in other organs of the snail (such as kidney, lung, genitals or foot sole) that to be affected by the test compound.

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