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Effects of stress on brain acetylcholinesterase activity of a centipede *Eupolybothrus nudicornis*

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Abstract

With a view to using the centipede *Eupolybothrus nudicornis* as a bioindicator in biomonitoring programs we studied the effects of environmental pollution on the activity of the enzyme acetylcholinesterase (AChE) as neurotoxicity marker. Males and females were collected during the spring breeding season at two sites located in the eastern part of Algeria: Guelma (36°25'N, 7°25'E) and Annaba (36°52' N, 7°45' E). The Guelma site was considered as the healthy reference site as it was away from any source of pollution. On the other hand, the Annaba site was considered polluted due to the presence of an important steel complex. Individuals of both sexes from Annaba showed a lower brain AChE activity than those from Guelma which could probably be due to chemical pollutants from the steel complex. Moreover, as AChE is considered as an exposure biomarker of pesticides contamination, we tested the effect of a bisacylhydrazine ecdysteroid analog (RH-0345) used as insecticide on brain AChE activity of *E. nudicornis*. Brain AChE activity was determined 5, 10 and 15 days after injection of 10 µg of RH-0345. A significant decrease of brain AChE activity was observed in both males and females but the effect was much more pronounced in females.

Keywords: AChE activity, Myriapoda, biomarker, ecdysteroids, neurotoxicity

1. Introduction

Soils of today are under pressure of various pollutants including inorganic and organic chemicals such as pesticides. Soil fauna plays an essential role in several soil ecosystem functions. Soil pollutants can affect soil animals and have deleterious consequences for ecosystems. Many arthropods make their home in the soil. Centipedes (Chilopoda), one of the four major lineages of myriapods, are an important group of predatory arthropods in many terrestrial habitats [1]. Due to their major role in terrestrial ecosystems centipedes could be used as sentinel organisms in order to assess soil quality.

With a view to using the centipede *Eupolybothrus nudicornis* (Gervais, 1837) (= *Eupolybothrus elongatus*, = *Bothropolys elongatus*) as a bioindicator in biomonitoring programs we studied the effects of environmental pollution on the activity of the enzyme acetylcholinesterase (AChE) as neurotoxicity marker. AChEs are widely distributed in the animal kingdom because they are essential for the transmission of nerve impulses. AChEs are involved in synaptic transmission and their main function is hydrolysis of acetylcholine, the mediator of cholinergic synapses in the nervous system. The inhibition of the activity of AChE provokes an accumulation of the neurotransmitter; its action is enhanced and it finally causes death. AChE activity is considered as an exposure biomarker to organophosphate and carbamate pesticides and also to other contaminants such as metals, synthetic detergents, some components of fuel oils and algal toxins [2]. So, the aim of this study was to test the influence of a bisacylhydrazine ecdysteroid analog (RH-0345) used as insecticide on brain AChE activity of *E. nudicornis* which could be directly affected by exposure to this compound or following ingestion of prey containing this molecule.

2. Materials and methods

Experiments have been conducted on mature females and males *Eupolybothrus nudicornis* collected during the spring (reproduction period) of 2012.

2.1 Study sites

Two sites located in the eastern part of Algeria were selected for this study (Figure 1). The first site is located north of the province of Guelma (36°25'N, 7°25'E). This province lies 290 meters above sea level and 537 km from Algiers. This is a fertile land due to Seybouse Oued and its large dam that provides a wide irrigation system. The climate is humid and sub-humid,

rainfall is estimated at 450-600 mm/year. This site is completely open and is considered as a healthy reference site as it is away from any source of pollution. The second site is located in the north-eastern part of the Annaba area (36°52' N, 7°45' E) near a steel complex (Figure 1). The climate is

humid, rainfall is estimated at 300-500 mm/year. This site is completely open and is characterized by the presence of some grasses and stones. Due to the presence of this steel complex, this site is considered as polluted.



Fig 1: Map of North Africa showing location of sampling sites near Annaba (polluted site) and Guelma (reference site) in Algeria.

2.2 Physico-chemical analysis of soil

At sampling sites, 5 soil cores were taken to a depth of 20 cm with a manually-powered Edelman auger. After removal of coarse material (leaves, stones, roots and snail shells) cores were combined. Homogenization was performed by hand. The samples were placed in clear polythene bags which were labeled clearly and transported to the laboratory for subsequent analysis. Once in the laboratory samples were dried in the open air and subsequently analyzed.

Soil texture was determined by densitometry based on the variation of the volumic mass of a suspension of 50 g of soil in 25 ml of sodium hexametaphosphate 5% during sedimentation with a ASTM hydrometer No. 1. 152H-Type (Bouyoucou Hydrometer Method).

The pH of the soil was measured with a pH meter (HANNA instruments P209) using a suspension of 50 g of soil (dry weight) in 100 ml of distilled water.

2.3 Collection of individuals

At each study site, five pitfall traps made of plastic cups (8.5 cm, in diameter by 17 cm in length) were used to collect individuals of both sexes during the spring breeding season sites in order to assess the effects of the environmental stress on the activity of the enzyme acetylcholinesterase as neurotoxicity marker.

Individuals were maintained individually in the laboratory on regularly moistened filter paper. They were fed three times a week with insects (cockroach larvae, flies and mosquitoes) and spiders.

2.4 Chemical and toxicity test

Only individuals collected from the reference site were used to test the effect of the ecdysteroid analog RH-0345 on AChE activity. RH-0345 (halofenozide) was developed by Rohm & Haas Company (Pennsylvania, USA). It was a gift of G. Smaghe (Laboratory of Agrozoology, University of Gent, Belgium). It is a bisacylhydrazine ecdysteroid agonist of 20-hydroxyecdysone and exhibits its insecticidal activity via interaction with ecdysteroid receptor proteins^[3].

RH-0345 was dissolved in acetone to a concentration of 3.33 µg/µl for experimental use. 3 µl were injected by means of a microsyringue between the third and fourth dorsal segment. Toxicity tests were performed during a 15-day period on females and males collected from the reference site. Dose level injected was based on previous finder range test^[4] and was

chosen in order not to be lethal during the experiment. Control animals were maintained in the same conditions and injected with 3 µl of acetone.

Animals were treated in accordance with the guidelines of the local ethics committee.

2.5 Protein content and brain AChE activity

The brain AChE activity was carried out following the method of Ellman *et al.* (1961)^[5] using acetylthiocholine (ACTH) as a substrate. Pooled head (each containing 2-3 heads per series) were homogenized in the following solution containing 38.03 mg ethylene glycol tetraacetic (EGTA), 1 ml Triton X-100, 5.845 g NaCl and 80 ml Tris buffer (10 mM, pH 7). After centrifugation (9000 g, 15 min), the AChE activity was measured in aliquots (100 µl) of resulting supernatants added to 100 µl of 0.01M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in Tris buffer (0.01 M, pH 8) and 1 ml Tris (0.1 M, pH 8). After 5 min, 100 µl of 0.075 M ACTH was added. Measurements were conducted at a wavelength of 412 nm with a run time of 20 minutes. Bradford's method^[6] was used for quantitative determination of proteins with bovine serumalbumin as the standard. Brain AChE activity was expressed as nmol ACTH hydrolysed/min/mg protein.

2.6 Statistical analysis

Results have been expressed as mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test. The homogeneity of variance was controlled by the Levene method, 1960^[7].

3. Results and discussion

3.1 Abundance of *E. nudicornis* in the two studied sites

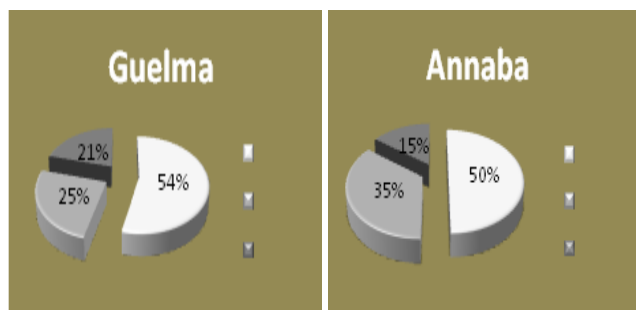
E. nudicornis was the most abundant species at the two study sites (Table 1) where it represented 24.68 and 32.96% of the individuals collected at Annaba and Guelma respectively. Nevertheless, the number of individuals collected in the reference site was almost the double than that of the polluted site (Table 1, Figure 2). Although the total number of myriapoda individuals collected was more important in Guelma (728) than in Annaba (632), the diversity was much more important in Annaba (9 species) than in Guelma (4 species). The Guelma site was specially characterized by the absence of Diplopoda (Table 1).

Table 1: Abundance of *E.nudicornis* at the two studied sites

Order	Species	Annaba		Guelma	
		Number	Percentage	Number	Percentage
Chilopoda	<i>Lithobius forficatus</i>	55	8.70%	0	0%
	<i>Eupolybothrus nudicornis</i>	156	24.68%	240	32.96%
	<i>Scolopendra morsitans</i>	98	15.50%	144	19.78%
	<i>Cryptops hortensis</i>	22	3.48%	0	0%
	<i>Orya barbaric</i>	99	15.66%	184	25.27%
	<i>Geophilus ferrugineus</i>	56	8.86%	0	0%
Diplopoda	<i>Scutigera coleoptrata</i>	15	2.37%	160	21.97%
	<i>Ommatoiulus malleatus</i>	18	2.84%	0	0%
	<i>Brachydesmus proximus</i>	113	17.87%	0	0%

3.2 Physico-chemical analysis of soil

The soils of the two studied sites had a high percentage of clay particles (50 and 54 percent for Annaba and Guelma respectively) and can be considered as clay. Nevertheless, the content of sand was more important in Annaba (35%) than in Guelma (25%) and inversely the content of silt was more important in Guelma (21%) than in Annaba (15%) (Figure 2). The soil pH in Annaba was near-neutral (pH = 7.2) while the soil pH in Guelma was slightly alkaline (pH = 7.8)

**Fig 2:** Soil texture at the two studied sites.

3.3 Brain AChE activity

3.3.1 Comparison of brain AChE activity in males and females during the spring breeding season from the two studied sites

Measurements of AChE activity provide information on contaminant exposure [8]. No significant difference was observed in brain AChE activity between males and females collected from the reference site (Table 2). On the contrary, brain AChE activity was significantly lower in females collected from the polluted site compared to males ($P < 0.05$). Brain AChE activity was significantly lower in females ($P < 0.001$) and males ($P < 0.05$) collected from the polluted site compared to the reference site (Table 2).

Animal's neurological and behavioral activities can be extremely sensitive to environmental contamination [9, 10, 11, 12]. Acetylcholinesterase (AChE) has a key role in neurotransmission by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses of the nervous system. Many authors reported that enzymatic activities of soil organisms can be extremely sensitive to seasonal variations of the environment, as well as the probable contamination of the environment [13]. In that context, we showed significant decreases of brain AChE activity in females and males from the polluted site (Annaba) compared to those from the reference healthy site (Guelma). AChE inhibition may be an indicator of metal pollution [14, 15, 16]. In addition, other authors emphasize the potential impact of several factors, such as age, sex, size or physiological conditions on AChE activity after exposure to polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [17] or pesticide [18].

Table 2: Comparison of AChE activity in males and females from the two studied sites.

Site Sex	Guelma	Annaba
Females	0.14±0.006	0.038±0.009***
Males	0.12±0.007	0.057±0.006***

Values are represented as Mean ± SD of five replicates

*** = 0.001 ≥ P

3.3.2 Influence of RH-0345 on brain AChE activity in females

Brain AChE activity of control females was stable throughout the experiment. A significant decrease ($P < 0.001$) of brain AChE activity in females was observed 5 days after exposure to RH-0345. This decrease was maintained throughout the experiment (Table 3).

Anticholinesterase compounds can affect locomotion and equilibrium of exposed organisms [19, 20, 21, 22], which usually lead to muscle tetany and death [23, 24].

Table 3: Effect of RH-0345 on AChE activity in females.

Observation Period (days)	AChE activity in Controls	AChE activity in Treatment (RH-0345)
0	0.14±0.006	0.14±0.006
5	0.17±0.03	0.04±0.002***
10	0.16±0.011	0.037±0.001***
15	0.19±0.001	0.029±0.005***

Values are represented as Mean ± SD of five replicates

*** = 0.001 ≥ P

3.3.3 Influence of RH-0345 on brain AChE activity in males

Brain AChE activity of control males remained stable throughout the experiment and was comparable to that of control females confirming previous results. We noticed a significant decrease of brain AChE activity 5 days ($P < 0.01$) and 15 days ($P < 0.05$) after intoxication while no effect was detected 10 days after intoxication (Table 4).

So, RH-0345 significantly reduced brain AChE activity in both males and females but the effect was much more pronounced in females. Anticholinesterases compounds are a class of chemicals which can take the place of acetylcholine (ACh) at the active site of AChE, significantly reducing the neurotransmitter's ability to bind with AChE and essentially limiting the rate at which ACh can be broken down [25, 26]. Indeed, inhibition of AChE has been frequently used in toxicology exposure to anticholinesterase chemicals [27, 28].

Table 4: Effect of RH-0345 on AChE activity in males.

Observation Period (days)	AChE activity in Controls	AChE activity in Treatment (RH-0345)
0	0.12±0,007	0.12±0,007
5	0.11±0,01	0.07±0.001**
10	0.12±0.022	0.09±0.002
15	0.11±0.026	0.065±0.003*

Values are represented as Mean ± SD of five replicates

* = 0.05 ≥ P > 0.01, ** = 0.01 ≥ P > 0.001

It is well known that organophosphate and carbamate pesticides inhibit AChE activity and AChE activity is considered as an exposure biomarker to organophosphate and carbamate pesticides [29]. However, the responsiveness of AChE to other chemicals including metals and other compounds has also been reported and several studies showed the potential use of this enzyme activity as a useful biomarker for detecting general physiological stress caused by exposure to contaminants [30, 31]. It should be noticed that our results were obtained with individuals (females and males) collected during the spring period, period during which the complex frontal lobe neurosecretory cells and plays an important role on the development of reproductive cycles [32].

Our results are in agreement with previous works who showed that in addition to be sensitive to OP and C pesticides and heavy metals, AChE of arthropods are also sensitive to insecticide such as azadirachtin [33, 34, 35], chlorfluzuron [36], oxydemeton [36], spinosad [36, 2] and indoxacarb [2].

5. Conclusion

Except the presence of a steel complex which being the source of pollution in Annaba, the physico-chemical properties of the two studied sites are comparable (fine-textured soils, near-neutral pH) and *E. nudicornis* is the more abundant Chilopoda species. So, the difference observed in brain AChE activity could probably be due to chemical pollutants from the steel complex. During this study we also showed that exposure of individuals (females and males) to an analog of the molting hormone (RH-0345) causes significant inhibition of brain AChE activity in *E. nudicornis*. The decrease in AChE activity suggests a neurotoxic action of RH-0345.

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