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The Use of Biomarkers to Evaluate the Toxicity of Metaldehyde and Methiocarb Baits to the Terrestrial Isopod *Porcellionides pruinosus* Brandt, 1833

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Abstract—Biochemical indicators (biomarkers) are known to provide early warning signs of environmental pollution or stress conditions to the organisms, by measuring cellular or molecular responses of the target organism to xenobiotic agents. In this study, the terrestrial isopod *Porcellionides pruinosus* was exposed to these two molluscicides and three different enzymes, glutathione *S*-transferase (GST), acetylcholinesterase (AChE) and catalase (CAT) were analyzed to evaluate the effects of the application of the two molluscicides in single exposures and binary mixtures tests. Results indicate that the carbamate methiocarb inhibited significantly AChE activity, but no effects were observed in CAT and GST levels. The exposure to the metaldehyde had no effects on AChE, but a decrease in the higher exposure period was observed in GST levels as well as a general increase in CAT activity. The combined exposure of the two molluscicides resulted in a general decrease in AChE and CAT activity, but no visible effects were observed in terms of GST. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species.

Keywords: *Porcellionides pruinosus*, methiocarb, metaldehyde, GST, AChE, catalase

INTRODUCTION

The most commonly used molluscicides are metaldehyde and the carbamate methiocarb, which represent more than 90% of all European sales for molluscicides (Henderson and Triebskorn, 2002). The modes of action of these two molluscicides in snail and slugs are well documented, with metaldehyde inducing severe alterations and ultrastructural destruction in mucocytes which leads to dehydration and subsequent death (Triebskorn *et al*., 1998), whereas methiocarb acts upon the central nervous system inhibiting acetylcholinesterase (AChE), which can cause overstimulation of the nervous system and ultimately the death of the animal (Stanek *et al*., 2003). As a synantropic species the terrestrial isopod *Porcellionides pruinosus* can get into contact with these molluscicides, thus the use of these

pesticides could pose a risk to these soil organisms. This species has been used in ecotoxicological tests (Loureiro *et al.*, 2005) and is considered a good test-species to evaluate pernicious effects of xenobiotics (Loureiro *et al.*, 2009).

Biochemical indicators, know as biomarkers, can serve as early warning signs of environmental pollution or stress indication to soil organisms, and can be divided in three classes: exposure biomarkers, effect biomarkers, and susceptibility biomarkers (Schlenk, 1999). Biomarkers of exposure are related with cellular or molecular responses indicating an interaction between an organism and a xenobiotic agent (Roberts and Oris, 2004). Several studies have been made using biomarkers as tools to assess the effects of different pollutants to terrestrial isopods, as heavy metals (Köhler *et al.*, 1996), PAHs (Stroomberg *et al.*, 1999), organochlorine pesticides (Köhler *et al.*, 1999), organophosphorous pesticides (Stanek *et al.*, 2003) or titanium nanoparticles (Jemec *et al.*, 2008). Three enzymes were chosen to perform this study, based on their specific action, relevance and sensitivity to xenobiotic compounds: Acetylcholinesterase (Fulton and Key, 2001), glutathione *S*-transferase (GST) (Schreck *et al.*, 2008) and catalase (CAT) (Brown *et al.*, 2004).

The aim of this study was to evaluate the effects of two molluscicides on the terrestrial isopod *Porcellionides pruinosus* (Brandt, 1883). This assessment was made in two different steps: first, to detect changes on the activity of three enzymatic biomarkers AChE, GST and CAT when exposed to molluscicidal baits of methiocarb and metaldehyde, and second to identify the combined effect of the two chemicals to this terrestrial isopod.

MATERIALS AND METHODS

Test organisms

The isopods used in this experiment were obtained from a laboratorial culture, maintained in a climatic chamber at 25°C, 60% moisture content, and with a 16h:8h light:dark photoperiod. Only adult animals (15–25 mg wet weight) with antenna were used. In the beginning of the test, no sex differentiation was done, although pregnant females were not used in the experimental procedure.

Test chemicals

Two molluscicides were used in the experiment: metaldehyde and methiocarb, as the commercial formulations of CARAKOL® (Kontact™) and MESUROL® (Baye™) respectively. Tests were performed in LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer. The soil used was characterized by the following properties: pH of 5.5, organic matter content of 3.9%, and 6% of clay, 17% of silt and 77% of sand.

Experimental procedure

Single chemical exposure

Each animal was placed individually in a plastic box, with a surface area of

0.0064 m^2 containing 30 g wet soil. In this experiment we used 5 baits of metaldehyde per test-box and tested 3 methiocarb pellets per test-box.

In this experimental setup isopods were collected 8, 16, 24 and 32 hours after metaldehyde exposure, and 1, 2, 3 and 4 hours after methiocarb exposure. Ten isopods were sampled per time of exposure. After each of these sampling times 5 isopods were collected from a control test-box, maintained under the same conditions although without any molluscicide baits. Thus, a total of 60 isopods was analysed per molluscicide. Isopods were then frozen at –80°C until the biomarker analyses were performed. In addition, several sets with different exposure periods to control situations were also included in this experiment to determine if there were any shifts in the enzymatic activities throughout time.

Experimental procedure—Mixture toxicity exposure

For the mixture toxicity tests, isopods were exposed to the two molluscicides in six different combinations for time of exposure. Each combination comprised 10 isopods exposed individually to 3 baits of methiocarb (Mb) and 5 baits of metaldehyde (Md), alternatively and never simultaneously. In the first combination, isopods were exposed to Mb for 1 hour, and after this period the Mb baits were removed and the isopods were exposed to Md for 16 hours (Mb1hMd16h). In the second combination the isopods were exposed to Mb for 1 hour and afterwards were exposed to Md for 32 hours (Mb1hMd32h). The third combination was made by exposing the isopods to Md for 32 hours and afterwards to Mb for 1 hour (Md32hMb1h). The fourth combination was made through the exposure of Md for 32 hours and afterwards to Mb for 2 hours (Md32hMb2h). The fifth combination was made with the exposure of Mb for 1 hour and afterwards to 24 hours of Md (Mb1hMd24h). Finally, in the sixth combination the isopods were exposed to Mb for 2 hours and afterwards for Md for 24 hours (Mb2hMd24h). In addition, several isopods were also kept in a test-box without any contamination (control conditions) as the mixture experiment went on. 5 isopods were collected after 17 hours, 5 isopods were collected after 25 hours and finally 10 isopods were collected after 34 hours. Again, after each sampling time, animals were stored at –80°C until the biomarkers analyses were performed.

Preparation of the animals

For the biomarker analysis isopods were divided in two sections: head and body. The head was used for the AChE assay and the remaining body was used for GST and CAT assays. Homogenisation of the animals was made using a sonicator (KIKA Labortechnik U2005 Control™).

Determination of AChE activity

After sonication, samples using isopods' heads were centrifuged at 1700g for 3 min at 4°C. The obtained supernatant was immediately assayed for AChE activity according with the Ellman *et al.* technique (1961) adapted to the microplate (Guilhermino *et al.*, 1996). The enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36×10^{-3} $M^{-1}cm^{-1}$.

GST analysis

Glutathione *S*-transferase activity was determined based on the method described by Habig *et al*. (1974) and adapted to microplate (Diamantino *et al*., 2001). The enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6×10^{-3} $M^{-1}cm^{-1}$.

CATALASE analysis

Catalase activity was determined based on the method described by Clairborne (1985). The enzyme activity is expressed as unit (U) per mg of protein. A U corresponds to a μmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

Statistical analyses

Enzymatic activities obtained in the different controls (different time periods) were compared using a one way ANOVA (SIGMA STAT 3.5) to detect possible differences between animals collected in different periods from the control test-boxes. If enzymatic activities in control animals were not different in any of the experiment periods, these values were pooled and used as a total mean control to compare changes in enzymatic activities of animals exposed to the molluscicides also using a one way ANOVA (SIGMA STAT 3.5).

RESULTS AND DISCUSSION

AChE activity

No statistical differences were found between activities observed in controls periods of metaldehyde (P = 0.473), methiocarb (P = 0.824) and combined experiments (P = 0.463) exposures.

Metaldehyde did not have any effects in the activity of AChE of the isopod *Porcellionides pruinosus*. After comparing the four exposure periods with the correspondent control values no differences were found in this enzyme activity. The results in AChE activity were homogenous during the 32 hours of exposure and no variations were found between the animals exposed (Table 1). Although some authors indicate a diminution in AChE activity in the nervous tissue of the snail *Lymnaea acuminata* (Tiwari *et al*., 2008) due to the application of the molluscicide metaldehyde, others (Putchakayala and Ram, 2000) found that although metaldehyde was responsible for an excitatory effect in muscle contraction in the zebra mussel, this did not enhance or inhibit the effects of acetylcholine, probably because the two stimulants activate different cells within the organism.

Methiocarb significantly inhibited AChE activity in *P. pruinosus* already after the first hour of exposure to the molluscicide baits and throughout the experimental period. In all sampling times the enzymatic activity of exposed animals was smaller than the control, with its lowest activity being observed after

Table 1. Biomarkers activity in homogenates of *Porcellionides pruinosus* exposed to methiocarb, metaldehyde and six different combinations of methiocarb and metaldehyde baits. Results are expressed as the mean value (U/mg protein).

Treatment	Exposure period	AChE	GST	CAT
Methiocarb	no exposure	127.1	48.7	137.2
	1 h	64.5*	52.3	137.2
	2 h	67.1*	50.6	97.9
	3 h	76.4*	48.4	87.2
	4 h	53.9*	48.3	132.3
Metaldehyde	no exposure	128.3	51.3	86.6
	8 h	121.9	53.6	156.13
	16 h	164.3	63.7*	140.6
	24 h	139.2	37.7*	409.1*
	32 h	117.2	39.9*	228.5*
Mixtures	no exposure	125.9	53.0	187.6
	Mb1hMd16h	59.5*	56.7	104.0
	Mb1hMd24h	80.0*	49.5	56.3*
	Mb2hMd24h	56.8*	65.1	171.2
	Mb1hMd32h	76.7*	40.5	62.2*
	Md32hMb1h	132.8	53.4	180.9
	Md32hMb2h	297.8*	53.6	251.2

*Indicates significant differences between control/no exposure and treatments ($P \leq 0.05$).
No exposure—relates to the mean of several control situations.

4 hours of exposure (53,997 U/mg protein), showing an inhibition of 42% compared with control activity (Table 1). The strong decrease in AChE activity within a small exposure period is also in agreement with previous studies that illustrated the decrease in the activity of this enzyme when terrestrial isopods were exposed to organophosphate insecticides, that have a similar inhibitory influence in AChE as carbamates, even at sublethal concentrations (Fischer *et al.*, 1997; Ribeiro *et al.*, 1999; Stanek *et al.*, 2003; Engenheiro *et al.*, 2005).

In the joint toxicity experiments a significant inhibition of AChE activity was found in almost all the combinations performed, with the exception of the combination f Md32hMb1h, where the activity obtained in the exposed animal (132.81 U/mg protein) was similar to control animals. Although not expected, a significant increase in AChE activity in comparison to the control was observed in the combined effect of 32 hours of metaldehyde plus 2 hours of exposure to methiocarb (Table 1). In this combination the value calculated (281.47 U/mg protein) was more than the double observed in the control (130.50 U/mg protein). This experiment was repeated twice and the pattern of response of increased AChE activity in the referred combination was consistent in all experiments made. The result obtained in the present work seems to indicate that the application metaldehyde has an impact in the CNS of *Porcellionides pruinosus*,

and that increasing time of contact with the pellets induces an augment of AChE activity. One can hypothesize that the increase in AChE activity might be a result of intra-specific responses of the CNS of this isopod species to the application of metaldehyde baits in combination with methiocarb baits.

GST activity

No statistical differences were found between activities observed in controls periods of metaldehyde (P = 0.853) methiocarb (P = 0.581) and combined experiments (P = 0.682).

An increase in GST activity after giving metaldehyde baits to the isopods was observed after 16 h of exposure, being the values in the exposed animals 125% of the activity calculated for the control animals (Table 1). Metaldehyde decreased GST activity in *Porcellionides pruinosus* after the 24 and 32 h of exposure to metaldehyde. In these two experimental periods the enzymatic values calculated for 24 h and 32 h was 37.98 and 39.92 U/mg protein, resulting in an inhibition of 73% and 78%, respectively. In adults of the isopod species *Porcellio scaber* a decreased GST activity was also found after application of the neonicotinoid imidacloprid (Drobne *et al*., 2008), which is in conformity with our results. It is important to state that this induction on GST activity and subsequent decrease with time of exposure not only reveals the role of this detoxifying enzyme in the biotransformation of metaldehyde, but is a valuable contribution to understand the mode of action of this molluscicide to *Porcellionides pruinosus*.

Methiocarb did not have any effects in GST activity; values calculated for each treatment and respective control period were consistent during the experimental period without any oscillation of the calculated values of activity. In addition, the combination of the two molluscicides did not provoke any effects in GST activity, in all combinations the values were similar to those observed in the respective control (Table 1). The lack of sensitivity of GST to some classes of insecticides may be related to intrinsic and molecular properties of this enzymatic complex (Crane *et al*., 2002), and in some cases the time of exposure or concentration of the xenobiotic is not enough to activate this enzymatic complex.

The response in all binary combinations tested, with no variations found along the exposure periods, seems to confirm that this enzymatic conjugate can not always be used to detect an impairment caused by pesticide application, and thus results should be interpreted carefully (Hyne and Maher, 2003).

CAT activity

No statistical differences were found between activities observed in control periods of metaldehyde (P = 0.506) methiocarb (P = 0.122) and combined experiments (P = 0.433).

The application of metaldehyde baits caused an increase in CAT activity in all exposure periods but the 16 h sampling time (Table 1). This increase in CAT activity was more evident after the periods of 24 h, where the mean values in

exposed animals (409.05 U/mg protein) were 6 times higher than the values in the control (86.63 U/mg protein). Also in the last sampling time (32 h) the mean values of the antioxidant enzyme in the animals exposed to the baits were twice the values in control situation (228.47 U/mg protein). This result is in conformity with previous studies that also observed a significant increase in CAT activity in terrestrial snails after the administration of metaldehyde (El-Wakil and Radwan, 1991), and might be indicative of a defence mechanism towards cellular damage occurring in the organism as a consequence of reactive oxygen species (ROS) formation.

CAT activity values were consistent along the four hours of exposure to methiocarb baits, since no variation in this enzyme activity was observed. Although an inhibition of 71% and 63% of CAT activity was found after 2 h and 3 h of exposure to this molluscicide, no significant differences were found after the statistical procedure. This non-activation of CAT activity in this isopod species might also be related to the results obtained in the enzyme GST, which was also not induced by the exposure to methiocarb and the fact that the short period of exposure to the carbamate was not sufficient to induce changes, since it is known that carbamate pesticides do cause oxidative stress and are responsible for the activation of CAT (Maran *et al*., 2009).

In the mixture experiments, only two combinations (Mb1hMd24h and Mb1hMd32h) were statistically different from the control (Table 1), showing and inhibition of 30% and 33% when compared to control mean values. An inhibitory pattern of CAT activity was observed in the combinations experiment where the animals first received methiocarb baits. In only two of the sampling times the decrease on CAT activity was statically significant (Mb1hMd24h, Mb1hMd32h), and the following period of exposure to metaldehyde baits was not enough to reverse the effects obtained in these combinations. In the combinations where metaldehyde baits was the first chemical to be applied, no differences in CAT activity were found in comparison with the enzymatic levels observed in control. These results were not in accordance with the extreme increase in CAT activity found in single toxicity experiments with metaldehyde, but probably the subsequent exposure to methiocarb baits could be responsible for the mitigation of an increased CAT activity during the exposure to metaldehyde baits.

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