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Antioxidant and oxidative stress related responses in the Mediterranean land snail *Cantareus apertus* exposed to the carbamate pesticide Carbaryl

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ABSTRACT

The aim of the present work was to study the alterations of the antioxidant defenses and the overall susceptibility to oxidative stress of the terrestrial snail *Cantareus apertus* exposed to the carbamate pesticide Carbaryl at a low environmentally realistic concentration.
The animals were exposed to *Lactuca sativa* soaked for 1 h in 1 μM Carbaryl. The temporal dynamics of the responses was assessed by measurements at 3, 7 and 14 days of exposure.
C. apertus exposed to Carbaryl activates a number of enzymatic antioxidant responses, represented by the early induction of catalase, glutathione peroxidase, glutathione reductase, followed by a delayed induction of superoxide dismutase. Concomitantly, a derangement of the total oxyradical scavenging of the tissues was observed, suggesting an overall impairment of the tissue capability to neutralize ROS probably resulting from the overall negative balance between enzymatic antioxidant defense capability and oxidative stress intensity. This negative balance exposed the animals to the risk of oxidative stress damages including genotoxic damage. Compared to acetylcholinesterase inhibition, the antioxidant responses developed to Carbaryl exposure at the low concentration utilized showed a greater percentage variation in exposed organisms.
The results pointed out the high sensitivity of the antioxidant and oxidative stress related responses to Carbaryl exposure at an environmental realistic concentration, demonstrating their usefulness in environmental monitoring and risk assessment. The study highlights also the usefulness of the terrestrial snail *C. apertus* as potential bioindicator species for assessing the risk of pesticide environmental contamination.

1. Introduction

The environmental pollution rising from the widespread use of pesticides is of great concern in the last years. Although the Directive, 2009/128/EC and the Regulation (EC) n., 1107/2009 established the European framework for the sustainable use and the ecological risk assessment of pesticides, a lack of data and considerations is found about the effects induced by pesticides on terrestrial fauna, with particular relevance for invertebrates that occupy levels of high sensitivity in the trophic chain (Mantecca et al., 2006).

Among pesticides carbamates represent the second class of the most used pesticides in Europe and the United States (Eurostat, 2007). Often, these compounds cannot be easily detected by chemical analysis in environmental matrices because of their relative short life, although the products of their environmental degradation can be persistent, retaining toxic activity on non-target organisms (WHO, 2006).

To date the most known molecular mechanism of carbamate toxicity is represented by the reversible inhibition of cholinesterase activity (Lionetto et al., 2011). As emerging in the last years, the toxic action of carbamates can involve also other molecular or cellular targets beyond cholinesterases, including overproduction of reactive oxygen species (ROS) (Milatovic et al., 2006). Oxidative stress resulting from the enhancement of ROS and perturbation of antioxidant efficiency is recognized as an important general toxicity mechanism of many lipophilic xenobiotics, including pesticides (Regoli et al., 2002, 2006). It is known that oxidative stress often preludes the onset of long term effects such as degenerative processes, impairments of immune response and reproduction, premature aging and lower survival rate (Banerjee et al., 1999). Therefore, the measurements of oxidative induced alterations can provide useful biomarkers of exposure/effect to environmental pollution in ecological risk assessment, allowing detection of early pollutant effects that may have reflexes later in time at higher levels of biological organization.

To date only a few numbers of pesticides and only a few numbers of species, mainly vertebrates, have been considered in relationship to the effects on the anti-oxidant defenses and to the oxidative induced damages (Maran et al., 2009; Kubrak et al., 2012; Ozden et al., 2013). As regards terrestrial gastropods which are important nontarget organisms

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for pesticides very few information are available. Snails are profoundly affected by pollution coming from intensive use of pesticides and fertilizers in agriculture, industrial activities and atmospheric deposition (Regoli et al., 2006). These organisms can absorb pollutants by dermal contact with soil, ingestion of soil, vegetation, water, and inhalation of air. Therefore, they play an important role in directly transferring pollutants to higher trophic levels of terrestrial food chains being prey or hosts for a variety of other animals. In the recent past, terrestrial snails have been used as bioindicator organisms for assessing the effects induced by environmental pollutants on key components of terrestrial food web (Notten et al., 2005). However, only limited numbers of studies are available on the use of terrestrial snails as bioindicator organisms (Regoli et al., 2006; El-Gendy et al., 2009; Itziou et al., 2011).

The present work was addressed to study the alterations of antioxidant defenses and the overall susceptibility to oxidative stress conditions of the snail *Cantareus apertus* exposed to the carbamate pesticide Carbaryl at a low environmentally realistic concentration in view of the development of sensitive biomarkers for the detection and assessment of effects caused by pesticides on non-target species in terrestrial environments. While most of the studies on the biological effects of pesticides are based on acute exposure conditions, very few investigate the effect exerted by more environmentally realistic pesticide concentrations. This aspect is of relevance if we consider that most of the pesticide environmental contamination is represented by non-point source contamination.

The study focuses on Carbaryl as prototype carbamate, according to Ecobichon (2001). Although Carbaryl is banned in some European countries it is still widely used all over the world, and it is still present in a number of formulated products.

The animals were exposed to *Lactuca sativa* soaked for 1 h in 1 μM Carbaryl. This concentration is one order of magnitude below the EC50 value for acute exposure of mollusks and aquatic worms (SERA TR-052-01-05a, 2008). Moreover, it is far below the concentration observed during direct overspray events (Norris et al., 1983) and it is included in the range of environmental Carbaryl concentrations resulting from peripheral spray, runoff, or insecticide breakdown (Bulen and Distel, 2011).

The temporal dynamics of the responses was assessed by measurements at 3, 7 and 14 days of exposure.

Our attention was focused on variations in antioxidant defenses such as catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), superoxide dismutase (SOD), reduced (GSH) and oxidized (GSSG) glutathione ratio, as very sensitive parameters in revealing pro-oxidant conditions, as indicated by Regoli et al. (2002). Moreover, in order to evaluate the global capacity of tissues to neutralize different forms of reactive oxygen species (peroxyl radical, hydroxyl radical and peroxynitrite), the Total Oxyradical Scavenging Capacity (TOSC) was quantified (Regoli, 2000). To support the battery of biomarkers analyzed, other cellular and biochemical parameters were investigated: lysosome membrane stability, sensitive to the variation of the induced redox status (Regoli, 1992), lipofuscin levels which reflect the intensity of lipid peroxidation processes (Moore and Allen, 2002), neutral lipids level, as indicator of xenobiotic-induced lipidosis, Acyl-CoA (AOX) as a group of enzymes that catalyzes the hydrolysis of acyl-CoAs to the free fatty acid, and micronuclei frequencies (MN) to detect possible genotoxic damage related to oxidative stress. In parallel acetylcholinesterase (AChE) inhibition was analyzed in both control and exposed organisms. It represents the most known molecular mechanism of carbamate toxicity in both vertebrates and invertebrates to date (Calisi et al., 2009; Calisi et al, 2011; Lionetto et al., 2011). AChE inhibition has been previously demonstrated to be related to alterations of antioxidant enzymes in other species (Lionetto et al., 2003; López et al., 2007),

The temporal dynamics of the responses were analyzed in order to deepen the mode of action of the pesticide on this terrestrial organism.

The study wants also to shed light on the performance of *C. apertus* as bioindicator organism for pesticide pollution monitoring and assessment in terrestrial environments. *C. apertus* is a central Mediterranean species, inhabiting maritime influenced Mediterranean habitats. It is also a commercial value species (Novelli et al., 2002; Avagnina, 2011). Therefore, the assessment of pesticide induced oxidative alterations in this organism could also be of concern for potential effects of human health due to the human consumption of this species.

2. Materials and methods

2.1. Experimental design

A homogeneous population of *C. apertus* (mean wt. 2.325 +/− 0.2 g), obtained from a local farm, was acclimatized in a plastic box (55 × 39 × 25 cm) under controlled conditions of temperature (20 +/− 2°c), photoperiod (18/6 light/dark regime) and humidity (85%) (De Vaufleury and Gimbert, 2009). The floor of the cage was covered with blotting paper and dampened with tap water; *L. sativa* was administered ad libitum as food and cleaned twice a week.

192 adult snails were chosen and starved for 2 days before starting the exposure experiment. 96 specimens were used as control and 96 were exposed to Carbaryl (test specimens). Biomarker responses were monitored in control and treated animals through the time. A two orthogonal factor experimental design was chosen: factor (A) "Carbaryl exposure" included two levels ("not exposed" or control animals and "exposed"), and factor (B) "time of exposure" included four levels (0, 3, 7 and 14 days). Eight boxes for each condition were utilized and three animals were added in each box (plastic box dimensions: 14 × 10 × 7 cm). All the groups were held in controlled conditions of temperature, photoperiod, and humidity (see above). Three times a week the animals were fed with *L. sativa*. Test specimens were exposed to *L. sativa* soaked for 1 h in 1 μM Carbaryl, according to the method described by Dallinger et al. (2005). Foliar consumption is one of the main natural pesticide exposure pathways in terrestrial snails. Carbaryl is considered to be a relatively immobile compound under conditions of foliar application with a high persistence on the foliar surface (FAO/WHO, 1967; Xu, 2002). In the case of foliar exposure the bioavailable pesticide is mainly represented by the pesticide adsorbed on the foliar surface, while the amount absorbed by the leaves is about 2% (Xu, 2002).

Not consumed food was removed at each cleaning time. At times 3, 7 and 14 (days), dead organisms were removed and recorded. Mortality of snails remained low throughout the 14-day exposure period (<20%), with no statistically significant differences between control and exposed animals.

Surviving organisms underwent hemolymph sampling by puncturing the shell with a sterilized hypodermic syringe. Then, each specimen was cold anesthetized at 4 °C for 30 min; then, foot and hepatopancreas were dissected, immersed in liquid nitrogen and maintained at −80 °C until used for biomarker analysis. The analyses were performed on pools each composed by tissues obtained from three animals exposed in the same box.

2.2. Biological responses

2.2.1. Digestive gland sample preparation

CAT, GR, GPX, SOD, and GSH/GSSG were measured in digestive gland samples homogenized (1:5 wt/vol) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/m aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, NaCl 0.6% and centrifuged at 12,000 ×*g* for 30 min at 4 °C, to yield the postmitochondrial fraction. Aliquots of this fraction were stored at −80 °C until their use for the assay. Each sample represented a pool of digestive glands dissected from 3 snails.