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Effects of azinphos-methyl on enzymatic activity and cellular immune response in the hemolymph of the freshwater snail *Chilina gibbosa*

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ABSTRACT

The use of a battery of biomarkers, especially those more closely related to species integrity, is desired for more complete ecotoxicological assessments of the effects of pesticide contamination on aquatic organisms. The phosphorodithioate azinphos-methyl has been intensively used in agriculture worldwide and have been found in the habitat of *Chilina gibbosa*, a freshwater snail endemic to South America. This snail has been proposed as a good model organism for ecotoxicity bioassays on the basis of studies focused mainly on enzymatic responses in whole tissue homogenates. Our aim was to evaluate the effect of an acute 48 h exposure to an environmental concentration of azinphos-methyl on *C. gibbosa* hemolymph enzymatic activity and cellular immune response. Our results show that cholinesterase activity was strongly inhibited (94%) in hemolymph of exposed snails. Carboxylesterase activity measured with *p*-nitrophenyl butyrate and glutathione S-transferase activity were augmented 47% and 89% respectively after exposure. No differences were found for hemolymph carboxylesterase activity measured with *p*-nitrophenyl acetate. These results differ from those reported for whole tissue homogenates and reveal that tissue-specific responses of enzymatic biomarkers exist in this species. Regarding immune cell response, hemocytes were identified for the first time for *C. gibbosa*. Their viability and phagocytic activity decreased after azinphos-methyl exposure although total number of circulating cells did not differ between treatments. We conclude that concentrations of azinphos-methyl that can be found in the environment can compromise both hemolymph cholinesterase activity and the immune system of *C. gibbosa*. Furthermore, we propose that carboxylesterase and glutathione S-transferase activities measured in hemolymph and hemocyte viability and phagocytic activity could be incorporated as sensitive biomarkers to evaluate the effects of pesticide exposure on this and related species.

1. Introduction

Chilina gibbosa (Sowerby 1841) is a freshwater snail from the family Chilinidae (Pulmonata) endemic to southern Argentina and Chile [6, 21, 51]. Adults are usually found aggregated in shallow areas and have limited mobility, which makes them easy to collect and handle for bioassays [6, 41]. The family Chilinidae is considered vulnerable due to the deterioration of its habitat related to different anthropogenic perturbations, such as the presence of toxic contaminants [61]. In Argentina, *C. gibbosa* is commonly found in rivers, lakes and reservoirs of the Río Negro and Neuquén provinces, North Patagonia [6, 51, 61]. This snail plays an important role in the native ecosystem as food source for birds and different fish species, some of which have commercial value like the native silverside *Odontesthes hatcheri* [1]and the rainbow trout *Oncorhynchus mykiss*.

One of the main economic activities in the Upper Valley of Río

Abbreviations: AcSCh, acetylthiocholine iodide; AZM, azinphos-methyl; CE, carboxylesterase; ChE, cholinesterase; CDNB, 2,4-dinitrochlorobenzene; CR, Congo Red; DTNB, 5,5-dithio-2-bis-nitrobenzoate; GS, Giemsa solution; GS-DNB, S-(2,4-dinitrobenzyl)glutathione; GST, Glutathione S-transferase; OP, organophosphate insecticide; p-NPA, *p*-nitrophenyl acetate; p-NPB, *p*-nitrophenyl butyrate; TB, Trypan Blue

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Negro and Neuquén is fruit production, which involves the use of large amounts of pesticides. The phosphorodithioate insecticide azinphos-methyl (AZM) has been one of the most commonly and intensively applied in this area. This insecticide is converted by oxidation to the organophosphate (OP) oxon-derivate. Maximum recorded concentrations of AZM in subsurface water vary from 0.25 μg L^{-1} to 79.30 μg L^{-1} between control and application periods, respectively [35, 36]. Previous studies in our laboratory have characterized the effects of acute exposures to AZM on *C. gibbosa* and evaluated recovery responses, based on the neurotoxicity, activity of B-esterases and oxidative stress parameters [5, 13]. Bianco et al. [5] provided a thorough evaluation of *C. gibbosa* whole tissue enzymatic response to a 48 h exposure to AZM. They reported an IC_{50} of 0.02 ± 0.01 μg L^{-1} for cholinesterases (ChEs) and higher than 1000 μg L^{-1} for carboxylesterases (CEs), with no effect of 20 μg L^{-1} AZM on the activity of glutathione S-transferase (GST). Hence, it has been suggested that *C. gibbosa* could be included as a sentinel species in monitoring programs due to its sensitive ChE response to AZM.

ChEs are most commonly used as sensitive biomarkers for OP exposure [60], as they constitute the target of the mechanism of action of these pesticides. OPs inhibit ChEs by phosphorylation of a serine residue in the enzyme's active site, preventing it from hydrolyzing the neurotransmitter acetylcholine. The subsequent accumulation of acetylcholine leads to overstimulation of cholinergic receptors followed by depression or paralysis and eventual death. CEs have been postulated as participating in the metabolism and detoxification of many agrochemicals including OPs [45, 63], either by binding to the insecticide and thus removing large amounts of it, or by hydrolyzing carboxylester bonds present in some OPs [27, 28, 55]. Hence, the combined use of ChEs and CEs as biomarkers has been proposed as a more suitable strategy to obtain a detailed evaluation of the effect of exposure to OPs [56, 64]. Another important enzyme that is habitually measured in toxicological studies that pursue a multi biomarker approach is GST [15]. It plays a vital role in detoxification of OPs by catalyzing the conjugation of xenobiotics or their metabolites with glutathione, which favors their excretion [30, 59].

There is evidence that both enzyme basal activity and sensitivity of ChEs, CEs and GST vary between tissues, therefore the response to compounds such as OPs can be tissue-specific. Thus, a more accurate assessment of pesticide effects could be obtained by determining biomarkers in different tissues [43]. For example, in the mussel *Mytilus edulis*, ChE activity is higher in hemolymph than in whole tissue homogenates, whilst the opposite is true for CE activity [23]. In the bivalve mollusk *Scapharca inaequivalvis* higher GST activity has been found in the digestive gland than in foot tissue or gills [4]. Regarding tissue-based differences in enzyme sensitivity, Cacciatore et al. [10] and Otero and Kristoff [43], have found variations in ChE and CE activities between whole tissue homogenates, pulmonary region, digestive gland and hemolymph of the freshwater snail *Planorbarius corneus* exposed to the OPs AZM and chlorpyrifos.

The study of other biomarkers directly related to species integrity and survival, such as effects on immune system or reproduction, is especially relevant. Nevertheless, no studies on this kind of biomarkers have been carried out yet for *C. gibbosa*. Invertebrates present a relatively simple immune system, which makes them good models for immunotoxicity studies [19, 20, 22]. Although a wide variety of specific host defense strategies exist, they are mainly based on phagocytosis by freely circulating blood cells such as hemocytes or coelomocytes [14, 38, 46, 50]. In this sense, different immune parameters such as quantity, viability and phagocytic activity of circulating cells can be considered as potential biomarkers of pesticide exposure [3, 7, 8, 22, 44]. For instance, OP and carbamate compounds have been shown to impair lysosome activity or integrity and inhibit phagocytosis in different invertebrates [17, 48, 62]. AZM specifically, resulted an important modulator of immune and detoxification responses in the Patagonian freshwater mussel *Diplodon chilensis*, enhancing responses it was challenged with *E. coli* [12]. Thus, our aim was to study hemolymph enzymatic and cellular immune responses of *C. gibbosa* after an acute 48 h exposure to AZM. We focused on ChE, CE and GST activities and total hemocyte number, hemocyte viability and phagocytic activity as enzymatic and immune response biomarkers, in order to provide more comprehensive knowledge of the acute toxic effects of AZM on non-target organisms.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AcSCh), *p*-nitrophenyl acetate (p-NPA), *p*-nitrophenyl butyrate (p-NPB), 5,5-dithio-2-bis-nitrobenzoate (DTNB), 2,4-dinitrochlorobenzene (CDNB), azinphos-methyl PESTANAL® (97.2% pure) were purchased from Sigma–Aldrich. Trypan Blue (TB), Giemsa solution (GS) and Congo Red (CR) were purchased from Biopack (Argentina). All other chemicals used were also of analytical reagent grade.

2.2. Organisms

Adult *C. gibbosa* individuals were hand collected from a site on the river Chimehuin (39°54′57.15”S 71°06′23”W; province of Neuquén, Argentina). The sampling site can be considered free from agrochemical or other kinds of pollution since there are no population centers upstream from it and the river Chimehuin originates in the Lanín National Park, where agricultural and industrial activities are banned [5, 13, 34].

2.3. Bioassays

Two bioassays were carried out in order to study hemolymph enzymatic activity and cellular immune response. Snails were exposed for 48 h to Chimehuin water with 0.002% acetone, as solvent control, or with 20 μg L^{-1}AZM, in 1 L glass vessels containing 500 mL of the corresponding solution. The concentration of 20 μg L^{-1}AZM represents possible real case scenarios by being within the range of pesticide found in freshwater bodies of Argentina (see Introduction). It was also chosen to match the concentration used in previous studies for evaluating enzymatic activity in whole tissue homogenates [5, 13], for comparison purposes. Acetone concentration was set at 0.002%, which is 5-fold lower than the concentration recommended by the Organization for Economic Cooperation and Development (OECD) [40] for aquatic toxicity testing. A Chimuehuin water control without acetone was included for the cellular immune response bioassay as there are no previous records of such studies in this species.

AZM working solution was obtained by diluting the stock solution of the insecticide prepared in acetone with Chimehuin water. Solutions were not renewed during exposure based on the results of stability studies of our laboratory in which concentration values measured at time 0 remained constantly after 48 h [9]. Always the AZM concentrations measured were within 97–102% of the nominal values.

In both bioassays, $N = 6$ (6 glass vessels per each treatment). Each replicate consisted of a pool of hemolymph from 12 snails for enzymatic activity (144 snails in total)or 3 snails (54 snails in total)for cellular immune response according to the amount of sample needed for measuring biomarkers. Snails were not fed during the bioassays.

After 48 h of exposure lethality and neurotoxicity were recorded. Snails were considered dead when they failed to respond mechanical stimuli or if they remained constantly retracted into the shell. The signs of neurotoxicity registered were the decrease or lack of adherence and the abnormally protruded head-foot region from the shell.