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Environmental concentrations of azinphos-methyl cause different toxic effects without affecting the main target (cholinesterases) in the freshwater gastropod *Biomphalaria straminea*

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ABSTRACT

Organophosphate insecticides (OPs) are commonly used in Argentina and around the world for pest control in food crops. They exert their toxicity through the inhibition of the enzyme acetylcholinesterase. In the present study, we aimed to evaluate biochemical and reproductive effects in *Biomphalaria straminea*, a freshwater gastropod naturally distributed in Argentina, of subchronic exposures to environmental azinphos-methyl concentrations (20 and 200 μg L^{-1}). For biochemical parameters, adult organisms were exposed for 14 days and the activity of cholinesterases (ChEs), carboxylesterases (CEs), glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), the production of reactive oxygen species (ROS), the total antioxidant capacity (TAC), glycogen and proteins were determined. For reproductive parameters, the egg masses of *B. straminea* were exposed to azinphos-methyl for one month, and the hatching time and success as well as the offspring survival were registered. We found different toxic effects elicited by the insecticide on the studied biomarkers. CEs activity was significantly inhibited while CAT and GST activities, ROS production and TAC were significantly increased, with respect to the solvent control group. ChE and SOD activities and protein and glycogen contents were not altered by azinphos-methyl. The hatching time and success were not statistically different from control. Nevertheless, the offspring survival was severely affected by the insecticide. Our results show that the primary target of the insecticide (ChE) was not inhibited but CEs, GST, CAT, ROS, TAC and offspring survival were sensitive biomarkers and valuable endpoints for subchronic toxicity assessments in this species.

1. Introduction

The levels of aquatic pollution have increased worldwide, requiring control strategies and routine monitoring of contaminants in the aquatic environment. Many pollutants are released into the environment far upstream from coastlines causing diverse toxic effects in aquatic organisms (Aliko et al., 2015, 2018; Faggio et al., 2018; Savorelli et al., 2017; Sehonova et al., 2018; Torre et al., 2013). Anthropogenic sources of xenobiotics include urban runoff, sewage, traffic emissions, coal and oil combustion, industrial production, mining, the smelting of ores, among others (Burgos-Aceves et al., 2018; Capillo et al., 2018; Fiorino et al., 2018; Pagano et al., 2016, 2017; Vajargah et al., 2018).

Azinphos-methyl is an organophosphate (OP) insecticide commonly used for agricultural pest management. In Argentina, it is mainly applied to control the codling moth *Cydia pomonella* L. (Cichón et al., 2014), an important pest in apple and pear production (Soleño et al., 2008). The excessive use of this OP has led to detect concentrations of azinphos-methyl in environmental compartments other than agricultural soil. For example, Loewy et al. (1999) detected a maximum

Abbreviations: AcSCh, acetylthiocholine iodide; CAT, catalase; CE, carboxylesterase; ChE, cholinesterase; DTNB, 5,5´-dithio-2-bis-nitrobenzoate; GST, Glutathione S-transferase; OP, organophosphate; p-NPA, p-nitrophenyl acetate; p-NPB, p-nitrophenyl butyrate; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity

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concentration of azinphos-methyl of 79.30 μg L^{-1} in groundwater of the Northern Patagonian Region (Argentina), whereas concentrations of up to 3000 μg L^{-1} have been reported in other parts of the world (Shayeghi et al., 2001).

Azinphos-methyl, like all OP insecticides, is known to act on the nervous system as an irreversible acetylcholinesterase (AChE) inhibitor (Pope, 1999). This enzyme is responsible for the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft. The inhibition of AChE causes the accumulation of the neurotransmitter, and thus an overstimulation of acetylcholine receptors. As cholinesterases (ChEs) are widely distributed in nature, OPs can have toxic effects not only on targeted pests but also on non-target organisms.

Environmental monitoring programs have implemented biological approaches together with the detection and/or quantification of contaminants, which allow for a more complete outlook in the assessment of environmental risk (Blasco and Picó, 2009; Burgess et al., 2013; Guasch et al., 2012). The use of sentinel or bioindicator species, through the study of the alteration of a battery of biomarkers, can provide valuable information about lethal and sublethal effects of contaminants at the individual level and, moreover, at the population and community levels. The use of invertebrates as model organisms to assess effects of OP exposure on different biomarkers, such as ChEs, carboxylesterases (CEs), detoxifying enzymes, antioxidant defenses and reproductive parameters has considerably increased over the last years (Amiard-Triquet et al., 2013; Cacciatore et al., 2015; Khalil, 2015; Latanville and Stone, 2013; Narra et al., 2014; Ochoa et al., 2013; Patetsini et al., 2013; Revathi and Bindhuja, 2008). The inhibition of ChEs is the most frequently used biomarker as these enzymes are the main target of OPs (Amiard-Triquet et al., 2013; Walker et al., 2012). However, the use of CE activity as an OP exposure biomarker has increased as several studies reported a higher affinity of OPs to CEs than to ChEs (Cacciatore et al., 2013; Ochoa et al., 2013; Wheelock et al., 2008). Furthermore, some authors have documented that CEs play a key role in the metabolism and detoxification of many xenobiotics including pesticides and pharmaceuticals (Potter and Wadkins, 2006; Wheelock et al., 2005).

Glutathione S-transferase (GST) is a phase II detoxifying enzyme that catalyzes the conjugation of electrophilic compounds (e.g., OPs or their metabolites) with glutathione (GSH) (Faggio et al., 2016; Strange et al., 2000). Therefore, GST is extensively used as an effect biomarker of OP exposure (Cacciatore et al., 2015; Khalil, 2015; Velki and Hackenberger, 2013). Several studies suggest that OPs are also capable of inducing oxidative stress processes as they trigger reactive oxygen species (ROS) production and/or affect the antioxidant capacity of organisms (Cacciatore et al., 2015; Lavarías et al., 2013; Narra, 2014; Patetsini et al., 2013). Antioxidant systems involve enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (e.g., glutathione, ascorbic acid, and tocopherols) defenses that work cooperatively and are commonly used as biomarkers of pesticide exposure (Amiard-Triquet et al., 2013).

Some authors have also evaluated changes in energy reserves measured as protein and glycogen content after exposure to OPs. For example, Rambabu and Rao (1994) and Revathi and Bindhuja (2008) found a decrease in both parameters in the freshwater invertebrates *Bellamya dissimilis* and *Macrobrachium malcolmsonii*, respectively, after acute and chronic exposures to OPs.

Environmental pollutants may have major consequences in reproduction, affecting the abundance and distribution of individual organisms and so, endangering the species' survival. The number of egg masses or capsules (covering that contains the eggs in some aquatic invertebrates), the number of eggs per egg mass, egg viability, the hatching time and offspring survival are frequent parameters studied as potential effect biomarkers in oviparous invertebrates (Kagley et al., 2014; Navis et al., 2013). Many authors have reported reproductive alterations in aquatic invertebrates caused by OPs (Kristoff et al., 2011; Latanville and Stone, 2013; Marcial and Hagiwara, 2007; Rivadeneira et al., 2013).

Biomphalaria straminea (Dunker, 1848) is a freshwater pulmonate snail (Gastropoda: Planorbide) that was first recorded in South America, but its distribution rapidly expanded to other locations (Paraense, 2001; Pointier et al., 2005; Rumi et al., 2008). It is a simultaneous hermaphrodite, and it is able to reproduce by both self-fertilization and cross-fertilization. After copulation, the snails lay their eggs in a gelatinous capsule that they adhere to hard substrates. The embryos develop inside the egg and after a few days juveniles hatch (Yipp, 1983). *Biomphalaria straminea* snails are easily maintained in laboratory cultures, have a great reproductive potential, rapid embryonic development and growth, and early sexual maturation (Yipp, 1983), which makes them ideal for carrying out bioassays.

In previous studies in our laboratory, Bianco et al. (2014) evaluated the acute and subchronic effects of azinphos-methyl on B-esterases (ChEs and CEs) of *B. straminea* after exposure of up to 10,000 μg L^{-1} (48 h) and to 20 and 200 μg L^{-1} (21 days). Interestingly, no inhibition of the activity of ChEs was observed despite being the primary target of OPs. Therefore, following these results, several questions came up: Could there be other biochemical effects related to the toxicity of azinphos-methyl? Is this insecticide capable of impairing reproduction in this species? Which could be sensitive biomarkers to provide reliable data on environmental exposure of *B. straminea* to azinphos-methyl? To answer these questions, we evaluated, on the one hand, the response of adult *B. straminea* B-esterases (ChEs and CEs), GST, catalase (CAT), superoxide dismutase (SOD), ROS, total antioxidant capacity (TAC), glycogen and protein content after 14 days of exposure to environmental concentrations of azinphos-methyl. On the other hand, we studied the effect of the insecticide on the hatching time and success and on the offspring survival of egg masses and juveniles of *B. straminea* after one month of exposure.

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), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), nitroblue tetrazolium (NBT), riboflavin, methionine, anthrone, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP), 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA), bovine serum albumin, standard glycogen, azinphos-methyl PESTANAL® (97.2% PURE) were purchased from Sigma-Aldrich. Other chemicals used were also of analytical reagent grade.

An azinphos-methyl stock solution (2300 mg L^{-1}) was prepared in acetone. This nominal concentration was tested by HPLC coupled with an UV detector set at 230 nm. The measured values were always within the range 95–102% of the nominal values. For the bioassay, working solutions of azinphos-methyl were obtained by dilution of the stock solution in dechlorinated tap water.

2.2. Organisms

Since 2012, a population of the gastropod *B. straminea* has been bred in our laboratory. The organisms are maintained in aerated glass aquaria (7–16 L) at a temperature of 22 ± 2 °C and under a photoperiod regime of 12:12 h (Light:Dark). Physicochemical parameters of dechlorinated tap water were: turbidity (NTU) = 1.6; alkalinity = 44 mg $CaCO_3$ L^{-1}; pH = 8.0; conductivity = 246 μS;cm^{-1}; UV 254 = < 5 l m^{-1}; ammonium = < 0.05 mg L^{-1}; chloride = 43 mg L^{-1}; aluminum = < 0.05 mg L^{-1}; calcium = 15 mg L^{-1}; dissolved oxygen = 8.8 mg L^{-1}; oxygen consumption = 1.1 mg L^{-1}. The snails are fed three times a week with *Lactuca sativa* var. *capitata* L. (butterhead lettuce).

2.3. Biochemical parameters

2.3.1. Experimental design and sample processing

The bioassay was conducted for 14 days with 224 adult snails of similar sizes (0.72 ± 0.07 cm; mean ± standard deviation) under controlled conditions of temperature (23 ± 1 °C) and photoperiod 12:12 h (Light:Dark). Four treatments (8 glass vessels per treatment holding seven snails each one) were designed and organisms were randomly assigned to each one: dechlorinated tap water (water control), acetone in dechlorinated tap water (solvent control), and two concentrations of azinphos-methyl in dechlorinated tap water (AZM 20 for 20 $\mu g\,L^{-1}$ and AZM 200 for 200 $\mu g\,L^{-1}$). The total acetone concentration was 0.0087%, below the concentration recommended by the Organization for Economic Cooperation and Development (OECD, 2010) for aquatic toxicity testing (0.01%). During the experiment, 200 mL of the treatment solutions were placed in 250 mL glass vessels and were renewed every 96 h by fresh solutions according to previous stability studies carried out in our laboratory with azinphos-methyl (Cacciatore, 2009). Organisms were fed ad libitum with *Lactuca sativa* var. *capitata* L. (butterhead lettuce). During the bioassay, mortality was registered daily. Organisms were considered dead when they displayed no movement in response to mechanical stimuli.

After 14 days, one snail from each glass vessel was frozen and stored at −80 °C to be later homogenized for ROS and TAC determinations. The remaining snails were immediately homogenized for ChE, CE, GST, CAT, SOD, glycogen and protein measurements.

For homogenate preparation, the snails were cold anesthetized on ice for 6–8 min; then, shells were carefully removed over ice with dissecting forceps. The soft tissues were rinsed out with distilled water, blotted on a paper towel to drain extra fluids, and weighed. To obtain the supernatants used for ChE, CE, GST, CAT, SOD, glycogen and protein determinations, between 4 and 5 snails were homogenized together (because of their small size) (1:10 w/v) with a Potter-Elvehjem homogenizer in 20 mM Tris/HCl buffer (pH = 7.5) containing 0.5 mM EDTA. An aliquot of each homogenate was separated and stored at −20 °C for glycogen measurements. The remaining homogenates were centrifuged for 20 min at 11,000 × *g* at 4 °C and supernatants were aliquoted and stored at −20 °C. Supernatants used for ROS and TAC determinations were prepared by homogenizing one snail (1:5 w/v) with a plastic pestle in 100 mM Tris/HCl buffer (pH = 7.75) with 2 mM EDTA and 5 mM $MgCl_2$ (Gallagher et al., 1992). Homogenates were centrifuged for 20 min at 10,000 × *g* at 4 °C. Eight homogenates per treatment were obtained for ROS and TAC and eight for the other biomarkers analyzed.

2.3.2. B-Esterases activity

The activity of ChEs was determined spectrophotometrically according to Ellman et al. (1961), with slight modifications for this species (Bianco et al., 2014). Reactions were performed in 100 mM phosphate buffer (pH = 8.0), 0.2 mM DTNB, 0.75 mM AcSCh and 100 µL of the supernatant, resulting in a final volume of 1.4 mL. The absorbance was read at 412 nm. The molar extinction coefficient of 2-nitro-5-thiobenzoate anion at 412 nm (13.6 $mM^{-1}\,cm^{-1}$) was used to calculate ChE activity, that was expressed as µmoles of 2-nitro-5-thiobenzoate anion produced per min per mg of protein.

The activity of CEs was determined through the hydrolysis of p-NPA and p-NPB, following the procedure described in Kristoff et al. (2010), with slight modifications introduced for *B. straminea* by Bianco et al. (2014). The reaction medium included 100 mM phosphate buffer (pH = 8.0) containing 5% of acetone, 1.5 mM p-NPA or p-NPB and 80 µL of the supernatant, resulting in a final volume of 2.5 mL. The absorbance was recorded at 400 nm. The activity of CEs was expressed as µmoles of p-nitrophenol produced per min per mg of protein, using the molar extinction coefficient for p-nitrophenol (18.6 $mM^{-1}\,cm^{-1}$).

2.3.3. GST activity

The activity of GST was measured according to Habig et al. (1974). The reaction mixture contained 100 mM phosphate buffer (pH = 6.5), 1 mM CDNB, 1 mM GSH and 100 µL of the supernatant, in a final volume of 3.1 mL. The formation of CDNB-GSH conjugate was monitored at 340 nm. The activity was calculated using the molar extinction coefficient of CDNB conjugate at 340 nm (9.6 $mM^{-1}\,cm^{-1}$) and expressed as µmoles per min per mg of protein.

2.3.4. CAT activity

CAT activity was evaluated by a spectrophotometric method based on the decomposition of hydrogen peroxide by CAT, as described by Claiborne (1985). The assay mixture consisted of 50 mM phosphate buffer (pH = 7.0), H_2O_2 (0.07%) and 150 µL of the supernatant, giving a final volume of 3.05 mL. The decomposition rate was evaluated by monitoring the decrease in absorbance at 240 nm (molar extinction coefficient of H_2O_2: 0.0436 $mM^{-1}\,cm^{-1}$). The enzymatic activity was expressed as µmoles of H_2O_2 degraded per min per mg of protein.

2.3.5. SOD activity

The activity of SOD was measured by the method of Beauchamp and Fridovich (1971), based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction medium consisted of 50 mM phosphate buffer (pH = 7.8), 13 mM methionine, 0.1 mM EDTA, 75 µM NBT, 20 µM riboflavin and 5, 10 and 15 µL of the supernatant, in a final volume of 3 mL. After light exposure (10 min), the absorbance at 560 nm was monitored. A negative control (dark-exposed) and a positive control without enzyme (light-exposed without supernatant) were conducted. Values were expressed as SOD units per mg of protein, where 1 Unit of SOD is defined as the amount of supernatant necessary to produce 50% inhibition of NBT.

2.3.6. ROS and TAC

TAC against peroxyl radicals was determined through the formation of ROS, according to the method of Amado et al. (2009), with modifications (Pérez et al., 2015). To quantify ROS production, the reaction buffer (30 mM HEPES at pH 7.2, 200 mM KCl and 1 mM $MgCl_2$) and 5 µL of the supernatant were added to a 96-well microplate. Two wells were used per sample: 4 mM 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP) was added to one well and the same volume of ultrapure water to the other well, reaching a final volume of 145 µL in each well. Fluorescence was determined at 37 °C using a spectrofluorometer microplate reader (Fluoroskan Ascent FL 2.6 equipped with Ascent Software Version 2.6). Before reading, 10 µL of the fluorogenic probe 40 µM 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA) were added to each well. The fluorophore DCF was detected at λ_{ex} = 488 nm and λ_{em} = 525 nm. Total fluorescence production was calculated by integrating the fluorescence units (FU) over the time of measurement. The results were expressed as the area difference of FU/min/wet weight in the same sample with and without ABAP and standardized to the ROS area without ABAP (background area). The antioxidant competence of the snails was calculated as follows: TAC = 1/[(ROS $area_{ABAP}$ - ROS $area_{background}$)/(ROS $area_{background}$)].

2.3.7. Energy reserves

We assessed the energy reserves by measuring glycogen and protein content. Glycogen was determined following the method by Van Handel (1965). Tissues (10 µL of supernatant) were digested with 33% potassium hydroxide and glycogen was precipitated with 75% ethanol and sodium sulfate in heated water for 5 min at 40 °C and then cooled in an ice-bath. After centrifugation at 3000 × *g* for 10 min, the precipitate was taken up in 2% anthrone diluted in sulfuric acid and heated for 10 min at 100 °C. The absorbance was measured at 620 nm. The results were expressed as mg of glycogen per g of wet tissue, using a standard curve with standard glycogen for the calculations.

Protein concentration was measured by the method of Lowry et al. (1951), with bovine serum albumin as a standard. The results were expressed as mg of protein per g of wet tissue.

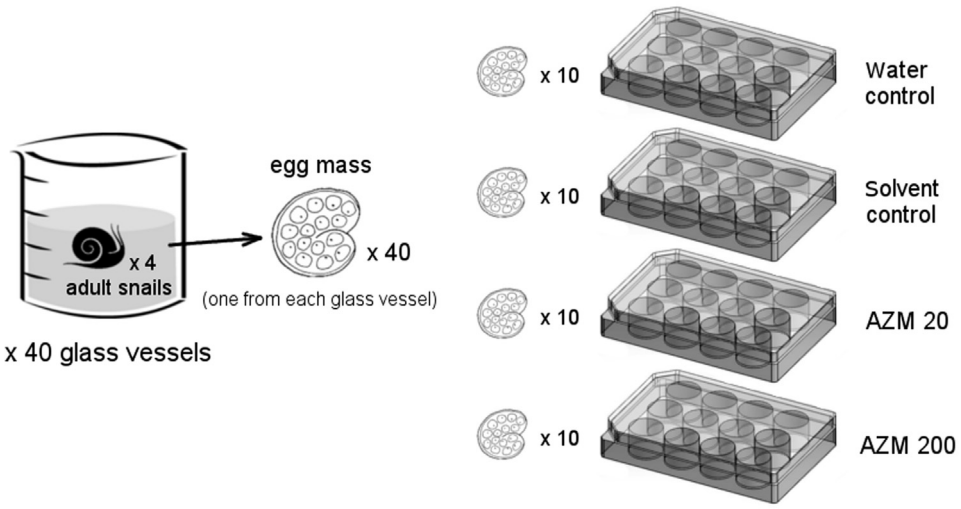

Fig. 1. Schematic representation of the bioassay carried out to study the subchronic effects of 20 and 200 μg L^{-1} azinphos-methyl (AZM 20 and AZM 200) on the egg masses and offspring of *Biomphalaria straminea*.

2.4. Reproductive parameters

2.4.1. Experimental design and data processing

Forty egg masses laid on the same day were separated for the bioassay (Fig. 1). Adult snails were separated in 40 glass vessels of 250 mL with dechlorinated water (4 individuals per vessel) in order to ensure that egg masses came from different parent snails, because this species can cross-fertilize. Snails were left for one week for depuration to ensure they released any egg mass they could have been carrying from the aquaria. Then, glass vessels were checked daily until at least one newly laid egg mass was found in each one on the same day. During this time, adult organisms were fed ad libitum with *Lactuca sativa* var. *capitata* L. (butterhead lettuce). Once the independent 40 egg masses were obtained, the bioassay was deemed to be ready to carry out.

The assay was performed under controlled conditions of temperature (23 ± 1 °C) and photoperiod 12:12 h (Light:Dark) with the same four experimental treatments as those for adult snails: water control, solvent control, AZM 20 μg L^{-1} and AZM 200 μg L^{-1}. One plaque with ten wells was used per treatment, with a single newly-laid egg mass in each well (ten egg masses per treatment) (Fig. 1; 2a). The egg masses were randomly assigned to each treatment. The bioassay lasted one month and solutions were renewed every 96 h (Cacciatore, 2009). The egg masses were examined daily under a stereoscopic microscope until hatching time (Fig. 2b). The hatching time and success were registered for each egg mass. Hatching was considered to have initiated when at least one egg hatched and a juvenile emerged (Fig. 2c). The hatching success was determined by counting the number of hatched eggs per egg mass. Once hatched, organisms were fed ad libitum with *Lactuca sativa* var. *capitata* L. (butterhead lettuce). The offspring survival was evaluated after one month of the bioassay. Juveniles were considered dead when they displayed no movement in response to mechanical stimuli or when shells were empty. The hatching success and the offspring survival were expressed as percentages, relative to the number of embryonated eggs and to the number of hatched ones, respectively.

2.5. Statistical analysis

Assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) were verified. For ChE, CE, GST and SOD activities, ROS, TAC, protein content, hatching success and offspring survival the differences between treatments were tested using a one-way ANOVA. Transformations of the data were applied when necessary. Significant differences were analyzed by Tukey multiple comparisons test. As the assumptions required by the parametric test were not met, mortality, CAT activity, glycogen content and hatching time were analyzed by a non-parametric test (Kruskal-Wallis or Mann-Whitney). All statistical tests were performed using 0.05 as the level of significance. The packages Statistica 7.1 and GraphPad Prism 5.0 were used for statistical analysis.

3. Results

The percentage of mortality of *B. straminea* snails at the end of the bioassay (14 days) was lower than 7% for all treatments and did not differ significantly (Kruskal-Wallis; $H = 4.85$; $P > 0.05$).

No significant differences were found between the water and the solvent control for the measured parameters ($P > 0.05$), with the exception of CAT activity ($P < 0.05$). Therefore, solvent control results are shown in the figures and table.

3.1. Biochemical parameters

3.1.1. B-Esterases

After 14 days of exposure to azinphos-methyl, no significant

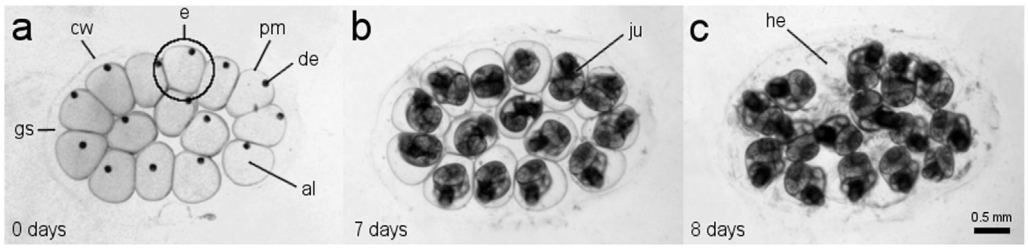

Fig. 2. An egg mass from *Biomphalaria straminea* that contains fifteen eggs at different times of development; **(a)** day 0 (when the egg mass was laid), **(b)** day 7 and **(c)** day 8 (almost all eggs hatched). al = albumen or perivitelline fluid; cw = capsular wall; de = developing embryo; e = egg; gs = gelatinous substance; he = hatched egg; ju = juvenile; pm = perivitelline membrane.

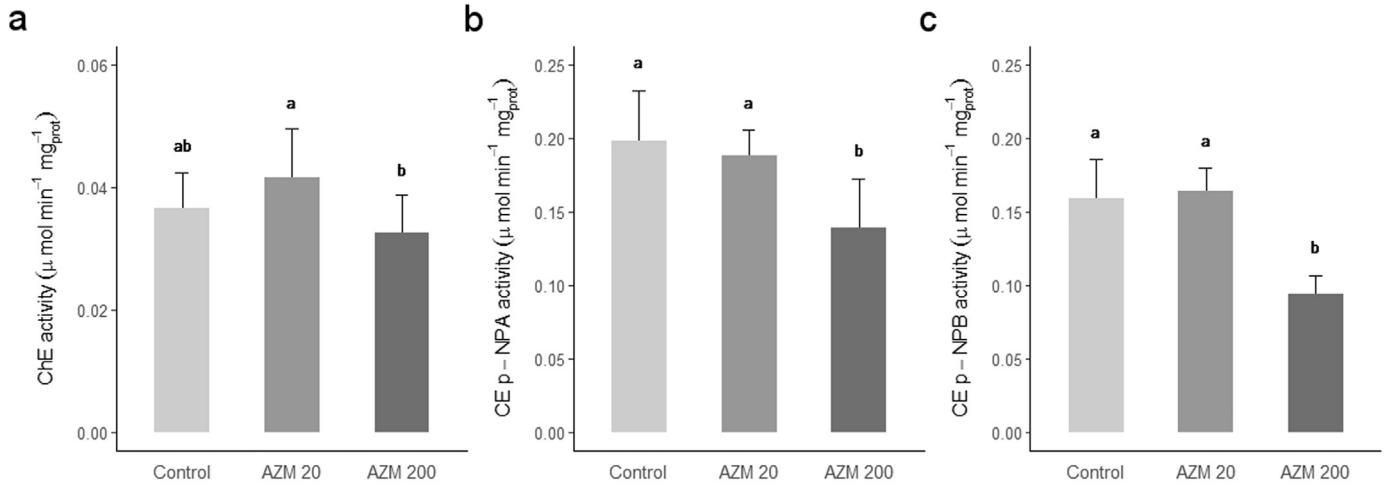

Fig. 3. (a) Cholinesterase (ChE) activity (mean ± SD) and carboxylesterase (CE) activity (mean ± SD) using **(b)** p-NPA and **(c)** p-NPB as substrates, in *Biomphalaria straminea* after 14 days of exposure to solvent control, 20 and 200 μg L^{-1} azinphos-methyl (AZM 20 and AZM 200). Statistical differences between treatments are indicated with different letters.

differences were observed in the activity of ChEs with respect to the control group. Nevertheless, both insecticide concentrations differed significantly amongst themselves (Fig. 3a; one-way ANOVA; F = 3.61; df = 2; P < 0.05).

The exposure of the organisms to AZM 200 μg L^{-1} caused a significant inhibition in the activity of CEs, using both substrates (p-NPA and p-NPB), with respect to the control snails and the lowest concentration of the insecticide (Fig. 3b,c; p-NPA: one-way ANOVA; F = 9.31; df; = 2; P < 0.05; p-NPB: one-way ANOVA; F = 33.42; df = 2; P < 0.05). The percentage of inhibition in relation to the control was 30% with p-NPA and 41% with p-NPB.

3.1.2. GST

The activity of GST significantly increased with both insecticide concentrations in comparison to the control group (Fig. 4; 33% and 31%; one-way ANOVA; F = 9.67; df = 2; P < 0.05).

3.1.3. CAT and SOD

The activity of the antioxidant enzyme CAT presented a significant increase with AZM 20 μg L^{-1} in relation to the control group (24%) (Table 1; Kruskal-Wallis; H = 7.07; P < 0.05). As stated above, this parameter presented significant differences between the water and the solvent controls (7.24 ± 1.60 and 5.52 ± 0.31 μmol of H_2O_2 degraded per min per mg of protein, respectively; Mann-Whitney; U = 0.00; P < 0.05) despite the acetone concentration being lower than the one recommended by the OECD (2010).

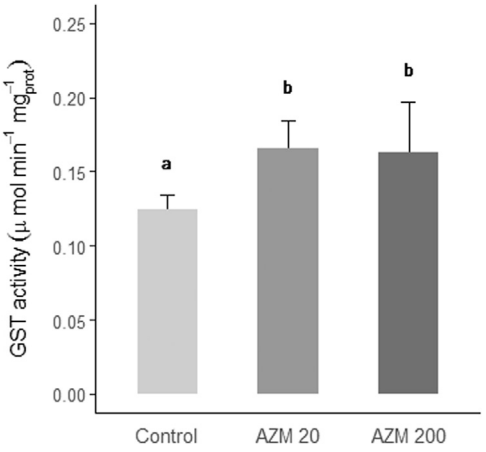

Fig. 4. Glutathione S-transferase (GST) activity (mean ± SD) in *Biomphalaria straminea* organisms after 14 days of exposure to solvent control, 20 and 200 μg L^{-1} of azinphos-methyl (AZM 20 and AZM 200). Statistical differences between treatments are indicated with different letters.

Table 1
Catalase (CAT) and superoxide dismutase (SOD) activities, and glycogen and protein content in *Biomphalaria straminea* after 14 days of exposure to solvent control, 20 and 200 μg L^{-1} azinphos-methyl (AZM 20 and AZM 200).

Treatment	Antioxidant enzymes		Energy reserves	
	CAT[1] Mean ± SD	SOD[2] Mean ± SD	Glycogen[3] Mean ± SD	Protein[3] Mean ± SD
Control	5.52 ± 0.31[a]	15.16 ± 1.84	1.47 ± 0.49	30.30 ± 1.27
AZM 20	6.74 ± 1.29[b]	17.62 ± 2.87	2.04 ± 0.52	26.78 ± 4.12
AZM 200	5.74 ± 0.38[a]	16.34 ± 2.15	2.05 ± 0.48	29.93 ± 2.49

Statistical differences between treatments are indicated with different letters.
[1] CAT; μmol per min per mg protein.
[2] SOD; U SOD per mg protein.
[3] glycogen, protein; mg per g tissue.

The insecticide did not significantly affect the activity of SOD as there were no differences between the azinphos-methyl treatments and the control group (Table 1; one-way ANOVA; F = 2.07; df = 2; P > 0.05).

3.1.4. ROS and TAC

Exposure to the highest concentration of azinphos-methyl produced a significant increase in ROS production compared to the control group (47%) and to AZM 20 μg L^{-1} (Fig. 5a; one-way ANOVA; F =;13.58; df = 2; P < 0.05). TAC also showed a significant increase with AZM 200 μg L^{-1} with respect to the control group (45%) and to the lowest concentration of the insecticide (Fig. 5b; one-way ANOVA; F = 6.99; df = 2; P < 0.05).

3.1.5. Energy reserves

Neither the glycogen nor the protein content varied significantly between azinphos-methyl treatments and the control group (Table 1; Kruskal-Wallis; H = 1.85; P > 0.05 and one-way ANOVA; F = 2.89; df = 2; P < 0.05, respectively).

3.2. Reproductive parameters

The exposure to AZM 20 and 200 μg L^{-1} did not cause a significant

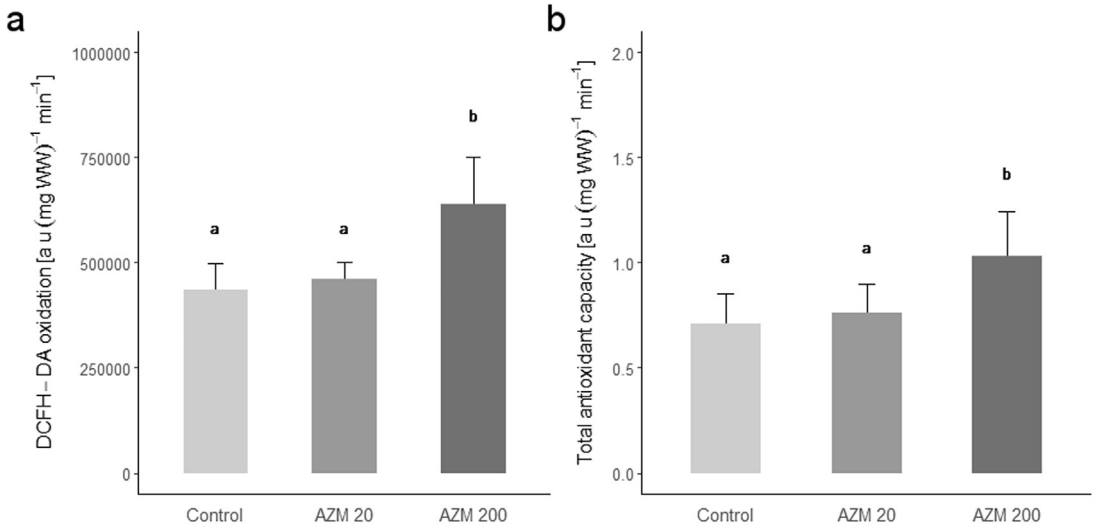

Fig. 5. (a) Reactive oxygen species (ROS) production (mean ± SD) and **(b)** total antioxidant capacity (TAC) against peroxyl radicals in *Biomphalaria straminea* organisms after 14 days of exposure to solvent control, 20 and 200 μg L^{-1} of azinphos-methyl (AZM 20 and AZM 200). Statistical differences between treatments are indicated with different letters.

inhibition in the hatching time nor in the hatching success when compared to the control group (Kruskal-Wallis; H = 3.22; P > 0.05 and one-way ANOVA; F = 2.48; df = 3; P > 0.05, respectively). The hatching time was eight days for all treatments (Fig. 2c; Fig. 6a) and the mean hatching success of the egg masses was similar between treatments (between 92% and 98%) (Fig. 6b).

After one month of exposure, a significant offspring mortality was observed with both insecticide concentrations (one-way ANOVA; F = 15.71; df = 3; P < 0.05). The lowest concentration of azinphos-methyl produced a mortality of 91% of the offspring, while the highest concentration 67%, with respect to the control group, and there were no statistical differences between them (Fig. 6c).

4. Discussion

Our results show that a 14 day exposure to environmental concentrations of azinphos-methyl caused sublethal toxicity in terms of CEs, GST, CAT, ROS and antioxidant capacity. Also, the findings in this study suggest that the exposure of the offspring to this OP leads to a significant mortality of juveniles of *B.straminea*.

Bianco et al. (2014) reported that a 21 day exposure period to 20 and 200 μg L^{-1} azinphos-methyl caused lethality and a decrease in the protein content in *B. straminea*. For this reason, in this study, we chose to work with a 14 day exposure time not finding lethality or effects in the protein content.

Despite being the primary target of OP insecticides, the activity of ChEs was not inhibited by azinphos-methyl. This is an interesting fact as the general response to OP exposure reported in invertebrates is ChE inhibition (Cossi et al., 2015; Ramírez Mora et al., 2000; Varó et al., 2002; Xuereb et al., 2007). Few species of aquatic invertebrates have been shown to have ChEs relatively insensitive to OP exposure (Fulton and Key, 2001; Gagnaire et al., 2008). Hence, we emphasize the importance of measuring a battery of biomarkers to obtain a more complete assessment of pesticide effects.

We measured CE activity using two different substrates: p-NPA and p-NPB. In aquatic gastropods, it has been reported that CE activity of total soft tissue resulted to be more sensitive to OPs when using p-NPA and p-NPB compared to other substrates (Cacciatore et al., 2013; Otero and Kristoff, 2016). *B. straminea* CE activity was significantly inhibited with both substrates with the highest concentration of azinphos-methyl and the decrease in its activity was greater with p-NPB.

The higher sensitivity of CEs compared to ChEs found in this study has also been reported in other organisms and related to a protective role that CEs could be carrying out by competing with ChEs for the binding site of the insecticide (Barata et al., 2004; Sanchez-Hernandez, 2007). Moreover, several authors have associated this greater sensitivity of CEs with the absence of neurotoxic signs (Anguiano et al., 2014; Cacciatore et al., 2013; Otero and Kristoff, 2016). Consistently, in this study, no neurotoxic signs were observed.

The activity of GST showed a significant increase with both insecticide concentrations. This could be associated with a detoxifying response of the enzyme against azinphos-methyl. Several authors reported an alteration in GST activity in other invertebrates exposed to OPs. The freshwater snails *Planorbarius corneus* (Cacciatore et al., 2015) and *Lanistes carinatus* (Khalil, 2015) and the earthworm *Eisenia andrei* (Velki and Hackenberger, 2013) showed an increase in GST activity after exposure to 2 (chlorpyrifos), 3 (chlorpyrifos) and 7–21 (pirimiphos-methyl) days, respectively. Nevertheless, a prolonged exposure produced a decrease in the activity of the enzyme in L. *carinatus* and *E. andrei*. Also, other organisms did not show any alteration in GST activity, such as the gastropods *Biomphalaria glabrata* pigmented and non-pigmented (Kristoff et al., 2008) and *C. gibbosa* (Bianco et al., 2013), both exposed to azinphos-methyl. Therefore, the exposure time, the type and concentration of the insecticide and the susceptibility of the species could be key in the response of GST.

In *B. straminea*, the highest concentration of azinphos-methyl caused an increase in ROS production. The antioxidants enzymes SOD and CAT are considered the first line of defense against ROS (Mates, 2000). In our study, the exposure to azinphos-methyl did not cause any alteration in SOD activity but resulted in an increase in CAT activity with the lowest concentration tested. The unaltered response of SOD might mean that this enzyme does not participate in the antioxidant defense in this case or, that there is a balance between its induction and inactivation resulting in a non-observable effect. There could also be an early SOD response before day 14 of the bioassay. Regarding CAT, its activity increased with AZM 20 μg L^{-1} but with AZM 200 μg L^{-1} is comparable to its control value. Similar results have been reported by other authors, such as Bianco et al. (2013) that found an increase in CAT activity at 0.02 μg L^{-1} azinphos-methyl and no response of the enzyme at 20 μg L^{-1}. Supporting these results, Cao et al. (2003) reported that low ROS levels are able to stimulate CAT activity while an increase in ROS levels can lead to dysfunction of the enzyme.

Antioxidant defenses are numerous and non-specific and may lead to contradictory results by the combination of induction and

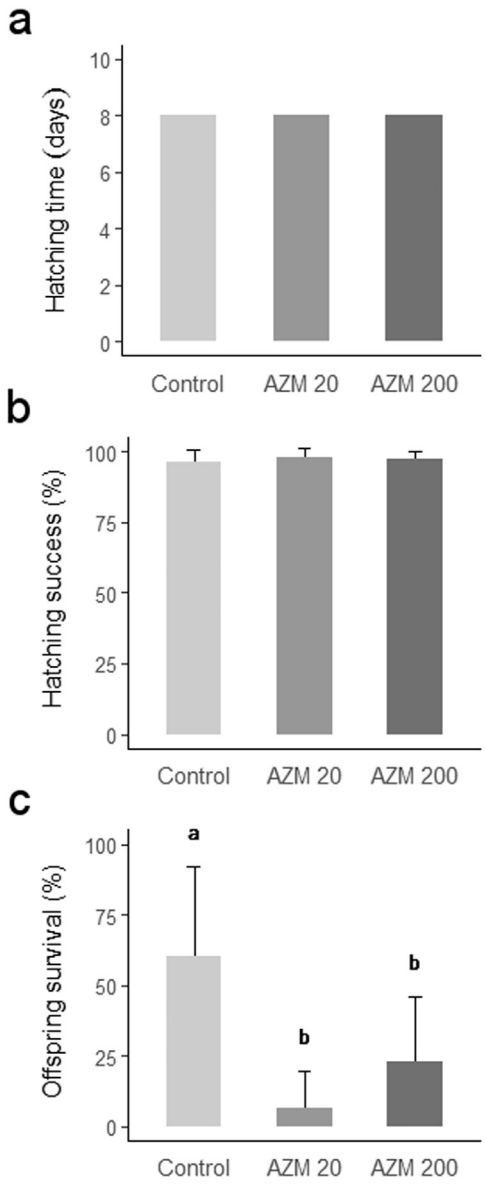

Fig. 6. (a) Hatching time, **(b)** hatching success and **(c)** offspring survival of *Biomphalaria straminea* egg masses and juveniles exposed to solvent control, 20 and 200 $\mu g\,L^{-1}$ of azinphos-methyl (AZM 20 and AZM 200). Statistical differences between treatments are indicated with different letters.

inactivation in the presence of pollutants (Amiard-Triquet et al., 2013). The determination of the total antioxidant capacity (TAC) instead of the traditional measurement of a limited number of antioxidant defenses could provide a clearer overview of an organism's response against contaminants (Amado et al., 2009). The increased TAC against peroxyl radicals with AZM 200 $\mu g\,L^{-1}$ could be reflecting an augmented total antioxidant capacity of the organism that is not enough to counter the elevated ROS production. Other antioxidant defenses than CAT and SOD could be responsible for this augmented TAC.

Despite some authors reporting altered glycogen and/or protein content in freshwater invertebrates exposed to OPs (Bianco et al., 2014; Rambabu and Rao, 1994; Revathi and Bindhuja, 2008), in our study we did not find significant differences between treated and untreated animals on both parameters. Therefore, glycogen and protein content are not altered by the exposure time and the concentrations of azinphos-methyl tested in this study.

Impairments in reproduction can lead to a decrease in the number of specimens of a species and therefore to its near extinction at a local scale (Chromcova et al., 2015). The exposure of the egg masses for one month to azinphos-methyl, despite not affecting the hatching time and success, produced a significant decrease in the offspring survival of *B. straminea*. The developing embryos are surrounded by different coats and substances that may act as barriers, isolating and protecting them against harmful external factors. Fig. 2a shows the layers present in a typical egg mass of Biomphalaria sp.; the embryo is in direct contact with the albumen or perivitelline fluid, in turn enclosed by an inner or perivitelline membrane, constituting an egg. The eggs are embedded in a gelatinous substance and covered by a capsular wall (Nahabedian, 1992). The hatching time and success were probably not affected by azinphos-methyl because those barriers kept the embryos protected. Once the juveniles emerged, they entered in direct contact with the insecticide which then probably caused the high mortality observed. Though these barriers may be effective at protecting the egg masses from exposure to azinphos-methyl in the case of low concentrations of the insecticide in the medium (as in this study), at higher concentrations different results might be obtained. Negro et al. (2014) reported that the embryonic coat of the crab *Zilchiopsis collastinensis* might provide protection to the embryos at low concentrations of chlorpyrifos ($26.2 \pm 3.95\,\mu g\,L^{-1}$) after a permanent exposure; but, at higher concentrations ($74.7 \pm 4.76\,\mu g\,L^{-1}$ and above) they found a decrease in the effective hatching probably because the pesticide was able to enter the eggs. Other authors have also reported alterations in reproduction after subchronic exposures to OPs at environmental concentrations. For example, Rivadeneira et al. (2013) found, after 14 days of exposure to $5\,\mu g\,L^{-1}$ chlorpyrifos, an important decrease in the offspring survival of *Planorbarius corneus*.

B. straminea is a native species with a wide distribution in Argentina, registered from the provinces of Salta (northern Argentina) up to Río Negro (southern Argentina) (Rumi et al., 2008). Azinphos-methyl has been reported as the most frequently detected OP in waters of the Upper Valley Region (southern Argentina) (Loewy et al., 2011; Tosi et al., 2009) and it has also been registered in other regions of Argentina (Acosta, 2014; Castañé et al., 2015). Thus, *B. straminea* snails could be exposed to azinphos-methyl in their natural environments. The inclusion of local species rather than exogenous ones in ecotoxicological studies could be considered more ecologically relevant and more pertinent for extrapolation to natural scenarios (Baird et al., 2007; Cossi et al., 2017; Krull and Barros, 2010).

In view of our results, we emphasize the importance of evaluating subchronic effects, particularly when working with environmental concentrations, as it represents a more realistic picture of the impact of the presence of pesticides in the environment. Though these concentrations can be low, they can produce subchronic effects at physiological, behavioral or ecological levels, as was found in our study. Additionally to the study of traditional biochemical parameters, such as ChEs, CEs, antioxidant enzymes and ROS, we show that the incorporation of reproduction assays can provide a valuable endpoint for chronic toxicity assessments.

5. Conclusions

The subchronic exposure of *B. straminea* to environmental concentrations of azinphos-methyl elicited different effects on the studied biomarkers. In *B. straminea* the primary target of the insecticide (ChE) is not inhibited while CEs, GST, CAT, ROS, TAC and offspring survival are sensitive biomarkers. Our results also suggest that ROS production is an important route of toxicity of azinphos-methyl in this species. The impaired survival of *B. straminea* juveniles during developmental stages reveals an important effect of this insecticide on the offspring of this species. This study confirms the importance of evaluating subchronic effects through the measurement of a battery of biomarkers and of incorporating reproduction assays for chronic toxicity assessments. *B. straminea* could be included in local biomonitoring programs and in other regions where these gastropods are naturally distributed.

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