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Toxicity evaluation of the active ingredient acetamiprid and a commercial formulation (Assail® 70) on the non-target gastropod *Biomphalaria straminea* (Mollusca: Planorbidae)

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ABSTRACT

Neonicotinoids emerged as an environmentally safe alternative to previous generations of insecticides becoming one of the most widely applied in modern agriculture. Nevertheless, they have been reported to affect several non-target organisms. Most toxicity studies focus on the effects on pollinators or terrestrial invertebrates and evaluate either the active ingredient or the commercial formulation. In the present study, we aimed to assess the long-term effects of the active ingredient acetamiprid and a broadly used commercial formulation (Assail® 70) on the non-target freshwater gastropod *Biomphalaria straminea* using a battery of biomarkers. A 14 day-exposure of adult organisms to both active ingredient and commercial formulation increased carboxylesterase activity and glutathione content, inhibited superoxide dismutase activity and decreased reactive oxygen species levels. The commercial formulation additionally increased glutathione S-transferase activity and inhibited catalase activity. The results indicate a greater toxicity of the commercial formulation than that of the active ingredient alone. Cholinesterase activity, development and offspring survival of *B. straminea* were not impaired. We conclude that the toxicity of acetamiprid on this gastropod species is mainly related to effects on detoxification and oxidative metabolism responses. This study provides novel information about the adverse effects of the active ingredient and a commercial formulation of a widely used neonicotinoid on a non-target aquatic species.

1. Introduction

Anthropogenic contamination of aquatic systems has increased worldwide and is an issue of major global concern (Anderson et al., 2016; Ghorade et al., 2014; Rasmussen et al., 2015; SanJuan-Reyes et al., 2017). The intensive use of a wide variety of chemical products as pesticides in modern agriculture constitutes an important source of pollutants which enter into the aquatic environment often causing toxic effects on the organisms inhabiting it (Katagi, 2010; Sánchez-Bayo et al., 2016; Tišler et al., 2009; Wandscheer et al., 2017; Werner and Moran, 2008).

Neonicotinoids emerged as a new generation of environmentally safe insecticides to replace more toxic and persistent compounds such as organophosphates and carbamates (Simon-Delso et al., 2015; Tomizawa and Casida, 2005). They act on the central nervous system (CNS) of insects by binding irreversibly to the postsynaptic nicotinic

Abbreviations: AcSCh, acetylthiocholine iodide; ACP, acetamiprid; AI, active ingredient; CAT, catalase; CE, carboxylesterase; CF, commercial formulation; ChE, cholinesterase; DTNB, 5,5′-dithio-2-bis-nitrobenzoate; GSH, glutathione; GST, glutathione S-transferase; 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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acetylcholine receptors (nAChRs) which leads to the continuous transmission of the nerve impulse and, eventually, neuronal death (Ghanim and Ishaaya, 2011).

Despite their selectivity for insect nAChRs (Shimomura et al., 2006), they may also cause lethal and sublethal effects on non-target organisms. Most studies on the effects of neonicotinoids are focused on bees due to the considerable negative impact on their populations (Henry et al., 2012). However, some authors have reported effects on several biomarkers in non-insect invertebrates, such as bivalves, gastropods and annelids, after neonicotinoid exposure. Some of these effects involve depletion of energy reserves, alterations in cholinesterase activity (ChE), detoxifying enzymes, antioxidant defenses and immunological parameters and impairments in reproduction, development and offspring survival (El-Gendy et al., 2019; Feng et al., 2015; Ge et al., 2018; Laycock et al., 2012; Moncaleano-Niño et al., 2018; Naveen et al., 2018; Radwan and Mohamed, 2013). Nevertheless, due to their relatively recent introduction into the market, there is still limited data available on the impact of this class of insecticides on non-target species, especially in the long term (Anderson et al., 2015; Godfray et al., 2015; Main et al., 2018).

The neonicotinoid acetamiprid (ACP) is extensively used in worldwide agriculture. Nevertheless, it has been recently included in the EU watch list as a potential water pollutant to be monitored (2018/840/EC). In Argentina, it is mainly applied to control lepidopteran and hemipteran pests in a variety of crops (CASAFE, 2017/2019). Even though widely used, there are scarce data available on environmental concentrations of ACP in the aquatic environment (maximum measured concentrations: 0.027 μg L^{-1} in Thessaly, Greece; 44.1 μg L^{-1} in Texas, USA) (Anderson et al., 2013; Tsaboula et al., 2016) and, moreover, about the potential consequences on non-target aquatic invertebrates (Pisa et al., 2015; Sánchez-Bayo et al., 2016).

Pesticides are usually applied as liquid or solid formulations which consist of an active ingredient (AI; chemical substance responsible for killing, controlling or repelling the pest of concern) plus excipients. Since the excipients do not have a pesticidal activity they are considered as inert substances but, in some cases, their toxicological properties are even more relevant than those of the AI (Rozman et al., 2010).

Mollusks are considered excellent indicators of general ecosystem health due to their great sensitivity to environmental changes (Wells and Chatfield, 1992). The abundance, diversity and wide distribution of gastropods together with other characteristics that many species present, such as easy handling, rapid growth, short life cycles and great reproductive potential (Haszprunar and Wanninger, 2012), have led to an increase in their use as model organisms in ecotoxicology (Bhagat et al., 2016; Tallarico, 2015). The freshwater gastropod *Biomphalaria straminea* Dunker, 1848, gathers all these features and also belongs to a genus that has been recommended as a potential bioindicator in Argentina and Chile (Tallarico, 2015). This species is native to South America but has rapidly expanded to other regions (Núñez et al., 2010; Rumi et al., 2008; Yang et al., 2018). These organisms can survive within a wide variety of habitats, and hard water, warm temperatures and eutrophic habitats are optimal for their development (Yipp, 1983). They are simultaneous hermaphrodites and reproduce by self-fertilization and cross-fertilization (Costa et al., 2004). Upon reaching sexual maturity, they lay their eggs inside a capsule (egg mass) attached to hard substrates such as plants, rocks and snail shells. Embryos develop inside the egg and, after a few days, juveniles hatch (Yipp, 1983).

Our aim in this study was to contribute to the knowledge of the toxicity of a widely used neonicotinoid on a non-target freshwater invertebrate species. Therefore, we evaluated the effect of different concentrations of the AI and a commercial formulation (CF) of ACP in *B. straminea* using a battery of biomarkers. After 14 days of exposure, we analyzed ChE, carboxylesterase (CE), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) activities, glutathione (GSH) content, total antioxidant capacity (TAC) and reactive oxygen species (ROS) production in adult organisms and, after 30 days of exposure, we studied hatching time and success in egg masses and offspring survival in juveniles.

2. Material and methods

2.1. Test chemicals

All the chemicals used were of analytical reagent grade. The following were obtained from Sigma-Aldrich: 1-chloro-2,4-dinitrobenzene (CDNB), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP), 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA), 5,5′-dithio-2-bis-nitrobenzoate (DTNB), acetamiprid PESTANAL® (98% purity), acetylthiocholine iodide (AcSCh), bovine serum albumin (BSA), methionine, nitroblue tetrazolium (NBT), p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), reduced glutathione (GSH) and riboflavin. The CF Assail 70® WP was obtained from SummitAgro.

2.2. Biochemical biomarkers in Biomphalaria straminea adults

Adult snails (n = 210) of similar size (0.73 ± 0.07 cm; mean ± standard deviation) from a culture maintained in our laboratory under conditions described by Cossi et al. (2018) were used for the bioassay. Experimental design consisted of five treatments with seven replicates each: dechlorinated tap water (water control), 150 and 1500 μg L^{-1} of ACP active ingredient (ACP AI 150 and ACP AI 1500), and 150 and 1500 μg L^{-1} AI of a commercial formulation containing 70% of the AI (Assail 70® WP) (ACP CF 150 and ACP CF 1500; the CF exposure concentrations refer to AI concentrations). Each replicate consisted of a 250 mL glass vessel containing 200 mL of the treatment solution and six snails haphazardly assigned. Considering ACP AI and CF water solubility, stock solutions (3000 mg L^{-1} and 5000 mg L^{-1}, respectively) were prepared in double-distilled water (ACP solubility in distilled water: 4.25 g L^{-1} at 25 °C; log Kow: 0.8 at 25 °C; European Commission, 2004). The working solutions of ACP AI and CF used in the bioassay were obtained by dilution of the stock solutions in dechlorinated tap water.

The bioassay lasted 14 days and was carried out under controlled conditions of temperature (24 ± 1 °C) and photoperiod (12:12 h, L:D). The organisms were fed *ad libitum* (butterhead lettuce), and solutions were prepared and renewed every 48 h to ensure continuous exposure to the same concentration (ACP Disappearance Time 50 [DT_{50}] hydrolysis: stable at 22 °C, 35 °C and 45 °C, pHs 4, 5 and 7; ACP DT_{50} photolysis: 34 days at 20 °C, pH 7; European Commission, 2004). At the end of the bioassay, snails were homogenized to obtain the supernatants for the biochemical determinations.

The organisms were cold anesthetized for 6–8 min, and then shells were carefully removed over ice with dissecting forceps. For protein, ChE, CE, GST, SOD, CAT and GSH five snails per vessel were homogenized together, due to their small size, with a Potter-Elvehjem homogenizer in 20 mM Tris/HCl buffer (pH = 7.5) containing 0.5 mM EDTA (1:10 w/v). The homogenates were centrifuged for 20 min at 11,000 × *g* at 4 °C and the obtained supernatants were aliquoted and stored at −20 °C. For ROS and TAC one snail per vessel was homogenized with a plastic pestle in 100 mM Tris/HCl buffer (pH = 7.75) with 2 mM EDTA and 5 mM $MgCl_2$ (1:5 w/v) (Gallagher et al., 1992). Homogenates were centrifuged for 20 min at 10,000 × *g* at 4 °C.

Since seven replicates (vessels) were used for each treatment, a total of seven supernatants were obtained per treatment for protein, ChE, CE, GST, SOD, CAT and GSH determinations and seven for ROS and TAC.

2.2.1. Survival

The survival of the organisms was registered daily throughout the bioassay; when dead individuals were found (unable to respond to mechanical stimuli), they were removed from the vessel to avoid water quality deterioration. At the end of the bioassay, survival per vessel was

estimated and expressed as a percentage of the total individuals in the vessel at the beginning of the experiment. The survival percentage per treatment was calculated as the mean percentage of the seven vessels.

2.2.2. Protein content

Protein concentration was determined as described by Lowry et al. (1951), with BSA as a standard, and the results were expressed as mg protein per g tissue.

The protein content (mg/mL supernatant) was used to calculate the specific activities of the enzymes ChE, CE, GST, SOD and CAT.

2.2.3. B-esterases

ChE activity was measured following the method of Ellman et al. (1961), with minor modifications (Bianco et al., 2014). Briefly, 100 μL of the supernatant were added to 100 mM phosphate buffer pH 8.0, 0.2 mM DTNB and 0.75 mM AcSCh. The formation of the dianion 5-thio-2-nitrobenzoic-acid (TNB) was monitored at 412 nm.

CE activity was evaluated according to Kristoff et al. (2010), with slight modifications (Bianco et al., 2014). The reaction mixture contained 100 mM phosphate buffer pH 8.0 with 5% acetone, 1.5 mM p_NPA or p-NPB, and 80 μL of the supernatant. The formation of p-nitrophenol was measured at 400 nm.

2.2.4. GST

GST activity was determined by the method of Habig et al. (1974). The reaction consisted of 100 mM phosphate buffer pH 6.5, 1 mM CDNB, 1 mM GSH, and 100 μL of the supernatant. The formation of GS-DNB was followed at 340 nm.

2.2.5. Antioxidant defenses

SOD activity was determined through the technique of Beauchamp and Fridovich (1971). The reaction mixture contained 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. After exposure to light, reduction of NBT was measured at 560 nm. The results were expressed as SOD units per mg of protein (one unit of SOD is defined as the amount of enzyme that causes 50% inhibition of NBT reduction).

CAT activity was determined by the method of Claiborne (1985). The reaction medium contained 50 mM phosphate buffer pH 7.0, 0.3 M H_2O_2 and 70 μL of the supernatant. The decomposition of H_2O_2 was evaluated at 240 nm.

2.2.6. Non-enzymatic antioxidant

GSH content was measured by the method of Anderson (1985). The reaction consisted of 0.134 M phosphate buffer pH 7.5 with 6.3 mM EDTA, 6 mM DTNB, and 100 μL of the deproteinized supernatant (obtained by adding 10% sulfo-salicylic acid to the supernatant fraction for 20 min and centrifuging for 10 min at 11,000 × *g*). After incubation (30 min at room temperature), the absorbance was recorded at 412 nm. GSH content was determined using a calibration curve with standard GSH and the results were expressed as μmol of GSH per g of wet tissue.

2.2.7. ROS production and TAC

ROS production and TAC were determined by the method of Amado et al. (2009), with modifications (Pérez et al., 2015). ROS production was measured using 5 μL of the supernatant in the reaction buffer (30 mM HEPES pH 7.2, 200 mM KCl and 1 mM $MgCl_2$) with and without ABAP. Then, 10 μl of the fluorogenic probe H_2DCF-DA were added and the fluorophore DCF was detected at 37 °C at λ_{ex} = 488 nm and λ_{em} = 525 nm with a spectrofluorometer microplate reader (Fluoroskan Ascent FL 2.6 with Ascent Software Version 2.6). Fluorescence units (FU) were integrated over time and referred to the wet weight, and expressed as FU wet weight^{-1} min^{-1}. ROS were obtained from the sample without ABAP (area background) and TAC was calculated from the same sample with (area ABAP) and without ABAP as follows:

$$\frac{1}{(\text{ROS area}_{\text{ABAP}} - \text{ROS area}_{\text{background}}) / (\text{ROS area}_{\text{background}})}$$

2.3. Egg mass development and offspring survival

Prior to the bioassay, 200 adult snails of *B. straminea* were haphazardly selected and assigned to 50 glass vessels of 250 mL containing 200 mL of dechlorinated water (4 individuals per vessel). As this species can reproduce by cross-fertilization, with this protocol, we ensured that egg masses came from different parent snails. Snails were left for one week in dechlorinated water to ensure they released any egg masses they could have been carrying from the aquaria. Then, each vessel was checked daily until at least one newly laid egg mass was found. Once 50 independent egg masses (one per vessel) were obtained on the same day, the bioassay was deemed to be ready to carry out.

The experimental treatments were the same as those used for the first bioassay: water control, 150 and 1500 μg L^{-1} of ACP AI, and 150 and 1500 μg L^{-1} AI of a CF of ACP (Assail 70® WP). A sterile 12-well plate was used per treatment (a total of 5 plates) where ten wells were used to expose the egg masses to the experimental solutions (one egg mass per well, each well constituted a replicate). In each well, 3 mL of the corresponding solution was added, and egg masses were haphazardly assigned to each one. Solutions were renewed every 48 h (European Commission, 2004; pesticide degradation data). The assay lasted one month (30 days) and was performed under controlled conditions of temperature (24 ± 1 °C) and photoperiod (12:12 h, L:D).

2.3.1. Hatching time and success

Each egg mass was observed daily under a stereoscopic microscope since the beginning of the bioassay. The hatching time was registered when at least one egg hatched within the egg mass, and the hatching success was calculated per egg mass as follows: (number of hatched eggs/total number of embryonated eggs) x 100.

2.3.2. Offspring survival

After one month, the survival of the juveniles was determined per egg mass as follows: (number of living juveniles/total number of hatched juveniles) x 100. Organisms were considered dead when there was a lack of response to mechanical stimuli or when the shells were found empty.

2.4. Data analysis

The assumptions of normality and homogeneity of variances were verified by Kolmogorov-Smirnov and Levene's tests, respectively. The differences between treatments for protein content, ChE, CE, GST, SOD and CAT activities, GSH content, ROS production, TAC and offspring survival were evaluated through a one-way ANOVA analysis followed by Tukey's multiple comparisons test. Since data transformation failed to meet the assumptions required by the parametric test, hatching time and success were analyzed by the non-parametric test Kruskal-Wallis. The level of significance used was 0.05. The GraphPad Prism 5.0 package was used to perform all statistical analyses.

3. Results

3.1. Biochemical biomarkers in Biomphalaria straminea adults

3.1.1. Survival

At the end of the bioassay (after 14 days of exposure), the survival percentage of *B. straminea* adult snails was higher than 95% in all treatments (mean ± SD; control: 100%, AI 150: 95.24% ± 8.13%, CF 150: 97.62% ± 6.30%, AI 1500: 97.62% ± 6.30%, CF 1500: 100%) and did not differ significantly between them (Kruskal-Wallis,

Table 1
Protein content (mean ± SD) in *Biomphalaria straminea* adults exposed to 150 and 1500 μg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 μg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500).

Treatment	Protein content (mg g $tissue^{-1}$)
Control	42.86 ± 3.32
AI 150	44.46 ± 2.41
CF 150	44.89 ± 2.06
AI 1500	44.28 ± 1.51
CF 1500	42.58 ± 1.17

H = 3.84, P > 0.05).

3.1.2. Protein content

Protein content also did not vary significantly among treatments (one-way ANOVA, F = 1.49, df = 4, P > 0.05) (Table 1).

3.1.3. B-Esterases

After 14 days of exposure, the activities of ChEs and CEs (p-NPA) did not differ significantly between treatments (one-way ANOVA, F = 1.08, df = 4, P > 0.05; one-way ANOVA, F = 2.00, df = 4, P > 0.05, respectively) (Fig. 1a and b).

The activity of CEs (p-NPB) varied significantly between treatments (one-way ANOVA, F = 13.03, df = 4, P < 0.05) (Fig. 1c). A significant increase was found at the highest concentration (1500 μg L^{-1}) of the AI and at both concentrations (150 and 1500 μg L^{-1}) of the CF compared with the control group (32%, 18% and 37%, respectively) (P < 0.05). The activity of the enzyme was also significantly higher between the highest concentration of both AI and CF and the lowest concentrations of each (the AI by 23% and the CF by 18%) (P < 0.05). Within each concentration, there were no significant differences between the AI and the CF (P > 0.05).

3.1.4. GST

The activity of GST was significantly higher at both concentrations of the CF in comparison to the control group (17% with 150 μg L^{-1} and 15% with 1500 μg L^{-1}) (one-way ANOVA, F = 7.97, df = 4, P < 0.05) (Fig. 2) and, only within the lowest concentration (150 μg L^{-1}), GST activity was significantly higher with the formulation in relation to the AI (15%) (P < 0.05).

3.1.5. Enzymatic antioxidants

The activity of both enzymes SOD and CAT varied significantly among treatments (one-way ANOVA, F = 6.57, df = 4, P < 0.05; one-way ANOVA, F = 9.31, df = 4, P < 0.05, respectively) (Fig. 3a and b).

A significant inhibition of SOD activity was observed in the snails exposed to the lowest concentration of the AI with respect to the control (23%) and also to the lowest concentration of the CF with regards to the control and to the highest concentration of the formulation (25% and 30%, respectively) (P < 0.05). Within each concentration, there were no significant differences between the AI and the CF (P > 0.05).

Regarding CAT, significant inhibition of its activity was observed at the highest concentration (1500 μg L^{-1}) of the CF with regards to the control (25%), its respective lowest concentration (16%) and 1500 μg L^{-1} of the AI (18%) (P < 0.05).

3.1.6. Non-enzymatic antioxidant

The content of GSH showed significant differences among treatments (one-way ANOVA, F = 18.69, df = 4, P < 0.05) (Fig. 3c). A significant increase in the content of this tripeptide was observed at both concentrations of the AI in comparison with the control group (15% with 150 μg L^{-1} and 38% with 1500 μg L^{-1}), that differed significantly between both concentrations (24%) (p < 0.05). Likewise, both concentrations of the CF increased significantly its content with respect to the control (36% with 150 μg L^{-1} and 52% with 1500 μg L^{-1}) (p < 0.05). Within each concentration, there were no significant differences between the AI and the CF (P > 0.05).

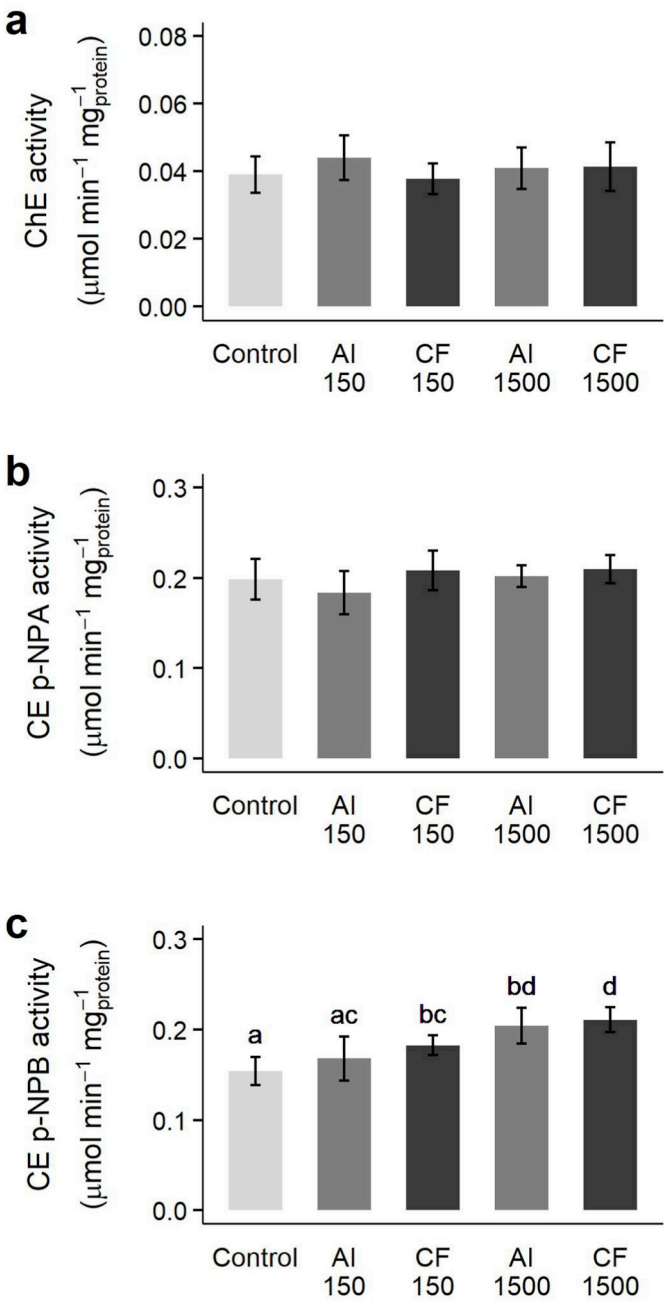

Fig. 1. (a) Cholinesterase (ChE) and **(b,c)** carboxylesterase (CE) activity (mean ± SD), using p-NPA and p-NPB as substrates, in *Biomphalaria straminea* after 14 days of exposure to 150 and 1500 μg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 μg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500). Different letters indicate statistical differences between treatments while the same letter indicates no differences between treatments.

3.1.7. ROS production and TAC

ROS content and TAC differed significantly between treatments (one-way ANOVA, F = 6.97, df = 4, P < 0.05; one-way ANOVA, F = 2.98, df = 4, P < 0.05, respectively) (Figs. 4 and 5).

The exposure to the highest concentration of both AI and CF caused a significant decrease in ROS production with respect to the control (18%) and the lowest concentration of the AI (22%) (P < 0.05).

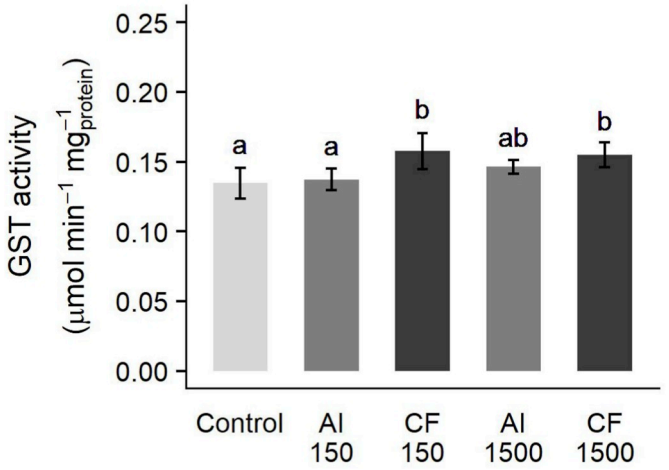

Fig. 2. Glutathione S-transferase (GST) activity (mean ± SD) in *Biomphalaria straminea* organisms after 14 days of exposure to 150 and 1500 µg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 µg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500). Different letters indicate statistical differences between treatments while the same letter indicates no differences between treatments.

Within each concentration, there were no significant differences between the AI and the CF ($P > 0.05$).

TAC was not significantly affected by the AI or by the CF in comparison with the control group ($P > 0.05$). Within each concentration, there were no significant differences between the AI and the CF ($P > 0.05$).

3.2. Egg mass development and offspring survival

3.2.1. Hatching time and success

The exposure to the AI and the CF did not produce a significant effect on the hatching time and success (Kruskal-Wallis, $H = 6.50$, $P > 0.05$; Kruskal-Wallis, $H = 0.32$, $P > 0.05$, respectively). The hatching time was approximately six days for all treatments and the hatching success of the eggs was between 96 and 98% (Table 2).

3.2.2. Offspring survival

The survival of the juveniles was not significantly affected by either the AI or the CF (one-way ANOVA, $F = 0.65$, $df = 4$, $P > 0.05$). After a month, the survival for all the treatments was between 89 and 96% (Table 2).

4. Discussion

Major concerns surrounding the use of insecticides include their distribution into the different environmental compartments and the threat they pose to non-target organisms. Currently, neonicotinoids are among the most commonly used class of insecticides, being registered worldwide in more than 120 countries (Kundoo et al., 2018). However, despite their success, some neonicotinoids have already been banned due to their high toxicity to pollinators (mainly bees) and beneficial insects (predators and parasitoids of agricultural pests) (Pietrzak et al., 2019; Pisa et al., 2015). Due to their intensive use, neonicotinoids began to be detected in water bodies around the world (Anderson et al., 2015; Pietrzak et al., 2019), but their impact on aquatic invertebrates has been relatively poorly studied (Sánchez-Bayo et al., 2016). Thus, further research on the adverse effects of neonicotinoids on potentially sensitive species is required to evaluate their impact on aquatic ecosystems and develop guidelines for the protection of aquatic wildlife.

Biomphalaria straminea is naturally distributed in freshwater bodies of Argentina, including highly productive agricultural regions where

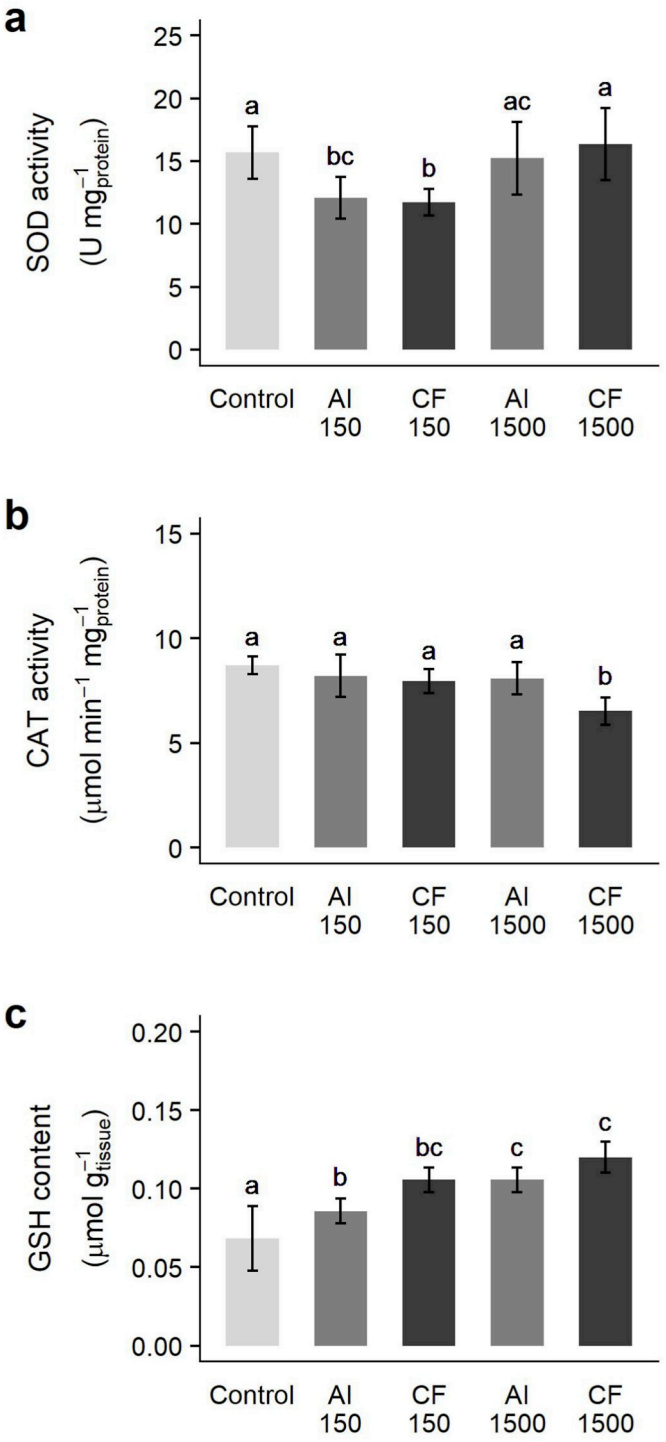

Fig. 3. (a) Superoxide dismutase (SOD) and **(b)** catalase (CAT) activity, and **(c)** glutathione (GSH) content (mean ± SD) in *Biomphalaria straminea* organisms after 14 days of exposure to 150 and 1500 µg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 µg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500). Different letters indicate statistical differences between treatments while the same letter indicates no differences between treatments.

ACP is applied (Núñez et al., 2010; Rumi et al., 2008; Vademécum Toxicológico Alimentario Argentino (VATOXA), 2018). Therefore, these gastropods can be exposed to this neonicotinoid insecticide in their natural environment. In this study, the lowest concentration of ACP tested falls within the range of concentrations of neonicotinoids found in the environment (e.g., imidacloprid: 320 µg L^{-1}; Van Dijk et al.,

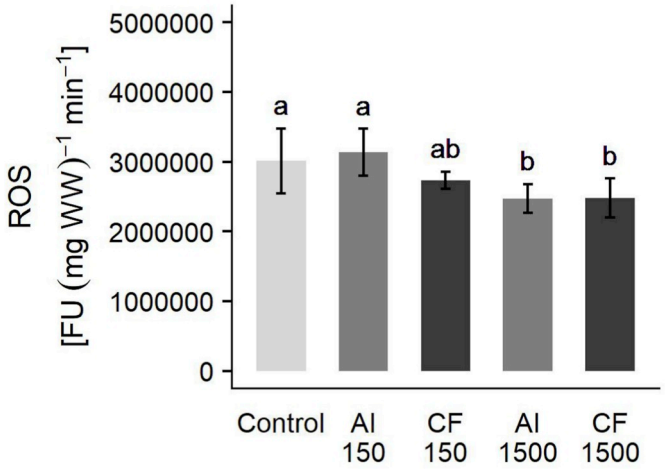

Fig. 4. Reactive oxygen species (ROS) production (mean ± SD) in *Biomphalaria straminea* organisms after 14 days of exposure to 150 and 1500 μg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 μg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500). Different letters indicate statistical differences between treatments while the same letter indicates no differences between treatments.

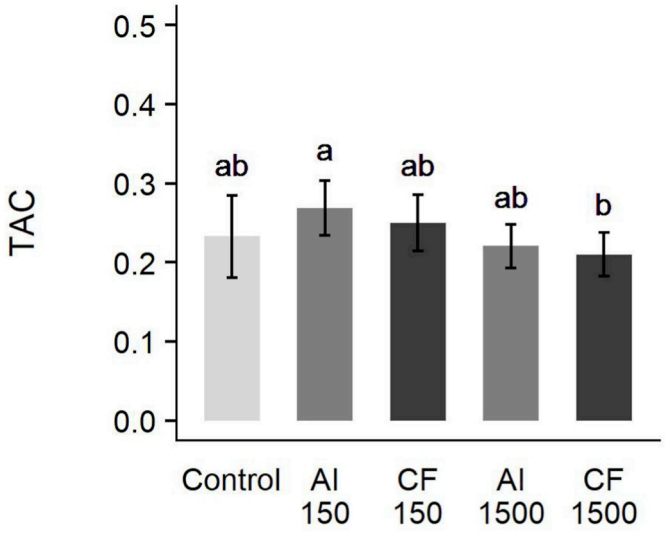

Fig. 5. Total antioxidant capacity (TAC) against peroxyl radicals (mean ± SD) in *Biomphalaria straminea* organisms after 14 days of exposure to 150 and 1500 μg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 μg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500). Different letters indicate statistical differences between treatments while the same letter indicates no differences between treatments.

Table 2
Hatching time, hatching success and offspring survival (mean ± SD) of *Biomphalaria straminea* egg masses and juveniles exposed to 150 and 1500 μg L^{-1} acetamiprid active ingredient (AI 150 and AI 1500), and 150 and 1500 μg L^{-1} acetamiprid commercial formulation (CF 150 and CF 1500).

Treatment	Hatching time (days)	Hatching success (%)	Juvenile survival (%)
Control	6	95.67 ± 10.66	92.02 ± 8.84
AI 150	6.2 ± 0.42	97.75 ± 4.78	88.53 ± 15.31
CF 150	5.9 ± 0.32	98.18 ± 3.83	88.96 ± 16.81
AI 1500	6.1 ± 0.32	97.23 ± 4.92	89.83 ± 26.75
CF 1500	6	97.19 ± 7.07	95.88 ± 6.16

2013). In the case of ACP, few studies reporting environmental concentrations can be found (a maximum of 44 μg L^{-1} was found in Texas, EEUU; Anderson et al., 2013), and particularly, in Argentina, there is a lack of data despite being intensively used. We also worked with a concentration ten times higher since concentrations of pesticides in water are highly variable in time and space, and monitoring data usually fails to capture peak concentrations of compounds (e.g., run off period immediately after pesticide application). Thus, non-target species can be exposed to a wide range of concentrations, even higher than the reported ones (Cacciatore et al., 2018; Hetrick et al., 2000). After a 14-day exposure, we found that ACP causes several toxic responses to adult organisms involving biochemical biomarkers related to detoxification and oxidative processes.

Neonicotinoids specifically bind to the nicotinic acetylcholine receptors (nAChRs) and thus, effects on the enzyme itself (AChE) are not typically expected. However, different responses of the enzyme were reported after exposure to neonicotinoids. The inhibition of AChE was observed in the snail *Helix aspersa* (Radwan and Mohamed, 2013) and the gills of the oyster *Saccostrea* sp. (Ewere et al., 2019; Moncaleano-Niño et al., 2018) after treatment with imidacloprid. Instead, no effects on the enzyme were found in the amphipod *Gammarus fossarum* (Malev et al., 2012) and the earthworms *Aporrectodea nocturna* and *Allolobophora icterica* (Capowiez et al., 2003), exposed to the same neonicotinoid. Similarly, in this study, ChE activity was not affected by ACP, which is consistent with the mechanism of action of neonicotinoids.

The enzymes CE and GST are involved in xenobiotic metabolism in living organisms through different mechanisms. CEs can hydrolyze carboxyl esters present in some insecticides while GSTs catalyze their conjugation with GSH which generates less toxic and more water-soluble compounds (Parkinson and Ogilvie, 2008; Potter and Wadkins, 2006). Hence, their activities are widely used as biomarkers of effect of different pesticides (Domingues et al., 2010; Wheelock and Nakagawa, 2010). Neonicotinoids have been found to cause different effects on these enzymes. An increase in GST activity was reported in *H. aspersa* (Radwan and Mohamed, 2013) and the earthworm *Eisenia fetida* (Wang et al., 2016), both exposed to imidacloprid. Conversely, a decrease in GST and CE activities was observed in *E. fetida* treated with thiacloprid (Feng et al., 2015) and in GST activity in the oyster *Saccostrea glomerata* exposed to imidacloprid (Ewere et al., 2019). In *B. straminea*, CE (p-NPB) activity increased at the highest concentration of the AI of ACP and with both concentrations of the CF. GST activity was also higher in the exposed organisms but only with both CF concentrations. Thus, these increases could be associated with the induction of different processes of detoxification and elimination of ACP.

The antioxidant defense system of organisms plays a critical role in protecting them against ROS. It involves enzymatic (CAT, SOD, GSH peroxidase, GSH reductase) and non-enzymatic antioxidants (GSH, vitamin C, vitamin E). An imbalance between ROS production and antioxidant defenses, in favor of the former, results in oxidative stress, with subsequent damage to macromolecules. Exposure to pollutants can increase ROS levels in aerobic organisms. It was reported that ROS generated at low or moderate concentrations can act as second messengers in cell signal transduction and gene regulation (Gloire et al., 2006) increasing antioxidant defenses, however, a significant increase in ROS levels can lead to a decrease in them (Amiard-Triquet et al., 2013; Scandalios, 2005). Therefore, the measured activities or contents of antioxidants can increase, decrease or remain unchanged as they depend on the balance between up-regulation and damage (Kristoff et al., 2008).

Altered antioxidant defenses were reported in non-target species after exposure to neonicotinoids. In the snails *Theba pisana* and *H. aspersa* an increase in CAT activity was observed after exposure to thiamethoxam and imidacloprid, respectively (Radwan and Mohamed, 2013; El-Gendy et al., 2019). In *E. fetida*, a decrease in CAT and SOD activities was found when exposed to thiacloprid (Feng et al., 2015) and a decrease in CAT and an increase in SOD when treated with

imidacloprid (Wang et al., 2016).

In *B. straminea* exposed to ACP, the most altered antioxidant defense was GSH content, its increase with both concentrations could be associated with its active participation in the conjugation catalyzed by GST and in several redox reactions. SOD and CAT are considered the first line of defense against ROS. Initially, SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which is then reduced to water by CAT (Mates, 2000). In *B. straminea*, their responses depended on ACP concentrations (AI and CF). At the lowest concentration, a decrease in SOD activity was observed while CAT was not affected. However, at the highest concentration of the CF, CAT activity decreased without any response in SOD. Antioxidant responses can greatly vary with different pollutant concentrations. Additionally, enzymatic antioxidants differ in substrates, products and gene regulation, and in consequence, up-regulation, synthesis and inactivation. Wang et al. (2016) have reported different responses of SOD and CAT in *E. fetida* after the exposure to imidacloprid. They observed an increase of SOD and inhibition of CAT when ROS content accumulated in cells. Several authors proposed that ROS could act inducing but also inhibiting enzyme synthesis (Liu et al., 2011; Wang et al., 2016; Xu et al., 2009). Liu et al. (2011) described that mRNA breakdown or cellular transcription mechanism might cause the inhibition of enzymatic synthesis by pollutants. So, at the same ROS level, some enzyme activities could increase by induction of synthesis, while others could decrease by inhibition of synthesis or inactivation. This could explain the different responses of SOD and CAT observed in B. straminea.

Eventually, the combined activity of the antioxidant defenses could have reduced, to a certain extent, the ROS generated in the exposed snails, leading to almost control levels of ROS at the lowest concentration and their decrease at the highest concentration of both AI and CF. The overall antioxidant status of *B. straminea*, evaluated through TAC, was not affected by the insecticide. This determination considers the interaction of several antioxidants that can be individually induced or inactivated by ROS and result in a constant TAC.

The impact of environmental pollutants on reproductive success and offspring survival of a species is critical as it can affect the abundance and distribution of organisms and, moreover, the perpetuation of a species. Effects of neonicotinoids on reproduction were reported in different soil invertebrates. In *E. fetida* a decrease in the mean cocoon number, mean cocoon weight and hatchability was reported after exposure to different neonicotinoid classes, including ACP (Wang et al., 2015; Ge et al., 2018). In *E. fetida,* the oligochaete *Enchytraeus crypticus* and the collembolan Folsomia candida a decrease in juvenile survival was observed after treatment with imidacloprid and thiacloprid (de Lima e Silva et al., 2017). Conversely, in *B. straminea* ACP did not produce an effect on hatching and offspring survival. Nevertheless, alterations in these parameters cannot be ruled out if, for example, snails are exposed for a longer period of time or at higher concentrations of ACP.

The evaluation of the toxicity of CFs represents a more realistic approach of the effects of pesticides on organisms since it is in this manner that AIs are applied. Several insecticides showed greater toxicity of the formulated product than the AI alone (Beggel et al., 2010; Demetrio et al., 2014; Mansano et al., 2016; Puglis and Boone, 2011). Accordingly, in this study, the CF of ACP (Assail® 70) resulted more toxic than the AI since it altered a greater number of biomarkers (GST, CAT) or produced a greater effect in the same biomarker at equivalent concentrations of the AI (CEs). The presence of excipients in the CF could be increasing the toxicity of the insecticide, either by their toxicological properties *per se* or by favoring the bioavailability of the AI. As these additional ingredients in the CFs are considered inert, their identity is often kept confidential and it is not informed in the labels of the products. However, as found in this and other studies, the effect of the excipients could be relevant in the overall toxicity of the CF and cannot be ignored or underestimated. Although these ingredients usually cannot be tested alone, an insight to their toxicity can be obtained when evaluating the effects of a CF together with its respective AI. Most of the few studies on ACP toxicity evaluate either the active ingredient (Ge et al., 2018; Raby et al., 2018) or a commercial formulation (Badawy et al., 2015; Bownik et al., 2017; Mishchuk and Stoliar, 2008). Our results report valuable data on the toxicity of both technical grade ACP and a CF.

In the present study, although survival was not affected, we found long-term effects on most of the biomarkers evaluated (CEs, GST, SOD, CAT, GSH and ROS) in *B. straminea* adults after exposure to the neonicotinoid ACP. The obtained results contribute to 1) the understanding of the mechanism of action of neonicotinoids in aquatic species, 2) the knowledge of toxic effects that these insecticides could cause in organisms that inhabit contaminated water bodies, and 3) the possibility of using these organisms for evaluation of water quality. From our results follows that oxidative stress would be a key mechanism to understand the toxicity of ACP in *B. straminea*, that organisms naturally exposed to contaminated freshwater environments could suffer alterations at the biochemical level, and that these organisms are sensitive to ACP. They were also sensitive to other insecticides studied by our group (carbaryl: Cossi, 2019; azinphos-methyl: Cossi et al., 2018) appearing to be a suitable pollution indicator. The study of multiple biomarker parameters in bioindicator organisms is a useful tool to evaluate water quality in addition to physical and chemical analysis. It is important to emphasize that detection and quantification of all possible contaminants is difficult and expensive and do not reflect the bioavailability or toxicity to organisms. Also, many contaminants can be found in undetectable concentrations and yet alter biomarkers that result from early alarm. Water quality, either directly or indirectly, is vital for environmental and human health. The alterations caused by contaminants in a particular species may impact other species, for example, through the food web. Thus, the effects on a species can negatively impact ecosystems, and affect economically relevant species that feed on it, such as silversides and trouts, important predators of gastropods (Martínez-Palacios et al., 2019; Ríos et al., 2015). This fish species are relevant for local consumption, commerce and tourism, livelihood sources of coastal populations in several regions of our country.

5. Conclusions

Our study shows that both the active ingredient and the commercial formulation (Assail® 70) of the neonicotinoid acetamiprid elicited long-term toxic effects on *Biomphalaria straminea*. The commercial formulation resulted more toxic than the active ingredient alone since it altered to a greater extent most of the biochemical biomarkers evaluated. We highlight the relevance of including commercial formulations in toxicity studies of insecticides to assess the effect of excipients since their impact on organisms cannot be ruled out. The insecticide affected mainly detoxifying (CE, GST) and antioxidant biomarkers (SOD, CAT, GSH) indicating a route of toxicity related to the detoxification and oxidative metabolism of *B. straminea*. These results confirm the importance of evaluating effects through the measurement of a battery of biomarkers for the understanding of the mechanism by which insecticides cause toxicity in a given species. Particularly, in this species, detoxifying and antioxidant biomarkers should be included to evaluate the impact of pesticides. This study provides the first data on the adverse effects of the active ingredient and a commercial formulation of acetamiprid on the aquatic invertebrate *B. straminea*. Our results contribute to the scarce knowledge of the impact of neonicotinoids on aquatic biota.

CRediT authorship contribution statement

Paula Fanny Cossi: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Lucila Thomsett Herbert:** Investigation. **María Soledad Yusseppone:** Investigation. **Analía Fernanda Pérez:** Methodology,

Resources. **Gisela Kristoff:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision.

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