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Ecotoxicological effects of the emerging contaminant ivermectin on *Rhinella arenarum*: A comparative study of active ingredient and commercial formulation

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ABSTRACT

Ivermectin (IVM) is a broad-spectrum veterinary antiparasitic used worldwide in cattle breeding. The aim of this study was to evaluate the lethal effects of the active ingredient and a commercial formulation of IVM (1 % active ingredient) in the embryonic stage (S. 4–6) and larval stage (S. 25) of the South American amphibian *Rhinella arenarum* through chronic standardized bioassays. Also, behavior analysis and oxidative stress and cholinergic effects biomarkers were analyzed at 1, 10 and 100 μg IVM/L concentrations. For the embryonic stage, the active ingredient (96 h- LC_{50}: 15900 μg/L) was more toxic than the commercial formulation (96 h-LC_{50}: 51230 μg/L) during the acute period, while at chronic exposure the commercial formulation was more toxic (504 h-LC_{50}: 10.25 μg/L), compared to the active ingredient (504 h-LC_{50}: 312.80 μg/L). For the larval stage, in acute exposure, the active ingredient (96 h-LC_{50}: 800 μg/L) was more toxic than the commercial formulation (96 h-LC_{50}: 1550 μg/L). In the chronic exposure, the commercial formulation (504 h-LC_{50}: 77.33 μg/L) was more toxic than the active ingredient (504 h-LC_{50}: 195.25 μg/L). Overall, larvae exhibited greater sensitivity to both the active ingredient and the commercial formulation. However, during chronic exposure, embryos were more sensitive to the commercial formulation than larvae. The commercial formulation primarily induced oxidative stress, and both forms of the compound affected behavior and cholinergic effect biomarkers, even at low environmentally relevant concentrations (1 μg/L). These results highlight the potential impact of IVM on aquatic ecosystems.

1. Introduction

The high demand of meat products led to the need to keep production levels high. In consequence, new production strategies were generated (García et al., 2015), such as the intensification of livestock production by fattening in pens (feedlot). In this method, the area for breeding is reduced, and a large number of animals are concentrated in small areas (Rodrigues Capítulo et al., 2010). Intensive breeding led to an increase in the percentage of sick animals due to overcrowding, and therefore necessitating an increase in the amount of veterinary drugs used (Mathew et al., 2007).

Ivermectin (IVM), an avermectin derivative, is a broad-spectrum veterinary antiparasitic used worldwide in cattle. It is produced by the bacteria *Streptomyces avermitilis* and acts by blocking the neurotransmitter γ-aminobutyric acid, which in invertebrates control the peripheral muscles (Hennessy and Alvinerie, 2002) and in vertebrates acts at synapses of the central nervous system (Jorgensen, 2005). After the administration of IVM to animals, its metabolic decomposition is generally low. So, between 62 and 98 % of the administered antiparasitic is excreted unchanged, mainly in the feces (Verdú et al., 2020). Given its poor metabolism and the high doses of the drug that are generally administered to ensure successful treatment, IVM is considered an emerging contaminant with considerable potential to cause harmful effects on non-target organisms (Nunes et al., 2021). In Argentina, a concentration of 1.24 μg/L was reported in surface water from a wetland of a cattle breeding area in the Paraná River basin (Mesa et al., 2020).

Previous studies have demonstrated the negative effects of IVM on

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nontarget organisms, from marine and terrestrial ecosystems (Ambrožová et al., 2021; Barrett, 2022). In freshwater organisms, studies are scarce. For instance, the toxicity of IVM was evaluated on freshwater invertebrates, such as *Daphnia magna* and *Chironomus riparius* (Schweitzer et al., 2010), algae (Garric et al., 2007), and some fish species, such as *Danio rerio* and *Salmo salar*, with lethal concentrations ranging between 0.0057 and 17 μg/L (Carlsson et al., 2013; Ucán-Marín et al., 2012). On the other hand, Sanderson et al. (2007) observed a significant decline in zooplankton richness after 10 days of applying varying concentrations of IVM (0.03–1 μg/L) in mesocosms. Also, the lethal toxicity of IVM was assessed on four amphibian's species from Asia with lethal concentrations ranging between 6 and 29 μg/L (Zhang et al., 2018).

Toxicity bioassays assess the potential risk of exposure to chemical substances of various origins, constituting a realistic diagnostic tool. Among the species employed in toxicity bioassays, amphibians are widely used due to their high sensitivity, tegument and gill permeability, and biphasic life cycle (Pérez Coll et al., 2017). The standardized AMPHITOX test uses embryos and larvae of a native amphibian, *Rhinella arenarum*, for the evaluation of the toxicity of different matrices (Pérez Coll et al., 2017). *Rhinella arenarum* is a representative species of the Argentine herpetofauna due to its relative abundance and wide distribution. As a complement of toxicity bioassays, the use of biomarkers is a useful tool that provides information about the sublethal effects produced by the exposure to different matrixes (Rodrigues et al., 2021). Behavioral, oxidative stress and cholinergic effects biomarkers have proved to be useful in monitoring the negative effects of xenobiotics (Lourido et al., 2022). Behavioral biomarkers are helpful for assessing sublethal toxicity during acute toxicity studies since behavioral changes frequently appear before decreasing survival (Scott and Sloman, 2004). The analysis of behavioral alterations provides understanding of the complex links between an organism's internal biochemistry and its external environment. Some studies have demonstrated that aquatic pollutants can alter several aspects of animal behavior, including motility (Walls and Gabor, 2019). On the other hand, oxidative stress biomarkers are commonly employed among aquatic species (Valavanidis et al., 2006). Aerobic organisms have defense mechanisms to prevent cellular damage from reactive oxygen species (ROS). Exposure to some xenobiotics may produce an imbalance between endogenous and exogenous ROS and in consequence a decrease the antioxidant defenses, which causes biological systems to experience oxidative stress, tissue damage, inflammation, degenerative illnesses, and aging (Finkel and Holbrook, 2000; Sohal et al., 2002). The activity of catalase (CAT) and glutathione S-transferase (GST) and the levels of reduced glutathione (GSH) and lipid peroxidation (TBARS) are among the most commonly employed to analyze oxidative stress (Lourido et al., 2022). In particular, some studies have indicated that IVM could lead to an increase in oxidative stress, highlighting potential adverse effects (Ogueji et al., 2020). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two enzymes from the family of cholinesterases (Prokić et al., 2016). Certain substances, such as pesticides, drugs and metals can alter these enzymes and in consequence affect the organism's behavior (dos Santos Carvalho et al., 2020; Güngördü et al., 2010).

To our knowledge, there are no previous studies that compare the effects of the active ingredient and a commercial formulation of IVM on any species. Most studies have employed the active ingredient (Garric et al., 2007; Lopes et al., 2009; Zhang et al., 2018) and only a few used a commercial formulation (Sands and Noll, 2022). Commercial formulations of drugs include not only the active ingredient but also excipients, which are generally unknown compounds. Comparative investigations of the negative effects produced by commercial formulations and active ingredients are relevant for a more accurate and proper risk assessment, even though commercial formulations from different countries may differ regarding the unknown excipients. In the case of IVM, most commercial formulations contain only 1 % of the active ingredient.

The aim of this study was to evaluate the lethal and sublethal effect of the active ingredient and a commercial formulation of IVM (1 % active ingredient) in the embryonic stage (S. 4–6) and larval stage (S. 25) of the native amphibian *R. arenarum* through standardized bioassays, behavior analysis and oxidative stress and cholinergic effects biomarkers.

2. Material and methods

2.1. Rhinella arenarum embryos and larvae

The ovulation of *R. arenarum* females, the in vitro fertilization for the obtention of embryos and the maintenance of individuals was performed following the AMPHITOX protocol (Pérez Coll et al., 2017). Individuals were kept in a physiological solution for amphibians, AMPHITOX Solution (AS, concentration in mg/L: HCO_3^- 1.45, Cl^- 22.71, Ca_2^+ 0.36, Na^+ 14.75 and K^+ 0.26), which was renewed every other day to ensure oxygen availability. Embryonic and larval stages were defined according to Del Conte and Sirlin (1952). The handling of the animals was carried out according to the regulations for the use of amphibians in laboratory research (Beaupre et al., 2004), controlled and approved by CICUAE-UNSAM (Res. 1/2022) and the Dirección de Flora y Fauna from Buenos Aires Province (Res. 01133069).

2.2. Test substances

Bioassays were carried out using either the active ingredient (CAS—No. 70288–86-7, purity grade 99 %, Sigma-Aldrich) or a commercial formulation of IVM (FACyT SRL, 1 % active ingredient).

For both the active ingredient and the commercial formulation, test solutions were prepared by diluting two stock solutions with AS. The stock solutions, at concentrations of 2000 μg/L and 500,000 μg IVM/L, were prepared by diluting either the active ingredient or the commercial formulation with AS. The concentrations of either the active ingredient or the commercial formulation are expressed as μg IVM/L. Then, for both compounds, a gradient of concentrations ranging from 1.5 to 150,000 μg IVM/L was performed for the lethality bioassays with embryos and between 1 and 100,000 μg IVM/L for the lethality bioassays with larvae. The concentrations range was based on preliminary bioassays and available data about the toxicity of IVM to other amphibians' species from Asia (Zhang et al., 2018). The IVM concentration in the stock solutions was determined by HPLC-MS using a Thermo Scientific Ultimate 3000 and a reverse phase C18 column (Hypersil Gold, USA, 1.9 μm, 2.1 mm × 50 mm) according to Peluso et al. (2023). The error between nominal and measured concentration did not exceed 5 %.

2.3. Bioassays for lethal and behavioral effects

Toxicity bioassays were carried out according to the AMPHITOX protocol (Pérez Coll et al., 2017). For each experimental condition, 10 embryos (from S.4–6) or 10 early larvae on complete operculum stage (S. 25) were placed in covered glass Petri dishes (diameter: 10 cm), in triplicate containing 40 mL of test solutions or AS (control) for 504 h. The medium was renewed every 48 h and the larvae were fed with balanced food for fish (TetraColor ®). The temperature and photoperiod were kept constant (20 ± 2 °C; 14/10 h light/dark). Lethal effects were evaluated every 24 h. At the 48, 96 and 168 h of exposure, the larvae exposed to the 1, 10 and 100 μg/L concentrations were recorded for 5 min, after a 5-min acclimation using a digital video camera (Gadnic CSC18) placed just above the Petri dish.

2.4. Biomarkers of oxidative stress and cholinergic effects

For the measurement of biomarkers, 50 embryos (S. 4–6) or larvae (S. 25) were exposed in triplicate to the control AS and to three low concentrations of active ingredient and commercial formulation of IVM (1.25, 5 and 10 μg/L) for 96 h. Once the exposure was over, the samples

were homogenized (0.154 M KCl with protease inhibitors) and centrifuged (20 min at 10000*g*). Proteins were measured on the supernatant according to the Bradford (1976) method. The enzymatic activities of catalase (CAT) and glutathione-S transferase (GST) were measured according to Lück (1965) and Habig (1974), respectively. CAT activity was expressed as U CAT (mmol*min)/mg protein and calculated using a molar extinction coefficient of 40/M × cm while GST activity was expressed as U GST mg (mmol*min)/protein and calculated using a molar extinction coefficient of 9.6/mM × cm. On the other hand, the concentrations of reduced glutathione (GSH) and lipid peroxidation (TBARS) were determined according to Anderson (1985) and Buege and Aust (1978), respectively and expressed as nmol GSH/mg protein or nmol TBARS/mg protein. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were determined only on the exposed larvae according to the Ellman et al. (1961) protocol. AChE activity was expressed as U AChE (nmol*min)/mg protein and calculated using a molar extinction coefficient of 14.15/mM × cm while BChE activity was expressed as BChE ((nmol*min)/mg protein and calculated using a molar extinction coefficient of 13.6/mM × cm.

2.5. Data analysis

Lethal concentrations 50 (LC_{50}) were calculated by the Probit transformation using the Statgraphics Centurion XVI program. Significant differences ($p \leq 0.05$) between LC_{50}s were estimated by the criterion of non-overlapping of the 95 % confidence intervals according to APHA (1989).

The speed (mm/S) and mobility rate (%) were estimated as behavioral parameters. The videos were analyzed with the free software ToxTrac, version 2.96 (Rodriguez et al., 2018). This software collects several parameters relating to activity and movement behaviors using automated image-based tracking. Also, it is frequently used to evaluate the behavior of different animal species, including rats and zooplankton (Rodriguez et al., 2018). The method employed by the software uses a threshold to remove objects (individuals) from the background.

Graphs and statistical analyses of biomarker data were conducted using GraphPad Prism 8. One-way ANOVA and Dunnett's post hoc test were applied when the assumptions were met; otherwise, non-parametric tests, such as Kruskal-Wallis and post hoc Dunn tests, were employed.

3. Results

3.1. Chronic toxicity bioassays of Rhinella arenarum embryos and larvae

The toxicity profiles of *R. arenarum* embryos and larvae exposed either to the active ingredient or the commercial formulation are shown in Fig. 1. For individuals exposed from the embryonic stage to the active ingredient, the LC_{50} was calculated from the 72 h of exposure, whereas for those exposed to the commercial formulation, the LC_{50} was calculated from the 96 h of exposure. On the other hand, for organisms exposed from the larval stage to the AI, the LC_{50} was calculated starting

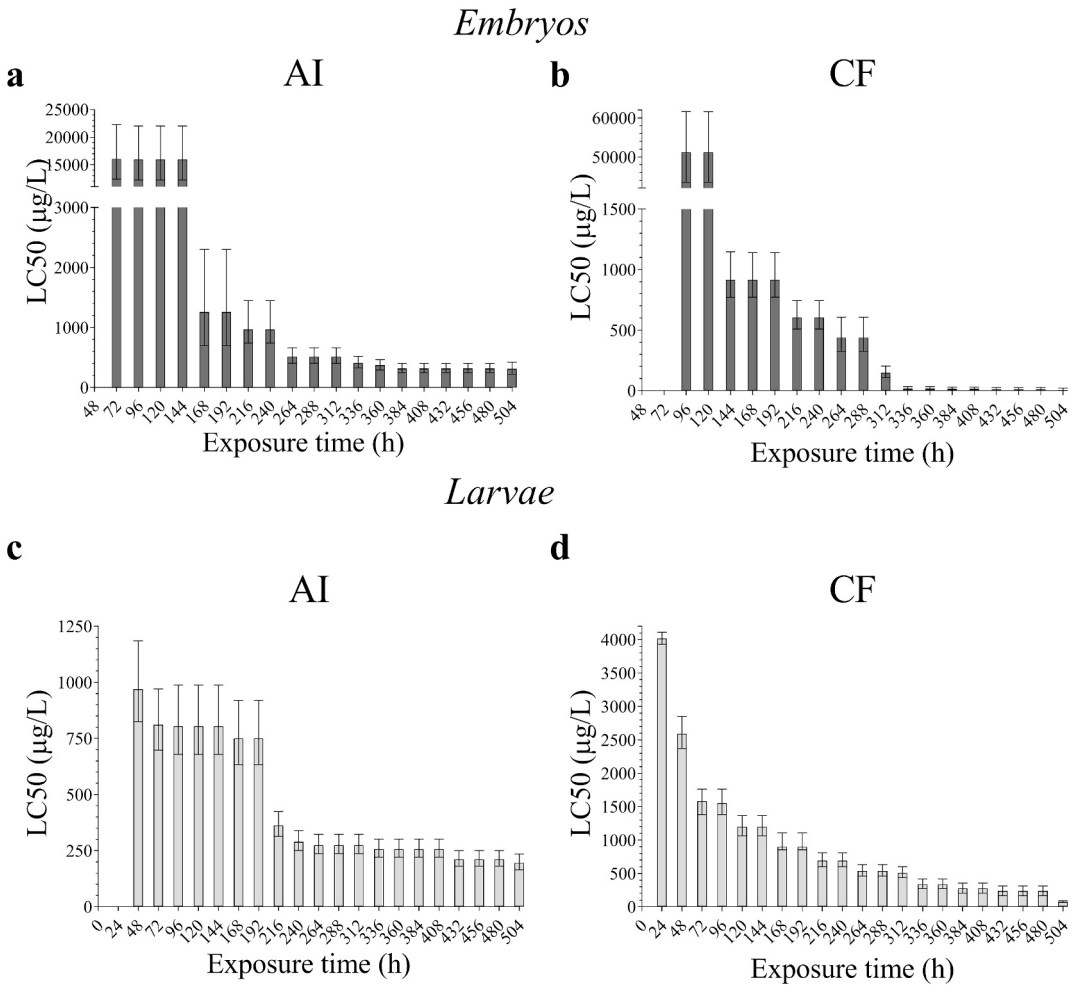

Fig. 1. Toxicity profile curves of IVM, with the Lethal concentration 50 (LC_{50}) for *Rhinella arenarum* embryos exposed: to the active ingredient (a) and commercial formulation (b) and larvae exposed to the active ingredient (c) and commercial formulation (d) continuously exposed for 504 h. Bars show the 95 % confidence intervals. "AI": Active ingredient, "CF": Commercial formulation.

from 48 h, whereas for those exposed to the commercial formulation, the LC_{50} could be calculated starting from 24 h.

For individuals exposed from the embryonic stage, the active ingredient (96 h-LC_{50}: 15,900 (12,220−22,010) μg/L) was more toxic than the commercial formulation (96 h-LC_{50}: 51,230 (43,490-61,620) μg/L) during the acute period, while at sub-chronic and chronic exposure times the commercial formulation was more toxic (168 h-LC_{50}: 915.92 (114.03–772.10) μg/L; 504 h-LC_{50}: 10.25 (2.31–25.85) μg/L), compared to the active ingredient (168 h-LC_{50}: 1258.69 (901.02–2301.69) μg/L; 504 h-LC_{50}: 312.80 (217.71–422.90) μg/L).

For individuals exposed from the larval stage, in acute and sub-chronic exposure times, the active ingredient (96 h-LC_{50}: 800 (680–990) μg/L; 168 h-LC_{50}: 750 (630–920) μg/L) was more toxic than the commercial formulation (96 h- LC_{50}: 1550 (1380-1760) μg/L; 168 h-LC_{50}: 890 (850–1110) μg/L). In the chronic exposure, the commercial formulation (504 h-LC_{50}: 77.33 (65.60–92.23) μg/L) was more toxic than the active ingredient (504 h-LC_{50}: 195.25 (164.64–235.27) μg/L).

When comparing both exposure periods, individuals exposed from the larval stage were more sensitive to both, the active ingredient and the commercial formulation, during acute exposure times. In chronic exposure time, individuals exposed from the embryonic period were more sensitive to the commercial formulation than larvae. However, when exposed to the active ingredient, larvae were more sensitive than embryos.

3.2. Behavior analysis of Rhinella arenarum larvae

Speed and mobility rate were evaluated on individuals exposed from the larval stage to the 1, 10 and 100 μg/L of the active ingredient and the commercial formulation for 48, 96 and 168 h (Fig. 2).

At the 48 h of exposure, the speed and mobility rates were significantly lower in comparison to the control group in larvae exposed to the 100 μg/L sublethal concentration of the active ingredient (Fig. 2a–b). At the 96 h of exposure, speed was significantly lower than the control group in larvae exposed to the 100 μg/L sublethal concentration of the active ingredient and commercial formulation and to the 10 μg/L sublethal concentration of the commercial formulation (Fig. 2c). Moreover, the mobility rate was significantly lower than the control group in larvae exposed to all concentrations of the commercial formulation and to the 100 μg/L concentration of the active ingredient (Fig. 2d). Finally, at the 168 h, the speed and mobility rate were lower in larvae exposed to all sublethal concentrations of the active ingredient and commercial formulation (Fig. 2e–f).

3.3. Biomarkers of oxidative stress on Rhinella arenarum exposed from the embryonic stages

An imbalance on the antioxidant defenses was observed on embryos exposed to the active ingredient and the commercial formulation (Fig. 3). However, the observed significant differences did not follow the expected dose response relationship. In particular, CAT activity was significantly higher than the control group only in embryos exposed to the 1.25 μg/L concentration of the commercial formulation (Fig. 3a). On the other hand, GST activity was significantly higher than the control group in embryos exposed to the 1.25 and 5 μg/L concentration of the commercial formulation and lower in embryos exposed to the 5 μg/L concentration of the active ingredient (Fig. 3b). The GSH levels were significantly lower than the control group in embryos exposed to the 5 μg/L concentration of the active ingredient (Fig. 3c) and the TBARS levels were significantly higher than the control group in embryos exposed to the 5 and 10 μg/L concentrations of the commercial formulation (Fig. 3d).

3.4. Biomarkers of oxidative stress and cholinergic effects on Rhinella arenarum exposed from the larval stage

In the exposed larvae, alterations on the oxidative stress and cholinergic effects biomarkers were observed (Fig. 4). Oxidative imbalance was observed in the exposed larvae since the CAT activity was significantly higher than the control group in larvae exposed to the 1.25, 10 μg/L of the active ingredient and 1.25 μg/L of the commercial formulation (Fig. 4a). Also, the GST and TBARS levels were significantly higher than the control group in larvae exposed to the 5 and 10 μg/L of the commercial formulation (Fig. 4b and d). The BChE activity was significantly higher than the control group in larvae exposed to the 1.25 μg/L of the active ingredient and commercial formulation (Fig. 4e). Finally, the AChE activity was significantly higher than the control group in larvae exposed to all active ingredient concentrations and the 1.25 μg/L of the commercial formulation (Fig. 4f).

4. Discussion

The antiparasitic IVM is commonly used in cattle farming and can potentially enter freshwater ecosystems. Research on its impact on amphibians is limited. This is the first study to report both lethal and sublethal effects, at varying levels, of both the active ingredient and a commercial formulation of IVM on a native South American amphibian species. In this way, the results obtained here provide relevant information about the negative effects of this antiparasitic on aquatic non-target organisms. Moreover, the present study compared the toxicity of the active ingredient and a commercial formulation, which results important since the majority of studies that analyzes toxicity of compounds uses commercial formulations (58 %), a lower percentage uses active ingredients (33 %) and only a few studies compare toxicity responses between both types of compounds (Bertrand and Iturburu, 2023).

The lethality of IVM differed between developmental stages and exposure period. For individuals exposed from the embryonic stage, the active ingredient was 3.22 times more toxic than the commercial formulation at acute exposure while at sub chronic and chronic exposure, the commercial formulation was more toxic (1.37 and 36.51 times, respectively). For individuals exposed from the larval stage, the active ingredient was more toxic than the commercial formulation at acute and sub chronic exposure (1.93 and 1.18 times, respectively). However, at chronic exposure, the commercial formulation was 2.52 times more toxic than the active ingredient. In both developmental periods, the commercial formulation was more toxic than the active ingredient at chronic exposure time. Also, IVM lethal toxicity resulted stage dependent since individuals exposed from the larval stage were generally more sensitive than the exposed from the embryonic stages to both, the active ingredient and commercial formulation. Nevertheless, individuals exposed from the embryonic stages were more sensitive than larvae to the commercial formulation at chronic exposure. In general terms, the commercial formulation was more toxic than the active ingredient, mainly at chronic exposure. This difference may be a consequence of the excipients present in the commercial formulation that enhance dissolution, stability, absorption, and action of the active ingredient (Cox and Surgan, 2006). Furthermore, these compounds have the potential to significantly enhance the toxicity of active ingredients to non-target organisms, and/or they may exhibit toxicity themselves, as was the case of the surfactant POEA (polyoxyethylene tallow amine) in glyphosate-based herbicides (Brausch and Smith, 2007; Pereira et al., 2009). Commercial formulation often becomes more toxic at chronic exposure due to slower degradation (Novelli et al., 2012). It should be noted that the 504 h-LC_{50} for embryos (10.25 μg/L) was only 10 times higher than the reported in wetlands from Argentina (Mesa et al., 2020). The obtained results highlight the importance of analyzing not only the toxicity of active ingredients but also commercial formulations. Moreover, it is interesting to evaluate different developmental stages in order

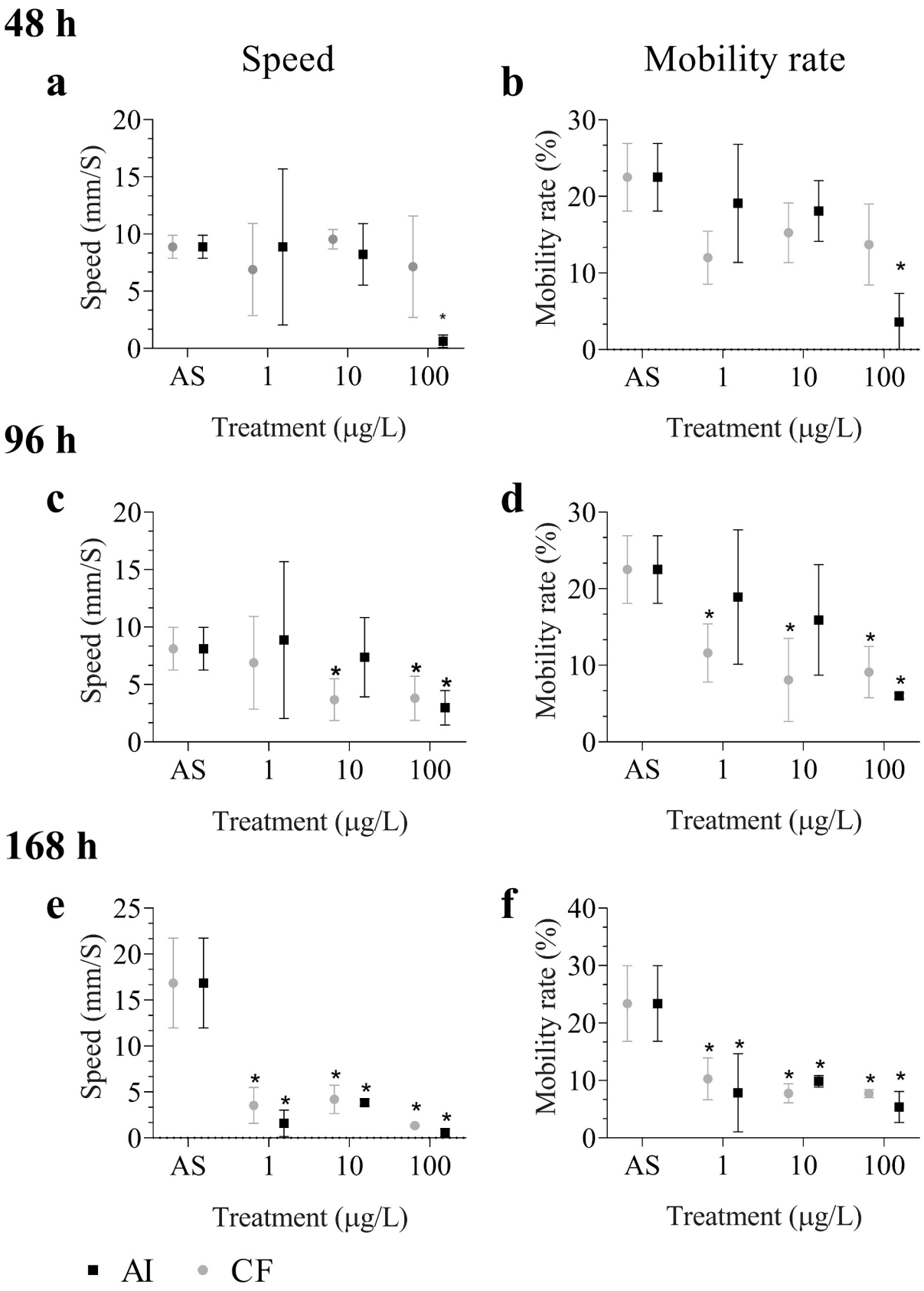

Fig. 2. Mean (±SD) speed (mm/S) and mobility rate values of *Rhinella arenarum* exposed from larvae stage to the control (AS), 1, 10 and 100 µg/L of the active ingredient and commercial formulation of IVM for 48 (a, b), 96 (c, d) and 168 h (e, f). "*": $p<0.05$. "AI": active ingredient, "CF": commercial formulation.

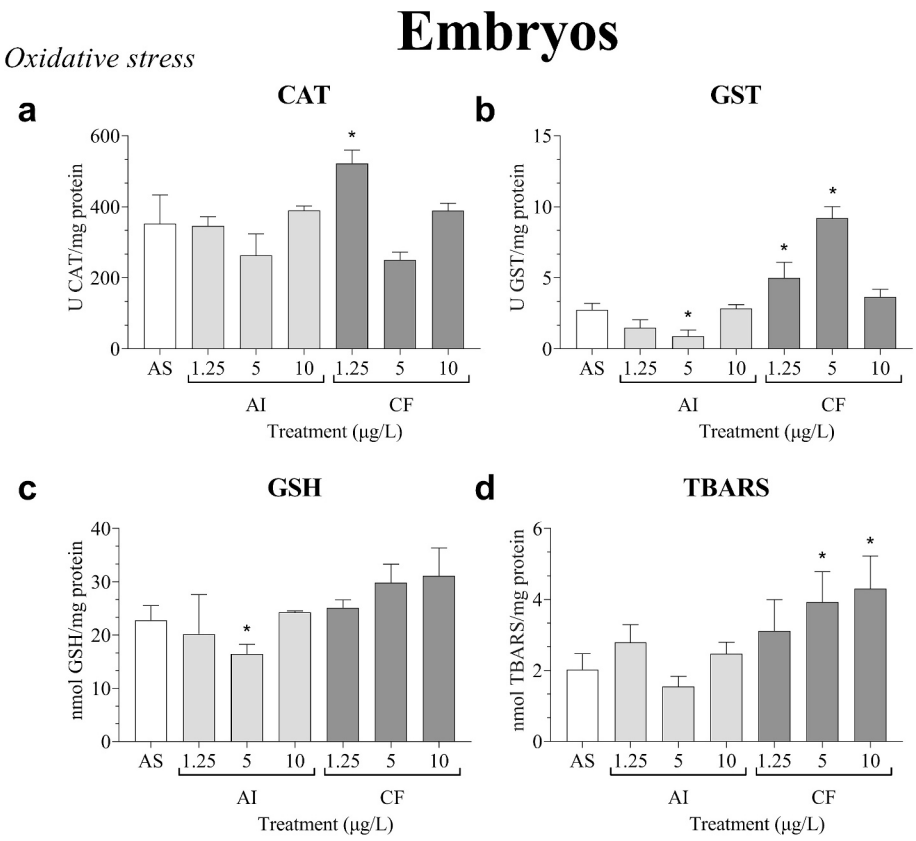

Fig. 3. Biomarkers of oxidative stress in *Rhinella arenarum* exposed from the embryonic stage for 96 h to 1.25; 5 and 10 µg/L of the active ingredient (AI) or the commercial formulation (CF): Catalase (CAT, a) and glutathione S-transferase (GST, b) activities; reduced glutathione (GSH, c) and lipoperoxidation (TBARS, d) levels. "*": $p<0.05$.

to detect a possible stage dependent toxicity and identify the most sensitive stage can be detected.

Previously, IVM toxicity was assessed mainly in aquatic invertebrates, such as *Daphnia magna* (48 h-LC_{50}: 0.0057 µg/L) (Garric et al., 2007), or fish, such as juveniles of *Danio rerio* (96 h-LC_{50}: 14 µg/L) and *Salmo salar* (96 h-LC_{50}: 17 µg/L) (Oliveira et al., 2016; Ucán-Marín et al., 2012). The lethal toxicity of IVM was also evaluated in amphibian species from Asia such as *Polypedates megacephalus* (96 h-LC_{50} = 12 µg/L), *Hyla ranaguentheri* (96 h-LC50 = 12 µg/L), *Duttaphrynus melanostictus* (96 h-LC_{50} = 29 µg/L) and *Microhyla heymonsi* (96 h-LC_{50} = 6 µg/L) (Zhang et al., 2018). *Rhinella arenarum* exposed from the embryonic and larval stages at acute exposure time (96 h) were more resistant for either the active ingredient or the commercial formulation. However, when extending the exposure times, individuals exposed from embryonic stages presented similar sensitivity to the commercial formulation than the other studies species (504 h-LC_{50}: 10.25 µg/L). Therefore, the need of prolonging exposure times is emphasized since it provides more accurate information about the tolerance limits for species preservation (Oliveira et al., 2016).

A sensitive approach for detecting the effects of harmful compounds on non-target species, even at environmentally relevant concentrations, is behavioral biomarkers (Kataba et al., 2020). Some research areas as ecology, medicine, ecotoxicology, and toxicology are giving greater attention to behavioral evaluation based on video recording (Henry et al., 2019). In the present study, behavioral parameters were affected on larvae exposed to the active ingredient and the commercial formulation of IVM. Larvae exhibited altered behavior as they were immobile, lying on the lateral or dorsal side, and eventually, they showed no reaction to stimulus. In addition to this general lack of response, the continuous presence of full pellets in the Petri dishes indicated a decrease in food consumption. Behavioral effects were observed even at the lowest concentration (1 µg/L) from the 96 h of exposure. Speed and mobility rates were affected by both, the active ingredient and the commercial formulation of IVM. In fish species exposed to IVM similar results were obtained. For example, on *Danio rerio* and *Catla catla* alterations in the stability and swimming pattern were observed at low concentrations (1–9 µg/L) (Nunes et al., 2021). Also, in *Fundulus heteroclitus* and *Sparus aurata* lethargy, postural changes, and loss of activity were observed at acute exposure (Bard and Gadbois, 2007; Mladineo et al., 2006). Weil et al. (2009) reported a complete lack of movement on embryos of *D. rerio* exposed for 48 h to concentrations higher than 250 µg/L. Since IVM interrupts the signal transmission of the neurotransmitter GABA, the observed effects may be a consequence of the anticholinergic effects produced by this antiparasitic (Kovecses and Marcogliese, 2005). Other study also reported that environmental concentrations (1.5 µg/L) of IVM altered the escape response of the fish *Prochilodus lineatus* (Lozano et al., 2021). The alterations on the behavior may impact on the refuge search, locomotion activities and feeding, which can increase the probability of predation (Thiripurasundari et al., 2014). It is important to highlight that the behavioral effects observed in this study occurred in environmentally relevant concentrations (Mesa et al., 2020).

The CAT, GST activity and GSH and TBARS levels are biomarkers extensively employed as tools for toxicity assessment (Domingues et al., 2010). However, the effects of IVM on these biomarkers were not studied on amphibian species. An imbalance on the antioxidant defenses was observed on the individuals exposed to IVM from the embryonic and larval stages although they did not follow the expected dose response relationship and there was an unclear pattern. The antioxidant enzyme CAT is involved in the detoxification of hydrogen peroxide. In amphibians this enzyme has an important role during the first stages since is one of the main antioxidant defenses (Pavlović et al., 2020). In the

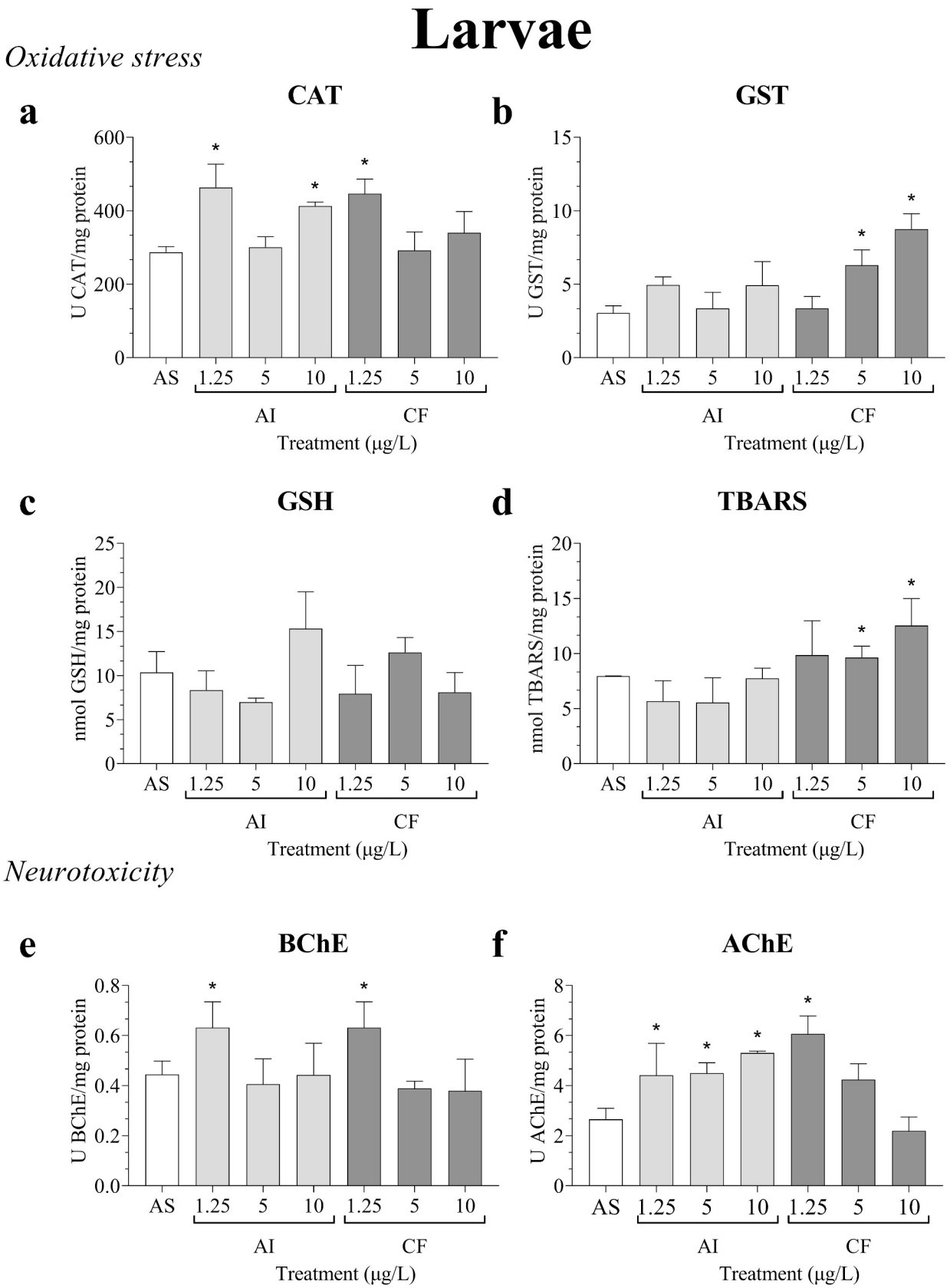

Fig. 4. Biomarkers of oxidative stress and neurotoxicity in *Rhinella arenarum* exposed from the larval stage for 96 h to 1.25; 5 and 10 µg/L of the active ingredient (AI) or the commercial formulation (CF): Catalase (CAT, a) and glutathione S-transferase (GST, b) activities; reduced glutathione (GSH, c) and lipoperoxidation (TBARS, d) levels; butyrylcholinesterase (BChE, e) and acetylcholinesterase (AChE, f) activities. "*": $p<0.05$.

present study, CAT activity was increased in embryos exposed to the lowest concentration of the commercial formulation and in larvae exposed to the lowest concentration of the active ingredient and commercial formulation and the highest of the active ingredient. However, in other studies with fish species, CAT activity was inhibited (Oliveira et al., 2016). The GST is a family of enzymes that has an important role in the phase II detoxification process by conjugating xenobiotics and/or endogenic compounds with reduced glutathione (GSH) (Mazari et al., 2023). Since IVM is a lipophilic substance, GST may have a relevant part in its detoxification (Oliveira et al., 2016). The commercial formulation increased GST activity in both, embryos and larvae. However, the active ingredient inhibited its activity in embryos (5 μg/L). This differential response may be a consequence of the bell-shape curve of response of these enzymes or the time it takes the enzymes to respond (Oliveira et al., 2016). Also, it might be related to some of the excipient present in the formulation or the interaction of the substances. A decreased in the GSH level was observed in embryos exposed to 5 μg/L of the active ingredient. So, the inhibition of GST on embryos exposed to that concentration may be the result of a depletion of GSH, which is also an antioxidant that neutralizes reactive oxygen species and is converted into oxidized glutathione. So, there is less available GSH for the conjugation with GST (Oliveira et al., 2016). Also, TBARS levels were higher in embryos and larvae exposed to the 5 and 10 μg/L concentrations of the commercial formulation of IVM. These findings are consistent with other studies that showed that IVM caused biochemical, oxidative stress, and biometric changes in juveniles of the fish *Clarias gariepinus* (Odo et al., 2020). Our results indicate oxidative damage to lipids and may be a result of the saturation of the antioxidant defense system as a consequence of some of the excipient present in the commercial formulation and/or a possible interaction between the substances.

BChE and AChE activity are useful biomarkers of cholinergic effects and widely employed in amphibians (Martins-Gomes et al., 2022). These enzymes had been associated with organophosphorus and carbamates exposure (Martins-Gomes et al., 2022). However, in previous years, it has been demonstrated that their activity may be affected by other compounds, such as metals and other organic compounds (dos Santos Carvalho et al., 2020; Ucán-Marín et al., 2012). In this case, the lowest concentration of the active ingredient and the commercial formulation of IVM increased BChE activity on the exposed larvae. On the other hand, AChE was increased in the larvae exposed to all concentrations of the active ingredient and the lowest concentration of the commercial formulation. This difference on anticholinergic effects might be related to the excipients present in the formulation and possible interactions. In contrast, another study reported an inhibition of cholinesterase (ChE) in *D. rerio* exposed to high concentrations of IVM (80 μg/L) (Oliveira et al., 2016). However, and similar to our results, an increased AChE activity was observed in *Salmo salar* exposed to IVM (Ucán-Marín et al., 2012). A possible consequence of the activation of AChE is a decrease in the acetylcholine (ACh) levels, which could be relevant due to the need for a precise level of ACh in the brain to preserve cognitive capacities (López et al., 2015). Since the disruption of the cholinergic system could affect the locomotor performance, exposure to IVM may have detrimental effects on organism's behavior, such as impairing their capacity to hide or escape from predators and altering their pursuit of food and safety, which may lower their fitness. Since IVM can cross the blood–brain barrier in fish (Katharios et al., 2004), it may affect the activity of AChE (Beauvais et al., 2000). To our knowledge, there are no previous studies about the effects of IVM on BChE and AChE in amphibians. Comparable results were observed for esterase in beetles (*Paederus fuscipes*) exposed to emamectin benzoate, a synthetic derivative of avermectin (Khan et al., 2021). Additionally, a similar response was previously reported for *Salmo salar* exposed to IVM (Ucán-Marín et al., 2012). The behavioral alterations observed might be consequence of the affected enzyme activities. However, behavioral effects were observed at low concentration of both, active ingredient and commercial formulation, probably due to the effect on another nervous pathway as GABA receptors as IVM is known as an important inhibitor. More studies might be done to elucidate the anticholinergic effects of IVM.

5. Conclusions

The analyzed set of biomarkers allowed characterizing the effects of the active ingredient and commercial formulation of IVM on a native amphibian specie. According to the lethality, the commercial formulation was generally more toxic than the active ingredient. Despite that for both stages, the acute LC_{50}s were high, the sublethal parameters responded at low concentrations and acute exposure time. The commercial formulation caused an oxidative defense imbalance and both forms of the compound caused cholinergic effects affecting mainly affecting AChE levels and the swimming behavior even at environmentally relevant concentrations (1 μg/L). The behavioral parameters analyzed showed to be an important and sensitive endpoint. The obtained results highlight the negative impact of IVM even at concentrations previously reported in wetlands from the distribution area of the study native amphibian species.

CRediT authorship contribution statement

Julieta Peluso: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Agostina Martínez Chehda:** Methodology, Investigation, Formal analysis. **Melisa S. Olivelli:** Methodology, Investigation. **Carolina M. Aronzon:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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