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The integrated biomarker response in three anuran species larvae at sublethal concentrations of cypermethrin, chlorpyrifos, glyphosate, and glufosinate-ammonium

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Abstract

The aim of the present study was to evaluate the response in larvae of the anuran species *Rhinella arenarum*, *Rhinella dorbignyi* and *Odontophrynus americanus* exposed to glyphosate (GLY, 2.5mg L⁻¹), cypermethrin (CYP, 0.013mg L⁻¹), chlorpyrifos (CP, 0.1mg L⁻¹) and glufosinate-ammonium (GLU, 15mg L⁻¹) using two behavioral endpoints: mean speed (MS) and total distance moved (TD); and two enzymatic biomarkers: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In order to assess a global response and to determine the most sensitive species, an integrated biomarker response (IBR) index was calculated. Behavioral biomarkers were tested at 1 and 60 min, and the enzymes at 60 min after exposure. The results

showed that: (1) there were statistical differences between species in a series of responses in swimming behavior, and cholinesterase activities within the first-hour of exposure to CYP, GLY, and CP at environmentally relevant concentrations (ERC); (2) IBR determined that *Rhinella* species were the most sensitive of the species tested and (3) IBR provided a comprehensive assessment of the health status of species exposed to ERC of a wide variety of agrochemicals globally and frequently used.

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Introduction

Contaminants such as pesticides and fertilizers are known to contribute to freshwater pollution and have become an important threat to aquatic species worldwide.^[1] Among these, amphibian larvae

became a critical tool for determining the ecotoxicity of single ^[2] or complex chemical mixtures as a model organism ^[3], and in recent years, they have been used in numerous studies that integrate several biological endpoints. ^[4-5] Amphibian larvae are particularly sensitive to contaminants because their skin is highly permeable and physiologically involved in gas, water, as well as electrolyte interchange with the environment.

In the last few decades, the need for biological tools in environmental monitoring became evident ^[6] leading the search for biochemical variables that could be related to environmental parameters ^[7] together with the development of integrative tools such as the integrated biomarker response (IBR) by Devin ^[8]. In addition, the inhibition of Cholinesterases (ChE) is a validated endpoint used in monitoring some neurochemicals like organophosphates and carbamates. This group of enzymes includes acetylcholinesterase (AChE, EC 3.1.1.7), responsible for the degradation of the neurotransmitter acetylcholine in cholinergic neurotransmission and butyrylcholinesterase (BChE, EC 3.1.1.8), involved in the detoxification of several neurochemicals and neurodegenerative diseases. ^[9] At the individual level, behavioral endpoints integrate exogenous and endogenous factors proving to be effective indicators of contamination. ^[10] In amphibian larvae, impairment of swimming has been linked to disturbances in neural, endocrine, and metabolic processes. ^[11] It has also been used as an indicator of sublethal effects associated with exposure to several chemicals. ^[12] Moreover, novel video tracking software offers the opportunity to accurately quantify locomotor behavior in aquatic organisms ^[13-15], as well as to detect effects at chemical concentrations that are orders of magnitude below those that can cause mortality. ^[12]

According to their metabolic and biochemical pathways of action, several pesticides commonly applied in different crops worldwide have immediate effects in target and non-target species (e.g.

wild fauna).^[16, 17] In addition, mixtures of active and auxiliary ingredients in commercial pesticide formulations may accelerate the permeation rate and lag-time. However, few studies analyze the immediate effects of pesticides on wild fauna, such as amphibians, which are the most sensitive aquatic vertebrates because of high permeable skin, particularly at larval stages. Thus, the present study evaluates two behavioral and two enzymatic biomarkers in three anuran species exposed to environmentally relevant concentrations (ERC) of commercial formulations of glyphosate (GLY), cypermethrin (CYP), and chlorpyrifos (CP) at 1 and 60 min. Additionally, a sublethal concentration of glufosinate-ammonium (GLU) was tested as a positive control. Likewise, biomarker responses were incorporated in one integrated biomarker response index (IBR) to determine the most sensitive species. The current study examined the hypothesis that at short time exposure behavioral biomarkers would be more effective at detecting different types of pesticides than ChEs and that GLU would induce higher IBR scores.

Materials and methods

Experimental design and chemicals

Experiments were carried out under static conditions, with ten replicates (one larva of each species individually exposed), for each of the pesticide treatments and to a negative control (N = 50 tadpoles per species), with dechlorinated tap water (DTW). When dealing with pesticide treatments, each larva was placed at the center of the arena (15-cm diameter, 2-cm deep glass Petri dish), with 100 mL of the solution. In the case of control treatment, each larva was in DTW. Behavioral biomarkers were evaluated at one and 60 min while enzymatic biomarkers were tested at 60 min. Tadpoles used for the biochemical analyses were exactly the same to those used in the behavioral tests.

The study of active ingredients (a.i.) without coadjutants overlooks real environmental effects of massive pesticide application at a global scale, therefore, commercial formulations of CYP (cyano-3-phenoxyphenyl)-2,2dimethylcyclopropane-1-carboxylate, trade name Mamboretá Z® 25% w/v), GLY (Nphosphonomethyl glycine, trade name Roundup Ultramax®, 74.7% a.i.), CP (0,0-diethyl-0,3,5,6-trichloropyridin-2yphosphorothioate, Tauro®L.O, 48% w/v), and GLU (3-amino-3-carboxypropyl) methyl phosphinate Liberty® 20 % a.i.), were used to evaluate pesticide effects. The active ingredient was not analyzed without coadjutants since the field application is not done in this form. These agrochemicals were selected like test substances because they are massively used in soybean crops.^[18] Tested concentrations of GLY (2.5mg L⁻¹), CYP (0.013mg L⁻¹), and CP (0.1mg L⁻¹) were considered ERC according to Wagner et al.^[19], Greulich and Pflugmacher^[20], Manjunatha, and Philip^[21], respectively. A sublethal concentration (15mg L⁻¹) of GLU was used as a positive control according to Peltzer et al.^[5] to test IBR sensitivity.

Species selected

Rhinella arenarum (Bufonidae), *Rhinella dorbignyi* (Bufonidae) and *Odontophrynus americanus* (Odontophrynidae), eggs were collected using sweep nets in water from temporary ponds, far from any agricultural activities in the nearby areas (31°10'21.10"S; 60°15'31.73"W Cayastá, Garay Department, Santa Fe Province, Argentina). Previous reports had determined that these ponds were free of anticholinesterase chemicals.^[22, 23] All larvae were collected with authorization from the Ministry of Water, Public Services and Environment of Santa Fe Province, Argentina. These anuran species with benthic larvae are widely distributed in the Neotropical region (Argentina, Paraguay, Uruguay, Bolivia, and Brazil).^[24] In Argentina, these amphibian species from two different families are categorized as “Least Concern”^[25], and they are frequently found in modified environments such as rice fields, soybean crops and urban areas, where they are likely exposed to contaminants during their breeding season and early development.^[26-28] For each species, eggs of different clutches were mixed to homogenize genetic variation, and then kept to a 12 h-light/dark cycle, with dechlorinated tap water (DTW) in 15-L glass tanks, with a pH of 7.4 ± 0.05 , a conductivity of $165 \pm 12.5 \mu\text{S cm}^{-1}$, a dissolved oxygen concentration of $6.5 \pm 1.5 \text{ mg L}^{-1}$, light $>200 \text{ lx}$, and a hardness of 50.6 mg L^{-1} of CaCO_3 at $24 \pm 2^\circ \text{C}$ until stages 30-33.^[29] Larvae were fed with boiled lettuce *ad libitum* until the experiments started. The average size (snout-tail tip) of larvae varied among sampled species (*R. arenarum*: $18.95 \pm 0.57 \text{ mm}$, *R. dorbignyi* $14.18 \pm 0.50 \text{ mm}$ and *O. americanus* $26.68 \pm 1.11 \text{ mm}$), but Gosner stages (stages 30-33) were similar for

all individuals. These stages were chosen because of their relative stability in key traits, despite some reservations regarding hind limb development. ^[29]

Swimming activity analysis

The larvae swimming behavior was evaluated at one min and at 60 min of exposure. Ten larvae per species per treatment were individually exposed to each pesticide's concentration and one negative control with dechlorinated tap water. Swimming behavioral of the same individual was recorded during one min at one min, and 60 min exposure. The videos were recorded with a digital camera (Genius® HD 2.0 MP) placed just above the arena, and then analyzed using MedeaLab 5.9.0.1 tracking System (Medea AV GmbH, Germany) after 15 s acclimation period. Two behavioral endpoints were analyzed at one min and 60 min: mean speed (MS) (cm sec^{-1}), and total distance (TD) i.e. total distance moved (cm) in 1 min.

Enzymatic determination

Immediately after 60 min exposure, control and exposed larvae were euthanized according to the criteria reported in ASIH et al. [30] with the approval from the Bioethics Committee of the School of Biochemistry and Biological Sciences (FBCB-UNL). Larvae exposed for 60 min were homogenized on ice in 0.1% t-octylphenoxypolyethoxy ethanol (triton X-100) in 25 mM Tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using an homogenizer. The homogenates were centrifuged at 10000 rpm at 4°C during 15 min, and the supernatant was collected and frozen at -80°C until assayed for enzymatic determination. Total protein concentrations in the supernatants were determined in duplicate according to the Biuret method. [31]

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) of 10 individuals per species and per condition were used as biomarkers of pesticide exposure and their activities were measured following Ellman et al. [32] The reaction mixture consisted of 0.01 mL extract, 2 mM dithiobis 2-nitrobenzoic acid, 20 mM acetylthiocholine, and butyrylthiocholine iodide, 25 mM Tris-HCl, and 1 mM CaCl₂ (pH 7.6). Assays were conducted at 25°C. Variation in optical density was recorded at 410 nm at 25°C for 1 min using a Jenway6405 UV-VIS spectrophotometer. The enzymes AChE and BChE were expressed as nmol min⁻¹ mg⁻¹ protein using a molar extinction coefficient of 14.15 x 10³ M⁻¹cm⁻¹ for AChE, and 13.6 x 10³ M⁻¹cm⁻¹ for BChE.

Integrated Biomarker Response

The Integrated Biomarker Response (IBR) was calculated for the four biomarkers at 60 min exposure following the method developed by Devin et al. ^[8], initially described in Beliaeff and Burgeot ^[33]. The first step consisted of the standardization of the mean value of each biomarker obtained for an X condition, using the mean value for all conditions (m) and the standard deviation for all conditions (s) to produce a value called Y: $Y=(X-m)/s$. After this standardization, the Z value was computed: $Z = Y$ or $Z = -Y$ whether an activation or an inhibition of the biomarker was expected in response to a contamination. The S value was lastly computed, with $S = Z+|Min|$, where the minimum value (Min) was observed for all exposure conditions for each biomarker, and finally, plotted on a radar diagram. These S values, thus, represent the gradient of values for each biomarker in the different exposure conditions, with the highest values corresponding to the highest biological effects. Since induction of their activity was expected in exposed organisms, positive coefficients were associated with AChE and BChE. Behavioral responses (MS and TD) were associated to negative coefficient, since an inhibition of movements was expected in exposed organisms. The IBR was calculated as the total area displayed by the radar diagram. The higher the IBR, the higher the stress level of the organisms.

IBR was computed independently for each species since the four markers raw levels were too different to allow direct comparisons. Moreover, IBR calculation integrated different biomarker datasets and standardized values on a common scale to compare contaminants and species.

Statistical analyses

Overall, a two-way Multivariate Analysis of Variance (MANOVA) was performed with species and treatment as independent variables, and MS, TD as dependent variables at one min. The same MANOVA (species*treatment) was performed at 60 min including MS, TD, AChE, and BChE as dependent variables. This analysis was used to test whether the considered biological responses allowed for identifying significant differences between species and pesticides treatments, at 1 min and at 60 min exposure for behavioral biomarkers, and after 60 min exposure for enzyme biomarkers. In addition, a one-way analysis of variance (ANOVA) was performed independently for each species on each biological response (MS, TD, AChE, and BChE) to identify more thoroughly pesticide exposure effects. When suitable, Dunnet post hoc tests were performed to determine differences in pesticide treatments against control. Finally, the IBR values computed through the permutation procedure, standardized biomarkers values across species. A two-way ANOVA was performed, with species and treatment as independent variables, to assess interactions between these two variables, and to define whether the different species exhibit the same response patterns to the different tested contaminants. Values were always normally distributed, and homoscedasticity was respected. All statistical analyses were performed with RStudio version 1.1.463, with R version 3.6.3.^[34]

Results

MANOVA using Pillai's trace indicated that tadpoles swimming behavior (MS and TD) differed between amphibian species ($\lambda = 0.276$, $F = 60.18$, $p < 0.0001$), and between pesticide treatments ($\lambda = 0.81$, $F = 3.61$, $p < 0.001$), with no interactions ($\lambda = 0.87$, $F = 1.22$, $p > 0.05$) after 1 min exposure. MANOVA results at 60 min indicated differences between amphibian species ($\lambda = 1.27$, $F = 55.77$, $p < 0.0001$) and between pesticide treatments ($\lambda = 0.43$, $F = 3.92$, $p < 0.0001$), with significant interactions ($\lambda = 0.73$, $F = 3.68$, $p < 0.0001$) regarding to MS, TD, AChE and BChE.

Statistical differences among treatments for MS ($F = 4.98$, $p = 0.002$) after exposure to CYP ($p = 0.041$) at 1 min were observed in exposed *R. arenarum* (Table 1), whereas no statistical differences were found for TD in none of the evaluated pesticides ($F = 1.48$, $p > 0.05$). Statistical differences among treatments for MS ($F = 2.714$, $p = 0.041$), TD ($F = 7.99$, $p = 0.0001$) and AChE ($F = 11.25$, $p = 0.0001$) were observed at 60 min (Table 1, Fig. 1). No differences were found for BChE ($F = 1.00$, $p > 0.05$). CYP produced a reduction in MS scores ($p = 0.043$), and in TD ($p = 0.0001$). GLY also produced a reduction in TD ($p = 0.0001$), GLU induced AChE ($p = 0.001$), whereas CP reduced TD ($p = 0.001$) and increased AChE activity ($p = 0.011$) respect to control (Table 1, Fig. 1).

There were no statistical differences for *R. dorbignyi* MS ($F = 2.35$, $p > 0.05$) and TD ($F = 2.48$, $p > 0.05$) exposure in none of the pesticides evaluated at 1 min. Moreover, statistical differences for MS ($F = 7.94$, $p = 0.0001$), TD ($F = 16.97$, $p = 0.0001$), AChE ($F = 13.445$, $p = 0.0001$), and BChE ($F = 19.062$, $p = 0.0001$) at 60 min were observed (Table 1, Fig. 1).

Dunnett's post hoc test revealed that CYP induced MS ($p = 0.0001$), and TD ($p = 0.0001$). In addition, GLU increased AChE ($p = 0.0001$) and BChE ($p = 0.0001$) activities, while CP only induced AChE ($p = 0.012$) respect to control (Table 1, Fig. 1).

Differences were noticed for MS ($F = 3.18$, $p = 0.022$) in exposed *O. americanus* at 1 min. The TD values showed statistical differences among treatments ($F = 6.583$, $p = 0.0001$) that decreased after GLY exposure (Dunnett's post hoc test $p = 0.001$) at 1 min also. Similarly, the values of MS and TD showed statistical differences ($F = 6.793$, $p = 0.0001$; $F = 5.308$, $p = 0.001$; respectively) at 60 min. Dunnett's test revealed that CYP inhibited MS ($p = 0.001$) and TD ($p = 0.0001$) as shown in Table 1 and Figure 1. No statistical differences were observed in AChE ($F = 1.16$, $p > 0.05$) or BChE ($F = 0.41$, $p > 0.05$) in none of the evaluated pesticides.

IBR Results

Radar plots (Fig. 2) evidenced important differences between species responses. CYP showed a marked response in behavioral endpoints for *R. arenarum*, but low effects on *R. dorbignyi*. In addition, when *O. americanus* was exposed to CYP, they presented important effects in enzymatic and behavioral endpoints. With respect to behavioral biomarkers, the most sensitive species to GLY was *R. dorbignyi*. Biological responses in both *Rhinella* species were similar when exposed to CP, except for *R. arenarum* which showed a lower response in MS after exposure to CP. *O. americanus* seemed less affected by CP.

The IBR ANOVA table evidence a highly significant effect of the interaction between pesticide and anuran species (Fig. 3, Table 2), indicating that the effect of contaminants differed for each species. Control IBR levels were similar for the three species. These results confirmed those that had been described through the radar plots, with GLU high toxicity in the three considered species. In addition, *Rhinella* species appeared to be the most sensitive since three contaminants induced a significant increase of the IBR when compared to the control; *O. americanus* was only affected by CYP and GLU.

Discussions

The results of our study showed that short-term exposure at environmentally relevant concentrations of GLY, CYP, and CP, and NOEC of GLU affects swimming performance and cholinesterase activities of amphibian larvae; all these variables are essential for self-preservation.

Ecological death is defined as the inability of an organism to function in an ecological context that might lead to premature death.^[35] In this context, the shift from a normal swimming behavior may limit the ability to find food, shelter and affect the outcome of a predator-prey relationship.^[36] In general, Dunnett tests showed more differences between treatments and control in behavioral biomarkers than enzyme biomarkers. All swimming parameters were affected by CYP at ERC, in all the species included in this experiment. The xenobiotic showed the highest behavioral toxicity among the four tested species, even after 1 min exposure. This insecticide is

lipophilic, which facilitates its rapid access to several organs and tissues leading to a high affinity to the central nervous system and favoring body accumulation.^[37] Xenobiotics diffuse into the body of amphibians through the skin depending on their hydrophobicity,^[38] and the high CYP hydrophobicity may explain its rapid effect on tadpole behavior. The findings of the present study, agrees with many other studies, which showed that pyrethroid affects the swimming behavior of amphibian larvae such as twisting, writhing, and not coordinated swimming.^[39-41] Noticeably, similar behavioral changes were described for fish species such as *Poecilia reticulata*, presenting loss of general activity and equilibrium^[42]; in *Oncorhynchus mykiss*^[43] presented loss of coordination thus, showing that CYP similarly affects the behavior of phylogenetically distant organisms from communities in aquatic ecosystems.

Thus, in this study, *R. arenarum* presented alterations in TD, showing irregular swimming and immobility when exposed to CP (Table 1), as it was observed in leopard frog tadpoles (*Rana blairi*) by Ruiz de Arcaute.^[44] In addition, Widder and Bidwell^[11] observed reduced swimming speed in two of four anuran larvae species exposed to the same CP concentration during a longer period of exposure; this implies that the different inter-species sensitivity registered in this study can be noticed as early as at 60min of exposure and that the deleterious effects could persist for at least up to 12 days as reported in the same study. Ceschin^[45] found that exposure of *R. arenarum* larvae to two organophosphates (CP and azinphos methyl) at sub-lethal concentrations produced changes in the expression of hundreds of genes as early as 6 h after exposure, though it did not affect detoxification enzymes. Moreover, exposure to GLU reduced 45% the MS and the TD by the *O. americanus* tadpoles in this assay (Table 1). Although these results were not statistically significant ($p=0.063$; $p=0.095$ respectively), IBR results showed a marked response of these behavioral biomarkers which are discussed below (Fig. 2 and 3). The target of GLU, glutamine

synthetase, is present both in plants and animals. Glutamine synthetase is mainly located in astrocytes which main role is to protect neurons against excitotoxicity resulting in excessive stimulation of receptors. ^[46] Previous studies evidenced that GLU is toxic and has a detrimental impact by reducing behavioral responses of the amphibian tadpole *Boana pulchella* at sublethal concentrations, which might be explained by the build-up of ammonia and glutamate in the brain affecting neuronal functions. ^[5] It has also been pointed out that GLU increases zebrafish embryo mortality, induces yolk sac edemas, and causes spinal deformities at concentrations ranging from 2-10 $\mu\text{g L}^{-1}$, considered ERC, ^[47] and inducing immunotoxicity and oxidative stress in zebrafish adults. ^[48] In addition, Ebert et al. ^[49] reported neurological symptoms in rats following exposure to GLU and suggested that its neurotoxic symptoms may result from GLU interfering with the neurotransmitter role of glutamate (due to their structural similarity) or by the inhibition of the enzyme glutamine synthetase.

Moreover, TD was significantly reduced by GLY in *O. americanus* and *R. arenarum* larvae tested in this study. The observed behavioral alterations in exposed individuals are consistent with previous reports on glyphosate-based herbicides. ^[50-52] Likewise, Nwani et al. ^[53] demonstrated that fish exposed to GLY presented erratic swimming, with jerky movements and hyperactivity. Consequently, GLY sublethal effects include accelerated ^[54] or delayed development, ^[55] reduced size at metamorphosis, ^[54, 55] developmental malformations, ^[56] and symptoms of oxidative stress. ^[57] Remarkably, the most active species *R. arenarum* ^[58], seemed more sensitive to behavioral biomarkers than *R. dorbignyi* ^[41] and *O. americanus* ^[59], considering that these species showed behavioral effects by CYP, GLY, and CP (Table 1).

Additionally, a significant induction of AChE activity was detected in *R. arenarum* and *R. dorbignyi* exposed to GLU and CP after being exposed for 1 h, while BChE activity was only

induced in *R. dorbignyi* tadpoles exposed to GLU. Induction ^[23] and inhibition ^[60] of ChE were previously reported in tadpoles from agricultural areas. Lajmanovich et al. ^[61] found induction of AChE of *R. arenarum* after 48 h exposure to a calculated NOEC of 10mg L⁻¹ GLY. However, AChE of *Scinax squalirostris* tadpoles was inhibited after 48 h exposure to 1.26, 3.12, and 6.25 mg L⁻¹ GLY. ^[62] Interestingly, in the same experiment Lajmanovich et al. ^[62] found that GLU mixed with polyethylene microplastic particles (PEMPs) inhibited AChE of *S. squalirostris* when exposed to 1.26, 3.12 and 6.25 mg L⁻¹ GLU during 48h, but GLU alone reduced AChE only at 6.25 mg L⁻¹. Farchi et al. ^[63] linked elevated levels of neuronal AChE to muscle exhaustion by a combination of modest aberrations in synaptic transmission, muscle function, and muscle morphology. In addition, Lev-Lehman et al. ^[64] suggested that both, the increase, and the reduction of synaptic activity imposed physiological stress on the muscle. The muscle stress is then translated into changes in gene expression having long-term implications for neuromuscular function. All these effects on ChE can potentially affect the behavior of tadpoles and impair their survival in an ecological context. Interestingly, there are some published studies that have examined this relation in amphibians and have found a correspondence between behavioral responses and AChE inhibition after a long-time (from 2 to 12 days) of exposure. ^[11, 65, 66] Likewise, inhibition of ChE's by CYP injected subcutaneously in amphibians after 24 h exposure ^[67] and by GLY after 48 h exposure has been already reported by different authors. ^[57] The sublethal concentrations used in the present study plus the relatively short time of exposure of tadpoles (1 h) could be a feasible explanation for the lack of AChE and BChE inhibition by GLY and CYP found during this study. Also, smaller tadpoles possess higher metabolic rates than larger tadpoles ^[68], while metabolically active organisms metabolize toxicants more readily ^[69]. In this context, smaller *R. dorbignyi* tadpoles with presumably higher metabolic rates as well as more

active tadpoles like *R. arenarum* may explain why their ChEs were affected after 1 h exposure to GLU and CP, while those of *O. americanus* were not affected. Also, *O. americanus* has higher ChE activity levels than *Rhinella* species (Fig. 1). However, this leads us to conclude that, for agrochemicals, the concentrations examined were sufficient to exert an effect on tadpoles swimming activity in a very short time of exposure. Indeed, this may also evidence that the impairment of swimming skills of tadpoles precedes the detectable effects on ChE activity, indicating that behavioral parameters are early indicators of short-term effects produced by pesticides and they could display the action of other underlying physiological pathways of toxicity.

Moreover, some authors have found that body size modifies the tolerance parameters of exposed individuals to several toxicants,^[70-72] while others found the opposite, i.e. that size has no significant effect on the tolerance.^[73, 74] Tadpoles of *R. dorbignyi* are considerably smaller than those of *R. arenarum* and *O. americanus*. Smaller tadpoles possess a greater surface area relative to body volume^[75]. The amphibian skin high permeability^[38] could explain the relative toxicant rate uptake. Furthermore, the ChE response in bufonids could be explained by a higher uptake because of their rudimentary lungs and higher cutaneous gas exchange when compared with species like *O. americanus* with a more developed pulmonary breathing system.^[76]

The IBR provided both a graphical synthesis and a numeric value that summarize different biomarker responses. Since the first step of IBR calculation, a scaling of each dataset is present, allowing comparing species with raw basal levels that differ and determining the most sensitive one. IBR results suggests that both *Rhinella* species are the most vulnerable among the three species included in the present study. In a previous report regarding relative risk^[22], *R. dorbignyi* and *O. americanus* larvae ranked second and fourth among 11 Neotropical anuran species due to their low enzyme activities (BChE and carboxylesterases), and the high overlap between their

geographical distribution and reproductive period with soybean land use and soy phenology (*R. arenarum* was not tested). Interestingly, *Rhinella* species belong to the Bufonidae family which was globally categorized as over-threatened by Bielby et al.^[77] although not when analyzed at a national level Vaira et al.^[78] *R. arenarum* is a non-threatened, widely distributed, still abundant, and one of the most frequently used Neotropical species in ecotoxicology tests. Nevertheless, this work highlights its sensitivity to widely used agrochemicals.

The highest IBR scores induced by GLU in the three anuran species evaluated, indicated that IBR was able to distinguish between lower ERC and higher sub-lethal concentrations. This was an expected result since IBR allows to distinguish between sites with different levels of contamination.^[8] High IBR values might be explained by the high concentration of GLU chosen as a positive control, which induced AChE of both *Rhinella* species and BChE in *R. dorbignyi*. The insecticide CYP was the second biggest inducer of IBR scores, mainly because of the changes produced in larval MS and TD of the three species studied. In addition, *Rhinella* species showed similar IBR responses, with GLY IBR scores mainly determined by changes in the behavioral parameters (in *R. arenarum* these alterations were not detected by IBR but by Dunnett's post hoc test), and CP IBR scores mostly affected by cholinesterases activities.

These results highlight the importance of testing multiple amphibian species that differ in natural history, such as behavior and metabolic traits, to evaluate the environmental risk of contaminants. In addition, IBR appears as a proper tool to provide a suitable characterization of different anuran species health status exposed to sub-lethal and environmentally relevant concentrations of pesticides. The integration of those biomarkers at short time of exposure provides an efficient and non-expensive tool for environmental monitoring.

Conclusions

Overall, the present results demonstrate that (1) there are statistical differences between species in a series of responses in swimming behavior and ChE concentrations within the first-hour of exposure to CYP, GLY, and CP at ERCs; (2) IBR determined that *Rhinella* species were the most sensitive of the species tested and (3) IBR appears as a suitable tool to provide a comprehensive assessment of the health status of species exposed to ERC and sub-lethal concentrations of a wide variety of agrochemicals globally and constantly used.

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Compliance with Ethical Standards

Animals used in this research have been treated according to ASIH et al. (2011) criteria and with approval from the animal ethics committee of the [School](#) of Biochemistry and Biological Sciences.

<http://www.fcb.unl.edu.ar/pages/investigacion/comite-deetica.php>

Competing interest statements

The authors declare that they have no competing interests.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure captions

Figure 1. Bar plot (mean \pm ci) of acetylcholinesterase (left) and butyrylcholinesterase (right) studied in the three different anuran species *R. arenarum*, *R. dorbignyi* and *O. americanus* exposed to cypermethrin (0.013mg L^{-1}), glyphosate (2.5mg L^{-1}), chlorpyrifos (0.1mg L^{-1}) and glufosinate ammonium (15mg L^{-1}). Asterisk represents significant differences against control ($p < 0.05$).

Figure 2. Radar plot of the standardized values S computed for the IBR calculation for three different Neotropical species exposed to sublethal concentrations of cypermethrin, glyphosate, chlorpyrifos and glufosinate ammonium. Plots are an example of the 3 possible permutations with 4 endpoints.

Fig. 2 legend. The IBR value was calculated as the total area displayed by the radar plot, so the bigger the area, the higher the level of response by the organisms. CYP showed a marked response in behavioral endpoints for *R. arenarum* but low effects on *R. dorbignyi*. *O. americanus* presented important effects in enzymatic and behavioral endpoints when exposed to CYP. *R. dorbignyi* was the most sensitive species to GLY respect to behavioral biomarkers. Biological responses of both *Rhinella* species were similar when exposed to CP while *O. americanus* seemed less affected by CP. As expected, the three species analyzed showed high sensitivity to GLU.

Figure 3. Barplot (mean \pm sd) of the 3 computed IBR values for each species of anuran larvae in each exposure condition. Letters refers to between-groups differences assessed with the Tukey HSD test performed on the results of a two-way ANOVA. Groups sharing the same letters are not significantly different.

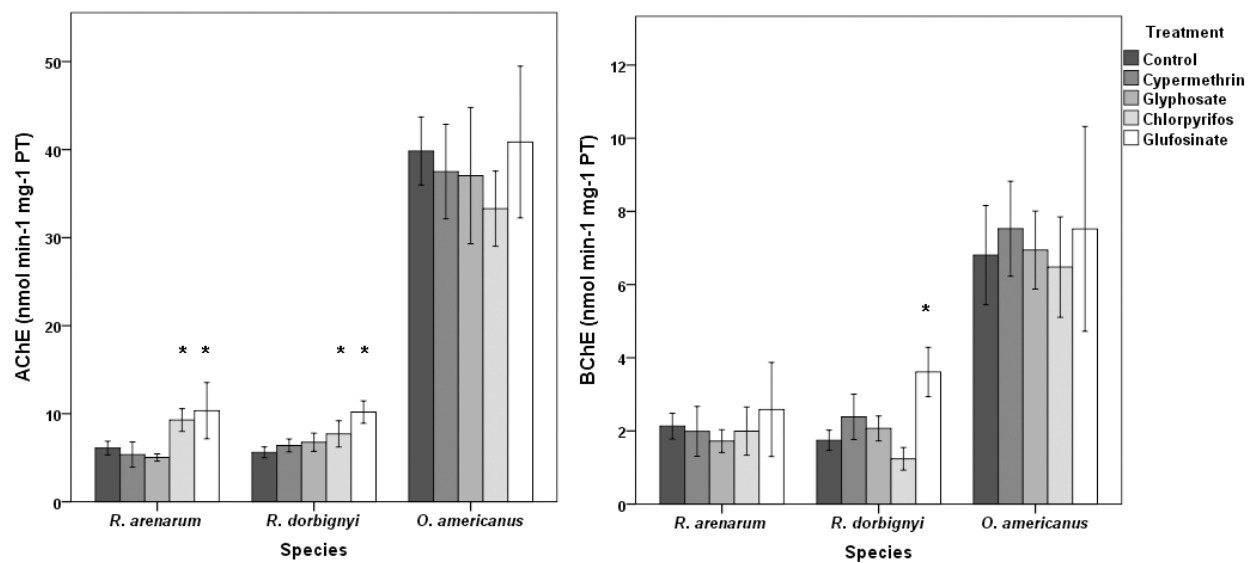


Fig. 1

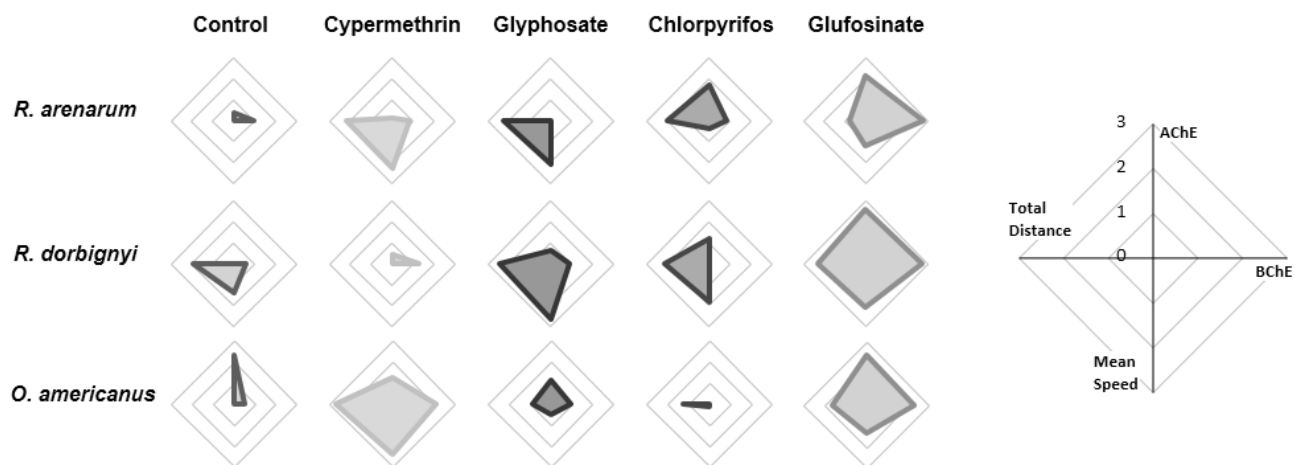


Fig. 2

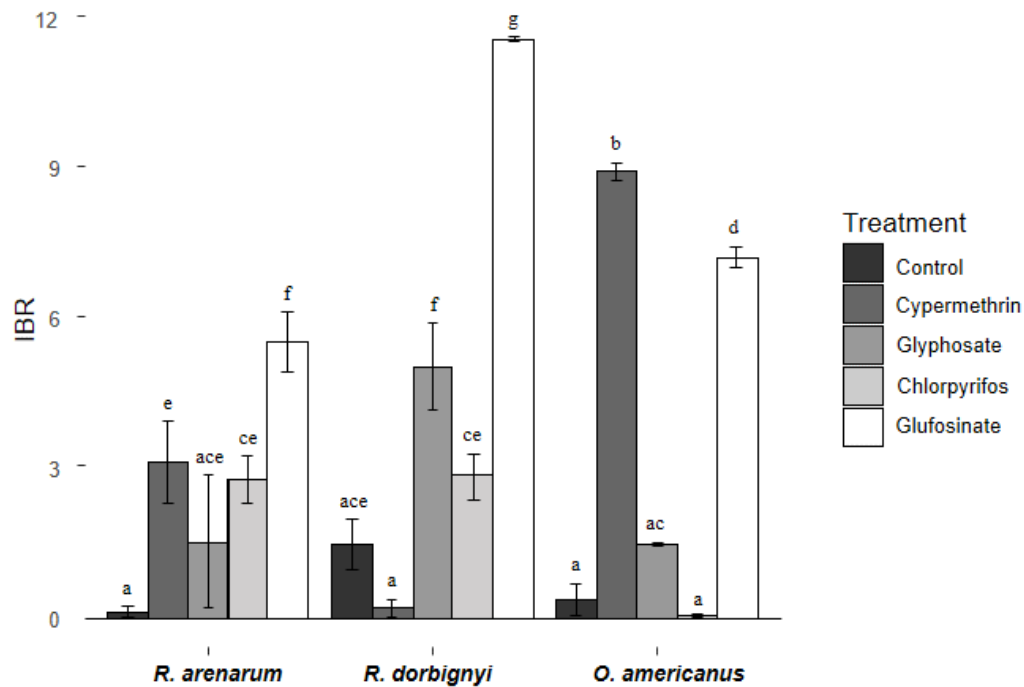


Fig. 3

Table 1. Mean and standard deviation values of mean speed (MS) and total distance traveled in one minute (TD) of *Rhinella arenarum*, *Rhinella dorbignyi* and *Odontophrynus americanus* exposed to cypermethrin (0.013mg L⁻¹), glyphosate (2.5mg L⁻¹), chlorpyrifos (0.1mg L⁻¹) and glufosinate ammonium (15mg L⁻¹) during 1 min and 60 min.

Species	Treatment	1min		60min	
		MS	TD	MS	TD
<i>R. arenarum</i>	Control	3,63 ± 0,59	117,61 ± 54,30	3,50 ± 0,69	97,04 ± 50,22
	Cypermethrin	4,32* ± 1,12	153,57 ± 90,93	2,12* ± 0,51	29,16*** ± 11,55
	Glyphosate	3,23 ± 0,47	120,29 ± 40,66	2,22 ± 1,95	28,02*** ± 24,94

	Chlorpyrifos	2,92 ± 0,48	94,74 ± 32,51	3,31 ± 1,29	34,77** ± 20,98
	Glufosinate	3,51 ± 0,88	112,63 ± 42,99	2,79 ± 1,11	74,01 ± 50,54
<i>R. dorbignyi</i>	Control	1,68 ± 1,13	9,63 ± 8,88	0,61 ± 1,34	1,62 ± 4,03
	Cypermethrin	1,24 ± 1,32	6,62 ± 8,07	3,38*** ± 1,64	44,8*** ± 25,72
	Glyphosate	0,97 ± 1,12	6,78 ± 9,49	0,03 ± 0,07	0,10 ± 0,20
	Chlorpyrifos	1,00 ± 1,55	4,67 ± 4,99	1,08 ± 1,61	5,42 ± 9,76
	Glufosinate	2,36 ± 0,69	15,90 ± 11,34	0,73 ± 1,53	2,62 ± 6,15
<i>O. americanus</i>	Control	3,43 ± 0,67	47,74 ± 20,91	3,33 ± 0,99	43,22 ± 31,44
	Cypermethrin	2,65 ± 1,47	34,35 ± 15,50	0,62** ± 0,81	6,31*** ± 6,83
	Glyphosate	2,30 ± 1,32	11,98** ± 11,33	2,77 ± 1,56	35,69 ± 17,77
	Chlorpyrifos	2,19 ± 1,34	31,22 ± 24,19	3,19 ± 2,01	26,61 ± 13,96
	Glufosinate	3,64 ± 0,76	51,81 ± 21,70	1,86 ± 1,21	23,58 ± 15,43

Asterisk represents significant differences against control *** p< 0.0001; ** p < 0.001; * p < 0.05

Table 2. Results of the two-way ANOVA on IBR values with species and treatment as independent variables.

	Df	Sum Sq.	Mean Sq.	F	p-value
Species	2	20.01	10.00	33.74	<0.001
Treatment	4	294.86	73.72	248.66	<0.001
Species*Treatment	8	200.38	25.05	84.49	<0.001
Residuals	30	8.89	0.30		