Environmental Toxicology and Chemistry—Volume 43, Number 10—pp. 2134–2144, 2024
Received: 18 March 2024 | Revised: 7 May 2024 | Accepted: 26 June 2024

Environmental Toxicology

Biomarkers at the Individual and Biochemical Level: Effects of Pure and Formulated Lambda-Cyhalothrin in *Boana pulchella* Tadpoles (Duméril and Bibron, 1841)

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Abstract: We compared the effects of lambda-cyhalothrin as the pure active ingredient and as a formulated product (Zero®), on the larval stage of the autochthonous species *Boana pulchella*. We evaluated ecotoxicological endpoints, behavioral and developmental alterations, and the biochemical detoxifying, neurotoxic, and oxidative stress responses, covering a wide concentration range from environmental to high application levels. Both pyrethroid preparations displayed similar ecotoxicity (median lethal concentration of ~0.5 mg/L), with the lethal effect of Zero® being more pronounced than that of the active ingredient. Sublethal behavioral alterations in natatory activity were observed at 1000 times lower concentrations, indicating the ecological hazard of tadpole exposure to this pyrethroid at environmentally relevant concentrations. Biochemical endpoints in *B. pulchella* larvae showed significant responses to lambda-cyhalothrin in the ng/L range; these responses were different for the pure or the formulated product, and they were variable at higher concentrations. Principal components analysis confirmed the prevalence of biochemical responses as early endpoints at the lowest lambda-cyhalothrin concentrations; the Integrated Biomarker Response Index proportionally increased with pyrethroid concentration in a similar way for the pure and the formulated products. We conclude that lambda-cyhalothrin is of concern from an environmental perspective, with particular emphasis on autochthonous anuran development. The battery of biochemical biomarkers included in our study showed a consistent integrated biomarker response, indicating that this is a potent tool for monitoring impacts on amphibians. *Environ Toxicol Chem* 2024;43:2134–2144. © 2024 SETAC

Keywords: Pyrethroids; Environmental impact; Aquatic organisms; Amphibians; Development; Lethal effects; Sublethal effects

INTRODUCTION

Pyrethroids have been used worldwide for pest control since 1980, generally as an alternative to organophosphates (Tang et al., 2018). Pyrethroids are synthetic insecticides with neurotoxic effects on both the peripheral and central nervous systems (Davies et al., 2007). Lambda-cyhalothrin (LCT) is a highly toxic pyrethroid with well-documented collateral ecotoxicity on nontarget organisms (He et al., 2008; Kumar et al., 2007). The expansion of extensive crops has increased the use of pesticides, especially in the Pampa Húmeda region, where most of Argentina's agricultural activities are concentrated (Leguizamón, 2014). Agricultural pollution is widespread, and impacts on nontarget biota such as invertebrates, fish, and amphibians have been reported (Demetrio et al., 2014; Giddings et al., 2009; Pérez-Iglesias et al., 2023).

Amphibians are declining around the world, and one of the proposed causes is the increasing presence of environmental pollutants (Alroy, 2015; Mann et al., 2009). Amphibians are a group of nontarget organisms particularly prone to ecotoxicological impairments as a consequence of direct or indirect exposure to a vast majority of pesticides, given their highly permeable integument and biphasic life cycle (Pérez-Iglesias et al., 2023). Even so, studies evaluating the effects of LCT on native anuran species from Argentina are surprisingly scarce.

This article includes online-only Supporting Information.
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Published online 2 August 2024 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5961

Boana pulchella is a native species representative of the Pampean region, previously used in ecotoxicological studies and suggested as a good bioindicator for pesticide contamination evaluation (Araújo et al., 2014; Barreto et al., 2020; Natale et al., 2006; Pérez-Iglesias et al., 2023). Considering the extensive use of LCT in the Pampean region, where considerable environmental residue levels are found in sediments (≥5 mg/kg) and superficial water (≥10 ng/L; Mac Loughlin et al., 2022), along with the wide distribution of *B. pulchella* and its high sensitivity toward different pesticides, it is reasonable to conduct ecotoxicological studies on this species.

In addition to the primary toxicity mechanisms described for the different pesticide families, there are also secondary mechanisms involved in both acute and chronic toxicity. Among them, oxidative stress with alteration of the antioxidant responses and a general disturbance of the antioxidant potential (Abdollahi et al., 2004) may be considered the most common mechanisms associated with pesticides and other contaminants (Venturino, 2017). Pesticides can induce oxidative stress during their activation or detoxification by the mixed oxidases system, through the generation of reactive oxygen species (ROS). In turn, ROS impact diverse biomolecules such as DNA, proteins, and lipids, eliciting oxidated products. The intrinsic antioxidant molecule glutathione (GSH) acts as a first protective agent by removing part of the ROS and triggering antioxidant responses through nuclear erythroid-related factor 2. This transcription factor regulates the expression of antioxidant enzymes genes through the antioxidant responding elements (Jaiswal, 2004). Thus, antioxidant enzymes such as superoxide dismutases, catalase (CAT), and GSH reductases and peroxidases are induced to overcome the pro-oxidative state elicited by xenobiotics (Venturino & D'Angelo, 2005). Depending on the intensity of the exposure, oxidative damage may become irreversible and lead to cell death. Recently, the role of oxidative stress in the toxic effects of LCT has been highlighted (Xu et al., 2023). Given the high variability in the responses of oxidative stress biomarkers to toxicant exposure (Ferrari et al., 2009; Lascano et al., 2011; Liendro et al., 2015), the use of a battery of biomarkers such as detoxifying enzymes, transporters, and neurotoxic targets has been proposed to adequately cover the different effects of contaminants (Rosenbaum et al., 2012).

The need to compare the toxicological effects between the active ingredients (a.i.) using a pure preparation and the corresponding commercial product has been amply recognized. In a recent review, two-thirds of the revised papers comparing both preparations concluded that the formulated products were more toxic than the pure pesticide alone, attributing the differences to adjuvants favoring bioavailability (Nagy et al., 2020). Fewer studies (20%) reported a decreased toxicity for the commercial product. Regarding LCT, one study reported increased genotoxicity and cytotoxicity for the Karate® formulation compared with the pure a.i. in CHO-K1 cell culture (Laborde et al., 2023). Thus it is relevant from an ecological perspective to compare the toxicity of pure LCT with the commercial formulation in autochthonous aquatic organisms.

We evaluated the toxicity of pure LCT and its Zero® 5% formulation on *B. pulchella* larvae at Gosner stage 25 (Gosner, 1960). We performed acute toxicity bioassays at presumed lethal and sublethal LCT concentrations, and evaluated effects at the individual level (mortality, swimming alterations, and morphological abnormalities). We also evaluated endpoints at the biochemical level, such as enzymatic activity and metabolism related to oxidative stress and the antioxidant response.

MATERIALS AND METHODS

Lambda-cyhalothrin standard solutions and exposure media

Pure LCT (CAS Number 91465-08-6; technical grade 95.4%), and the commercial formulation Zero® (5% LCT) were provided by Gleba, Argentina. The stock solution of the formulated product was prepared by dispersion and dilution of Zero® to 5 mg/L LCT (nominal concentration) in filtered and dechlorinated tap water (25 ± 1° C, pH 7.8–8.2, hardness 96–144 mg $CaCO_3$/L, alkalinity 94–176 mg $CaCO_3$/L, dissolved oxygen 6.3 ± 0.3 mg/L). The different exposure media were prepared by serial dilution from this stock emulsion. A solution of pure LCT was prepared by dissolving the a.i. in analytical-grade acetone to 50 g/L (nominal concentration). From this stock solution, the pure a.i. exposure media were prepared by serial dilution in filtered and dechlorinated tap water, starting from 5 mg a.i./L with 0.1% final acetone concentration as a vehicle. This procedure was followed exactly as for the formulated product. The effective LCT concentration was evaluated for both preparations by gas chromatography (GC) as indicated below.

Initial study of LCT preparations in experimental conditions

The real concentrations in the pure a.i. and the formulated LCT solutions were checked at three levels (50–5000 µg/L), by quadruplicate preparations of 500 mL of media. The stability of these preparations was also determined by LCT quantitation in the remaining solutions after 24 and 48 h. In all instances, 50-mL samples were extracted, processed, and analyzed by GC (adapted from Mac Loughlin et al., 2022). Briefly, water samples were extracted 3 times with dichloromethane (20 mL in total), and then the extracts were dried under nitrogen gas and reconstituted in n-hexane (500 µL). The resulting solutions were analyzed with a DANI Master™ gas chromatograph with a Phenomenex® Zebron ZB-SemiVolatiles™ column (30 × 0.25 × 0.25 µm), coupled to a time-of-flight mass spectrometry (TOF–MS) device. Sedimentation of formulated LCT at 48 h was analyzed by collecting the deposited material with 2 mL analytical grade methanol, followed by QuEChERS extraction with 15 mL acetonitrile, 2 g NaCl, and 6 g anhydrous $MgSO_4$. The extracts were dried, redissolved, and analyzed by GC–TOF–MS as just described (Mac Loughlin et al., 2022).

Study species and experimental design

The native anuran *B. pulchella* at larval stage 25 (Gosner, 1960) was chosen as the experimental model. Fertilized eggs (10% of 10 clutches randomly selected) were collected in Autumn in an unexposed pond located on the outskirts of La Plata City (Buenos Aires, Argentina) within the flood valley of the El Pescado stream (35.020621° S, 57.857203° W). The clutches were transported to the laboratory to continue their development up to stage 25, in controlled conditions according to Barreto et al. (2020). Animal treatment and experimental designs complied with the ARRIVE 2.0 guidelines and were approved by the Institutional Committee for Animal Care and Use (#003-00-22), La Plata National University, Argentina. The collection permit number was 22500-41820/18 Provision 73.

We performed two series of experiments for each LCT preparation, in an attempt to cover lethal and sublethal (environmental) concentration effects, respectively. All the exposures were performed in quadruplicate, each experimental unit consisting of 500 mL of the media and 10 larvae (<1 g fresh weight/L), in 1-L glass vessels (10.5 × 13.0 cm), at 25 ± 1° C, 16:8-h light:dark photoperiod and without aeration. The nominal LCT concentrations selected for the "lethal" range were: 0, 1, 5, 10, 50, 75, 100, 500, and 1000 μg/L for pure a.i. and for the formulated product. The nominal LCT concentrations selected for the "sublethal" range were: 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μg/L for pure a.i., and: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 μg/L for the formulated product.

The exposures lasted for 96 h under semistatic conditions with media renewals every 24 h and no feeding. Before each media renewal, lethal and sublethal endpoints were evaluated. Mortality was assessed with a binocular stereoscope as absence of movement and cardiac beat, and dead larvae were removed. Swimming alterations were documented as irregular, circular, or absent natatory activity (Brunelli et al., 2009). Morphological alterations were registered under a binocular stereoscope and categorized according to Bantle et al. (1991).

Biochemical endpoints

After the 96-h exposures, the larvae from each experimental unit were collected, rinsed with cold distilled water, and stored in potassium phosphate buffer (143 mM, pH 7.5) Na-EDTA 6.3 mM at −20 °C until processing. For this, larvae were homogenized in the same buffer (final volume: 2 mL) and aliquoted for immediate analysis of reduced GSH and lipid peroxide contents. The remaining homogenate was centrifuged at 10,000 *g*, and the supernatant was used to measure enzymatic activities. Reduced GSH was determined as acid-soluble thiols as described in Venturino, Anguiano, et al. (2001). Lipid peroxide content was measured as thiobarbituric acid-reactive species (TBARS) by fluorescence spectrophotometry (Mardirosian et al., 2017). The enzymatic activities of GSH-reductase (GR), GSH-transferase (GST), CAT, and acetylcholinesterase (AChE) were kinetically assessed by spectrophotometry (Ferrari et al., 2008; Venturino, Gauna, et al., 2001). Carboxyl aryl-esterase (CabE) activity was kinetically determined following the hydrolysis of α-naphthyl acetate (3 mM) as a substrate in Na phosphate buffer (25 mM, pH 6.5), coupled to Fast Garnet salt (0.4 mg/mL) to form the azo product absorbing at 490 nm (modified from the method described by Dary et al., 1990). Total protein quantitation was adapted from Lowry et al. (1951).

Statistical analyses

The percentage of LCT determined in the different concentration levels was compared using analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test, at both 0 and 48 h and for pure and formulated preparations, by separate analyses. The responses of biochemical endpoints at 96 h were also analyzed by an ANOVA-LSD test (Statistica 7).

Nonlinear regression models fitting to data

We performed nonlinear regression fittings of logistic models to the experimental data to assess the equation parameters associated with ecotoxicological endpoints (RNL BASIC program; Venturino et al., 1992, 2007). Quadruplicate to octuplicate data (considering those concentration levels repeated in both experimental sets) for mortality and behavioral and morphological alterations, from the acute lethal and sublethal assays together, were used for model fitting to data. In addition to a logistic equation to determine the median effective concentration (EC50), we developed a more general equation enabling the estimation of any EC*x* with the corresponding standard error (SE).

From the sigmoidal function used in the logistic model:

$$E\% = 100 - \frac{100}{1 + \left(\frac{CC}{EC50}\right)^{S}} \quad (1)$$

where *E*% is the effect in response to the contaminant concentration *CC*; with the parameters *EC50* and the slope of the sigmoid function, *S*, the EC50 can be expressed as a function of any EC*x* and replaced in Equation 1 to obtain a general expression as:

$$E\% = 100 - \frac{100}{1 + \left(\frac{Ex}{100 - Ex}\right)\left(\frac{CC}{ECx}\right)^{S}} \quad (2)$$

where Ex is a constant corresponding to the level of effect of EC*x*.

We used this equation to calculate EC10 and EC1 values as predictors of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC), respectively, with the corresponding SE.

Principal components analysis

The response of *B. pulchella* larvae to either pure a.i. or formulated LCT at the ecotoxicological, behavioral, and biochemical levels was analyzed using principal components analysis (PCA; NTSYS software package). Mean values of all the

endpoints at a 96-h exposure to the whole range of LCT concentrations were used for the multivariate analysis.

Integrated biomarker response analysis

We calculated an integrated biomarker response (IBR) index corresponding to each LCT concentration level using the Integrated Biological Responses Ver. 2 approach (Sanchez et al., 2013). The standardized and centered responses of the different endpoints at each LCT level were displayed in star plots to identify different response patterns, and the IBR indices for pure and formulated LCT were represented as a function of the effective concentration.

RESULTS AND DISCUSSION

GC checking of LCT concentrations and removal dynamics

The real LCT concentrations in pure a.i. preparations were, on average, 50.1% of the nominal values. The highest concentration of 5 mg/L a.i. LCT showed solubility problems and could not be included in the ecotoxicological analyses. We also checked LCT concentrations for the formulated product, which was, on average, 87.0% of the nominal values. No significant differences were observed between the measured versus nominal ratios of the different initial concentrations for either a.i. or formulated LCT (Figure 1). With respect to the removal dynamics, the formulated LCT proved to be stable up to 48 h, whereas, for the pure LCT, the initial concentration decayed to 46% (Figure 1; see the raw and processed data in the Supporting Information, S1).

A fine film of deposited material was observed in the flasks containing the highest levels of formulated LCT at 24 and 48 h. The quantitation of this material resulted in 35.1 µg LCT, a nonsignificant amount of the initial LCT mass (1.6%).

An early review of LCT reported a half-life of 22 days in the aquatic environment. Its elevated sorption to particulate matter and sediments would lead to its removal from surface water and would mitigate its toxicity toward aquatic organisms by reducing its bioavailability (He et al., 2008). Our results suggest that LCT remains more stable as a formulated product compared with the pure LCT preparation, which showed a global disappearance rate of approximately 2 days.

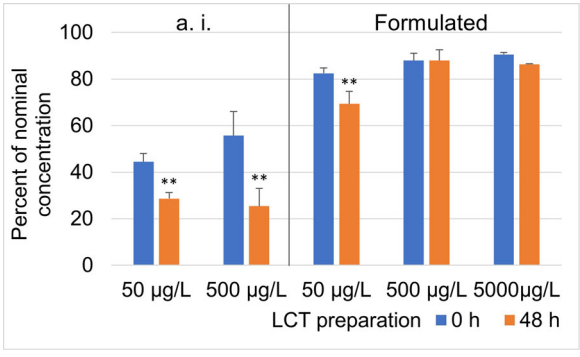

FIGURE 1: Measured lambda-cyhalothrin (LCT) concentrations in pure a.i. and formulated (Zero®) fresh preparations (0 h) and after 48 h. Data expressed as % of the nominal concentrations for the mean ± SE of quadruplicate samples. Factorial analysis of variance: No significant differences between concentration levels. Significant differences at 48 h versus fresh media, $^{**}p \leq 0.01$.

Ecotoxicity of LCT in *B. pulchella* larvae

We verified that the presence of 0.1% acetone in the control media did not cause lethal or sublethal effects on *B. pulchella* (mortality: $p = 0.43$; behavioral or morphological alterations: $p = 1.00$, compared with water controls). We estimated lethal endpoints for the pure a.i. LCT toxicity through the logistic model fitting to data, obtaining a median lethal concentration (LC50) of 470 µg/L (SE 118 µg/L). In turn, the formulated LCT was slightly less toxic, with an LC50 of 655 ± 26 µg/L, although its lethal effects were more clearly defined by a slope of 6.13 (Table 1). From the corresponding logistic fittings to the data, the LC10 and LC1 were also estimated as predictors of the LOEC and NOEC, respectively. For pure LCT, the LC10 was estimated as 70 ± 19 µg/L, and the LC1 was 8.7 ± 6.4 µg/L. For Zero® formulated LCT, the LC10 and LC1 were closer to the LC50, at 458 ± 32 µg/L and 310 ± 36 µg/L, respectively. The entire range of experimental data and nonlinear regression fittings of logistic models for Zero® and pure a.i. LCT are shown in the Supporting Information, S1.

The acute lethal effects of LCT in *B. pulchella* were more clearly defined for the formulated preparation, as indicated by the steeper slope compared with the pure a.i. This could be the result of improved absorption and distribution processes in the animals exposed to Zero®. The 96-h LC50 results were quite similar for both preparations, in the order of sub-milligrams/L. These values are relatively close to those reported for other anurans such as the common African toad *Amietophrynus regularis* larvae (24-h LC50 of 3.7 mg/L; Nkontcheu et al., 2017) but are far from others such as *Xenopus laevis* (168-h LC50 4 µg/L; Aydin-Sinan et al., 2012). The similarity in the LC50 for pure and formulated LCT in *B. pulchella* tadpoles

TABLE 1: Ecotoxicological, behavioral, and morphological endpoints for pure and formulated lambda-cyhalothrin evaluated at 96 h in *Boana pulchella* tadpoles

	Endpoint (µg a.i./L)		
Effect	EC50 (SE)	EC10 (SE)	EC1 (SE)
Mortality			
Pure LCT	469.9 (117.8)	69.6 (19.3)	8.71 (6.38)
Zero®	655.5 (25.8)	458.0 (32.1)	309.6 (35.5)
Narcosis			
Pure LCT	0.549 (0.133)	0.463 (0.096)	0.374 (274)
Zero®	0.829 (0.048)	0.586 (0.225)	0.399 (0.305)
Tail flexure			
Pure LCT	43.51 (16.12)	0.377 (0.277)	0.0021 (0.0033)

Parameter values were estimated from nonlinear regression fittings of logistic models to data (raw data shown in the Supporting Information, S1).
EC50 = median effective concentration; EC10 and -1 = effective concentration at 10% and 1%; LCT = lambda-cyhalothrin.

was not expected, because formulated biocides are usually better absorbed by organisms, causing more drastic effects. Cell culture experiments have also indicated that formulated LCT Karate® induced larger genotoxic and cytotoxic effects in CHO-K cells, compared with the pure a.i. (Laborde et al., 2023). Ecotoxicological data for more sensitive aquatic organisms have been reported in the nanogram/L range, which is closer to the environmental concentrations of LCT found in several superficial water courses. Aquatic macroinvertebrates were among the most sensitive organisms, with 96-h LC50 values on the order of 1 ng/L, the hazard-concentration limit protecting 95% of the species (HC5). Fish were next in sensitivity to LCT, with an HC5 of 41 ng/L (Giddings et al., 2009). In Argentina, environmentally relevant concentrations of LCT in superficial waters were found ranging up to 10 ng/L (Mac Loughlin et al., 2022), which is directly related to the rapid dissipation of this compound in the environment and its adsorption to particulate matter. The same authors report particulate and sediment LCT concentrations of 5.0 and 2.6 mg/kg, respectively, confirming this assumption. This means that aquatic organisms are likely to be exposed to high LCT levels through suspended particles. Higher water concentrations of LCT have been reported in other countries, between 40 and 140 ng/L (Budd et al., 2020; He et al., 2008; Papadakis et al., 2015).

Behavioral and morphological alterations

The percentages of individuals showing either subtle or conspicuous swimming alterations were used to fit logistic models by nonlinear regression. The pure a.i. LCT showed a sharp increase in the percentage of swimming alterations, with an EC50 of approximately 0.55 ± 0.13 µg/L; the NOEC and LOEC values were estimated as 0.37 ± 0.27 µg/L and 0.46 ± 0.10 µg/L, respectively (Table 1). At approximately 1 µg/L pure LCT, all the individuals showed altered swimming. The formulated LCT caused behavioral alterations at the same concentration range, with an EC50 of 0.83 ± 0.05 µg/L; the EC10 (NOEC) was estimated as 0.59 ± 0.23 µg/L, and the EC1 (NOEC) was 0.40 ± 0.30 µg/L, similar to the pure LCT.

Tail flexures were also detected in individuals exposed to pure LCT treatments. The EC50 for this effect was estimated as 43.5 ± 16.1 µg/L. The estimated LOEC and NOEC values for tail flexure were 0.38 ± 0.28 µg/L and 0.0021 ± 0.0033 µg/L, respectively (Table 1; Supporting Information, S1).

Narcotic effects and altered swimming in *B. pulchella* exposed to LCT occurred at concentration levels well below those causing acute (lethal) toxicity. In fact, behavioral alterations were observed at concentrations approximately 3 orders of magnitude lower than lethal concentrations, 856 times for pure LCT and 791 times for formulated LCT. Again, both LCT preparations showed a similar range for the effects, with EC50 values in the order of sub-microgram/L. It has been recognized that the sublethal effects elicited by pyrethroid insecticides are highly relevant from an ecological perspective (Werner & Young, 2018). Similarly, in the present study these effects are visible at very low concentration levels, thus acting as better biomarkers to assess ecological impacts, compared with acute toxicity endpoints. As a consequence of impaired or altered behavioral responses, the organisms may display poor performances in feeding, growing, reproduction, and predator avoidance (Werner & Young, 2018).

Biochemical toxicity endpoints

To gain insights into the differential effects of the a.i. preparation and the formulated product Zero®, the results for each biochemical parameter were displayed in the same graph. Lipid peroxidation was significantly increased by 50% to 60% by the a.i. LCT in the low-intermediate range of the pesticide (0.03–3.0 µg/L), while the effect was more pronounced at the high concentration range (0.25–0.50 mg/L), increasing TBARS production by 2.1 times compared with controls. In turn, LCT in the Zero® formulation increased lipid peroxidation only at the high concentration range (0.09–0.44 mg/L), but this was 3.3 times the control values (Figure 2A).

Levels of the antioxidant GSH showed a progressive increase at the high concentration range of pure LCT (0.05–0.5 mg/L), which was 3 times higher than in the controls. In turn, GSH response to the formulated LCT was approximately opposite to lipid peroxidation, with a protective increase in GSH levels, at the 0.4 to 8.7 µg/L range, up to 3.2 times that of control values, and a second rise to 2.3 times at the highest formulated LCT concentration of 0.87 mg/L (Figure 2B).

The GSH-dependent antioxidant enzyme GR was significantly affected by Zero® formulated LCT at the low and intermediate ranges, from 0.04 to 44 µg/L, for an increase of approximately 2 times. Pure a.i. LCT did not significantly affect GR activity. The high concentration ranges were not evaluated for GR (Figure 2C).

The enzyme GST, which includes GSH as a substrate, was in turn affected by pure LCT at the low-intermediate ranges (0.05–2.5 µg/L), significantly increasing its activity by approximately 2.2 times (Figure 2D). Zero® did not significantly affect GST activity.

Another detoxifying enzyme, CabE, showed response patterns similar to those of GST. Pure LCT caused an increase in CabE activity of nearly 2 times, at the low-intermediate concentration range (0.25–2.5 µg/L). In turn, Zero® formulation did not significantly affect CabE activity (Figure 2E). The activity of the antioxidant enzyme CAT was also affected by tadpole exposure to pure LCT, being significantly increased (by 1.8 times) at the low-intermediate range (0.05–2.5 µg/L). However, both very low (0.0005 µg/L) or high (25 µg/L) LCT concentrations caused CAT activity inhibition. The effect of formulated LCT was always inhibitory, being significant at all the different concentration ranges, up to approximately 50% decrease in CAT activity (Figure 2F).

Finally, the effects on AChE activity as a neurotoxicity biomarker, were variable and tended to increase such activity, but not significantly, for both the pure LCT and the Zero® formulation (data available in the Supporting Information, S2).

Three main aspects should be highlighted regarding the biochemical biomarker responses to LCT in *B. pulchella* tadpoles. The first is the remarkable variability in response depending on

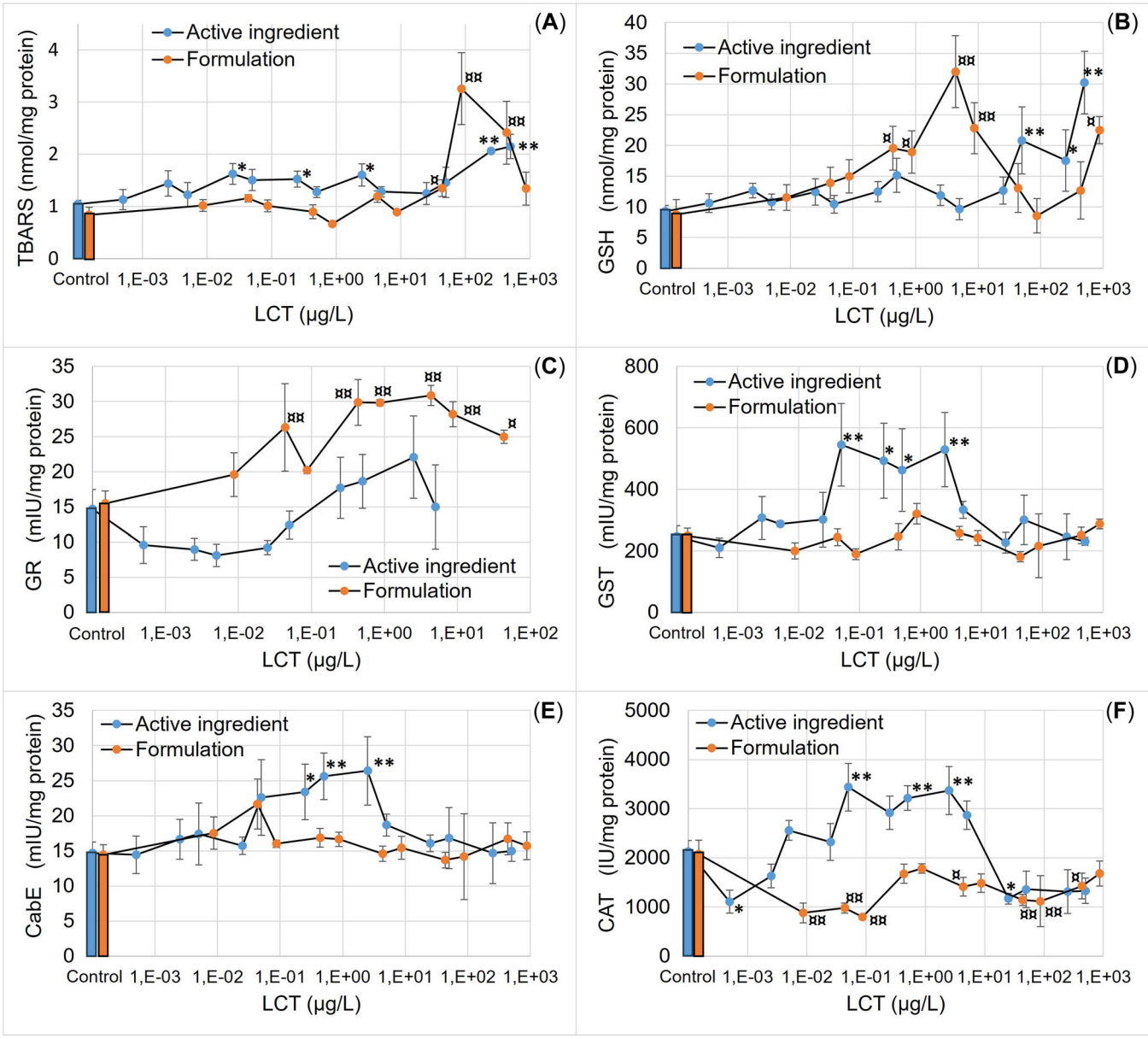

FIGURE 2: Effect of lambda-cyhalothrin (LCT) on biochemical parameters determined in exposed *Boana pulchella* tadpoles. Stage 25 larvae were exposed to pure LCT or to Zero® during 96 h, and responses to the following biomarkers were determined in quadruplicate: (**A**) thiobarbituric acid-reactive species (TBARS); (**B**) glutathione (GSH); and enzyme activities (**C**) GSH-reductase (GR); (**D**) GSH-transferase (GST); (**E**) carboxyl aryl-esterases (CabE); and (**F**) catalase (CAT). Significant differences (analysis of variance least significant difference post hoc test) versus controls: $^{*,\text{¤}}p < 0.05$; $^{**,\text{¤¤}}p < 0.01$.

the exposure concentration, with monophasic or biphasic peaks of responses even in opposite directions, instead of a linear response according to the concentration. The second is the variability of responses to the LCT preparation as pure a.i. or as the formulated product. The third aspect is in fact the most remarkable: that the responses of some biochemical parameters were elicited at very low LCT concentrations, in the ng/L range.

The high variability in biomarker responses is not a new finding, in particular when oxidative stress biomarkers are studied after exposure to toxicants, as has been previously reported and reviewed in amphibians and other aquatic organisms (Ferrari et al., 2009; Lascano et al., 2011; Liendro et al., 2015). Therefore, the simultaneous measurement of a battery of biochemical biomarkers is recommended to assess any impact from contaminated sites (Mardirosian et al., 2017; Rosenbaum et al., 2012; Venturino, 2017). The convenience of biochemical and molecular biomarkers to assess contaminant effects at very low concentrations in exposed aquatic organisms has been previously recognized (Mardirosian et al., 2017). The most sensitive biochemical biomarker in the response of *B. pulchella* to LCT exposure was the antioxidant activity of CAT, which showed a pronounced reduction, in the ng/L range. However, the response changed to a high increase in activity at higher LCT concentrations for the pure preparation, whereas CAT activity remained

inhibited for the formulated LCT. The response of CAT activity to oxidative stress results from the balance between the induction of protein synthesis as an antioxidant response and its inactivation by the attack of ROS in the active center of CAT (Kono & Fridovich, 1982; Venturino & D'Angelo, 2005). While low LCT concentrations might be producing ROS levels sufficient to cause CAT inactivation but not enough to induce CAT protein synthesis, at higher a.i. concentrations protein synthesis would prevail. This is consistent with the observed lipid peroxidation (also related to ROS levels), which increased regularly at intermediate concentrations for pure LCT. For the Zero® LCT formulation, CAT activity remained greatly reduced at practically the whole LCT concentration range, but no correlation was seen with TBARS levels. The ROS levels in *B. pulchella* tadpoles exposed to Zero® might also be controlled in other ways, such as a GSH-related antioxidant pathway, as is suggested by the increases in GR activity and GSH contents at the low and intermediate LCT levels. A recent review highlights the role of oxidative stress in the toxic actions of LCT, involving mitochondrial damage, an increase in ROS levels, DNA damage, lipid peroxidation, and protein inactivation through carbonylation (Xu et al., 2023). Among a series of biochemical responses, the increase in TBARS and the decrease in CAT activity as well as other antioxidant enzymes, are mentioned as a consequence of LCT exposure. Technical-grade LCT dissolved in ethanol as a vehicle caused oxidative stress in fish spermatozoa at the μg/L range, with an increase in TBARS levels and a decrease in CAT activity, and an antioxidant response shown by and increase in GR and GSH-peroxidase activity (Kutluyer et al., 2015). The commercial formulation Karate® caused hepatic toxicity and neurotoxicity in fish at μg/L levels mainly when associated with piperonyl butoxide, increasing TBARS and GSH levels and GR and GST activities (Piner & Üner, 2012, 2014). In our study, only the pure a.i. preparation of LCT was able to induce GST activity in *B. pulchella* tadpoles. The exposure of an Amazonian fish (*Brycon amazonicus*) to the formulated LCT Trinca Caps® caused CAT inhibition in the μg/L range, together with an increase in TBARS and GSH levels (Venturini et al., 2019), similar to the effects we observed with Zero® in *B. pulchella* tadpoles.

Apart from GST detoxification, LCT is hydrolyzed by different CabE activities (Aouey et al., 2017; Crow et al., 2007). We observed an induction of CabE activity by pure LCT in *B. pulchella* tadpoles exposed at the sub-μg–μg/L range. The exposure of *X. laevis* tadpoles during 24 h to the commercial preparation Karate® at similar LCT concentrations caused a significant inhibition of CabE activity (Aydin-Sinan et al., 2012). The biomarker AChE has been currently assessed for neurotoxic effects even when direct enzymatic outcomes are not expected from the mode of action of a biocide. In the present study, LCT did not affect AChE activity in *B. pulchella* tadpoles over a wide concentration range extending from the sub-ng/L to the mg/L level, for either the pure a.i. or the formulated product Zero®. This was also the case for *X. laevis* tadpoles exposed to Karate® in the μg/L range (Aydin-Sinan et al., 2012).

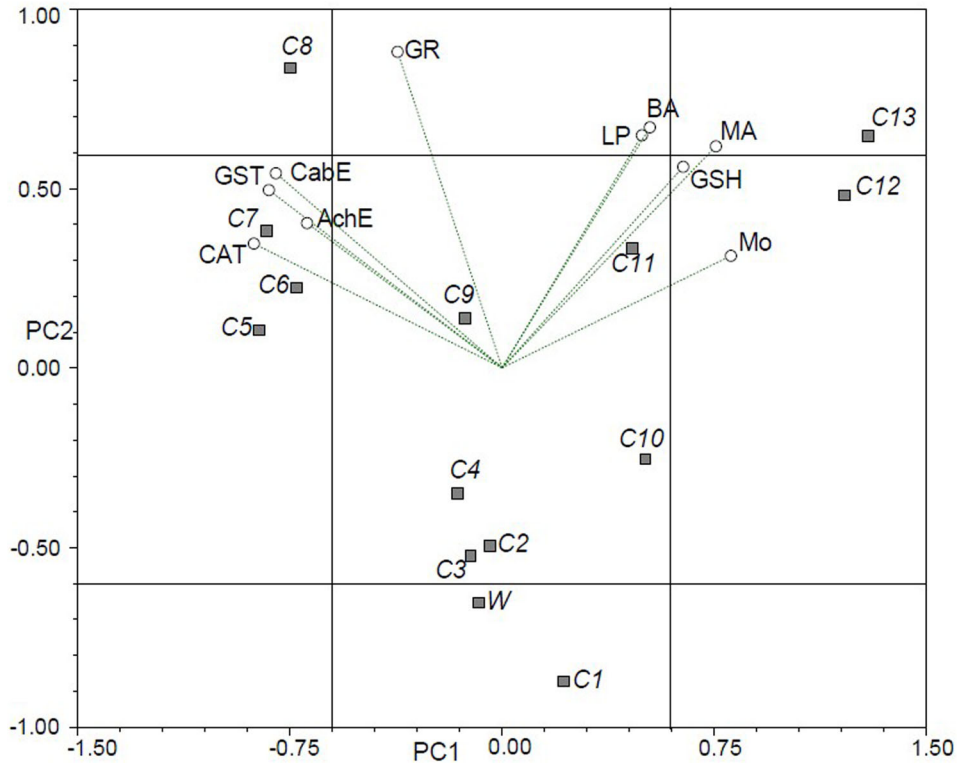

FIGURE 3: Principal component analysis of biomarker responses assessed in *Boana pulchella* larvae exposed to pure lambda-cyhalothrin (LCT). Codes *C1–C13* correspond to measured concentrations of 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5, 2.5, 5.0, 25.0, 50.1, 250.4, and 500.7 μg/L. *W* = water control. Endpoint codes: MA = morphological alterations; BA = behavioral alterations; Mo = mortality; LP = lipid peroxides; AChE = acetylcholinesterase; CabE = carboxyl aryl-esterase; CAT = catalase; GSH = glutathione.

Exposure of the fish *B. amazonicus* to formulated LCT at μg/L concentrations did not affect brain AChE activity (Venturini et al., 2019). Finally, a significant inhibition of AChE activity was observed in carp exposed to sub-μg/L concentrations of LCT (Chatterjee et al., 2021), and in the brain of tilapia exposed to Karate® (Piner & Üner, 2012).

PCA and integrated biomarker response

Using PCA, we looked for integrated responses in the different parameters evaluated for the pure LCT and Zero® formulation, and their relationship with the concentration levels of the pesticide. For pure LCT exposures, the first component C1 explained 48.5% of the total variability of the responses, involving CAT, GST, CabE, and AChE enzymes on one side, and on the opposite side the GSH content, mortality, and morphological alterations. The second component C2 contributed 32.6% of the variability, cumulating 81.2% together with C1 of the total variability in response to pure LCT. In this case, all the response variables were on the same side, with the most representative being GR activity, lipid peroxidation, behavioral effects, and morphological alterations. When the concentration levels were projected onto the plot, the controls and the lowest values in the ng/L range segregated together, in a sector where the influence of the response variables was minimal, and opposite to TBARS levels according to the lowest lipid peroxidation observed in the assays, and the absence of behavioral alterations. The low-to-intermediate range represented by sub-μg/L–μg/L concentrations was associated with the higher detoxifying enzyme activities in the C1 (negative) quadrant. The next intermediate range, comprising 5 to 50 μg/L, was found dispersed in the middle of the biplot, where in general no clear effects were seen in the response variables. Finally, the high concentration range near the mg/L values was found in the upper-right corner of the biplot, where high TBARS levels, all the high ecotoxicity indicators, and, curiously, the highest GSH concentrations were also present (Figure 3).

The PCA applied to the Zero® formulation responses showed a more diffuse behavior, with the first two components C1 and C2 explaining only 57% of the total variability (35.3% and 21.7%, respectively). Consequently, the third component, C3, was also considered, and it explained up to 71.5% of the total variability. Nearly all the response variables were involved in the C1 variability, in the same quadrant of the biplot, except CabE activity and lipid peroxidation, which weighed each to opposite sides on the C2 component. The behavioral alterations were seen in both C2 and C1 variability. The C3 component had CabE activity on the positive side, and CAT activity on the opposite side. When treatment levels were projected in the 3D-variability plot, the control (W) was segregated alone. In an opposite cut plane in C3, the lowest concentrations in the ng/L range (F1–F3) were found, in relation to the increase in CabE activity (F2); the intermediate range concentrations (F4–F7), approximately μg/L, were close to GR and GST activities together with GSH levels; the intermediate to high levels (F8–F10), approximately 40 to 400 μg/L, were close to the highest TBARS values; and finally, the highest concentration (F11) was associated with CAT and AChE activities, and fundamentally with the noticeable effects on behavioral alterations and mortality (Figure 4).

Next, we proceeded to calculate the IBR values, primarily to visualize the evolution of the biomarkers as a function of LCT

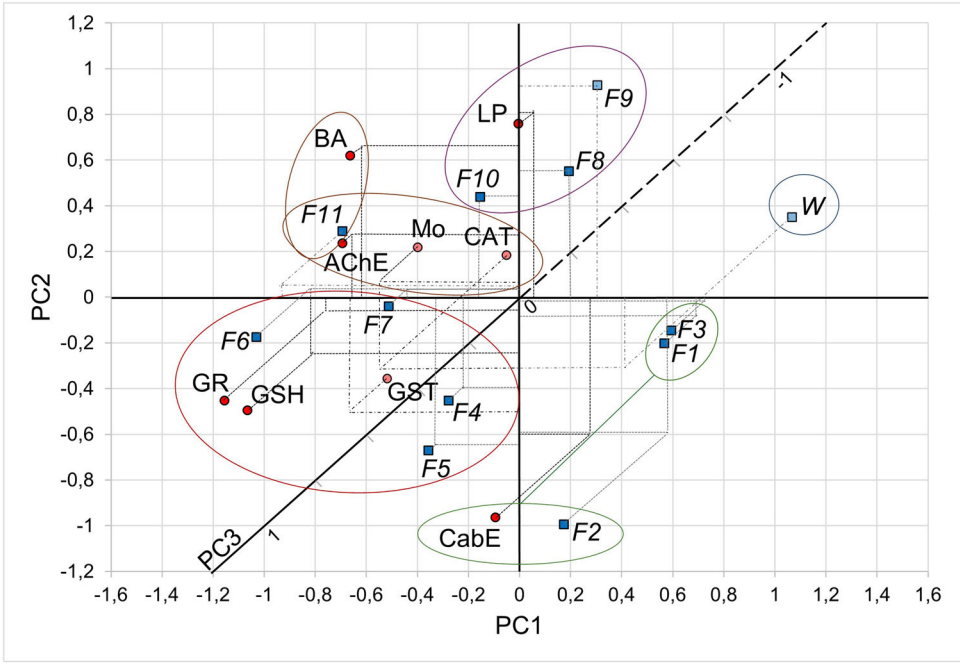

FIGURE 4: Principal component analysis of biomarker responses assessed in *Boana pulchella* larvae exposed to Zero® formulation of lambda-cyhalothrin (LCT). Endpoint codes as in Figure 5. Codes *F1–F11* correspond to measured concentrations of 0.009, 0.044, 0.087, 0.44, 0.87, 4.4, 8.7, 43.5, 87.0, 435.0, 870.1 μg/L; *W* = water control. BA = behavioral alterations; Mo = mortality; LP = lipid peroxides; AChE = acetylcholinesterase; CabE = carboxyl aryl-esterase; CAT = catalase; GSH = glutathione.

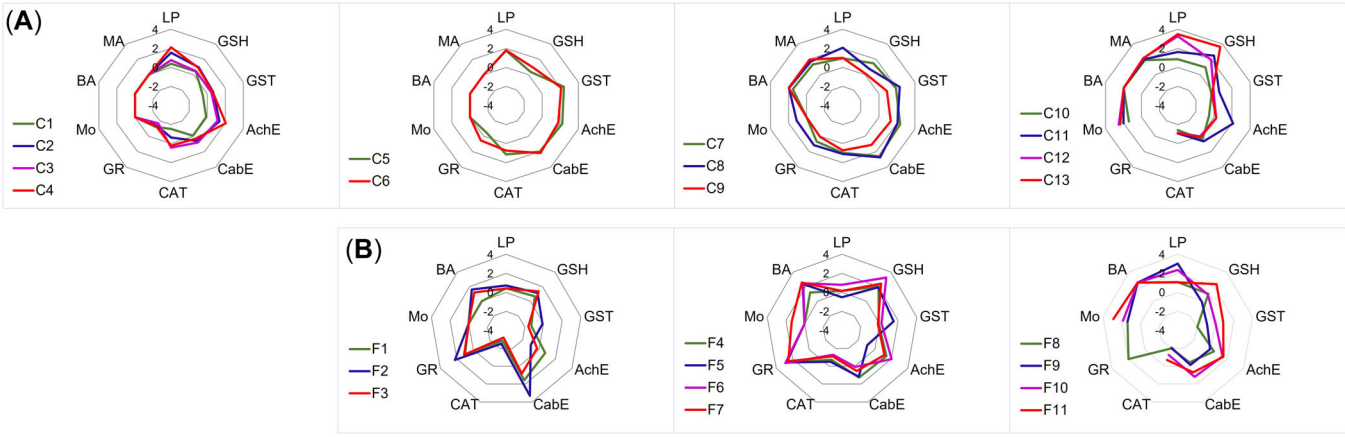

FIGURE 5: Star-plot of the biomarker responses in *Boana pulchella* for the different exposure concentrations corresponding to pure lambda-cyhalothrin (LCT) and the Zero® formulation. Data correspond to the modified values previous to integrated biomarker response calculations, as in Sanchez et al. (2013). (**A**) The concentration codes C1–C13 for pure LCT stand for the measured values of 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5, 2.5, 5.0, 25.0, 50.1, 250.4, and 500.7 µg/L. (**B**) Concentration values for F1–F11 determined as µg/L LCT in Zero®: 0.009, 0.044, 0.087, 0.44, 0.87, 4.4, 8.7, 43.5, 87.0, 435.0, and 870.1 µg/L. For abbreviations, see Figure 3 legend.

concentration and the different profiles for both the pure and formulated preparations (Figure 5). It can be seen that these profiles are similar within the same concentration range and also that they gradually differ from the other ranges. It is also noticeable that these profiles vary for the same concentration ranges when the pure and formulated LCT exposures are compared. In general, the specific biomarker information was similar to that from the PCA analysis when compared in a complementary way. The IBR values were then calculated and plotted as a function of LCT concentration (Figure 6). The profiles for the pure a.i. and the Zero® formulation were roughly similar, showing an ascending trend with the concentration values.

Since its proposal two decades ago (Beliaeff & Burgeot, 2002), the IBR concept has been used in different approaches to improve the methodology for calculating representative indices as tools for environmental monitoring management. The basic idea is to summarize the multiple responses triggered by any particular contamination episode in a graphical design and/or an index capable of displaying the magnitude of these effects on organisms. In this way, through biomarker responses, highly complex comparisons become possible among contaminated sites, concentration effects, or any different exposure conditions. It is interesting to note that in the present study, the combined value of the IBR complied with the expected requirement for a biomarker to show a semiquantitative response according to the level of a contaminant (Adams et al., 2001). Another interesting finding is that the IBR turned the negative (decreasing) responses of individual biomarkers into positive values and smoothed the complex picture of multiple biomarkers fluctuating in biphasic responses; thus the IBR finally revealed an impact on the exposed *B. pulchella* tadpoles. In this sense, the proposed use of a battery of biomarkers to adequately cover the varying effects of contaminants in exposed organisms (Rosenbaum et al., 2012) would be excellently complemented with an IBR analysis as performed in the present study. Chatterjee et al. (2021) integrated multiple biomarker responses in carp exposed to LCT, using PCA and IBR approaches for hematological and biochemical parameters to assess the impacts at a physiological and biochemical level as well as the effects of oxidative stress. Similarly, PCA and IBR approaches were applied for hematological and biochemical biomarker responses in carp exposed to another pyrethroid, to facilitate the interpretation of sublethal effects (Bej et al., 2021).

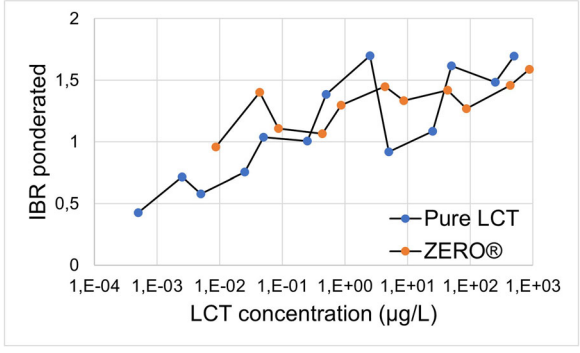

FIGURE 6: Integrated biomarker response (IBR) profile in *Boana pulchella* tadpoles exposed during 96 h to different lambda-cyhalothrin (LCT) concentrations.

CONCLUSIONS

The formulated LCT Zero® proved to be more stable in emulsion than the pure a.i., at least in the short term of 48 h. However, we are aware that in an aquatic environment there is a fast removal of the compound into suspended solids and other lipophilic compartments. Both LCT preparations were toxic to *B. pulchella* tadpoles, with comparable LC50 values, Zero® being slightly less toxic in this sense, but with a clearly more homogenous response (steeper toxicity–concentration slope). The narcotic effects elicited by both LCT preparations would be more crucial in terms of survival ability, observed at concentrations 3 orders of magnitude lower than those of lethal effects and in the range of environmentally expected levels. These behavioral

alterations would strongly impede tadpoles in terms of feeding and predator avoidance. In terms of biochemical responses, there was no "golden" biomarker as the best monitor for low LCT concentrations. Lipid peroxidation (TBARS) as a marker of oxidative stress and GSH as an antioxidant response were mostly consistent with this biochemical—physiological path recognized as a LCT target. The increasing IBR profile, observed for both the pure and the formulated LCT preparations, even at the sub-nanogram/L level, was more consistent with the biomarker responses. The response of the battery of biochemical biomarkers included in our study represents a potent tool for impact monitoring because of its very high sensitivity.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5961.

Acknowledgements—The present study was supported by grants PICT 2017-1529 and PICT 2021-0569 from the Argentinian Foundation for Scientific and Technological Research.

Conflict of Interest—The authors declare no conflicts of interest.

Author Contributions Statement—**Evelina Barreto**: Formal analysis; Investigation; Methodology; Writing—original draft. **Jorgelina Villanova**: Investigation; Methodology; Writing—original draft. **Carolina Parra**: Investigation; Methodology. **Manuel Flores**: Investigation; Methodology. **Carolina Salgado Costa**: Conceptualization; Supervision; Writing—review & editing. **Cecilia Lascano**: Methodology; Supervision; Writing—review & editing. **Guillermo Natale**: Conceptualization; Funding acquisition; Project administration; Supervision. **Andrés Venturino**: Data curation; Formal analysis; Project administration; Resources; Supervision; Writing—original draft; Writing—review & editing.

Data Availability Statement—Data and calculations are available in the Supporting Information.

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