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Early Toxic Effects in a Central American Native Fish (*Parachromis dovii*) Exposed to Chlorpyrifos and Difenoconazole

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Abstract: In Costa Rica, agriculture is one of the most important economic activities. Chlorpyrifos and difenoconazole have been identified as agrochemicals widely used in banana and pineapple crops in the Caribbean area of the country and are constantly recorded in aquatic ecosystems. The toxicity of these pesticides in *Parachromis dovii* was studied. Median lethal concentrations (LC50s) for each substance were obtained from 96-h acute tests. Then, fish were exposed to sublethal concentrations of both substances (10% of LC50), individually and in mixture, to evaluate biomarker responses. Ethoxyresorufin-O-deethylase (EROD), catalase, and glutathione *S*-transferase activities as well as lipid peroxidation were measured in liver and gill tissues as markers of biotransformation and oxidative stress processes. Cholinesterase activity in brain and muscle tissue was also quantified as a biomarker of toxicity. The LC50s were 55.34 µg/L (95% confidence interval [CI] 51.06–59.98) for chlorpyrifos and 3250 µg/L (95% CI 2770–3810) for difenoconazole. Regarding the biomarkers, a significant inhibition of brain and muscle cholinesterase activity was recorded in fish exposed to 5.50 µg/L of chlorpyrifos. This activity was not affected when fish were exposed to the mixture of chlorpyrifos with difenoconazole. Significant changes in lactate dehydrogenase activity were observed in fish exposed to 325 µg/L of difenoconazole, whereas fish exposed to the mixture showed a significant increase in EROD activity in the liver. These results suggest harmful effects of chlorpyrifos insecticide at environmentally relevant concentrations. There is also evidence for an interaction of the 2 substances that affects the biotransformation metabolism at sublethal levels of exposure. *Environ Toxicol Chem* 2021;40:1938–1947. © 2021 SETAC

Keywords: Biomarkers; Biotransformation; Ecotoxicology; Mixture toxicology; Pesticides; Agriculture

INTRODUCTION

In Costa Rica, intensive agriculture is one of the most important economic activities, and a great variety of pesticides are used in crops (Arias-Andrés et al. 2018). Certain unsuitable agricultural practices, combined with the rainy weather in some areas of the country, with up to 4500 mm/yr (Instituto Meteorológico Nacional 2020) in regions of intensive agriculture, favor the transport of these substances toward aquatic environments by drift, runoff, or leaching. In these ecosystems, pesticide residues may become bioavailable to nontarget species such as fish or macroinvertebrates, which may be exposed and suffer toxic effects (Echeverría-Sáenz et al. 2018).

In traditional intensive agriculture, it is common to use several substances, with different biocidal actions, for the same crop. Because of that, it is common to detect pesticide mixtures in water bodies near agricultural land (Castillo et al. 2014; Yang et al. 2014). For example, some studies in the Caribbean zone of Costa Rica, where banana is the dominant crop, followed by rice and pineapple, have detected residues of more than 20 different pesticides in a single-point water sample (Arias-Andrés et al. 2018; Echeverría-Sáenz et al. 2018; Rämö et al. 2018). This is evidence that in the environment organisms are usually exposed to several substances simultaneously. Therefore, assessing the toxicity of mixtures is a constant challenge in environmental health research (Wang et al. 2017).

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Chlorpyrifos and difenoconazole are 2 pesticides widely used in banana production in the Caribbean region of Costa Rica (Bravo Durán et al. 2013), and residues of both are frequently found in aquatic ecosystems close to crops. The range of chlorpyrifos concentrations measured in water bodies in the Caribbean region of the country ranges from 0.009 to 1.42 μg/L and for difenoconazole, from 0.12 to 6.00 μg/L (Polidoro and Morra 2016; Arias-Andrés et al. 2018; Echeverría-Sáenz et al. 2018; Rämö et al. 2018). An organophosphate insecticide that causes neurotoxicity by inhibiting cholinesterase (ChE) activity, chlorpyrifos is mainly used in plastic bags that cover the bunches of fruit to protect them from cosmetic damage caused by insects (Polidoro et al. 2008). A triazole fungicide that affects the synthesis of sterols (Wijeyaratne and Pathiratne 2006; Mu et al. 2015), difenoconazole is applied weekly in banana plantations, mixed with other fungicides, to control the black sigatoka disease (Polidoro and Morra 2016). The intensive use of both substances favors their constant presence in the environment. As for their toxicity to fish, taking rainbow trout as a reference, chlorpyrifos is known to be 40 times more toxic than difenoconazole (Lewis et al. 2016), but the effects of a mixture of these compounds are unknown.

Apart from the pressing need to understand the toxicity of pesticidal mixtures to nontarget species, it is also paramount that sublethal effects of pesticides in agricultural runoff be investigated (Barata et al. 2008). Acute toxicity can be related to specific catastrophic events that cause massive mortality of organisms, but chemical monitoring has shown that freshwater ecosystems are more frequently exposed to mixtures of different substances at sublethal concentrations (Colin et al. 2016).

Regarding sublethal effect assessments, biomarker batteries in ecotoxicological studies are considered rapid and sensitive tools to be used as early warning signals in ecosystems. Such biochemical or physiological measurements allow for detection and intervention before more severe effects occur, either at the individual level or at higher levels of biological organization (Wu et al. 2014; Colin et al. 2016; Bonifacio et al. 2017).

These biochemical assessments include processes related to the metabolism and biotransformation of xenobiotics in the exposed organism. For example, 7-ethoxy-resorufin-O-deethylase (EROD), which is one of the cytochrome P450 enzymes that mediate reactions in phase I of biotransformation, can be triggered in the presence of contaminants (Heinrich et al. 2014). Another example is glutathione *S*-transferase (GST), a widely used biomarker that is involved in the detoxification and biotransformation of many xenobiotics. The conjugation reaction mediated by GST increases the solubility of electrophilic compounds, facilitating their excretion (Amiard-Triquet et al. 2011). Also, enzymes and processes related to oxidative stress, including antioxidant activities such as catalase (CAT), are related to the group of antioxidant enzymes that counteract the damage produced by hydrogen peroxide (Vasylkiv et al. 2011). In addition, the estimation of lipid peroxidation (LPO) has been used to study oxidative stress side effects because reactive oxygen species that are not neutralized can target biomolecules, such as membrane lipids (Sharbidre et al. 2011). The wide range of available protocols can also shed light on energy metabolism–related responses such as lactate dehydrogenase (LDH) activity. This enzyme is involved in the anaerobic pathway of energy production, catalyzing pyruvate reduction by nicotinamide adenine dinucleotide phosphate (NADH), resulting in lactate and NAD^+ (Benedetti et al. 2015). Biomarkers can also be associated with the mode of action of the contaminants, as is the case of inhibition of ChE activity by organophosphate pesticides, because they bind to the active site of the enzyme and have an important impact on the transmission of nerve synapses; therefore, they are related to neurotoxicity (Rao 2006; Wijeyaratne and Pathiratne 2006). Biomarkers have been used in laboratory investigations under controlled conditions to evaluate pesticide mixtures and find synergistic, additive, or antagonistic effects, making them useful methods for understanding the toxicity of the mixture (Osten et al. 2005; Jonsson et al. 2017; Rossi et al. 2017).

Considering the biotic communities that can be affected by the contamination of aquatic ecosystems with mixtures of pesticides, fish have frequently been used as model organisms for the evaluation of effects (Wu et al. 2014). In Costa Rica, the native cichlid guapote (*Parachromis dovii*) has a broad distribution, covering the northern part of both, the Atlantic and Pacific slopes, including areas of intensive banana production. The species occupies a high position in the trophic chain of freshwater ecosystems and has economic relevance because it is a resource for aquaculture as well as subsistence and sport fishing (Bussing 2002). The suitability of *P. dovii* for ecotoxicological studies has been previously tested (Mena et al. 2014; Navarro et al. 2014), and its use is favored by the possibility of breeding them in the laboratory, although the species is considered of least concern according to the International Union for Conservation of Nature (Lyons and Matamoros 2020). In this regard, the use of native species as models for ecotoxicological studies in the tropics may provide more relevant information than conventional models, like temperate species (Chiang et al. 2012; Diepens et al. 2014).

Focusing on the evaluation of subindividual effects associated with the 2 pesticides selected for the present study, chlorpyrifos is known to act by inhibiting ChE activity, and this effect has been observed in organisms exposed to sublethal concentrations of the insecticide (Wacksman et al. 2006). Meanwhile, the toxicity mechanism for difenoconazole in vertebrates is not clearly understood (Dong et al. 2013). Reports that exposure to this fungicide induces biotransformation phase I, through EROD activity currently offer the most leading evidence (Xing et al. 2014). We can therefore expect ChE inhibition in *P. dovii* after exposure to sublethal concentrations of chlorpyrifos, whereas exposure to difenoconazole would induce EROD activity. These responses were tested by exposing fish to a fraction (10%) of the median lethal concentration (LC50) of each substance individually. Then, a possible interaction of the substances was evaluated by a mixture exposure. Furthermore, because the activity of enzymes of the cytochrome P450 family can induce other biotransformation events, oxidative stress, and metabolic processes, GST and

CAT activities as well as LPO and LDH activity were measured in fish exposed to individual substances and the mixture.

Considering the ubiquity of chlorpyrifos and difenoconazole in aquatic environments associated with banana plantations in Costa Rica and the importance of elucidating the effects of mixture pollution, in the present study, our aim was to characterize early biochemical responses in *P. dovii* exposed to sublethal concentrations of chlorpyrifos and difenoconazole, individually and in mixture. We first determined the acute toxicity for both substances and then assessed the biochemical responses in fish exposed to a fraction of the LC50 of each substance and in a mixture of them.

MATERIALS AND METHODS

Acquisition and maintenance of fish

Fish of the species *P. dovii* (Ciclhidae; 3.3 ± 0.3 cm total average length and 0.6 ± 0.3 g total weight) were acquired from the Laboratory of Continental Aquaculture of the Biology Department, Universidad Nacional (UNA), Heredia, Costa Rica. These fish were reared on an adequate diet, and this was considered because the nutritional status of the organisms can affect the outcome of the bioassays (Lanno et al. 1989). Prior to any assay, the fish were acclimatized for 24 h in the Laboratory of Ecotoxicological Studies of the Regional Institute for Toxic Substances Studies (IRET-UNA), where the tests were conducted. During this period, the fish were placed in a glass aquarium with ultraviolet (UV) water (filtered with 1-µm pore + 5-µm pore + activated carbon filter and treated with UV light [Millipore]) and constant aeration.

Acute toxicity bioassay

Acute static 96-h toxicity tests were performed to find the LC50 of chlorpyrifos and difenoconazole. A minimum of 5 concentrations were tested in each assay (Table 1), in addition to a negative control (UV water) and a solvent control (acetone added to UV water in a volume equal to the amount added to the highest stock concentration of each pesticide). The fish were distributed individually and randomly in 1-L glass containers, each with 700 mL of exposure solution, with constant aeration, room temperature (24 ± 2 °C), and a 12:12-h light:dark natural photoperiod. For each treatment, 7 replicates were established, fish were not fed during the test, and mortality was recorded every 24 h (Organisation for Economic Co-operation and Development 1972).

TABLE 1: Nominal concentrations of chlorpyrifos and difenoconazole used for different assays carried out during the assessment

	Concentration ranges (µg/L)	
Bioassay	CPF	DFZ
Acute toxicity tests	72.32, 67.8, 63.28, 58.76, 54.24	4000, 3500, 3000, 2500, 2000, 1500
Sublethal assays	12.50, 6.25, 3.12, 1.56, 0.78, 0.39	2050, 1750, 1450, 1150, 850, 550
Assays with 10% of estimated LC50	5.50	325
Mixture	5.50 (CPF) + 325 (DFZ)	

CPF = chlorpyrifos; DFZ = difenoconazole; LC50 = median lethal concentration.

Bioassays with sublethal concentrations

Once the acute toxicity data were obtained, tests were performed with sublethal concentration ranges (Table 1) to find the lowest-observable-effect concentrations (LOECs) for chlorpyrifos and difenoconazole, respectively. Because no significant EROD induction was observed with any difenoconazole concentration tested, 10% of the LC50 was selected to perform the bioassays with the individual substances and the mixture (Table 1), following the conditions of the assays described (Narra et al. 2017). For chlorpyrifos, 10% of the LC50 was also equivalent to the LOEC obtained on ChE.

Solutions of chlorpyrifos and difenoconazole were prepared by dissolving each pesticide in acetone (gas chromatography quality) according to the advice of the Pesticide Residue Analysis Laboratory, IRET-UNA. In each definitive trial, a mixed sample of at least 3 concentrations at 0 and 96 h was collected to analyze the exposure concentrations. The liquid chromatographic analysis was done in the Pesticide Residue Analysis Laboratory with ACQUITY UPLC H-class (Waters) and tandem quadrupole mass spectrometry (XEVO TQ S-micro; Waters). For the acute toxicity test with chlorpyrifos, an average of 42 and 11% of the nominal concentrations was quantified at the initial and final times, respectively. In the case of difenoconazole, an average of 36.5 and 18% of the nominal concentrations at the initial and final times was quantified, respectively. For this reason, the estimate of the LC50 is also reported based on both nominal and quantified concentrations (Table 2). For the individual sublethal test with chlorpyrifos, an average of 65 and 1% of the nominal concentrations was quantified at the initial and final times, respectively. In the case of difenoconazole, an average of 72 and 32% of the nominal concentrations at the initial and final times was quantified, respectively. For the mixture sublethal test (chlorpyrifos + difenoconazole), an average of 16 and 1% of the nominal concentrations of chlorpyrifos were quantified at the initial and final times, respectively; and an average of 49 and 20% of the nominal concentrations of difenoconazole was quantified at the initial and final times, respectively (quantification available in Supplemental Data, Table 1).

Therefore, the tests should be interpreted as a static exposure of 96 h with an initial pulse of the nominal

TABLE 2: Toxicity of chlorpyrifos and difenoconazole to *Parachromis dovii* 96 h after a pulse exposure

	Pesticides	
LC50 (µg/L; 95% CI)	CPF	DFZ
Nominal values	55.34 (51.06–59.98)	3250 (2770–3810)
Quantified initial values	23.24 (21.44–25.19)	1180 (1010–1390)

LC50 = median lethal concentration; CI = confidence interval; CPF = chlorpyrifos; DFZ = difenoconazole.

concentrations. It was realistic as a pulse of pesticides arriving in a river from a runoff event, for example.

It is important to clarify possible factors that influence the low recovery of the initial concentrations in each test with respect to the nominal ones. For example, the samples for quantification at the initial time were taken approximately 30 min after placing the fish in each jar, allowing for evaporation in the preparation of exposure solutions, in addition to the aeration processes and the time that elapses when storing and preserving (–20 °C) the sample until analysis.

Four physicochemical parameters were monitored in exposure solutions during the test: pH (Hach®; HQ411pH/mV), dissolved oxygen (Hach; HQ440dmulti), temperature, and conductivity (WTW Cond 315i). The mean and standard deviation of these parameters maintained during the tests were pH 7.87 ± 0.01, dissolved oxygen = 7.16 ± 0.11 mg/L, conductivity = 466.33 ± 2.27 µS/cm, and temperature = 23.39 ± 0.15 °C.

Measurement of biochemical biomarkers

In each case, the fish were immobilized by submersion in ice-cold water, then weighed and measured (total length, standard length, and maximum height), before decapitation (Matthews and Varga 2012). The brain, liver, gills, and 2 muscle fractions of the dorsal myotome were removed, excluding scales, skin, and spines. Target tissues were placed in microtubes and stored at –80 °C until the quantification of biochemical biomarkers.

The tissues were weighed before homogenization, which was carried out with a Branson sonicator (SLPt). The livers were homogenized in buffer KH_2PO_4/K_2HPO_4 0.1 M (pH 7.4). To the homogenate used for LPO analysis, 0.2 mM butylated hydroxytoluene was added to avoid lipid oxidation. The rest of the homogenate was centrifuged at 15 300 *g*, 4 °C, for 20 min. The supernatant (postmitochondrial fraction) was used for the measurement of the enzymatic activities (EROD, CAT, and GST). The brains and muscles were homogenized in buffer KH_2PO_4/K_2HPO_4 0.1 M (pH 7.2) and centrifuged for 5 min at 10 600 *g*, 4 °C. Muscle samples for LDH measurement were homogenized in Tris-NaCl buffer, pH 7.2. Protein content was measured in all samples by the method of Bradford (1976), with a BIO-RAD® reagent and bovine serum albumin as standard.

Fluorometric measurement of EROD activity was determined according to Peters et al. (1994), with modifications. Briefly, a standard fluorescence curve of resorufin (concentrations 5–200 nM) was prepared by diluting a 50-µM resorufin stock solution in methanol with 0.1 M phosphate buffer of pH 7.4. The reaction solution was prepared using 0.1 M phosphate buffer, pH 7.4; aliquots of an NADH (0.10 mM) solution, prepared fresh in 0.1 M phosphate buffer, pH 7.4; and a 7-ethoxyresorufin stock solution, 415 µM in dimethyl sulfoxide. The kinetics of the reaction was measured in microplates, for 3 min, with readings every 20 s, in a Thermo Scientific™ Fluoroskan™ fluorometer, with filters of 530 nm (excitation) and 580 nm (emission). The EROD activity unit is defined as the formation of 1 nmol of resorufin per minute per milligram of protein.

Activity of CAT was measured according to Aebi (1984), by the decrease in absorbance at 240 nm for 20 s that is due to consumption of the substrate H_2O_2 6 mM and expressed as micromoles per minute per milligram of protein.

Conjugation activity of GST was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to the Habing et al. (1976) method. The reaction mixture was prepared in 200 mM phosphate buffer (pH 6.5), with CDNB 1 mM and GSH 1 mM. The increase in absorbance at 340 nm for 3 min was evaluated and expressed as nanomoles per minute per milligram of protein.

The determination of LPO was made by means of the thiobarbituric reactive species (TBARS) test following the method of Ohkawa et al. (1979). For the reaction, the sample was mixed with 12% trichloroacetic acid, Tris-HCl 60 mM at pH 7.4 with 0.1 mM diethylenetriaminopentaacetic acid and 0.73% thiobarbituric acid, and placed in a bath at 100 °C for 1 h. Subsequently, the reaction product was centrifuged at 13 500 *g* for 5 min at 25 °C, and the absorbance was measured at 535 nm in the supernatant. Levels of LPO were expressed as TBARS nanomoles per milligram of protein.

The spectrophotometric measurement of ChE activity was carried out following the method of Ellman et al. (1961), adapted to a microplate (Guilhermino et al. 1996). The reaction occurred in the presence of acetylcholine 1 mM as a synthetic substrate and dithiobis-2 dinitrobenzoic acid 5.5 mM 0.1 mM as a chromogenic reagent, measuring absorbance at 415 nm for 10 min. Activity of ChE was expressed as nanomoles per minute per milligram protein.

Activity of LDH was measured following the Vassault (1983) method, adapted to a microplate (Diamantino et al. 2001). The reaction mixture contained pyruvate 12 mM and NADH 0.25 mM in Tris-NaCl buffer, pH 7.2. Readings were made every 20 s for 3 min at 340 nm in the spectrophotometer. Activity was expressed as nanomoles per minute per milligram protein.

Biomarkers were measured at 96 h after the pulse exposure for the experiments with the range of sublethal concentrations of each pesticide. For the experiments with one-tenth of the LC50 of individual substances and the mixture, biomarkers were measured at 48 and 96 h after the pulse exposure.

Data analysis

The LC50 values were determined by means of a Spearman regression with RStudio, Ver 1.1423 (RStudio 2017), using the mortality data.

To know the interactions between the treatments and the biochemical determinations, a mixed linear model was fitted, and the group means were compared, using *p* values to evaluate the result of the comparisons, in RStudio. The heterogeneity of variance was assessed with the Breusch-Pagan test. The best model was chosen according to the interaction with time in accordance with Akaike information criterion values.

RESULTS AND DISCUSSION

The acute toxicity data for chlorpyrifos and difenoconazole (LC50) are presented in Table 2 (Figure 1). According to our

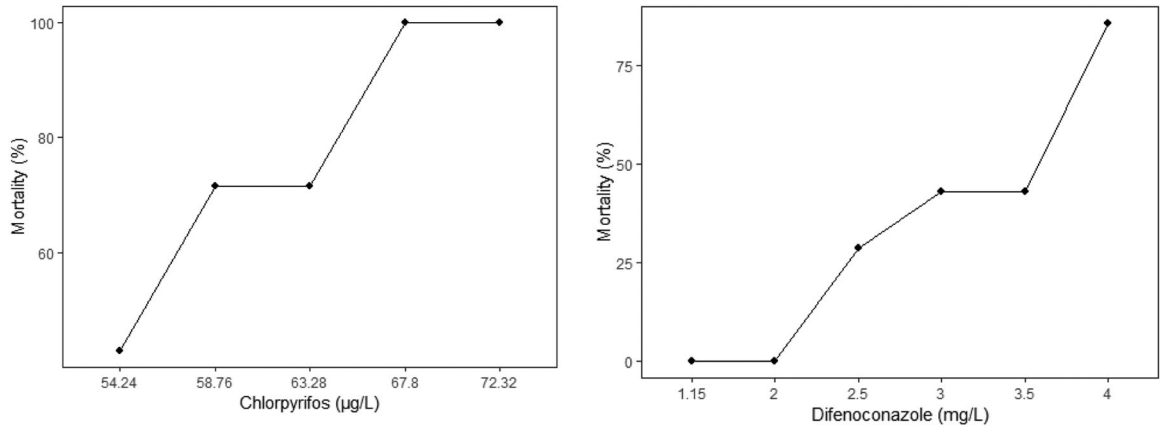

FIGURE 1: Toxicity of chlorpyrifos and difenoconazole in *Parachromis dovii* after 96 h.

results, chlorpyrifos is 50 times more toxic than difenoconazole to *P. dovii*. In comparison with a convential standard, rainbow trout (*Oncorhynchus mykiss*), the tolerance of *P. dovii* to chlorpyrifos (LC50 = 25 µg/L) was twice as high and that to difenoconazole 3 times as high (LC50 = 1100 µg/L; Lewis et al. 2016). However, when correction by chemical quantification is considered, the sensitivity of *P. dovii* is similar to that of *O. mykiss*.

A higher LC50 value for chlorpyrifos has been reported for *P. dovii* (Diepens et al. 2014): 117 (87–158) µg/L. Interestingly, it was calculated using the larval stage of the species. It is common to find variation in toxicity for different substances and species. The physiological and developmental states of the organism can affect its sensitivity. The differences between organisms relate to variations in the time of exposure, the size and age of exposed fish, as well as differences in the activities of xenobiotic-metabolizing enzymes, among other factors (Mu et al. 2013; Wu et al. 2014). They also relate to differences in the chemical structure of the substance, physical–chemical characteristics, mode of action, among other factors, influencing the intake and elimination by individuals as well as the environmental conditions of each test, such as the temperature (Diepens et al. 2014).

Referring to biochemical determinations, when fish were exposed to a range of sublethal concentrations of each pesticide individually, a clear dose-dependent trend in brain ChE inhibition was observed for chlorpyrifos (Figure 2A). In the case of difenoconazole, we expected a response by EROD because phase I induction has been previously reported in fish exposed to this fungicide (Zhang et al. 2017). However, no trend was observed in the activity of the enzyme after exposure to a range of concentrations of difenoconazole. In addition, a great variation in the enzyme activity was obtained among individuals (Figure 2B).

To assess the sublethal effects of chlorpyrifos and difenoconazole, individually and in mixture, *P. dovii* were exposed to one-tenth of the LC50 of each substance individually and a mixture of both at those concentrations. Then, biochemical biomarkers were measured in different tissues of exposed fish.

Fish exposed to chlorpyrifos showed significant inhibition in ChE activity in brain and muscle (Figure 3A and B). Activity of ChE is frequently used as a biomarker related to the neurotoxicity caused by organophosphate insecticides (Wijeyaratne and Pathiratne 2006). When ChE activity is inhibited, the neurotransmitter acetylcholine accumulates in the cholinergic synapses that affect the proper functioning of the nervous system, generating an overstimulation of the system that produces multiple generations of muscle impulses and contractions (Dutta and Arends 2003). In other studies, inhibition of ChE in brain and muscle tissue has been observed in fish exposed to higher concentrations of organophosphate insecticides (Rao 2006).

These results with chlorpyrifos highlight the environmental relevance of the research because the effects (significant ChE inhibition) were obtained in fish that were exposed to concentrations very close to those measured in the environment, even more so when the corrected concentrations according to the quantification are taken into account (Table 3). This is important because there is evidence that such levels of exposure and inhibition caused by organophosphate insecticides can affect vital functions such as locating prey, evading predators, and foraging, threatening the health of fish populations in their natural environment (Bonifacio et al. 2017; Sandoval-Herrera et al. 2019). Interestingly, fish exposed to the mixture showed no significant ChE inhibition (Figure 3A and B), which might suggest an antagonistic interaction.

Antagonistic interactions have been reported in organisms exposed to organophosphate and carbamate mixtures (Bonansea et al. 2016). In our case, fish exposed to a mixture of organophosphate and triazole had a significant induction of EROD activity, which might be related to an enhanced metabolism of chlorpyrifos that prevented its effect on ChE activity. Such interactions have been previously described (Hernández-Moreno et al. 2010; Wang et al. 2017).

In fish exposed to the pesticide mixture, a significant increase in EROD enzyme activity in the liver was observed with respect to controls and individual substances (Figure 3C). In general, mixtures of pesticides and their metabolites can generate interactive effects in fish and alter their metabolism (Clemente et al. 2010; Bacchetta et al. 2014; Xing et al. 2014). In the biotransformation process, both pesticides together activate the cytochrome P450 family, which is a large group of

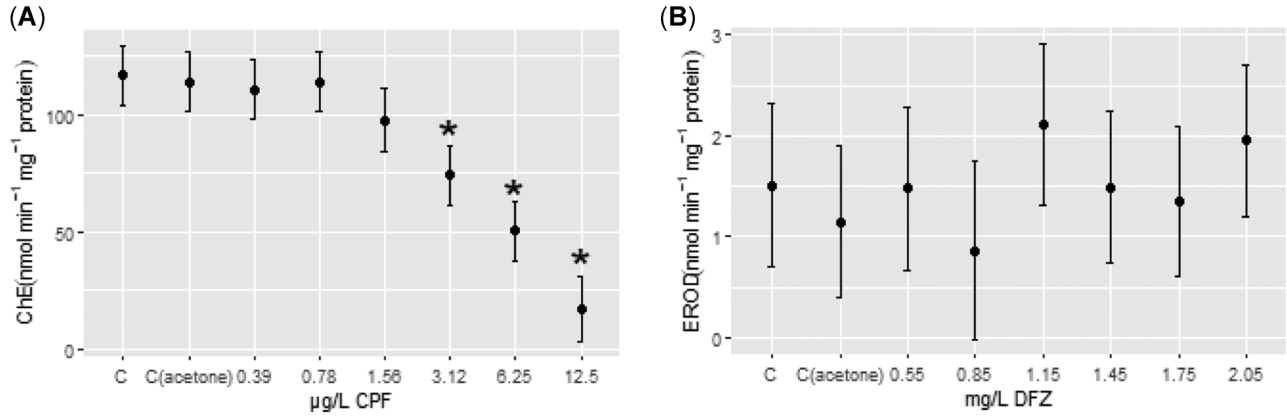

FIGURE 2: Cholinesterase activity in brain (**A**) and ethoxyresorufin-O-deethylase in liver (**B**) of *Parachromis dovii* exposed to chlorpyrifos and difenoconazole, respectively. *Significant difference between treatments and controls. ChE = cholinesterase; CPF = chlorpyrifos; EROD = ethoxyresorufin-O-deethylase; DFZ = difenoconazole.

enzymes involved in the catalysis of oxidation, bioactivation, and detoxification of various substances (Kim et al. 2013), in this case by induction of EROD, which could facilitate the excretion of formed products (Karaca et al. 2014). The measurement of this enzyme is considered a sensitive but nonspecific indicator and is commonly related to the identification of a possible exposure to xenobiotics, being considered a valuable biomarker (Heinrich et al. 2014).

Although no other studies with this mixture were found, EROD activation has been reported in fish exposed to pesticides (Dong et al. 2009; Mikula et al. 2009; Haluzová et al. 2011) and to some organophosphates (Almeida and Oliveira 2010) and triazoles (Celander 2011; Zhang et al. 2017).

Fish exposed to chlorpyrifos showed a significant increase in CAT activity in gills (Figure 3D), which generally indicates activation of the antioxidant defense system, preventing oxidative stress (Clemente et al. 2010; Ghisi et al. 2017). This recorded induction may be sufficient to cope with oxidative stress induced by the toxic substance because no significant increase was found in the levels of LPO, as has been reported in another study (Almeida and Oliveira 2010). Chlorpyrifos is considered to be an irritant substance to tissues such as fish gills. Significant activation of CAT has been reported in gills with other pesticides such as endosulfan (Bacchetta et al. 2014). Activation of these enzymes in liver, kidney, and brain has been reported in fish exposed to chlorpyrifos at similar concentrations, with longer periods of exposure (Kaur 2017).

A significant increase in LDH activity measured in muscle of fish exposed to difenoconazole was shown (Figure 3E). Lactate dehydrogenase is an enzyme involved in the anaerobic pathway of energy production, catalyzing pyruvate reduction by NADH, resulting in lactate and NAD^+ (Benedetti et al. 2015). It is involved in the intermediate metabolism of fish. When there is a reduction of hepatic glycogen, which might be caused by exposure to chemical stress, this activity provides a useful route for coping with additional energy demands for detoxification, indicating metabolic disorders (Almeida and Oliveira 2010; Sancho et al. 2010; Kaviraj and Gupta 2014). Our results show that the effect of the fungicide on LDH activity in fish is consistent with other studies where high LDH activity is highlighted as a marker of tissue damage in fish and has been considered a good diagnostic tool in ecotoxicology (Rao 2006). Similar results were found in zebrafish exposed to other triazole fungicides such as tebuconazole and triciclazol (Sancho et al. 2010). An important activation of this enzyme has also been reported with fish exposed to other pesticides such as pyrethroids (Kaviraj and Gupta 2014).

According to the analysis applied, time was not a significant factor regarding the biomarker responses throughout the experiment. However, we observed that significant inhibition of ChE activity in brain and muscle as well as CAT activity induction in gills of fish exposed to chlorpyrifos occurred as soon 48 h after exposure to the pulse. Significant changes in the EROD responses in the liver of fish exposed to the pesticide mixture and the LDH responses in muscle of fish exposed to difenoconazole were observed only after 96 h of exposure.

We consider that to expand the study on the metabolic capacities of aquatic organisms, the measurement of glycolytic capacities (pyruvate kinase), aerobic capacities (citrate synthase, cytochrome c oxidase), and lipid metabolism (glucose-6-phosphate dehydrogenase) should be included (Gauthier et al. 2011; Caron et al. 2016). Further, studies carried out with difenoconazole recommend the use of biomarkers more related to the mode of action of triazole fungicides, for example, the measurement of lipid levels in the liver and the expression of genes related to lipid synthesis and metabolism (Mu et al. 2015). Finally, to adequately reflect the toxicity of the chlorpyrifos/difenoconazole mixture in the natural environment, it is recommended that studies be complemented with chronic exposures and a biomarker battery, taking into account other levels of biological organization.

Only significant responses of biomarkers were included and discussed. However, we include in the Supplemental Data results of measurements where no significant differences were detected but trends could be observed. In this regard, higher values of CAT activity and LPO were measured in fish exposed to each pesticide individually, especially in liver (Supplemental Data, Figure 1A and C). Meanwhile, an apparent increase of

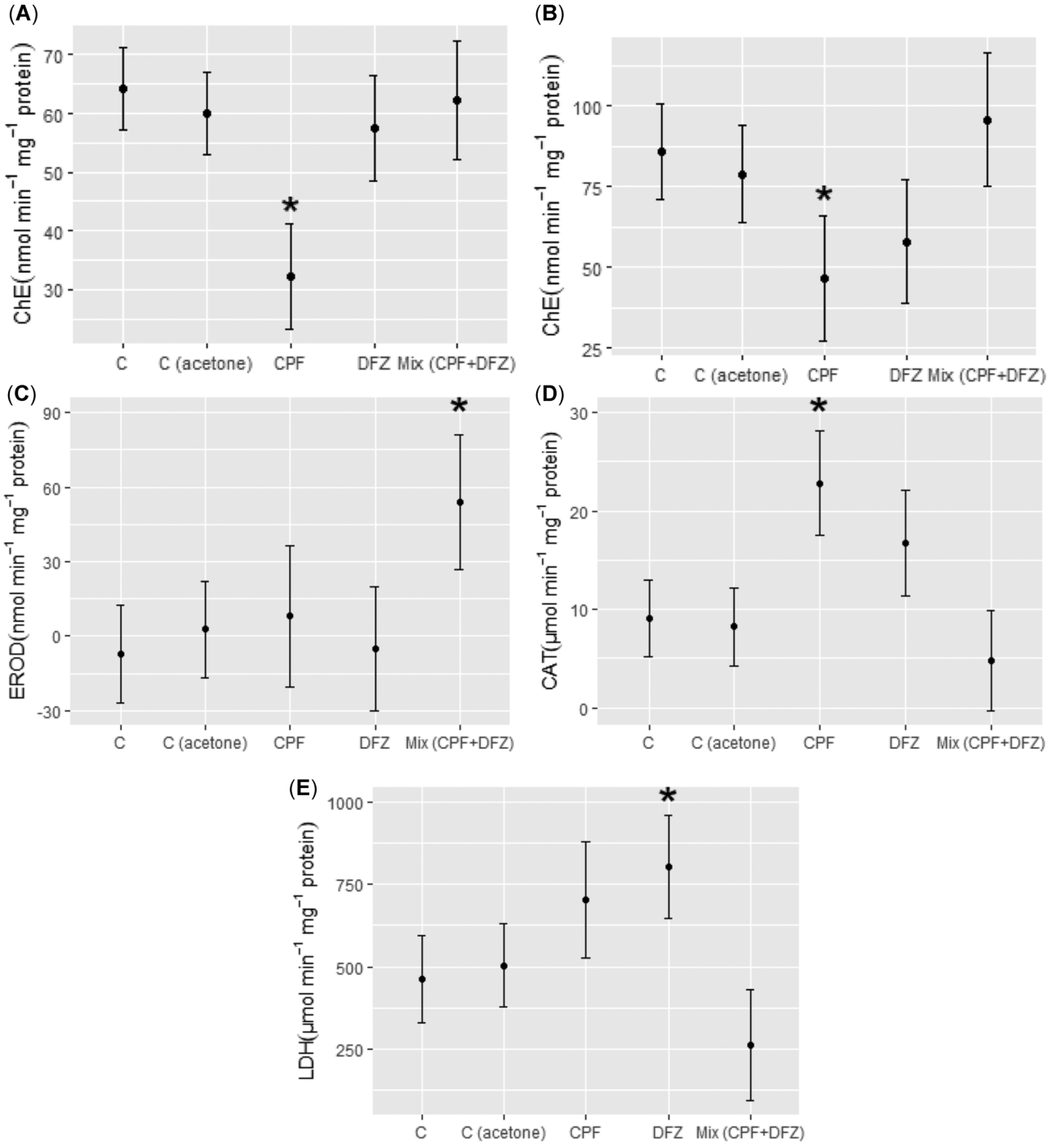

FIGURE 3: Cholinesterase activity in brain (**A**) and muscle (**B**), ethoxyresorufin-O-deethylase activity in liver (**C**), catalase in gills (**D**), and lactate dehydrogenase in muscle (**E**) in fish exposed to chlorpyrifos (CPF; 5.5 µg/L), difenoconazole (DFZ; 325 µg/L), and chlorpyrifos + difenoconazole. *Significant difference between treatments and controls. ChE = cholinesterase; EROD = ethoxyresorufin-O-deethylase; CAT = catalase; LDH = lactate dehydrogenase.

GST activity was observed in liver and gills of fish exposed to the mixture (Supplemental Data, Figures 1B and 2B).

The present study shows that exposure of fingerlings of *P. dovii* to 2 pesticides intensively used in banana production induces contrasting biochemical responses if the fish are exposed to the substances individually or in mixture. As expected, ChE inhibition was caused by chlorpyrifos; but contrary to our hypothesis, EROD activity was not induced by difenoconazole alone. Interestingly, when EROD was induced in fish exposed to the mixture of substances, the neurotoxic, antioxidant, and metabolic responses observed with exposure to individual compounds were absent. This suggests that the cellular events triggered by pesticides can change depending on what substances are in a cocktail, and this should be thoroughly studied to better characterize the risk that mixtures represent for aquatic ecosystems.

TABLE 3: Equivalent real concentrations by initial percentage of recovery of chlorpyrifos and difenoconazole used for different assays carried out during the assessment

	Concentration ranges (µg/L)	
Bioassay	CPF	DFZ
Acute toxicity tests	30.37, 28.48, 26.58, 24.68, 22.78	1460, 1277, 1095, 912, 730, 547
Sublethal assays	5.25, 2.62, 1.31, 0.65, 0.33, 0.16	748, 639, 529, 420, 310, 201
Assays with 10% of estimated LC50	3.6	234
Mixture	0.9 (CPF) + 159 (DFZ)	

CPF = chlorpyrifos; DFZ = difenoconazole; LC50 = median lethal concentration.

CONCLUSIONS

Transient exposure to an environmentally relevant concentration of chlorpyrifos elicited a significant neurotoxic response in *P. dovii* that can be observed early after the exposure. Although difenoconazole is less toxic than chlorpyrifos, it produces important enzymatic alterations related to metabolism and energy cost. Physiological responses provoked by exposure of *P. dovii* to the mixture of these pesticides diverge from the responses produced by exposure to individual compounds. Biotransformation, antioxidant, neurotoxic, and energy metabolism–related responses triggered by one exposure scenario can lead to no response in other scenarios.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5048.

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