Environmental Toxicology and Chemistry—Volume 41, Number 11—pp. 2688–2699, 2022
Received: 8 March 2022 | Revised: 12 April 2022 | Accepted: 15 July 2022

Environmental Toxicology

The Amphibian Short-Term Assay: Evaluation of a New Ecotoxicological Method for Amphibians Using Two Organophosphate Pesticides Commonly Found in Nature—Assessment of Biochemical, Morphological, and Life-History Traits

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Abstract: Amphibia is the most threatened class among vertebrates, with >40% of the species threatened with extinction. Pollution is thought to alter amphibian population dynamics. With the growing interest in behavioral ecotoxicology, the neurotoxic organophosphate pesticides are of special concern. Understanding how exposure to neurotoxics leads to behavioral alterations is of crucial importance, and mechanistic endpoints should be included in ecotoxicological methods. In the present study, we tested an 8-day assay to evaluate the toxicity of two organophosphates, diazinon and chlorpyrifos, on *Xenopus laevis*, that is, on biochemical, morphological, and life-history traits related to locomotion capacities. The method involves measuring biomarkers such as glutathione-*S*-transferase (GST) and ethoxyresorufin-*O*-deethylase (EROD; two indicators of the detoxifying system) in the 8-day-old larvae as well as acetylcholinesterase (AChE) activity (involved in the nervous system) in 4-day-old embryos and 8-day-old larvae. Snout-to-vent length and snout-to-tail length of 4-day-old embryos and 8-day larvae were recorded as well as the corresponding growth rate. Fin and tail muscle widths were measured as well for testing changes in tail shape. Both tests showed effects of both organophosphates on AChE activity; however, no changes were observed in GST and EROD. Furthermore, exposure to chlorpyrifos demonstrated impacts on morphological and life-history traits, presaging alteration of locomotor traits. In addition, the results suggest a lower sensitivity to chlorpyrifos of 4-day-old embryos compared to 8-day-old larvae. Tests on other organophosphates are needed to test the validity of this method for the whole organophosphate group. *Environ Toxicol Chem* 2022;41:2688–2699. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Amphibians; Organic contaminants; Aquatic toxicology

INTRODUCTION

For 30 years now, it has been widely accepted that amphibian populations worldwide have been facing a global decline (Barinaga, 1990; Collins, 2010; European Food Safety Authority [EFSA] Panel on Plant Protection Products and Their Residues et al., 2018). With >40% of species threatened with extinction, Amphibia is the most threatened class among the vertebrates (International Union for Conservation of Nature, 2022). Among other threats, such as habitat loss, disease, and invasive species, chemicals are thought to contribute to this global decline. In particular, amphibians are likely to be exposed to substances, such as pesticides used in farming. Indeed, these chemicals present in treatments sprayed on crops are taken up by rainwater flow directly in amphibian habitats (Chen et al., 2019). Thus, amphibians are exposed to pesticides at crucial periods of their life cycle: reproduction and metamorphosis (Sandin et al., 2018).

Pesticides have been demonstrated to have numerous effects on survival, reproduction, growth, development, and

This article includes online-only Supporting Information.

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Published online 20 July 2022 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5436

the immune and nervous systems of amphibians (EFSA Panel on Plant Protection Products and Their Residues et al., 2018; Peltzer et al., 2019). Among these compounds, those interacting with behavior are of crucial concern in amphibians. Indeed, individual behavioral traits are known to influence individual fitness and therefore population dynamics (Ballew et al., 2017; Ford et al., 2021). Recently, the necessity to develop approaches for evaluating the effects of pollutants on wildlife behavior was highlighted by an international workshop (Ford et al., 2021). More specifically, the workshop emphasized the mechanisms underlying behavioral alterations, which are barely understood but relevant to the adverse outcome pathway (AOP; Ankley et al., 2010); the AOP pictures the effects of a pollutant from its binding to a biological molecule to the impacts at the population, community, or ecosystem level. The workshop recommended adapting current protocols for disentangling the mechanisms underlying the behavioral changes.

Different protocols exist for testing amphibians. Among those, the frog embryo teratogenesis assay—*Xenopus* (FETAX) from ASTM International was originally developed "as an indicator of potential human developmental health hazards" (ASTM International, 1998). This protocol is a convenient test for screening molecules. Indeed, the 3Rs (Replacement, Reduction, and Refinement) do not apply to the embryonic stage in Europe (Sneddon et al., 2017), and its short duration (4 days) makes it suitable for a large substance screening assessment. Thus, it can be used as a preliminary test for screening the most toxic molecules and evaluating the relevant range of concentrations to test before a chronic behavioral assay. Nevertheless, the literature suggests that, in amphibians, 4-day-old embryos present a lower sensitivity to contaminants than subsequent stages (Berrill et al., 1998; Edginton et al., 2004; EFSA Panel on Plant Protection Products and Their Residues et al., 2018; Ortiz-Santaliestra et al., 2017; Yu, Wages, Cai, et al., 2013). This leads to an underestimation of chemical toxicity and thus could lead to unnecessary pain to animals. Indeed, if the range of concentrations used for a chronic assay were determined based on FETAX protocols, an overmortality of the subsequent stages could occur. One possibility to overcome these disadvantages is to develop a method that is still short but that covers a period >4 days.

In the present study, we used two organophosphate insecticides (OPIs) commonly found in nature for testing an 8-day protocol that could eventually be extended to other OPIs. These compounds form one of the largest groups of chemicals used as insecticides and represent ecotoxicological risks in both developed and developing countries (Derbalah et al., 2019; Malhat et al., 2018). For this test, we selected the diazinon and chlorpyrifos insecticides. Both target acetylcholinesterase (AChE) activity in insects, an enzyme involved in the nervous system. Although both are prohibited within the European Union and Switzerland, their use is still authorized in several countries including Brazil (Agência Nacional de Vigilância Sanitária, 2022), an important amphibian hotspot. Besides, numerous studies have illustrated their toxicity to amphibians and fish, representing a substantial source of information for developing this method (see Bonifacio et al., 2020; Colombo et al., 2005; EFSA Panel on Plant Protection Products and Their Residues et al., 2018). In this assay, we focused on examples of biochemical, morphological, and life-history traits because they are known to influence the behavior and thus represent good mechanistic endpoints. The species used in the present study was *Xenopus laevis*, a common model organism for amphibians.

As biochemical traits, we selected three enzyme activities. We measured the AChE activity as a potential mechanistic insight of behavioral changes because it reveals alterations of the nervous system. As the OPIs' target, we expect AChE's activity to be inhibited with increasing OPI concentrations. Glutathione-*S*-transferase (GST) and ethoxyresorufin-*O*-deethylase (EROD) activities were also measured as indicators of pesticide metabolism (Amiard-Triquet et al., 2012). These enzymes being involved in detoxification process, we expect their activities to be induced with increasing OPI concentrations. In addition to biochemical biomarkers, we quantified several morphological traits during the chlorpyrifos test. Because locomotion is highly related to body shape (Van Buskirk & McCollum, 2000), morphological traits represent potential endpoints for understanding the mechanisms behind behavioral changes. Snout-to-vent length (SVL) was measured on 4-day-old embryos (Nieuwkoop et al., 2020; Nieuwkoop-Faber [NF] Stage 45, hereafter referred to as *embryos*) and 8-day-old larvae (NF Stage 48, hereafter referred to as *larvae*). Growth rate was quantified from these two previous metrics as a life-history trait. Because of the reallocation of energy from growth to the detoxification function, we expect lower SVL and growth rate with increasing OPI concentrations. We also measured the snout-to-tail length (STL) on embryos and larvae and the fin width and tail muscle width on embryos. These parameters were used to compute the SVL-to-STL ratio for embryos and larvae as well as the fin width-to-muscle width ratio for embryos for testing potential effects of exposure on body shape. During the chlorpyrifos test, AChE activity and SVL were measured on both embryos and larvae. Because the literature suggests that embryos are less sensitive to chemicals (Berrill et al., 1998; Edginton et al., 2004; EFSA Panel on Plant Protection Products and Their Residues et al., 2018; Ortiz-Santaliestra et al., 2017; Yu, Wages, Cai, et al., 2013), we expect the larval responses to be of higher magnitude than those of the embryos.

MATERIAL AND METHODS

Test organisms and husbandry conditions

Egg acquisition. African clawed frog (*X. laevis*, Daudin, 1802) eggs were obtained from a wild-type breeding colony reared at the Centre Hospitalier Universitaire Vaudois, Switzerland (approval number A31113002); and the entire experimental procedure was approved by the veterinary and ethics committee (VD3521a). Adults were maintained under a 12:12-h light: dark cycle at a temperature of 21 °C and fed 6 g of fish food (Neo Grower) twice a week. To stimulate egg deposition,

the females were injected with human chorionic gonadotropin (15 IU 30 h prefertilization and 750 IU ~6 h prefertilization). When the females began laying eggs, the males were euthanized by intracelomic injection of 0.1 ml of 300 mg/ml pentobarbital. Once individuals were unconscious, the spinal cord was severed. Gonads were extracted after dissection and crushed in 2 ml F1 solution (4.56 g NaCl, 0.33 g KCl, 0.28 g $CaCl_2$, 0.03 g $MgCl_2$, 0.34 g $NaHCO_3$, and 5.96 g *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid in 1 L deionized water). Eggs were collected in a dry Petri dish by gently massaging the abdomen of the females. Once this step was completed, the eggs were sprayed with testis homogenate for fertilization. After 5 min of contact with sperm, eggs were covered with water to initiate egg membrane transformation. After the dorsoventral polarization phase, eggs were collected in a 50-ml vial and brought to the laboratory. The females and males used in each test were different individuals.

Egg dejellying. At stage NF 8, the egg mass was split into two equal batches. One of these batches was dejellied by bathing in a 2% L-cysteine solution buffered at pH 8.1, while the other batch was kept entire.

Testing

Test substances. Diazinon (Chemical Abstracts Service [CAS] no. 333-41-5, Pestanal; purity ≥98%) and chlorpyrifos (CAS no. 2921-88-2, Pestanal; purity ≥98%) were used as test compounds for the amphibian short-term assay and were supplied by Merck. Each pesticide concentration was quantified in 12-well plates (only for chlorpyrifos) and 125-ml plastic containers (diazinon and chlorpyrifos). The pesticide uptake by individuals is thought to influence the pesticide concentrations. Because we assumed that the maximum uptake occurs when the individual reaches the largest size, we quantified pesticides on samples collected during the last renewals of embryonic (days 3–4) and larval (days 7–8) stages. The quantification method consisted of triplicate injections using liquid chromatography coupled with tandem mass spectrometry. The limits of detection were 5 and 2.3 ng/L for diazinon and chlorpyrifos, respectively. Because diazinon and chlorpyrifos decreased over 24 h, the measured arithmetic mean between t_0 and $t_{24\,h}$ was used for the analyses and the figures. Concentration values are available in Supporting Information, Tables 1 and 2.

Stock solutions. The FETAX solution was used as the dilution water for the stock, test, and control solutions. The FETAX solution was composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \times 2H_2O$, and 75 mg $MgSO_4$ per liter of ultrapure water. As suggested by the larval amphibian growth and development assay (Organisation for Economic Co-operation and Development, 2015), the iodide concentration (I^-) was 10 µg/L. The pH of the final solution ranged from 7.7 to 7.9. A 20-mg/L diazinon stock solution was prepared in the FETAX solution, while a 1-mg/L chlorpyrifos stock solution was prepared in dimethyl sulfoxide (DMSO) with a proportion of 0.002% v/v DMSO/FETAX, as suggested by Hutchinson et al. (2006). Stock solutions were stored in the dark at ambient temperature during the tests. The test solutions were prepared daily by diluting the stock solutions. In the chlorpyrifos tests, the stock solution was diluted with a 0.002% v/v DMSO/FETAX solution.

Physicochemical parameters. During the tests, dissolved oxygen, pH, and conductivity were measured each day during medium renewal on fresh medium and 24-h-old medium. The average values for these parameters were 8.58 ± 0.11 mg/L, 7.8 ± 0.07, and 1647.12 ± 17.90 µS/cm, respectively.

Test conditions. Embryos were continuously exposed to six nominal diazinon concentrations (0, 0.0001, 0.001, 0.01, 0.1, and 1 mg/L) and five concentrations of chlorpyrifos (0, 0.0001, 0.001, 0.01, and 0.1 mg/L) in addition to a solvent control (0.002% v/v DMSO/FETAX). Exposure lasted from 5 h postfertilization (hpf) to 8 days postfertilization. The method was composed of two phases: the embryonic phase (from 5 hpf to day 4, stage NF 45) and the larval stage (from day 5 to day 8, stage NF 48). During the tests, individuals were reared in a climatic chamber at 21 ± 1 °C with a 12:12-h light: dark cycle and an illumination of 680 lx. The test/control solutions were renewed daily. The locations in the chamber were assigned randomly. From day 5 to the end of the experiment, individuals were fed once per day with 60 µl of a 1:1 (m/m) mixture of spirulin:tetrafin (24 g:24 g/L; JBL Spirulina Premium and JBL Novo Bel). Both tests were performed by the same operator.

For limiting adsorption of diazinon and chlorpyrifos, 12-well plates and 125-ml plastic containers were preconditioned 24 h prior to their use, and medium was renewed before the beginning of the exposure.

Diazinon test. Twelve embryos from each dejellying condition were exposed in 12-well plates filled with 2 ml of test/control solutions during the first phase. Each concentration/dejellying condition (e.g., exposure to 0.1 mg diazinon/L of nondejellied eggs) was run in one replicate only. At day 5, 10 individuals from each concentration/dejellying condition (120 in total) were randomly selected and individually transferred to 125-ml plastic containers filled with 90 ml of test/control solutions until the end of the experiment. At day 8, larvae were euthanized in a 2-mg/L tricaine mesylate solution buffered at pH 7, quickly frozen in liquid natrium, and stored at −80 °C for further biochemical biomarker measurements.

Chlorpyrifos test. Some improvements were made to this method. First, during the embryonic phase, each concentration/dejellying condition was run in three replicates. This choice allowed us to retain 120 supernumerary embryos, which were euthanized and stored at −80 °C for further biochemical biomarker measurements. When transferred to 125-ml plastic containers, individuals were pseudorandomly selected, including four random individuals from Plate 1 and three random individuals from Plates 2 and 3. Finally, pictures of individuals

were taken at day 4 and day 8 for morphological measurement of body length and growth rate.

Morphological traits

Pictures taken during the chlorpyrifos test were analyzed using ImageJ software. Individual SVLs were extracted from pictures of embryos and larvae. The SVL was measured as the distance between the middle of the mouth and the extremity of the intestines, as described in Figure 1. The growth rate was measured as the larvae SVL divided by the embryo SVL. Measurements of fin width and muscle width were recorded on embryos only because of larval shape, which avoided keeping individuals laid laterally while taking pictures. These metrics were, respectively, measured as the distance between the vent and the opposite fin edge and the overlapping distance between muscle edges, as described in Figure 1. This measurement was performed three times for each individual, and the individual means were used for the statistical tests. All morphological parameters were measured by the same operator.

Enzymatic activities

Levels of AChE, EROD, and GST were quantified in larvae. The low amount of biological tissue in embryos made the measurement of multiple biomarkers impossible in a single individual. Based on the results for 8-day-old larvae, we decided to quantify AChE only in embryos. All biochemical biomarkers were measured using the same operator.

Homogenization and protein quantification. Euthanized individuals were frozen at −80 °C in reinforced tubes with approximately 40 ceramic beads and one steel bead. On the day of measurement, individuals were thawed and homogenized at 7200 rpm for 60 s in phosphate-buffered saline (PBS; 100 mM, pH 7.8) supplemented with a cocktail of protease inhibitors (Thermo Scientific™ Halt™ Protease Inhibitor Cocktail).

Protein concentration. The proteins were quantified spectrophotometrically using a bicinchoninic acid (BCA) assay (BCA Assay Kit; QuantiPro™). The reaction medium consisted of 100 µl of BCA reagent and 100 µl of sample. Optical density was measured using a multiplate reader capable of measuring the absorbance at 562 nm.

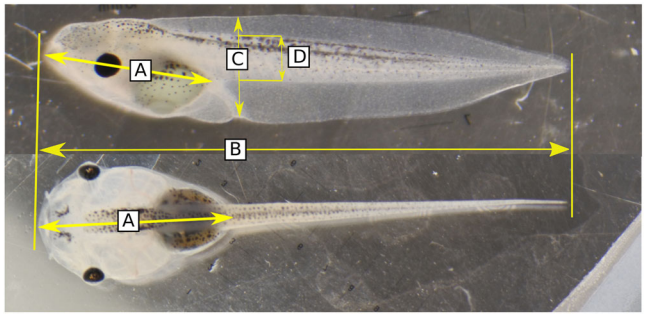

FIGURE 1: Morphological endpoint measurements on embryos (top) and larvae (bottom). (**A**) Snout-to-vent length, (**B**) snout-to-tail length, (**C**) fin width, (**D**) muscle width.

AChE. Activity of AChE was measured spectrophotometrically according to the method described by Ellman et al. (1961) and modified by Xuereb et al. (2009). The reaction medium consisted of 330 ml PBS (100 mM, pH 7.8), 20 µl of 0.425 mM 5.5-dithio-bis (2-nitro-benzoic acid; DTNB), 10 µl of acetylthiocholine iodide (1 mM), and 20 µl of sample. Kinetics were measured using a multiplate reader capable of measuring the absorbance at 405 nm. Readings were performed every 15 s for 6 min. Enzyme activity was expressed as moles per minute per milligram of protein, using a molar extinction coefficient of $1.36\ 10^{-4}$/M/cm.

EROD. Activity of EROD was measured using fluorescence based on the method described by Burke and Mayer (1974). The reaction medium consisted of 150 µl of 0.162 mM 7-ethoxyresorufin, 2.5 mM nicotinamide adenine dinucleotide phosphate, and 30 µl of sample. Kinetics were measured using a multiplate reader with the following parameters: excitation wavelength, 535 nm; emission wavelength, 590 nm; and kinetic duration, 30 min.

GST. Activity of GST was determined spectrophotometrically using the method described by Habig et al. (1974). The reaction medium consisted of 150 ml of PBS (100 mM, pH 6.5), 180 µl of a mixture of glutathione (200 mM), 1-chloro-2,4-dinitrobenzene (40 mM), and 20 µl of sample. The kinetics were measured using a multiplate reader capable of measuring absorbance at 340 nm. Readings were performed every 15 s for 6 min, and the enzymatic activity was expressed as moles per minute per milligram of protein, applying a molar extinction coefficient of 9.6 mM/cm.

Statistical analysis

All statistical analyses were performed using R software (R Foundation for Statistical Computing, 2021).

Extreme outliers were removed from the data set using the boxplot method. An extreme outlier was defined as a data point lying outside three times the interquartile range.

Test methods for diazinon and chlorpyrifos were different regarding the nature of replicates. So were the statistical approaches. During the diazinon test, the replicates were the individual larvae within a plate. We then performed simple linear models for assessing the effects of exposure and dejellying conditions, in addition to their interaction. During the chlorpyrifos test, individual larvae were considered to be pseudoreplicates because three replicated plates were used for each concentration/dejellying condition. We then performed linear mixed-model effects for assessing the effects of exposure and dejellying conditions, in addition to their interaction. In these mixed-effect models, the plate number was used as a random effect.

For each approach (i.e., simple and mixed-effects models), we compared three models using analysis of variance: Model 1

had concentration as a fixed effect, Model 2 had concentration and dejellying condition as fixed effects, and Model 3 had concentration, dejellying condition, and their interaction as fixed effects. For every studied parameter in the diazinon and chlorpyrifos tests (e.g., AChE, growth rate), comparisons showed no difference between Model 3 and Model 2 or between Model 2 and Model 1. This suggests no interaction between concentration and dejellying condition on the studied parameters nor a main effect of dejellying. A main effect is the effect of an explanatory variable on the response variable without taking into account another explanatory variable, in opposition to an interaction effect between two explanatory variables. Therefore, Model 1 was used for the whole of our study. Normality and homogeneity of residuals were graphically checked. When the residuals were not reasonably normally distributed, a log transformation was applied to data, and normality was tested again. After transformation, the dependent variable distributions were reasonably normally distributed. The p values resulting from the models were adjusted to control for the familywise error rate using the Bonferroni-Holm method (Holm, 1979). The α significance level of all tests was set at 0.05. In the pairwise comparisons, the reference group is composed of negative control individuals only.

RESULTS

Negative and solvent control

Comparison between the diazinon and chlorpyrifos tests reveals different magnitudes of AChE (respectively, Figures 2A and 3B) and GST (respectively, Figures 2C and 3D) activities in the negative controls for larvae. Activity of AChE in the diazinon test was approximately three times lower than that in the chlorpyrifos test (respectively, 5.88 and 17.95 nmol/min/mg protein). Activity of GST was more than two times lower in the diazinon test than that in the chlorpyrifos test (respectively, 0.188 and 0.448 nmol/min/mg protein). Activity of EROD was similar in both tests (respectively, 2.48 and 2.06 pmol/min/mg protein; Figures 2B and 3C).

Regarding chlorpyrifos results at both stages in negative controls, we observed a much lower AChE activity in embryos than in larvae, with 0.43 and 17.95 nmol/min/mg protein, respectively (Figure 3A,B). The embryos are not very active compared to larvae, and such results were expected because AChE is involved in muscle contractions.

With respect to the solvent control, no impact of DMSO was observed graphically or statistically for every recorded parameter except for the embryos' fin width to muscle width ratio. In this condition, individuals in the solvent control demonstrated a higher ratio (Figure 4) compared to the control.

Diazinon test

Biochemical biomarkers. The results are reported in Table 1. The mean AChE activity under the control condition was 5.88 nmol/min/mg protein (Figure 1A). Although non-significant, a decrease seems to start at a concentration of 0.095 mg/L, with a loss of 23% of activity (mean activity = 4.52 nmol/min/mg protein). At a concentration of 0.896 mg/L, we can observe a significant loss of 48% of AChE activity

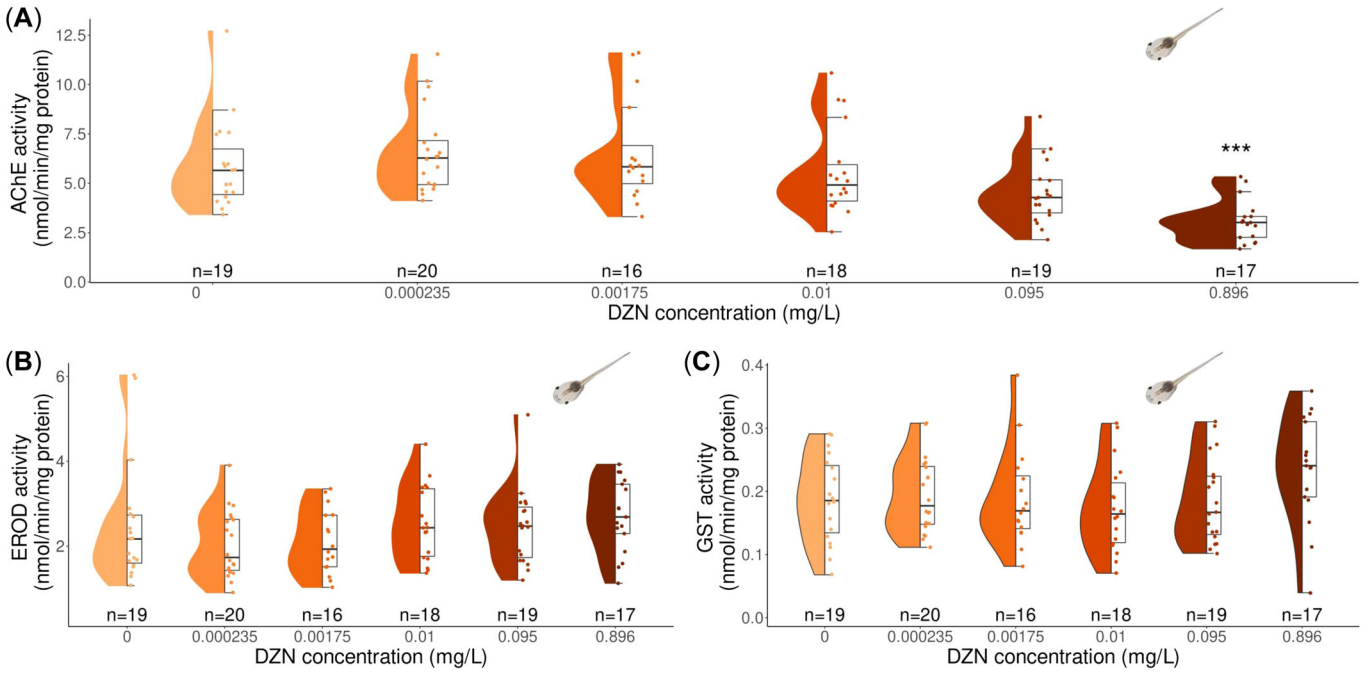

FIGURE 2: Acetylcholinesterase (**A**), ethoxyresorufin-*O*-deethylase (**B**), and glutathione-*S*-transferase (**C**) activities measured on 8-day-old larvae continuously exposed to six concentrations of diazinon (*$p = 0.01$–0.05; **$p = 0.001$–0.01; ***$p < 0.001$). p values are adjusted with the Bonferroni-Holm method. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histograms of the data distribution. Extreme outliers are not displayed in the graphs. AchE = acetylcholinesterase; DZN = diazinon; EROD = ethoxyresorufin-*O*-deethylase; GST = glutathione-*S*-transferase.

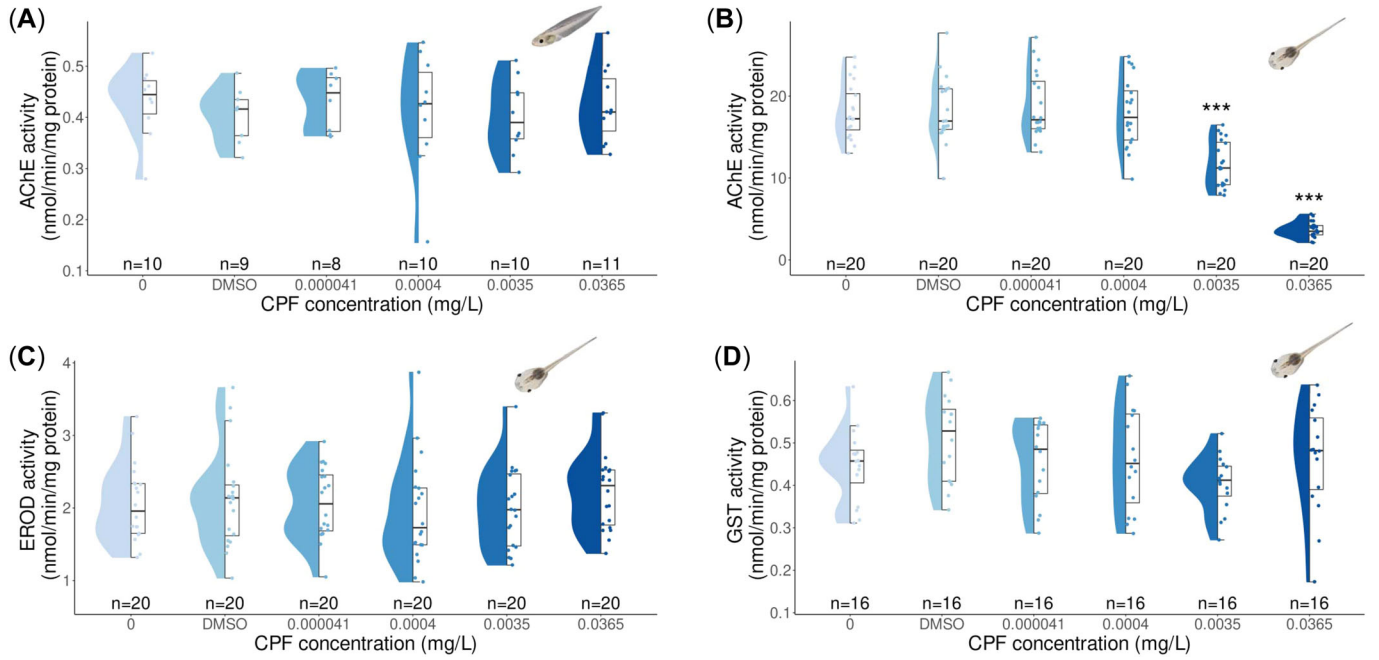

FIGURE 3: Acetylcholinesterase measured on 4-day-old embryos (**A**) and 8-day-old larvae (**B**) and ethoxyresorufin-*O*-deethylase and glutathione-*S*-transferase activities measured on 8-day-old larvae (**C**,**D**) continuously exposed to six concentrations of chlorpyrifos (*$p = 0.01$–0.05; **$p = 0.001$–0.01; ***$p < 0.001$). p values are adjusted with the Bonferroni-Holm method. Plates of origin are set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histograms of the data distribution. Extreme outliers are not displayed in the graphs. AchE = acetylcholinesterase; DMSO = dimethyl sulfoxide; CPF = chlorpyrifos; EROD = ethoxyresorufin-*O*-deethylase; GST = glutathione-*S*-transferase.

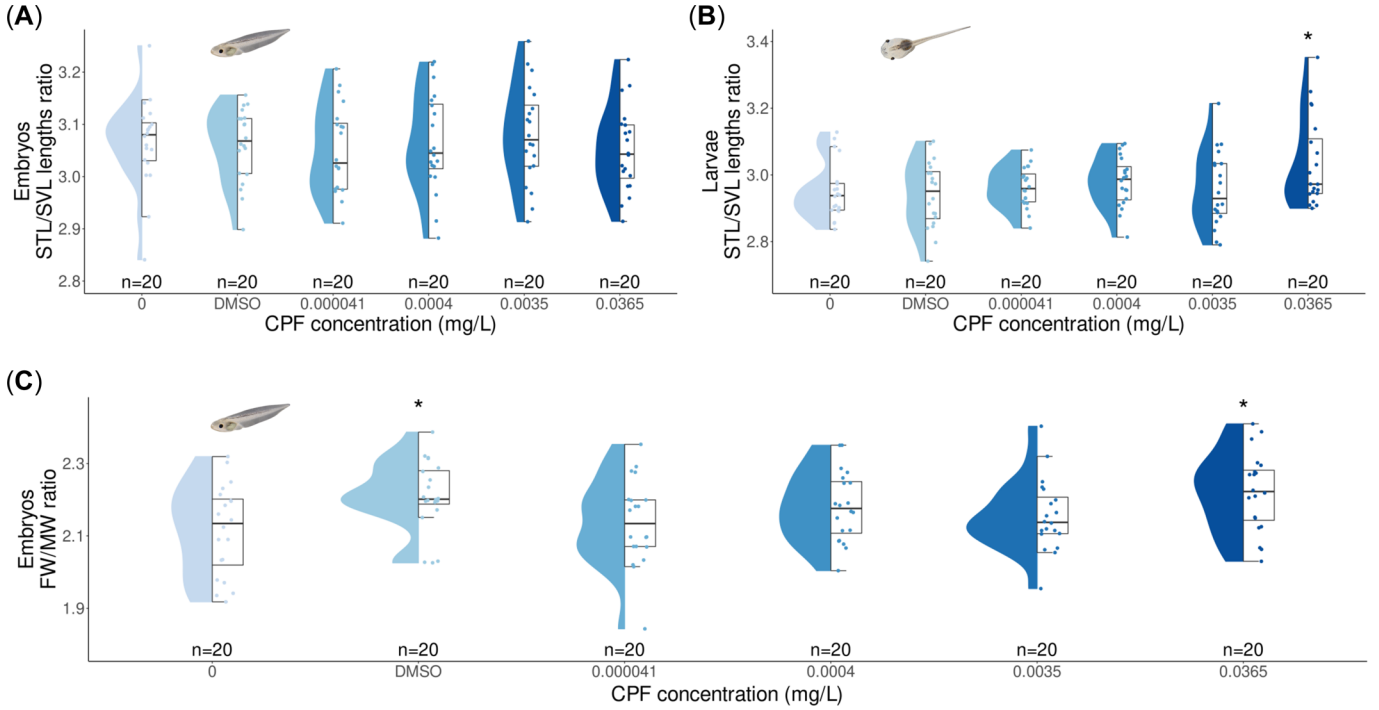

FIGURE 4: Snout-to-tail to snout-to-vent length ratio measured on 4-day-old embryos (**A**) and 8-day-old larvae (**B**) and fin width to muscle width ratio (**C**) measured on 4-day-old embryos continuously exposed to six concentrations of chlorpyrifos (*$p = 0.01$–0.05; **$p = 0.001$–0.01; ***$p < 0.001$). p values are adjusted with the Bonferroni-Holm method. Plates of origin are set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. STL = snout-to-tail length; SVL = snout-to-vent length; DMSO = dimethyl sulfoxide; CPF = chlorpyrifos; FW = fin width; MW = muscle width.

TABLE 1: Outputs of simple linear models testing the effects of diazinon on 8-day-old larval acetylcholinesterase, ethoxyresorufin-*O*-deethylase, and glutathione-*S*-transferase activities

	Log-transformed AChE activity			EROD activity			GST activity		
Predictors	Est.	95% CI	*p*	Est.	95% CI	*p*	Est.	95% CI	*p*
Intercept	1.72	1.56–1.87	**<0.001**	2.48	2.04–2.92	**<0.001**	0.19	0.16–0.22	**<0.001**
Conc. 0.000235 mg/L	0.12	−0.09 to 0.34	0.778	−0.45	−1.06 to 0.17	0.76	0.01	−0.04 to 0.05	1
Conc. 0.00175 mg/L	0.09	−0.14 to 0.32	0.846	−0.38	−1.02 to 0.27	1	0	−0.05 to 0.05	1
Conc. 0.01 mg/L	−0.06	−0.29 to 0.16	0.846	0.07	−0.56 to 0.70	1	−0.01	−0.06 to 0.03	1
Conc. 0.095 mg/L	−0.26	−0.48 to −0.04	0.078	−0.02	−0.64 to 0.60	1	0	−0.05 to 0.04	1
Conc. 0.896 mg/L	−0.65	−0.88 to −0.43	**<0.001**	0.23	−0.41 to 0.87	1	0.05	0.00–0.09	0.216
Observations		109			109			109	
R^2/R^2 adjusted		0.382/0.352			0.061/0.016			0.072/0.027	
Deviance		12.04			95.718			0.512	

p-value adjustment: Bonferroni-Holm method. Bold indicates significant *p* values.
AChE = acetylcholinesterase; EROD = ethoxyresorufin-*O*-deethylase; GST = glutathione-*S*-transferase; Est. = estimated; CI = confidence interval; Conc. = concentration.

(mean activity = 3.07 nmol/min/mg protein). Regarding EROD and GST activities, no significant impact of concentration was observed in this experiment (respectively, Figure 2B,C).

Chlorpyrifos test

Biochemical biomarkers. The results are reported in Table 2. Although no changes were observed in AChE activity in embryos (Figure 3A), results on larvae demonstrate significant decreases of 35.4% (mean activity = 11.59 nmol/min/mg) at a concentration of 0.0035 mg/L and 79.5% (mean activity = 3.67 nmol/min/mg protein) at a concentration of 0.0365 mg/L (Figure 3B). The mean AChE activity in the control was 17.95 nmol/min/mg protein. Regarding EROD and GST activities, no significant statistical impact of concentration was observed in this experiment (respectively; Figure 3C,D).

Morphological traits. The results are reported in Tables 3 and 4. No significant changes were observed on embryos' SVL (Figure 5A), while in larvae, SVL significantly decreased from 5.53 mm in the control condition to 5 mm at the highest concentration (Figure 5B). The consequence is a significantly decreased 96-h growth rate at the highest concentration (Figure 5C). The embryos' STL to SVL ratio demonstrated no changes (Figure 4A), while the larval STL to SVL ratio showed a significant increase at the highest concentration (Figure 4B) with respective values of 1.08 and 2.01. Lastly, embryos' fin width to muscle width ratio significantly increased at the highest concentration (Figure 4C) from 2.11 in the control condition to 2.22. Surprisingly, a significant increase of the fin width to muscle width ratio occurred in the solvent control, while no changes were detected at the lower concentrations.

DISCUSSION

In the present study, we tested an 8-day protocol with diazinon and chlorpyrifos to evaluate their toxicity to amphibians' biochemical, morphological, and life-history traits at early stages, alteration of which is known to impair some behavioral endpoints.

Impact of dejellying

No impact of dejellying was observed on any of the recorded parameters. Because the amphibian jelly coat was demonstrated to be involved in embryonic protection against pollution (Bosisio et al., 2009), the dejellying conditions were expected to show differences in exposure toxicity. Our results suggest that this does not affect diazinon or chlorpyrifos toxicity.

Solvent concentration

The use of a 0.002% v/v DMSO/FETAX solution did not affect the recorded parameters except for the fin width to muscle width ratio. In that case, the solvent control had a significantly higher ratio than the negative control, though no changes appeared at concentrations <0.0365 mg chlorpyrifos/L. Nevertheless, the low level of significance of the test ($p = 0.047$) leaves some uncertainty, and the toxicity of solvents used in ecotoxicity testing should be investigated further.

Biochemical traits

As expected, the present study demonstrates inhibition of AChE in larvae by both diazinon at a concentration of 1 mg/L and chlorpyrifos at concentrations of 0.01 and 0.1 mg/L. Inhibition of AChE activity has been documented for diazinon and chlorpyrifos on *X. laevis* as well as in other amphibian species (Colombo et al., 2005; Tongo et al., 2012). Although these activities were expected to increase with the level of exposure to diazinon and chlorpyrifos, no effect was observed in larvae. To our knowledge, few have studied the impact of diazinon and chlorpyrifos on larvae GST and EROD activities, with most research on organophosphates focusing on AChE inhibition. But Güngördü et al. (2013) suggest different patterns of correlation between AChE and GST activities. An assumption for the origin of such different patterns is the differential impact of organophosphate metabolites on the detoxification process. Furthermore, EROD is a biomarker used for evaluating the response of the cytochrome P450A1

TABLE 2: Outputs of linear mixed-effects models testing the effects of chlorpyrifos (CPF) on 4-day-old embryo and 8-day-old larval acetylcholinesterase activity and 8-day-old larval ethoxyresorufin-*O*-deethylase and glutathione-*S*-transferase activities

	Embryo AChE activity			Larval AChE activity			Larval EROD activity			Larval GST activity		
Predictors	Est.	95% CI	*p*	Est.	95% CI	*p*	Est.	95% CI	*p*	Est.	95% CI	*p*
Intercept	0.43	0.38–0.48	**<0.001**	17.95	16.19–19.72	**<0.001**	2.06	1.80–2.32	**<0.001**	0.45	0.40–0.50	**<0.001**
DMSO	−0.03	−0.10 to 0.05	1	0.28	−2.22 to 2.77	1	0.07	−0.30 to 0.44	1	0.06	−0.01 to 0.13	0.451
Conc. 0.000041 mg/L	0	−0.07 to 0.07	1	0.76	−1.74 to 3.26	1	0	−0.36 to 0.37	1	0.01	−0.06 to 0.08	1
Conc. 0.0004 mg/L	−0.02	−0.09 to 0.05	1	−0.14	−2.63 to 2.36	1	−0.12	−0.49 to 0.25	1	0.01	−0.06 to 0.09	1
Conc. 0.0035 mg/L	−0.03	−0.10 to 0.04	1	−6.36	−8.85 to −3.86	**<0.001**	−0.06	−0.43 to 0.30	1	−0.04	−0.12 to 0.03	0.873
Conc. 0.0365 mg/L	−0.01	−0.07 to 0.06	1	−14.28	−16.77 to −11.78	**<0.001**	0.17	−0.20 to 0.54	1	0.02	−0.05 to 0.09	1
Observations		58			120			120			96	
Marginal R^2/conditional R^2		0.025/NA			0.721/0.762			0.024/NA			0.086/NA	
Deviance		−139.633			626.899			207.55			−173.477	

Bold indicates significancet *p* values.
AChE = acetylcholinesterase; EROD = ethoxyresorufin-*O*-deethylase; GST = glutathione-*S*-transferase; Est. = estimated; CI = confidence interval; DMSO = dimethyl sulfoxide; Conc. = concentration; NA = not available.

TABLE 3: Outputs of linear mixed-effects models testing the effects of chlorpyrifos on 4-day-old embryo and 8-day-old larval snout-to-vent length and corresponding growth rate

	Embryo SVL			Larvae SVL			Growth rate		
Predictors	Est.	95% CI	*p*	Est.	95% CI	*p*	Est.	95% CI	*p*
Intercept	3.11	3.06–3.16	**<0.001**	5.53	5.42–5.64	**<0.001**	1.78	1.74–1.82	**<0.001**
DMSO	−0.01	−0.08 to 0.06	1	−0.09	−0.25 to 0.07	0.994	−0.02	−0.07 to 0.03	1
Conc. 0.000041 mg/L	0.01	−0.06 to 0.08	1	−0.09	−0.26 to 0.07	0.994	−0.02	−0.07 to 0.03	1
Conc. 0.0004 mg/L	−0.01	−0.08 to 0.06	1	−0.06	−0.22 to 0.10	0.994	−0.01	−0.06 to 0.04	1
Conc. 0.0035 mg/L	−0.03	−0.10 to 0.04	1	0	−0.16 to 0.16	0.994	0.02	−0.03 to 0.07	1
Conc. 0.0365 mg/L	−0.02	−0.10 to 0.05	1	−0.53	−0.69 to −0.36	**<0.001**	−0.16	−0.21 to −0.11	**<0.001**
Observations		120			118			119	
Marginal R^2/conditional R^2		0.012/NA			0.362/0.399			0.380/0.428	
Deviance		−187.163			−9.559			−285.817	

p-value adjustment: Bonferroni-Holm method. Bold indicates significancet *p* values.
SVL = snout-to-vent length; Est. = estimated; CI = confidence interval; DMSO = dimethyl sulfoxide; Conc. = concentration; NA = not available.

TABLE 4: Outputs of linear mixed-effects models testing effects of chlorpyrifos on 4-day-old embryo and 8-day-old larval snout-to-tail length to snout-to-vent length (SVL) ratio, and 4-day-old embryo fin width to muscle width ratio

	Embryo STL/SVL ratio			Larval STL/SVL ratio			Embryo FW/MW ratio		
Predictors	Est.	95% CI	*p*	Est.	95% CI	*p*	Est.	95% CI	*p*
Intercept	3.07	3.03–3.10	**<0.001**	1.08	1.07–1.10	**<0.001**	2.11	2.06–2.17	**<0.001**
DMSO	0	−0.06 to 0.05	1	0	−0.02 to 0.02	1	0.1	0.02–0.17	**0.047**
Conc. 0.000041 mg/L	−0.02	−0.07 to 0.04	1	0	−0.02 to 0.02	1	0.02	−0.05 to 0.10	0.526
Conc. 0.0004 mg/L	0	−0.05 to 0.05	1	0.01	−0.01 to 0.03	1	0.07	−0.01 to 0.14	0.239
Conc. 0.0035 mg/L	0.01	−0.04 to 0.07	1	0	−0.02 to 0.02	1	0.04	−0.03 to 0.12	0.526
Conc. 0.0365 mg/L	−0.01	−0.07 to 0.04	1	0.03	0.01–0.05	**0.041**	0.11	0.03–0.18	**0.027**
Observations		120			120			120	
Marginal R^2/conditional R^2		0.014/NA			0.091/NA			0.108/0.178	
Deviance		−257.61			−489.504			−198.723	

p values adjustment: Bonferroni-Holm method. Bold indicates significancet *p* values.
STL = snout-to-tail length; SVL = snout-to-vent length; FW = fin width; MW = muscle width; Est. = estimated; CI = confidence interval; DMSO = dimethyl sulfoxide; Conc. = concentration; NA = not available.

subfamily. Another assumption is that other cytochrome P450 subfamilies may be involved in detoxifying OPIs in amphibians.

Morphological traits

In the chlorpyrifos test, larvae exposed to the highest concentrations had a smaller body size. Similar results were reported in Richards and Kendall (2003), who showed a decrease in body lengths of what they defined as "metamorphs" (from stage NF 46 to a 96-h exposure) compared to "pre-metamorphs" (from stage NF 14 to a 96-h exposure). However, in their study, the effects occurred at a chlorpyrifos concentration of 0.0001 mg/L, while it occurred at 0.0365 mg/L in the present study. This difference might be explained by the higher number of individuals per exposure condition in their work (mean of 81 individuals per condition), thus increasing test power. Nevertheless, the absence of specification in their study regarding the actual measured concentrations and the method for adjusting the *p* values does not allow us to evaluate the relevance of this assumption. In addition, our method demonstrated the impact of exposure on growth rate. Previously, chlorpyrifos has been shown to disrupt the endocrine system (Ur Rahman et al., 2021). Nevertheless, to our knowledge, only

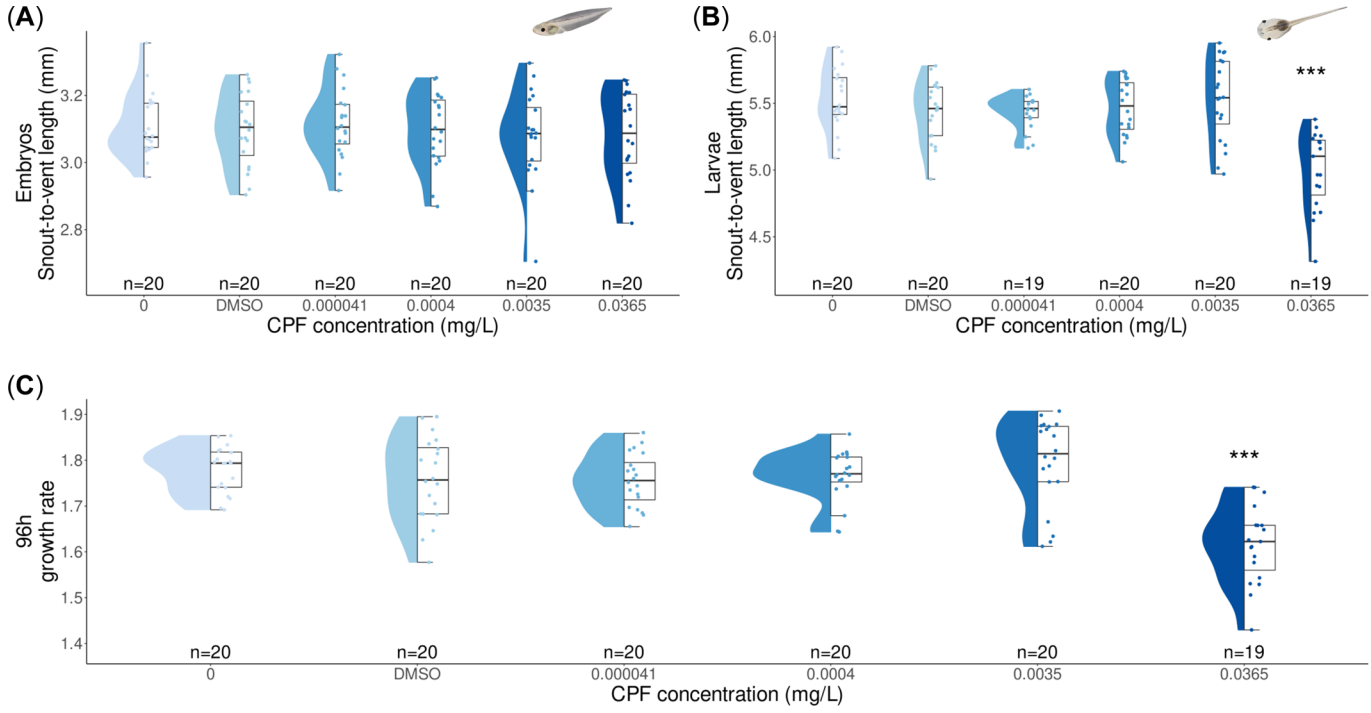

FIGURE 5: Snout-to-vent length measured on 4-day-old embryos (**A**) and 8-day-old larvae (**B**) continuously exposed to six concentrations of chlorpyrifos and the corresponding growth rate (**C**) (*$p = 0.01$–0.05; **$p = 0.001$–0.01; ***$p < 0.001$). *p* values are adjusted with the Bonferroni- Holm method. Plates of origin are set as a random effect in statistical models. The central bar of the boxplot is the group median. Upper and lower hinges correspond to the 25th and 75th quantiles, respectively. Upper and lower whiskers extend from the closest hinge, respectively, to the largest and the smallest values at most 1.5 times the interquartile range. Violins represent the smoothed histogram of the data distribution. Extreme outliers are not displayed in the graphs. DMSO = dimethyl sulfoxide; CPF = chlorpyrifos.

a few studies have investigated the impact of chlorpyrifos on amphibian growth rate (see Wijesinghe et al., 2011). Such changes are likely to have an impact on size at metamorphosis, a life-history trait known to influence behavior, fitness, and ultimately population dynamics (Bredeweg et al., 2019). Because of the decrease of SVL, the STL to SVL ratio was also affected, with larvae exposed to the higher concentration of chlorpyrifos demonstrating a higher ratio. No research demonstrated such effects of OPIs, but Yu, Wages, Cobb, and Maul (2013) documented a lower STL/SVL ratio in *X. laevis* embryos exposed to environmentally relevant concentrations of chlorothalonil, an organochlorine fungicide. The embryo tail shape was impacted as well at the highest chlorpyrifos concentration, presaging alteration of locomotion capacities.

Embryo sensitivity

Although no impact of chlorpyrifos was demonstrated on AChE activity in embryos in the present study, Colombo et al. (2005) showed significant statistical changes in *X. laevis* embryo AChE activity from day 3 at a nominal concentration of 0.1 mg chlorpyrifos/L. Important differences exist between their study and our measurement protocols that can explain this divergence. While the authors incubated pooled embryos (10 individuals per concentration) with DTNB for 10 min before measuring the absorbance, we incubated single individual homogenates for 4 min. Our results are supported by Richards and Kendall (2002), who demonstrated a higher sensitivity of "metamorphs" compared to "premetamorphs" with respective lower nominal concentrations affecting the whole cholinesterase activity of 0.01 and 0.1 mg chlorpyrifos/L. Regarding morphological traits, Richards and Kendall (2003) demonstrated significant decreases in "premetamorph" body length at nominal concentrations of 0.001 and 1 mg/L, while no changes were observed in the present study at similar concentrations. The higher sample size (an average of 83 individuals) in Richard and Kendall's study could explain such a difference. Nevertheless, as mentioned above, the concentrations measured and the method for adjusting the *p* values are not mentioned in their article. Contrary to embryos, the use of larvae allows the measurement of multiple biochemical biomarkers on a single individual. These findings highlight the interest of extending the exposure duration to 8 days. To summarize, our results suggest that larvae are more sensitive to chlorpyrifos than embryos regarding both AChE activity and morphological and life-history traits. More research is needed to extend this suggestion to other OPIs.

Environmental considerations

The concentrations at which the changes mentioned above occur are substantially higher than the concentrations usually measured in the environment. Nevertheless, some articles mention chlorpyrifos environmental concentrations up to 5.49×10^{-3} mg/L in Mexico (Ávila-Díaz et al., 2021) and 11.2×10^{-3} mg/L in Pakistan (Arain et al., 2018), while AChE inhibition occurred at 3.5×10^{-3} mg/L in the present study. This suggests a potential short-term risk for amphibian larvae from these regions. Moreover, diazinon and chlorpyrifos are usually applied together, and the literature suggests a synergistic effect of a mixture of these two pesticides on fish (Laetz et al., 2009).

Limits of the method

An issue highlighted in the present study is the different magnitudes of biochemical biomarker values between different runs of the method, suggesting differences in enzymatic activity between offspring from different breeders. Poor repeatability of biochemical biomarkers is a known issue in ecotoxicology, and factors such as sex ratio and genome are known to modulate between bred responses in fish, for instance (Wang, 2018). A commonly recommended means for diminishing this variability is to increase the sample size.

CONCLUSION

The method proposed in the present study is promising and demonstrates the capacity to evaluate the effects of two organophosphate pesticides on biochemical and morphological traits, both considered to provide possible insight into behavioral alteration mechanisms. To our knowledge, this is the first protocol proposing a set of different mechanistic endpoints related to behavior, a crucial component of amphibian ecology. However, this approach should be validated with other OPIs for testing its suitability to this pesticide group. Besides, although *X. laevis* seems to be very tolerant to pollutants (see Adams et al., 2021; Yu, Wages, Cai, et al., 2013), it is often used as a surrogate species for amphibians. Its capacity to cover other species has been barely investigated, and the difficulty of transferring these results to other species without more comparative studies is obvious. Nevertheless, transposition of the present method to other amphibians would allow for comparative studies and should be tested. We believe that it can be easily optimized for many other amphibian species because most of them have an aquatic development (~85% according to Nunes-de-Almeida et al. [2021]). Besides, AChE, EROD, and GST were already measured in other amphibian species (Venturino & de D'Angelo, 2005; Venturino et al., 2003), and the morphological traits measured in the present method are commonly used in amphibian ecology (see Van Buskirk & McCollum, 2000). Lastly, because this method demonstrated the effects of two OPIs on both the nervous system and morphology, we think that the implementation of behavioral tests should be considered with the aim of studying how the biochemical and morphological alterations are linked to behavioral changes.

Supporting Information—The Supporting information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5436.

Acknowledgment—We thank F. Tisserand for her biochemistry expertise and B. Guillet for her *X. laevis* expertise. We thank A. Ballu, M. Faria, and L. Monbaron for their assistance in the laboratory. We thank F. Breider and D. Grandjean for measuring the pesticide concentrations. The present study was supported by the Swiss National Science Foundation (FNS 205321_182182). Open access funding provided by Universite de Lausanne.

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

Author Contributions Statement—**Laurent Boualit:** Conceptualization; Methodology; Formal analysis; Investigation; Writing—original draft; Visualization; Project administration. **Hugo Cayuela:** Conceptualization; Methodology; Writing—review & editing; Visualization; Supervision. **Loic Cattin:** Investigation. **Nathalie Chèvre:** Conceptualization; Methodology; Writing—review & editing; Supervision; Funding acquisition.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (laurent.boualit@protonmail.com). The data set used in the present study is part of a wider data set currently used for a PhD thesis. As soon as the whole data set is analyzed, the Swiss National Science Foundation (funding this research) will make it mandatory to share the data set on its open-access platform and the link to it will be shared. Until then, the only means to share the data used in the present study is to contact the corresponding author.

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