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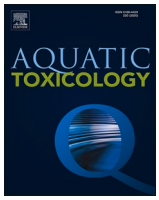

Weak effects of chlorpyrifos at environmentally relevant concentrations on fitness-related traits in agile frogs

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ABSTRACT

The widespread application of pesticides makes it important to understand the impacts of these chemicals on wildlife. Chlorpyrifos, an organophosphate insecticide that is still used *en masse* over large parts of the globe, can affect the development and behavior of non-target organisms and may thereby alter predator-prey interactions. To investigate whether environmentally relevant concentrations of chlorpyrifos affect survival, somatic, cerebral, and sexual development, as well as anti-predator behavior of the agile frog (*Rana dalmatina*), we exposed tadpoles to one of three treatments (0, 0.5, or 5 μg chlorpyrifos / L) either for three days (acute exposure) or throughout larval development (chronic exposure). We measured mortality, activity, and space use in the presence or absence of chemical cues of predatory fish, brain morphology, length of larval development, body mass at metamorphosis and two months later, and phenotypic sex. Compared to control individuals, tadpoles acutely exposed to 5 μg/L chlorpyrifos showed a shorter freezing response to predator cue on the first observation day. Also, chronic exposure to the same concentration decreased body mass at metamorphosis. Neither the chronically nor the acutely applied 0.5 μg/L chlorpyrifos concentration had any significant effect on the evaluated traits. Our results demonstrate that exposure to chlorpyrifos can induce changes in behavior and may result in lowered body mass of agile frog tadpoles, but only if the insecticide is present chronically at relatively high concentrations. Thus, agile frog tadpoles appear to be relatively tolerant to chlorpyrifos, but may suffer from its repeated high-dose application.

1. Introduction

Aquatic pollutants and pesticides in particular have the potential to affect animals via a variety of lethal and sublethal effects, including changes in physiology, development, and reproduction (Köhler and Triebskorn, 2013; Orton and Tyler, 2015). Also, aquatic pollutants can affect several aspects of animal behavior, including changes in microhabitat use (Tierney et al., 2007; Yu et al., 2014), foraging behavior (Pavlov et al., 1992; Semlitsch et al., 1995), and can also cause abnormal motion (Denoël et al., 2013; Levin et al., 2004). Moreover, pollutants can disrupt chemical communication and predator avoidance behavior (Bridges, 1999; Janssens and Stoks, 2012; Scholz et al., 2000). When predators capture, consume, and digest prey, the released chemicals can provide detailed information about predation risk (Bairos-Novak et al., 2019; Hettyey et al., 2015; Schoeppner and Relyea, 2009), which in turn can induce adaptive (and maladaptive) plastic changes in animals (Tollrian and Harvell, 1999). Chemical contaminants may disrupt this process by either acutely interfering with the prey's sensory systems or altering the development of the relevant sensory organs and brain regions (Lürling and Scheffer, 2007; Scott and Sloman, 2004; Tierney et al., 2010). Investigating these sensory and behavioral effects of sublethal exposures is relevant for several reasons. First, changes in behavior are generally the first signs of exposure to pesticides (Sparling et al., 2010), while behavior is an important life-history trait that

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directly influences fitness (Lind and Cresswell, 2005; Weis et al., 2001). Furthermore, measuring behavioral alterations is relatively easy and does not require sacrificing experimental animals. Also, pesticide concentrations that occur in the environment are usually lower than the LC/LD50 values of most chemical contaminants. Consequently, the examination of behavioral changes at environmentally relevant sublethal concentrations appears to be highly commendable, especially if the alternative is the estimation of effects at concentrations that are never experienced under natural conditions (Bridges, 1997; Ford et al., 2021).

Standard toxicity testing and authorization procedures require testing on fish but not on other aquatic vertebrates (Adams and Rowland, 2003; Nikinmaa, 2014). Therefore, the most often used vertebrate model animals are fishes in aquatic toxicology (Melvin and Wilson, 2013). Studying the effects of pesticides on amphibians is, however, similarly important because amphibians are the most severely threatened class of vertebrates (Luedtke et al., 2023), and they are especially susceptible to chemical pollution due to their highly permeable skin and complex life cycle. Furthermore, because many amphibians use small puddles, temporary ponds, and ditches as breeding sites, which are often adjacent to agricultural fields, they can easily become exposed to high concentrations of agricultural contaminants during their larval life (Bridges, 1997), which is the most sensitive life stage due to intense development and growth, and the re-organization of entire organ systems during metamorphosis (Ishizuya-Oka et al., 2010).

Chlorpyrifos (O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate; CPF) is a broad-spectrum organophosphate insecticide that has been applied in large quantities both in agricultural and urban settings since 1965 (Bernabò et al., 2011b). Although the European Union and several non-European countries have restricted or banned its use for food/feed-related applications in recent years, it continues to be applied in many places worldwide (Foong et al., 2020). Moreover, even the countries that banned it for food-related applications continue to use it for non-food applications such as pest control in forestry and mosquito control near water bodies (e.g. Environmental Protection Agency, 2021). These latter applications are especially relevant for many amphibians because many of their species live in wooded and wetland habitats. The primary mode of action of CPF is the inhibition of cholinesterases (ChEs), enzymes that are involved in the normal function of nerve synapses. Blocking of cholinesterases induces neurotoxic effects in insects as well as non-target organisms (Barron and Woodburn, 1995) via the buildup of excessive acetylcholine in the synapses, causing hyperactivity and uncontrolled muscle spasms and, eventually, paralysis, respiratory failure, and death (Barron and Woodburn, 1995; Ruiz de Arcaute et al., 2012). Aquatic environments are easily contaminated by CPF through direct application against mosquitoes, as well as spray drift and runoff from adjacent agricultural areas (Bernabò et al., 2011a; Widder and Bidwell, 2008). CPF concentration in surface waters generally ranges from 0.02 ng/L to 10.8 μg/L but can be as high as 96 μg/L (e.g. Americas: Delgado-Moreno et al., 2011; Jergentz et al., 2005; Kurt-Karakus et al., 2011; Marino and Ronco, 2005; Asia: Arain et al., 2018; Chowdhury et al., 2012; Xue et al., 2005; Europe: Claver et al., 2006; Rico et al., 2021).

CPF can be severely toxic to aquatic invertebrates and fish, and the highest measured environmental concentrations can also cause excessive mortality in amphibian tadpoles (Carriger and Rand, 2008; Giesy et al., 1999; Huang et al., 2020). In non-target aquatic vertebrates exposure to CPF at sublethal concentrations can cause oxidative stress (Oruç, 2010; Xing et al., 2012a, 2012b), histopathological changes (Pal et al., 2012; Xing et al., 2012a, 2012b), damage of the nervous and reproductive system (De Silva and Samayawardhena, 2005; Manjunatha and Philip, 2016; Venkateswara Rao et al., 2005), and genotoxicity (Ali et al., 2008). It can also influence behavior; for example, in fish, it is reported to disturb swimming activity (Levin et al., 2004; Tierney et al., 2007) and olfaction-mediated behaviors (Maryoung et al., 2015; Sandahl et al., 2004; Tilton et al., 2011). In amphibians, an apparent connection between the presence of CPF residues and reductions in population sizes has been reported both at the local (Fellers et al., 2004) and at the landscape scale (Sparling et al., 2001). Experimental studies reported reduced growth and development in tadpoles (Richards and Kendall, 2003; Widder and Bidwell, 2008, 2006), increased incidence of developmental malformations (Bernabò et al., 2011b; Rutkoski et al., 2020; Silva et al., 2020), altered behavior (Barreto et al., 2020; Basso et al., 2022; Henao et al., 2024; Rutkoski et al., 2020; Silva et al., 2020; Spirhanzlova et al., 2023; Widder and Bidwell, 2008) and brain morphology (McClelland et al., 2018; MacClelland and Woodley, 2022; Spirhanzlova et al., 2023; Woodley et al., 2024), as well as disrupted sexual development (Bernabò et al., 2011a). Nevertheless, these studies typically applied relatively high concentrations, so we still have scarce knowledge regarding the ecotoxicological effects of environmentally relevant concentrations of CPF on amphibians (Bernabò et al., 2011b; Giesy et al., 1999; Richards and Kendall, 2003, 2002).

In this study, our aim was to investigate the effects of CPF on the development and antipredator behavior of larval agile frogs (*Rana dalmatina*), a European species showing a decreasing population trend (IUCN, 2023). To achieve this, we exposed tadpoles to one of two ecologically relevant concentrations of CPF throughout their entire larval life and investigated the effects on mortality, length of somatic development, brain morphology, body mass at metamorphosis and two months later in juveniles, and phenotypic sex ratio. To assess if acute exposure to CPF can also cause changes in the abovementioned traits, we also applied the chemical treatments for a brief period of three days. Finally, to assess the effect of CPF on predator-avoidance behaviors, we observed activity and space use of CPF-exposed and control tadpoles in the presence or absence of chemical cues of predatory fish. The agile frog is a highly relevant study species for these questions because it occurs not only in woodlands but also near or in agricultural and urbanized areas where tadpoles develop in polluted waters (Nemesházi et al., 2020), and, because they lack chemical defenses that are efficient against predators, they rely on fine-tuned predator avoidance behaviors (Hettyey et al., 2016, 2011, 2010).

2. Methods

2.1. Experimental procedures

2.1.1. Animal collection

We conducted the experiment in 2018 when CPF was the insecticide used in the second highest quantity in Hungary, one year before it was banned (NÉBIH, 2019). In March we collected 50 eggs from each of ten freshly laid egg clutches from a pond (Szárazfarkas, 47°44′ 4.12″ N, 18°49′ 7.04″ E) in the Pilis mountains, a hilly woodland in Hungary, and transported them to the Julianna-major Experimental Station of the Plant Protection Institute in Budapest (47°32′ 52″ N, 18°56′ 07″ E). Until hatching, we kept each sibling group separately in the laboratory in 3-L containers holding 1 L of reconstituted soft water (RSW, USEPA, 2002) at 19 °C and a photoperiod mimicking outdoors light-dark cycles (starting with 12:12 h light:dark cycles in late March, which we gradually changed to 14:10 h by the end of April).

2.1.2. Experimental setup

When hatchlings reached the free swimming state, i.e. developmental stage 25 (Gosner, 1960), we started the experiment (day 1) by haphazardly selecting 26 healthy-looking tadpoles from six sibling groups and 32 tadpoles from the other four sibling groups (284 larvae in total) and placing them into rearing containers. We reared the experimental tadpoles at 20 °C individually in 1.5-L containers filled with 1 L RSW, arranged in a randomized block design. Twice a week we changed the rearing water and fed the tadpoles ad libitum with slightly boiled, chopped spinach. The remaining tadpoles were used to feed predators before behavioral trials (see below).

We distributed the 284 tadpoles among 10 treatment groups as follows. Besides the control group (Control, no CPF), we applied 0.5 or 5

μg/L CPF either acutely (i.e. for 3 days at the time of behavioral observations, CPF-Acute-0.5 and CPF-Acute-5, see below) or chronically (i.e. during the entire larval development, CPF-Chronic-0.5 and CPF-Chronic-5), and these 5 chemical treatments were combined with the presence or absence of chemical cues of predatory fish during behavioral observations (see below). In case of the Control and the Chronic treatments, we had 34 individuals per group, whereas in the Acute treatments, we had 20 individuals per group. We used larger sample sizes in the former treatment groups to ensure sufficient numbers of individuals for sex-ratio analysis. In the Control treatment, we kept tadpoles in CPF-free RSW throughout the experiment, to which we added 1 μL/L of 96% ethanol as solvent control. This ethanol concentration was lower by three orders of magnitude than the lowest concentrations observed to harm anuran embryos or tadpoles (Fainsod and Kot-Leibovich, 2018; Peng et al., 2005; Taylor and Brundage, 2013). The other treatment groups were exposed to one of the two nominal concentrations (i.e. 0.5 or 5 μg/L) of CPF dissolved in ethanol. We used an analytical standard from Sigma (Product number 45395) and prepared the stock solution by dissolving 50 mg CPF in 10 mL 96% ethanol. Tadpoles assigned to the CPF-Chronic treatments were exposed to the insecticide from developmental stage 25 to 42 (start of metamorphic climax), whereas the CPF-Acute treatments started at the beginning of the first behavioral observation on day 30 and ended 72 hours later, just before the second behavioral observation. We performed the CPF-Acute treatments the same way as the CPF-Chronic treatments, with the only difference being a shorter exposure period. In all treatment groups, we renewed the nominal concentrations at each water change. To measure actual concentrations in rearing containers, we took samples from the freshly prepared rearing water of tadpoles into amber PET flasks once per week over three weeks, taking one liter per nominal concentration (0, 0.5, or 5 μg/L CPF) on each occasion. The samples were immediately transported to an accredited analytical laboratory (SynTech Research Hungary Kft.) and were analyzed as described in Bókony et al. (2020). These analyses detected no CPF in the Control treatment (lowest quantification level: 50 ng/L), whereas the concentrations measured in samples treated with CPF corresponded well with the magnitude of the nominal concentrations (0.533, 0.498, and 0.35 μg/L in the treatment with a nominal concentration of 0.5 μg/L; and 4.76, 4.13, and 5.4 μg/L in the treatment with a nominal concentration of 5 μg/L). The concentrations given throughout the text henceforth are nominal.

2.1.2. Behavioral observations

Thirty days after starting the experiment we video-recorded the behavior of 200 individuals, 20 from each of the 10 treatment groups. Immediately before recording, we transferred tadpoles into new containers identical to the rearing containers but containing no food remnants, filled with 1 L RSW and containing 0, 0.5, or 5 μg/L CPF, depending on the treatment. Tadpoles in both the CPF-Acute and the CPF-Chronical treatments received CPF this time. After recording 20 min of baseline behavior, we abruptly poured 40 mL pure RSW or 40 mL predator-cue water into each container and recorded the behavior of the animals for another 20 min. The chemical cues were prepared as described in Hettyey et al. (2016), using water from a 130-L tank holding 4 European perch (*Perca fluviatilis*) that had been feeding on agile frog tadpoles. The fish were obtained and maintained as described in Üveges et al. (2021). Similarly prepared predator-cue water elicited clear antipredator responses in behavioral and chemical defenses of tadpoles in previous studies (Hettyey et al., 2019, 2016; Üveges et al., 2021), so we could safely assume that tadpoles would sense chemical cues of predation threat. The European perch is a native predator in our region, and agile frog tadpoles respond to chemical cues indicating its presence by decreasing their activity if fish had been feeding on conspecific tadpoles, even if the tadpoles are predator-naïve (Hettyey et al., 2016). To record the behavior of tadpoles we used three digital camcorders (Canon LEGRIA HF R66). Because ten rearing containers were fitted under each camera, we performed seven consecutive rounds of recordings between 9:00 and 16:00, where the spatial and temporal order of individuals was randomized. After recording, we returned containers to rearing shelves and we fed the tadpoles.

On day 33 we repeated the above process of recording tadpole behavior, except that this time none of the animals received CPF during the video recording (i.e. the CPF-Acute ended after 3 days, right before the second observation, and the CPF-Chronic groups spent the 40 min of recording without the chemical). With this approach, we aimed to test if the olfactory inhibition is temporary (occurring only in the presence of CPF, i.e. only in our first behavioral observation) or a permanent damage of the olfactory system. In the latter case, we expected that olfactory abilities would be inhibited in both observations of the CPF-Chronic animals, but for the CPF-Acute animals either only in the second observation or in neither if the Acute treatment is not long enough for causing permanent damage. For each individual, the predator-cue treatment was the same as during the first observation. After 40 min of observation, we changed the water in the CPF-Chronic groups to CPF-treated RSW, returned all containers to the rearing shelves, and fed the tadpoles. One day after the second behavioral observation we randomly chose 100 individuals, 10 from each treatment group, measured their body mass (± 0.1 mg) using an Ohaus PA114 digital scale and preserved them in 4% formalin - 0.1 M phosphate-buffered saline solution for later brain measurements. At this time, we terminated the two CPF-Acute treatments and released the tadpoles remaining in the CPF-Acute groups at their pond of origin. Recordings on behavior and brain samples were analyzed after termination of the experiment (see *Section 2.2* below).

2.1.3. Metamorphosis, froglet rearing and dissection

The remaining animals (the two CPF-Chronic groups and the Controls) were raised further for measuring developmental endpoints (Fig. 1). When a tadpole reached developmental stage 42 (start of metamorphosis, identified by the appearance of forelimbs), we noted the date, measured body mass to the nearest 0.1 mg and replaced the rearing water with 0.1 L pure RSW (i.e. containing neither ethanol nor CPF) and slightly tilted the container to allow the animal to leave the water. When metamorphosis was completed (developmental stage 46, identified by complete tail resorption), we moved the animal into a clean rearing box that contained wet paper towels as a substrate and a piece of egg carton as shelter, which we changed every other week. Metamorphosed animals were fed ad libitum with springtails and small (2–3 mm) crickets sprinkled with a 3:1 mixture of Reptiland 76280 (Trixie Heimtierbedarf GmbH & Co. KG, Tarp, Germany) and Promotor 43 (Laboratorios Calier S.A., Barcelona, Spain) containing vitamins, minerals, and amino acids.

We dissected the animals 59–86 days after completion of metamorphosis (102–131 days after they reached the free-swimming tadpole stage) when the gonads are already well differentiated in this species (Bernabò et al., 2011b; Ogielska and Kotusz, 2004). When all froglets of a family reached the age of two months after metamorphosis, we measured their body mass to the nearest 0.01 g and euthanized them using a water bath containing 6.6 g/L tricaine-methanesulfonate (MS-222) buffered to neutral pH with the same amount of Na_2HPO_4. We examined the gonads under an Olympus SZX12 stereomicroscope at 16 × magnification and categorized phenotypic sex as male (testes) or female (ovaries) based on the gross anatomy of the gonads. We have successfully used this method of identifying phenotypic sex in juvenile anurans before (Bókony et al., 2020; Mikó et al., 2021).

Experimental procedures were approved by the Ethical Commission of the Plant Protection Institute, permissions were issued by the Government Agency of Pest County (PE/KTF/3596-6/2016, PE/KTF/3596-7/2016 and PE/KTF/3596-8/2016), experiments were carried out according to recommendations of the EC Directive 86/609/EEC for animal experiments.

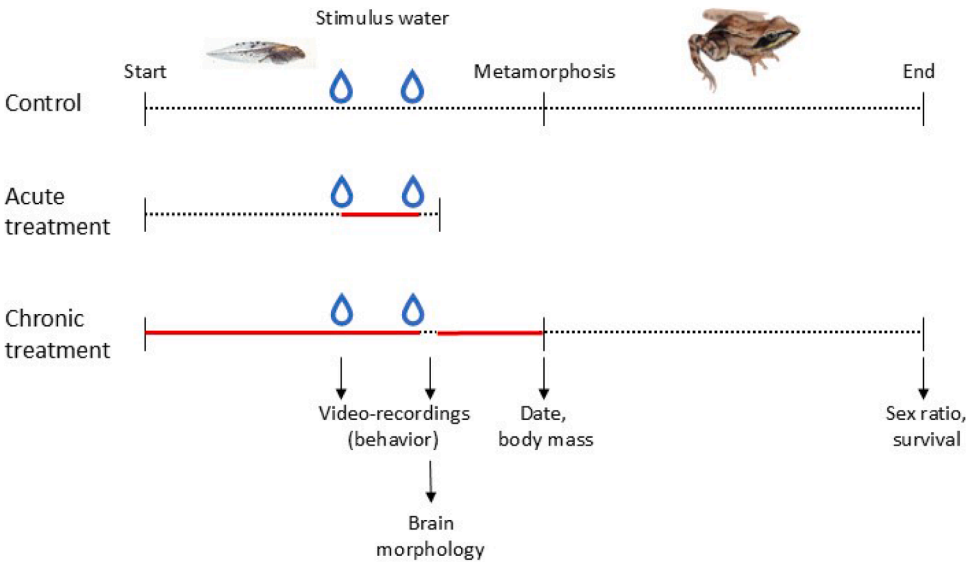

Fig. 1. Schematic illustration of the experimental procedures over time. Each horizontal line represents a treatment group: longer lines are the Chronic, shorter lines are the Acute treatments. Solid red lines denote the CPF exposure, dashed lines are the periods without CPF exposure. Water drops represent the chemical cue treatments (either predator cue or clean water), arrows below the lines mark the data-collection events.

2.2. Sample processing

2.2.1. Brain measurements

We dissected whole brains from the cranium of the animals stored in fixative solution under a Zeiss SteREO Discovery.V8 microscope, placed them on glass beads, and took photographs from dorsal, lateral (left), and ventral views with a Canon 600D digital camera. We analyzed photographs using the TpsDig 2.31 software (https://life.bio.sunysb.edu/ee/rohlf/software.html). For the whole brain as well as for each brain region (bulbus olfactorius, telencephalon, tectum opticum, medulla oblongata, diencephalon, and hypothalamus) we measured height, length, and width as the greatest distance enclosed by the given structure in the given direction, and converted numbers of pixels to mm using a size reference included in each picture. From these three size dimensions we estimated the volume of the brain and each brain region according to the ellipsoid model following the formulas (including the correction factor) of Pollen et al. (2007). We measured 20 randomly chosen brains three times; these measurements showed high repeatability (intra-class correlation coefficient $\geq$ 0.85). The medulla got damaged during dissection in two animals, so we could not estimate the total brain size in these two cases.

2.2.2. Video analyses

To analyze the behavior of tadpoles, we used a freely available video tracking program, ToxTrac (Rodriguez et al., 2018). We divided the video recordings into two parts, before and after the addition of the 40 mL stimulus water into the containers, yielding 400 recordings to be analyzed for each day (200 individuals $\times$ two 20-min recordings). At the start of each 20-min recording, we discarded those few seconds during which the experimenters left the room. One observer validated the results of the automated tracking on all recordings and found tracking errors in 21 recordings belonging to 13 individuals. Because movement tracks could not be constructed by hand in the same way as done by the software, we excluded these individuals from the statistical analyses. We extracted the following variables from each 20-min recording: mobility rate (proportion of time spent moving), swimming trajectory length (total distance traveled by the tadpole in kilopixels), and proportion of time spent close to the sides of the container (i.e. when the tadpole's body was within a 50-pixel wide stretch measured from the side, as the tadpoles had an average body length of ca. 40 pixels in the videos).

The tadpoles typically reacted to the disturbance at the start of each 20-min recording by a few-second burst of fast swimming, followed by freezing (i.e. a period of time spent immobile), and then swimming again. In case of the swimming trajectory length, we excluded the first 1 min from the analysis to avoid the distorting effect of the fright reaction of the tadpoles. We also extracted two further variables from the second 20-min recording: the time spent frozen after the addition of stimulus water and the movement intensity after freezing. The latter variable was scored by a single observer to describe the apparent fear of the tadpoles when they first started to move after the freezing response, categorized on a 1–3 scale as 1: slight movement, 2: swimming away apparently calmly, 3: swimming around fervently. We used these variables to test if predator chemical cues indeed affected the behavior of the tadpoles.

2.3. Statistical analyses

We analyzed the data in the R computing environment v4.0.3 (R Core Team, 2020). For mobility rate, swimming trajectory length, and the proportion of time spent close to the sides of the container we used linear mixed-effects models ('lmer' function of the 'lme4' package). For each variable we ran two models. In the first model we analyzed the baseline behaviors (first 20 min), where the behavioral variable before the addition of stimulus water was the dependent variable, the CPF treatment (5 groups), and the day of observation (first or second) were the fixed factors, and individual nested within sibling group and crossed with the position of the boxes were the random factors. We also included the time of day (observation session, ranging from 1 to 7) as numeric covariates, and the two-way interaction between the fixed factors. In the second model the behavioral variable after the addition of stimulus water was the dependent variable, the CPF treatment, the predator-cue treatment (presence/absence of chemical cues of predatory fish) and the day of observation were the fixed factors, and individual nested within sibling group and crossed with the position of the boxes were the random factors. The respective behavioral variables before the addition of stimulus water (i.e. in the first 20 min) and the time of day were numeric covariates, and we added all two-way interactions between the fixed factors as well as their three-way interaction. In case of the swimming trajectory length we used log10 transformation for better model fit. For the analysis of the time spent frozen, we used a generalized linear mixed model ('glmmTMB' function of the 'glmmTMB' package) with negative binomial error distribution (with quadratic parameterization), with the same fixed and random effects as in the

linear mixed-effects models. To analyze the movement intensity after freezing we used cumulative link mixed models ('clmm' function of the 'ordinal' package). We used the same fixed and random effects as above, except that sibling group could not be entered as a random factor due to convergence problems (however, we note here that the effect of sibling group was very small in the rest of the analyses).

In the analysis of total brain size, we used linear mixed-effects models where the fixed effects were the CPF treatment (5 groups) and predator-cue treatment along with their two-way interaction, body mass was a covariate, and sibling group was a random factor. These same predictors were included in the models of each brain region, except that we entered total brain size instead of body mass as a covariate. We graphically examined the relationship between total brain size and body mass, and also between each brain region and total brain size, both with and without logarithmically transforming the variables, and we found no indication that power-function relationships would fit the data better than linear relationships, so we used the latter (Pollen et al., 2007).

To analyze body mass at metamorphosis and body mass at dissection we used linear mixed-effects models ('lmer' function). For the analysis of time to metamorphosis we used a generalized linear mixed model with Conway-Maxwell Poisson error distribution ('glmmTMB' function). To analyze sex ratio (the probability of being male), we used a generalized mixed-effects model with binomial distribution ('glmer' function of the 'lme4' package). For the analysis of survival among the animals that were raised until the end of the experiment, we used a generalized linear model with binomial error distribution and bias-reduction adjustment for separation ('glm' function of the 'brglm2' package). We chose this approach because in some treatment groups all individuals survived until dissection (thus it was not possible to fit survival models like Cox regression). In this analysis, survival was treated as a binary variable, expressing whether the individual survived to dissection. In these five models, the fixed factors were chemical treatment (3 groups: Control, CPF-Chronic-0.5, and CPF-Chronic-5), predator-cue treatment, and their two-way interaction; and sibling group was entered as a random factor (except for the analysis of survival, because the bias-reduced analysis cannot accommodate random effects). In case of body mass at dissection we also entered age (days from the start of the experiment to dissection) as a covariate.

For the purpose of visualization, from the above models we calculated treatment-group means and corresponding 84% confidence intervals using the 'emmeans' package, since the absence of overlap between two 84% confidence intervals is equivalent to the difference between group means deviating significantly from zero at the 95% confidence level (Payton et al., 2003). To present the model results as type-2 analysis-of-deviance tables, we used the 'Anova' function of the 'car' package. Unless stated otherwise, we report the statistics from the reduced models after omitting the non-significant model terms stepwise, but never excluding the main effect of CPF treatment and those covariates that we used to control for differences in size (for brain morphology) or age (for froglet mass). Throughout the text we report estimated means ± standard errors (SE).

3. Results

Before the addition of stimulus water, none of the behavioral variables were significantly influenced by CPF treatment (Supplementary Material: Table S1). After the addition of stimulus water, out of the five behavioral variables, only the time spent frozen was significantly affected by CPF treatment, but only in interaction with the day of observation (Table 1, Fig. 2, Supplementary Material: Table S2). On the first observation day, tadpoles in the CPF-Acute-5 treatment spent less time frozen compared to the Control (Control: 141.5 ± 29.7 *sec*, CPF-Acute-5: 65.5 ± 13.8 *sec*), but there were no differences among treatment groups on the second day (in clean RSW). The presence of predator chemical cues had a significant effect on three behavioral variables (Table 1, Supplementary Material: Table S2, S3) as follows. Individuals that received predator cues spent less time along the edges (70.7 ± 1.47%) and showed more intensive movement reaction (2.22 ± 0.07) compared to individuals that did not receive predator cues (74.8 ± 1.46%, 1.66 ± 0.07 respectively). Also, on the first observation day tadpoles receiving predator cues froze for longer (147.7 ± 22.7 *sec*) than the group that received clean RSW as stimulus water (91.3 ± 14.2 *sec*), but this effect was not detectable on the second day (predator cue

Table 1
Type-2 analysis-of-deviance tables of the full models of the effects of CPF-Acute and CPF-Chronic treatments at a concentration of 0.5 or 5 µg/L on tadpole behavioral variables in the presence or absence of predator cues on the first and second observation day. Significant effects ($P < 0.05$) are highlighted in bold. Baseline behavior means the first 20 min of recording preceding the addition of predator cues. Round is the observation session, ranging from 1 to 7.

Dependent variable	Predictors	χ^2	df	*P*
Swimming trajectory length[1]	CPF treatment	8.01	4	0.091
	Predator cue	2.81	1	0.094
	Day	11.38	1	**<0.001**
	Round	6.28	6	0.392
	Baseline behavior	148.35	1	**<0.001**
	CPF treatment × Predator cue	2.01	4	0.734
	CPF treatment × Day	4.92	4	0.296
	Predator cue × Day	0.95	1	0.329
	CPF treatment × Predator cue × Day	5.68	4	0.224
Proportion of time near wall	CPF treatment	5.21	4	0.266
	Predator cue	4.47	1	**0.035**
	Day	0.65	1	0.419
	Round	4.81	6	0.659
	Baseline behavior	29.21	1	**<0.001**
	CPF treatment × Predator cue	4.54	4	0.337
	CPF treatment × Day	2.78	4	0.595
	Predator cue × Day	0.67	1	0.413
	CPF treatment × Predator cue × Day	0.17	4	0.997
Mobility rate of the tadpole	CPF treatment	6.88	4	0.142
	Predator cue	1.77	1	0.184
	Day	1.22	1	0.269
	Round	1.69	6	0.946
	Baseline behavior	116.32	1	**<0.001**
	CPF treatment × Predator cue	3.66	4	0.455
	CPF treatment × Day	1.79	4	0.774
	Predator cue × Day	0.37	1	0.546
	CPF treatment × Predator cue × Day	1.00	4	0.909
Time spent frozen	CPF treatment	4.83	4	0.306
	Predator cue	1.12	1	0.289
	Day	2.32	1	0.127
	Round	8.09	6	0.231
	Baseline mobility rate	0.77	1	0.381
	CPF treatment × Predator cue	4.43	4	0.351
	CPF treatment × Day	11.68	4	**0.019**
	Predator cue × Day	7.92	1	**0.005**
	CPF treatment × Predator cue × Day	0.97	4	0.914
Movement intensity after freezing	CPF treatment	2.14	4	0.710
	Predator cue	30.39	1	**<0.001**
	Day	1.56	1	0.212
	Round	20.53	6	**0.002**
	CPF treatment × Predator cue	2.86	4	0.582
	CPF treatment × Day	1.95	4	0.745
	Predator cue × Day	1.79	1	0.181
	CPF treatment × Predator cue × Day	3.63	4	0.458

[1] $\log_{10}$ transformed.

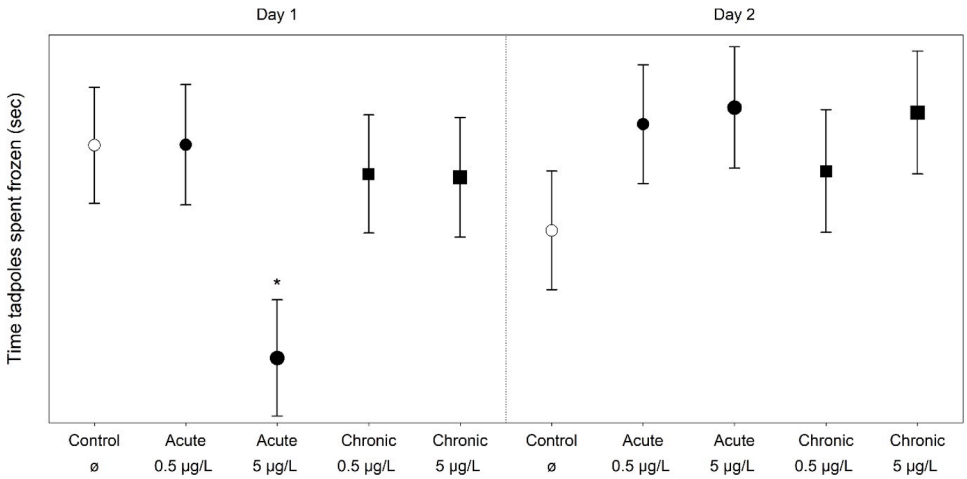

Fig 2. The time tadpoles spent frozen after the addition of stimulus water in each CPF treatment group (regardless of presence or absence of predator cues). Squares represent the Chronic, while circles represent the Acute exposure groups. Error bars show the means and 84% confidence intervals estimated from the model in Table 1. Asterisks mark the treatment groups that differed significantly from the Control ($P < 0.05$).

absent: 153.1 ± 24.5 *sec*, predator cue present: 127.2 ± 20.3 *sec*).

Exposure to CPF and/or predator cues had no effect either on total brain size or on the size of any brain region, except for the bulbus olfactorius, where the interaction between the two treatments was significant (Table 2). In the CPF-Acute-0.5 group, the animals that had been briefly exposed to predator cues exhibited decreased volumes of bulbus olfactorius, while there were no differences in other treatment combinations (Fig. 3). However, this effect appears to be caused by a single outlier data point; the CPF treatment × Predator cue interaction was no longer significant after excluding the outlier from the model (Table 2).

Time to metamorphosis did not differ between the Control (40.2 ± 0.69 days) and either the CPF-Chronic-0.5 (40.1 ± 0.69 days) or the CPF-Chronic-5 group (41 ± 0.71 days; Table 3). Chronical exposure (i.e. throughout the entire larval development) to CPF had a significant effect on body mass at metamorphosis (Table 3): individuals exposed to the CPF-Chronic-5 treatment had lower mass than the Control individuals (Fig. 4). However, this difference was no longer detectable in froglets: body mass at dissection (two months after metamorphosis) was not affected by CPF at either concentration (Table 3). Sex ratio was independent from the CPF-Chronic treatments (Table 3). Among the 135 individuals that reached the age for phenotypic sexing, we found 23 females and 24 males in the Control treatment, 21 females and 26 males in the CPF-Chronic-0.5 treatment, and 20 females and 21 males in the CPF-Chronic-5 treatment. Only seven individuals died during the experiment: six in the CPF-Chronic-5 treatment group (four after termination of the treatment) and one in the Control before metamorphosis. This difference in survival between treatment groups was not significant (Table 3). The brief exposure to predator cues had no significant effect on time to metamorphosis, body mass, sex ratio, or survival either alone ($P > 0.15$) or in interaction with CPF treatment ($P > 0.23$).

Table 2
Type-2 analysis-of-deviance tables of the effects of CPF-Acute and CPF-Chronic treatments at a concentration of 0.5 or 5 µg/L on total brain size and the size of each brain region. Significant effects ($P < 0.05$) are highlighted in bold. Body mass was measured just before sample collection.

Dependent variable	Predictors	χ^2	df	*P*
Total brain size	CPF treatment	1.77	4	0.779
	Body mass	10.83	1	**0.001**
Bulbus olfactorius	CPF treatment	6.02	4	0.198
	Predator cue	0.62	1	0.431
	CPF treatment × Predator cue	11.06	4	**0.026***
	Total brain size	12.49	1	**<0.001**
Telencephalon	CPF treatment	4.35	4	0.361
	Total brain size	20.21	1	**<0.001**
Tectum opticum	CPF treatment	1.24	4	0.872
	Total brain size	41.33	1	**<0.001**
Medulla oblongata	CPF treatment	5.87	4	0.209
	Total brain size	63.35	1	**<0.001**
Diencephalon	CPF treatment	3.09	4	0.544
	Total brain size	36.50	1	**<0.001**
Hypothalamus	CPF treatment	1.79	4	0.775
	Total brain size	7.05	1	**0.008**

* Without the outlier $P = 0.063$.

4. Discussion

In this study, we investigated the effects of larval exposure to ecologically relevant concentrations of CPF on a comprehensive suite of fitness-related traits in the agile frog, assessing changes in anti-predator behavior, brain morphology, growth, somatic and sexual development, and survival. We found that CPF had no significant effects on most of the measured variables, with two exceptions. First, CPF-Acute-5 treatment resulted in a shorter freezing response to stimulus water indicating predation threat on the first observation day. Second, CPF-Chronic-5 treatment decreased body mass at metamorphosis. Neither the CPF-Acute-0.5 nor the CPF-Chronic-0.5 treatments had any significant effects on the evaluated traits.

Tadpoles of many species show behavioral responses to predators that decrease their chances of being eaten (Alford, 2000). The most common behavioral responses are to quickly move away from the predator, to reduce foraging activity, and to stay motionless. Amphibian larvae are capable of detecting the presence of dangerous predators via a number of sensory cues, including tactile, visual, and chemical cues (Wells, 2007), but chemical contaminants can disrupt these sensory processes (Lürling and Scheffer, 2007). Indeed, previous studies found that CPF can disrupt the ability to detect and/or respond to predation risk in fish (McClelland and Woodley, 2022; Maryoung et al., 2015; Sandahl et al., 2004; Tilton et al., 2011). They suggest that the possible underlying mechanisms of this hyposmia (decreased ability to smell or detect odors) is that CPF inhibits the responsiveness of olfactory receptor neurons in the sensory epithelium (Sandahl et al., 2004), and/or that it lowers the regenerative capacity of the olfactory system (Tilton et al., 2011). In our experiment, the tadpoles reacted with several behavioral changes to the sudden appearance of chemical cues by a predatory fish, but these reactions were not altered by CPF treatment. This suggests that the environmentally relevant exposures that we applied caused no info-disruption for the prey in this predator-prey system. Although the tadpoles in the CPF-Acute-5 treatment spent less time immobile than the

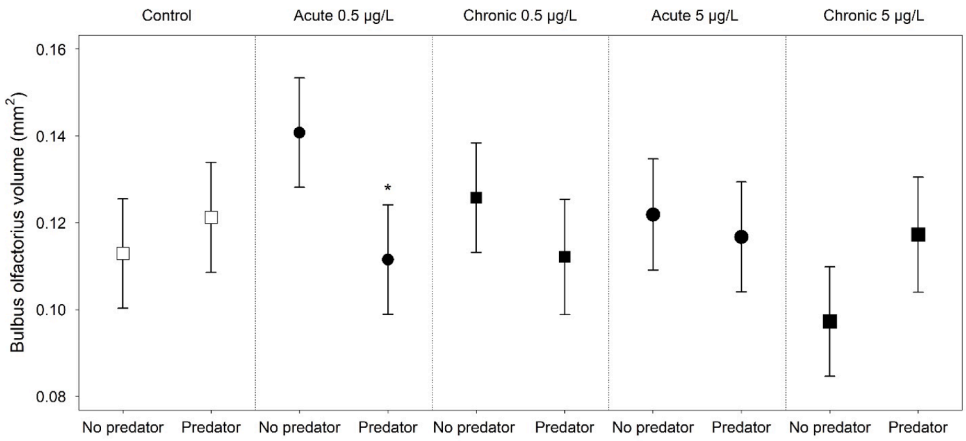

Fig. 3. Bulbus olfactorius volume in each CPF treatment group in the presence or absence of predator chemical cues. Squares represent the Chronic, while circles represent the Acute exposure groups. Error bars show the means and 84% confidence intervals estimated from the model in Table 2. Asterisk marks the treatment group that differed significantly from their predator free equivalent ($P < 0.05$).

Table 3
Effects of CPF-Chronic-0.5 and CPF-Chronic-5 treatments on development, sex ratio, and survival. Coefficients (*c*) represent the mean in the Control group and the differences of the mean in each remaining treatment group from the Control group; significant treatment effects ($P < 0.05$) are highlighted in bold.

Dependent variable	Parameter	*c*	SE	z	*P*
Time to metamorphosis[1]	Control	3.69	0.02	212.68	
	CPF-Chronic-0.5	−0.003	0.02	−0.14	0.886
	CPF-Chronic-5	0.02	0.02	0.99	0.320
Mass at metamorphosis (mg)	Control	512.33	9.69	52.89*	
	CPF-Chronic-0.5	−12.85	13.23	−0.97*	0.333
	CPF-Chronic-5	−35.39	13.29	−2.66*	**0.009**
Mass at dissection (g)	Control	−1.83	0.18	−10.31*	
	CPF-Chronic-0.5	0.01	0.03	0.43*	0.669
	CPF-Chronic-5	−0.01	0.03	−0.17*	0.863
	Age (days to dissection)	0.03	0.002	19.54*	**<0.001**
Sex ratio[2]	Control	0.04	0.45	0.08	
	CPF-Chronic-0.5	0.21	0.45	0.46	0.647
	CPF-Chronic-5	−0.03	0.47	−0.05	0.958
Survival[3]	Control	−3.46	0.84	−4.12	
	CPF-Chronic-0.5	−1.09	1.66	−0.66	0.509
	CPF-Chronic-5	1.43	0.95	1.50	0.133

[1] log scale coefficients (number of days).
[2] logit scale coefficients (probability of being male).
[3] logit scale coefficients (probability of surviving).
* t-value.

unexposed Control group, this response was shown not only by the individuals receiving predator cues but also by those that received clean stimulus water. Furthermore, this effect was detectable only on the first observation day, when CPF had just been added to the water of the CPF-Acute groups, but not on the second day when CPF had just been removed. This suggests that the sudden appearance of CPF might alter the tadpoles' general behavioral responsiveness to disturbance (including but not exclusive to predator cues), but this effect disappears if the pollutant either disappears after a short time (as on the second day in our CPF-Acute treatment groups) or if it is present for a long time (as in our CPF-Chronic treatment groups). This pattern might indicate that tadpoles can sense the presence of CPF and habituate to it, although it is unclear why it changes their sensitivity to disturbance; further experiments are needed to clarify this.

One of the greatest concerns about CPF is its developmental neurotoxicity in humans (Burke et al., 2017), and it may have similar effects in amphibians. Woodley et al. (2015) exposed leopard frog tadpoles to 5 µg/L CPF, which caused narrower and shorter brains compared to the control group. In a later study, they found that CPF induced relatively longer telencephalon lengths in the same species (Woodley et al., 2024). Conversely, McClelland et al. (2018) found that exposure to 1 µg/L CPF during larval development resulted in brains that were wider than those in the control individuals. In another study, they found that tadpoles exposed to 1 µg/L CPF had larger relative brain mass and changes in relative telencephalon shape (McClelland and Woodley, 2022). Spirhanzlova et al. (2023) also found altered brain morphology resulting from exposure to 35.1 ng/L and 3.51 µg/L CPF in *Xenopus laevis* tadpoles. Our results suggested no effect of CPF either at a concentration of 0.5 or 5 µg/L on brain morphology in agile frog tadpoles. These discrepancies among results may be due to interspecific differences in the sensitivity of amphibians or in the developmental stage of the exposed animals, but further investigations are clearly needed if we are to understand the heterogeneous effects of CPF on amphibian neurodevelopment and brain architecture.

We found that CPF-Chronic-5 treatment resulted in reduced body mass at metamorphosis. Previous studies also found that this chemical can have a negative effect on tadpole mass (Richards and Kendall, 2003; Widder and Bidwell, 2006; Woodley et al., 2015). This suggests that higher, but still ecologically relevant concentrations of CPF may decrease population viability, because metamorphic mass is an important determinant of survival to maturity in amphibians (Altwegg and Reyer, 2003; Berven, 1990; Smith, 1987; Üveges et al., 2016). However, in our study, the negative effect of CPF on body mass disappeared two months post-metamorphosis: by this time the CPF-exposed animals did not differ from control individuals. This result might be explained by compensatory growth, an accelerated growth that occurs if environmental conditions improve following a period of growth inhibition (Hector et al., 2012; Squires et al., 2010). Compensatory growth, however, can come at a cost via increased levels of oxidative stress (Kim et al., 2019) and compromised immune defenses (Gervasi and Foufopoulos, 2008; Murillo-Rincón et al., 2017). Consequently, even if not manifest in lowered body size of juveniles, larval exposure to CPF may still exert negative effects on amphibians by altering growth trajectories. Therefore, it would be important to monitor the delayed effects of CPF exposure in the long term, especially under ecologically realistic conditions where the potential costs of immune suppression can be

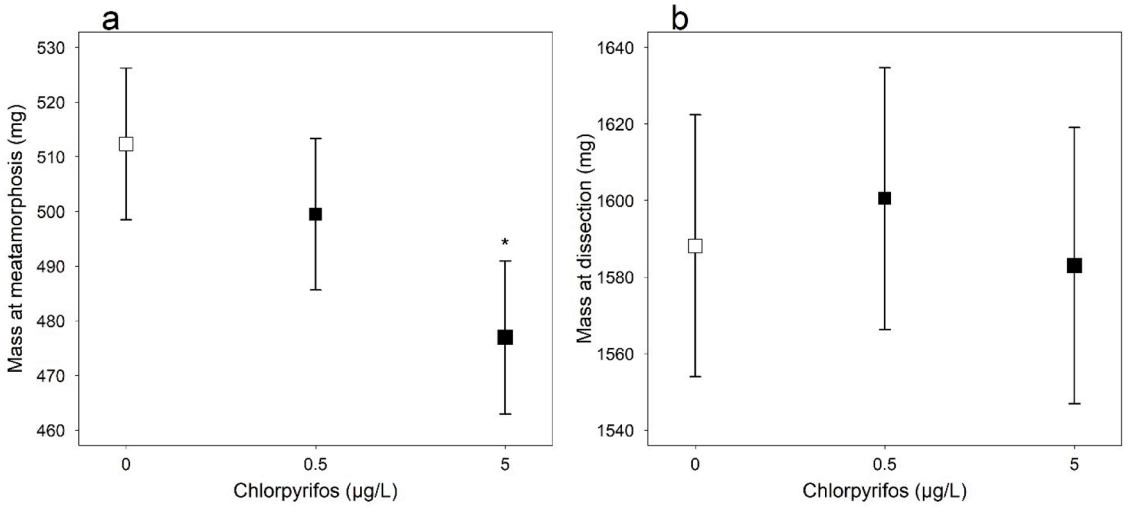

Fig. 4. Effects of CPF-Chronic-0.5 and CPF-Chronic-5 treatments on tadpole mass at metamorphosis (a) and froglet mass at dissection (b). Error bars show the means and 84% confidence intervals estimated from the model in Table 3. Asterisk marks the treatment group that differed significantly from the Control ($P < 0.05$).

detected.

In our experiment, CPF did not have significant effects on survival and the length of larval development. This contrasts with the results of some previous studies (Henao et al., 2024; Rutkoski et al., 2020; Silva et al., 2020) which found decreased survival rates of tadpoles, as well as morphological and enzymatic changes. However, in those experiments, the applied effective concentrations were much higher (i.e. between 250 and 500 µg/L) because they were based on surveys of natural water bodies located in the United States, Canada, and South America, where CPF was found to be present at approximately four times higher concentrations than in Europe (EUROSTAT, 2007; Grube et al., 2011). Studies that used similar concentrations as we did in our experiment also found no significant effects on survival and length of developmental period (Barreto et al., 2020; Bernabò et al., 2011a; Widder and Bidwell, 2008, 2006). Because legislation regarding the use of CPF-based insecticides varies widely across the globe, the concentrations that can be reached in natural habitats are region-dependent. This highlights the importance of studying locally relevant environmental concentrations in ecotoxicology.

CPF is considered a potential endocrine disruptor (Ubaid ur Rahman et al., 2021), but it did not affect the phenotypic sex ratio in our study. Bernabò et al. (2011a) obtained similar results when applying higher concentrations than we used here, but they reported an elevated incidence of intersex condition (testicular oocytes) in agile frogs exposed to 25–50 µg/L CPF during their larval development. We did not systematically screen for oocytes in the testes of all individuals in the present study, but for a related study (Bókony et al., 2021) we investigated the gonad histology of five phenotypic males per chemical treatment (15 in total), and we found only two intersex animals: one in the Control group and one in the CPF-Chronic-0.5 group, suggesting no major effect at the studied concentrations. Furthermore, for the purposes of the related study, we also examined the genotypic sex of the dissected froglets and found that the incidence of sex reversal (i.e. mismatch between genotypic and phenotypic sex) was unrelated to CPF treatment (Bókony et al., 2021). Thus, it seems that the ecologically relevant CPF concentrations that we used are not harmful to sexual development in agile frogs. A low but apparent prevalence of intersexuality may be a natural phenomenon in young post-metamorphic amphibians (Orton and Tyler, 2015), and, similarly, rare sex reversal may spontaneously occur in amphibians, including agile frogs (Lambert et al., 2019; Mikó et al., 2021; Nemesházi et al., 2020).

Taken together, our study revealed that environmentally relevant concentrations of CPF are not toxic to agile frog tadpoles in terms of direct mortality, time to metamorphosis, neural and gonadal development, and response to chemical cues of predation risk, although we detected a transient negative effect on body mass and a slight change in tadpole activity. Thus, larvae of this species appear to be relatively tolerant to CPF, suggesting that this insecticide might only harm them in extreme cases, i.e. upon repeated high-dose applications. However, it is worth noting that different species may vary greatly in their susceptibility to pesticides (Bókony et al., 2020; Bridges and Semlitsch, 2000), so ecotoxicological effects cannot be generalized across species. Also, commercially available formulations contain not only CPF as active ingredient but also adjuvants, which may have more detrimental consequences on various aquatic organisms (Demetrio et al., 2014; Mikó and Hettyey, 2023; Majumder, 2024), so that care has to be taken when extrapolating effects. Bearing these complexities in mind, our results highlight the importance of testing the ecotoxicological effects of concentrations that are ecologically relevant based on environmental surveys, because only such studies can provide realistic assessments of ecological risk.

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CRediT authorship contribution statement

Zsanett Mikó: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Veronika Bókony:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis. **Nikolett Ujhegyi:** Writing – review & editing, Investigation, Data curation. **Edina Nemesházi:** Writing – review & editing, Investigation. **Réka Erös:** Writing – review & editing, Methodology, Investigation, Data curation. **Stephanie Orf:** Writing – review & editing, Investigation. **Attila Hettyey:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107400.

Data availability

Data will be made available on request.

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