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Environmental Toxicology

Environmentally Relevant Concentrations of the Insecticide Fipronil Modulated Molecular Response in *Chironomus riparius*

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Abstract: Pesticides employed worldwide for crop protection easily reach aquatic systems, which act as the main reservoirs, and become a risk factor for aquatic fauna. Fipronil is a broad-spectrum insecticide acting on the insect nervous system; however, other effects and systems unrelated to this mechanism could be affected in non-target organisms. Thus, the present study aimed to assess the impact of fipronil on the suborganismal response (gene expression and enzymatic activity) of *Chironomus riparius* larvae as a model organism in ecotoxicology. To this end, short-term toxicity tests were carried out with fourth-instar larvae exposed to 0.001, 0.01, and 0.1 μg L^{-1} of fipronil for 24 and 96 h. Messenger RNA levels of 42 genes related to diverse metabolic pathways were analyzed by real-time polymerase chain reaction, complemented with catalase (CAT), glutathione *S*-transferase (GST), and acetylcholinesterase (AChE) activities. Few effects were observed at 24 h; however, after longer exposure (96 h), genes involved in the endocrine, detoxification, stress, and immune response pathways were altered. Moreover, fipronil at 96 h increased CAT and GST activity at 0.01 μg L^{-1} and AChE at the highest concentrations. The results demonstrate that even low environmentally relevant fipronil concentrations can modulate the molecular response of several cellular pathways in *C. riparius* after short-term exposure. These results bring new information about the underlying response of fipronil and its mode of action on a key aquatic invertebrate. Despite no effects on mortality, strong modulation at the suborganismal level emphasizes the advantage of biomarkers as early damage responses and the harmful impact of this pesticide on freshwater organisms. *Environ Toxicol Chem* 2024;43:405–417. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Chironomids; Insecticides; Molecular response; Real-time polymerase chain reaction (RT-PCR); Aquatic toxicity; Pesticides

INTRODUCTION

Aquatic pollution by pesticides is a global concern because of their increasing use over the years (e.g., for agriculture, veterinary, and domestic use) and presence in aquatic systems worldwide (de Souza et al., 2020). Fipronil has a half-life in soil that ranges from 3 to 7.3 months in field conditions (Bonmatin et al., 2015). Because of this relative persistence, it is maintained over time in soil and finally released into water. So, the insecticide fipronil has been reported in water bodies worldwide (Fang et al., 2019). Fipronil is a broad-spectrum insecticide from the phenylpyrazole group that acts as a blocker of the chlorine ion channels regulated by the gamma-aminobutyric acid (GABA) receptors in insects (Gunasekara et al., 2007). Recently, its use has significantly increased because of the ban and restriction on organochlorine and organophosphate insecticides and its higher selective toxicity (Gunasekara et al., 2007), so lower toxicity is expected in non-target organisms. Because of its agricultural and veterinary applications, fipronil has been detected in rural and urban areas in freshwater ecosystems (Fang et al., 2019; Mutzner et al., 2022). In aquatic environments, this insecticide occurs in dissolved and particulate forms in the water column and sediment (Peret et al., 2010), with a half-life ranging from 13.2 to

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87.9 and from 6.9 to 21 days in water and sediment, respectively, producing four metabolites (amide-, desulfinyl-, sulfide-, and sulfonyl-fipronil; Peret et al., 2010; Thuyet et al., 2013). The reported concentrations in freshwater environments worldwide ranged from 0.006 to 26.2 $\mu g\,L^{-1}$ (Fang et al., 2019; Marchesan et al., 2010).

Studies have verified toxic effects on non-target organisms after exposure to fipronil (Goff et al., 2017; Lee et al., 2018; Roat et al., 2017), thus refuting the expectation of its high selectivity. For chironomids, effects on mentum deformities, development, adult emergence, and population size over multi-generations were described after exposure to fipronil (Monteiro et al., 2019; Pinto, Moreira, et al., 2021; Pinto, Rocha, et al., 2021). Despite these effects reported on several species after exposure to fipronil, doubts remain, mainly related to cellular mechanisms different from the mode of action described for fipronil (inhibition of GABA receptors). In this way, molecular tools bring the opportunity to elucidate the underlying mechanistic responses at the suborganismal level, which could predict short-term effects (Verheijen et al., 2020). In the present study, several biological processes and systems, such as endocrine and detoxification systems, stress response, antioxidant defense, immunity, and DNA repair mechanisms, are evaluated, employing a specific array designed for *Chironomus riparius*, including 42 genes by real-time polymerase chain reaction (RT-PCR) analysis, to detect the impact of fipronil at the gene expression level.

Insects' main hormonal classes are ecdysteroids, especially 20-hydroxyecdysone (20-E), the juvenile hormones (JHs), and the peptide hormones (Soin & Smagghe, 2007). The present study explored genes associated with the 20-E and JH hormonal pathways (Figure 1A). The ecdysone hormones belong to the steroid group and are responsible for triggering and controlling the molt throughout the different developmental stages of insects (Gilbert, 2004; Truman, 2019). Juvenile hormones are crucial in regulating development and reproduction (Belles, 2020). In the larval stage, JH is responsible for molt regulation and triggering metamorphosis (Truman, 2019). Moreover, the detoxification response is an essential pathway to manage toxicant stress and is grouped into three phases (Figure 1B). Phase I comprises the transformation enzymes, including those from the cytochrome P450 (CYP) family; Phase II is composed of conjugation enzymes as the glutathione *S*-transferases (GSTs); and Phase III involves the transport of conjugated products and metabolites outside the cell for excretion by adenosine triphosphate–binding cassette (ABC) proteins (Berenbaum & Johnson, 2015; Hodges & Minich, 2015).

The next pathway selected is the stress response, employing diverse heat shock proteins (HSPs; Figure 1C). The HSPs are divided into families by molecular weight (Hsp110, Hsp100, Hsp90, HSP70, Hsp60, Hsp40, and small HSPs [sHSPs]). They are used as biomarkers because of their sensitivity to stress and potential as environmental pollution indicators (Gupta et al., 2010). Organisms present different levels of defense against oxidative stress (Figure 1F). The first line consists of critical enzymes, including superoxide dismutase (SOD) and catalase (CAT), which act by neutralizing the superoxide radicals and breaking down the hydrogen peroxides, respectively (Ighodaro & Akinloye, 2018). If the antioxidant defense fails, several macromolecules such as DNA may be oxidized; thus, cells present a robust system to repair damaged DNA (Figure 1E) in stressed organisms (Mota et al., 2019). Another important defense mechanism in organisms is the immune system, composed of innate and adaptive mechanisms (Figure 1D). The innate immune system of insects is characterized by cellular responses, mediated by hemocyte cells, and humoral mechanisms, composed of the myelinization and secretion of antimicrobial peptides (Sheehan et al., 2018).

In the present study, gene expression analyses were supplemented with enzymatic activity assays to provide a more complete assessment of the total impact caused by exposure to fipronil. The enzymatic assay is a helpful tool to verify the consequences of environmental pollution (Rao et al., 2014). The analyses include the activity of CAT and SOD, whose functions have already been described, and the acetylcholinesterase (AChE) enzymes. Acetylcholinesterase controls the nervous system's normal functioning once it acts by hydrolyzing the neurotransmitter acetylcholine and regulating this concentration at the synapses (Soreq & Seidman, 2001). Alterations in AChE activity are typically associated with organophosphate insecticides (Ghorab & Khalil, 2015); however, exposure to fipronil was already related to the effects of AChE on fish (Moreira et al., 2021). Regarding the gene expression and enzymatic analyses approach, the present study aimed to investigate new mechanisms associated with short-term (24 and 96 h) exposure to fipronil.

Thereunto, the midge *Chironomus riparius* was used as a biological model. This species is widely used in ecotoxicology as a bioindicator of environmental pollution and to assess the toxic effects of several xenobiotics (Monteiro et al., 2019; Muñiz-González et al., 2021). Moreover, it acts as a key aquatic organism in water systems, recycling organic matter, and as a base of the food web. Thus, the present work aimed to evaluate the impact of fipronil at environmentally relevant concentrations on the suborganismal response of *C. riparius* to determine the underlying mechanism of this pesticide. To do this, fourth-instar larvae of *C. riparius* were exposed to fipronil (0.001, 0.01, and 0.1 $\mu g\,L^{-1}$) for 24 and 96 h, using gene expression by RT-PCR and enzymatic activity as endpoints. In line with this, new mechanisms associated with the exposure of non-target organisms will be described, expanding the discussion about the ecological implications associated with fipronil in the environment.

MATERIAL AND METHODS

Test organisms

Chironomus riparius larvae were obtained from cultures maintained in the laboratory of the Biology and Environmental Toxicology group of the National Distance Education University (Madrid, Spain) according to Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2011). Organisms were maintained in a glass aquarium containing culture medium (0.5 mM $CaCl_2$, 1.0 mM NaCl, 1.0 mM

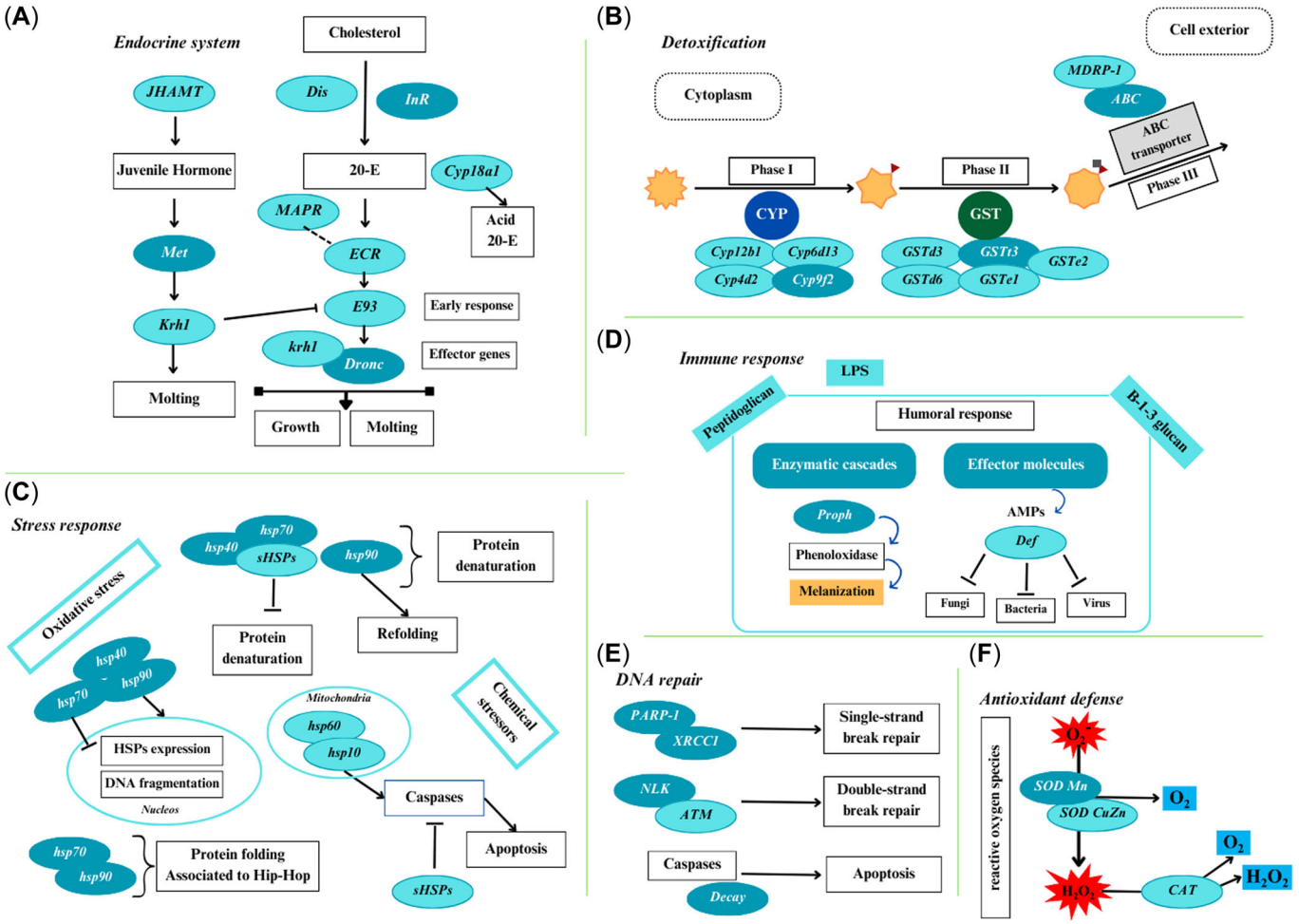

FIGURE 1: Scheme of the metabolic pathways studied in the present study after exposure of *Chironomus riparius* to the insecticide fipronil and their associated genes. The routes presented are (**A**) endocrine system, (**B**) detoxification mechanisms, (**C**) stress response, (**D**) immune system, (**E**) DNA repair, and (**F**) antioxidant defense. Genes with changes in messenger RNA expression are presented as light blue ovals with black bold text inside and those without changes are presented as dark blue ovals with text inside. *JHAMT* = juvenile hormone acid methyltransferase; *Dis* = disembodied; *Inr* = insulin receptor; 20-E = 20-hydroxyecdysone; *Cyp18a1* = cytochrome P450 18a1; *MAPR* = membrane-associated progesterone receptor; *Met* = methoprene-tolerant protein; *ECR* = ecdysone receptor; *Krh1* = Krüppel homolog 1; *E93* = ecdysone-induced protein 93; *Dronc* = death regulator Nedd2-like caspase; *MDRP-1* = multidrug resistance-associated protein 1; ABC = adenosine triphosphate–binding cassette; GST = glutathione *S*-transferase; *hsp* = heat shock protein; *sHSPs* = small heat shock proteins; LPS = lipopolysaccharide; *Proph* = prophenoloxidase; AMPs = antimicrobial peptides; *Def* = defensin; *PARP-1* = poly(adenosine diphosphate-ribose) polymerase 1; *XRCC1* = X-ray repair cross-complementing protein 1; *NLK* = Nemo-like kinase; *ATM* = ataxia telangiectasia mutated; SOD = superoxide dismutase; CAT = catalase.

$MgSO_4$, 0.1 mM $NaHCO_3$, and 0.025 mM KH_2PO_4) and cellulose tissue as substrate at 19 ± 1 °C, in a 16:8-h light:dark photoperiod, and constant aeration. Larvae were fed ad libitum with fish food (TetraMin®). For bioassays, egg masses were obtained from the cultures, and the hatched larvae were maintained separately (but under the same conditions) from the culture until reaching their fourth instar.

Fipronil solutions and chemical analyses

A stock solution was prepared by dissolving fipronil (technical grade, purity >97%; European Pharmacopoeia) with ethanol (analytical grade). After that, three intermediate solutions of 0.1, 1, and 10 mg L^{-1} were prepared by diluting the stock solution in ethanol. These intermediate solutions were prepared to ensure that the same proportion of solvent was used in all treatments. Three nominal concentrations of fipronil were prepared (0.001, 0.01, and 0.1 µg L^{-1}) by diluting the intermediate solutions with the culturing medium. In addition, a solvent control was prepared with a proportion of 0.001% ethanol, corresponding to the same amount of ethanol used in each dilution. The culture medium was prepared with ultrapure water, and the controls were manipulated independently of the treatments to avoid contamination by the insecticide.

The fipronil concentrations were confirmed in aliquots of the intermediate solutions (0.1, 1, and 10 mg L^{-1}) and solvent control by liquid chromatography coupled with mass spectrometry (Goulart et al., 2020). For that, samples were diluted in H_2O:MeOH 70:30 (v/v) and filtered (polytetrafluoroethylene syringe filter, 22 µm pore size, and 13 mm diameter). A calibration curve was applied to determine the concentrations of

fipronil. The limit of quantification (LOQ) was determined using a signal-to-noise ratio of 10:1, being 0.1 μg L^{-1}. Thus, the linear working range was 0.1 to 100 μg L^{-1} of fipronil. Because of the LOQ, the concentrations of fipronil were confirmed only in the intermediate solutions and control.

Toxicity tests

A 96-h toxicity test was conducted under the same temperature and light regime described above (Test organisms). Each treatment consisted of a glass bottle containing 100 mL of the test solution and 18 larvae. The test solutions were completely renewed 48 h from the test beginning once no significant degradation was expected in this period (Singh et al., 2021). Survival was monitored daily, and food was provided at test renovation (0.5 mg TetraMin $larvae^{-1}$). The experiments were repeated three times using different egg masses (populations). After 24 and 96 h from the test beginning, six larvae were taken, frozen, and stored (−80 °C) for gene expression ($n = 3$) and biochemical ($n = 3$) analyses per replicate. Thus, both analyses were performed with $n = 9$ for 24 and 96 h, three coming from each experiment.

RNA extraction, complementary DNA synthesis, and RT-PCR

The RNA was extracted from the frozen larvae with a Trizol reagent (Life Technologies) following the manufacturer's protocol. First, the RNA was treated with RNase-free DNase (Roche) and then purified using phenol:chloroform:isoamyl alcohol (Fluka), employing Phase Lock Light tubes (Quantabio). Finally, the RNA was precipitated and washed with isopropyl alcohol (0.5 v/v) and 75% ethanol, respectively; resuspended in diethylpyrocarbonate water; and stored (−80 °C) until further analysis. The quantity (in micrograms per milliliter) and quality of extracted RNA were assessed by absorbance spectrophotometry at 260 nm wavelength (Biophotomer; Eppendorf).

Reverse transcription was carried out using 1 μg of purified RNA, 100 units of Moloney murine leukaemia virus enzyme (Invitrogen), 0.5 μg of oligo dT20 primer (Sigma), and 0.5 mM of deoxynucleotide triphosphates (Biotools). The complementary DNA (cDNA) was stored (−20 °C) and used for the RT-PCR. All procedures used are described in more detail by Muñiz-González & Martínez-Guitarte (2020). Forty-two genes previously described for *C. riparius* were selected, besides six endogenous reference genes: glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein 11, ribosomal protein 13, phosphofructokinase, RNA polymerase, TATA-binding-box (see Supporting Information S1, Tables S1–S3). The most stable reference genes at each experimental time (24 and 96 h) were selected using the BestKeeper tool (Pfaffl et al., 2004). The levels of messenger RNA (mRNA) for the selected genes were determined by RT-PCR (CFX96 RT-PCR system; Bio-Rad; Muñiz-González, 2021). Each cDNA sample was run on two independent plates, and each primer was placed in two wells per plate. The mRNA levels were calculated using the $2^{-\Delta\Delta C_q}$ method according to Pfaffl (2001).

Biochemical analyses

The proteins were extracted from frozen larvae (individually) for biochemical analyses according to the protocol adapted to *C. riparius* (Muñiz-González & Martínez-Guitarte, 2020) using an extraction buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid, pH 7.9; 0.5 mM dithiothreitol; 0.1 mM ethylene glycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 8; 400 mM NaCl; 0.5 mM phenylmethylsulfonyl fluoride; and 5% glycerol). The extracted protein was quantified in a microplate reader (Multiskan Go; Thermo) with Bradford solution (Bio-Rad) according to Bradford (1976) and stored at −80 °C until the enzymatic activity analyses. The CAT activity was determined according to the procedure described in Li & Schellhorn (2007). The GST and AChE activities were measured based on the method described in Muñiz-González & Martínez-Guitarte (2020).

Data analyses

Responses on gene expression and enzyme activities in each treatment were compared with the solvent control group in each sampling period by using generalized linear models (GLMs) applying the Gaussian family with the identity-link function. Alterations in larval survival were also analyzed using GLM with the binomial family and the logit-link function. Multiple comparisons were made using Fisher's correction factor (least significant difference test). All analyses were carried out with a confidence interval of 95% ($p < 0.05$) in the software R (Ver 3.6.0) with the application of RStudio (Ver 1.2.1335; R, 2019).

RESULTS

Chemical analyses

The concentrations of fipronil quantified in the solutions were 11.07, 1.08, and 0.106 mg L^{-1} relative to the nominal concentrations of 10, 1, and 0.1 mg L^{-1}. Because the measured concentrations were within 20% of the nominal, the nominal concentrations were reported as the test concentrations (OECD, 2011). The insecticide was not detected in the control treatment.

Survival

No significant effects were detected on the survival of *C. riparius* larvae after exposure to any of the test doses of fipronil in any experimental period ($p > 0.05$), demonstrating that the molecular and biochemical responses evaluated were relative to sublethal concentrations.

Gene expression

Endocrine system. No significant alterations occurred in the expression of the 10 endocrine system–related genes in *C. riparius* larvae after 24-h exposure to fipronil (Figure 2; $p > 0.05$). In the same way, no alterations occurred in mRNA levels of the genes death regulator Nedd2-like caspase, insulin receptor, and methoprene-tolerant protein after 96-h exposure (Supporting Information S1, Figure S1). A down-regulation in the expression of the *Cyp18a1* and Krüppel homolog 1 (*Kr-h1*) genes was observed in larvae exposed to all of the concentrations. In the same way, 96-h exposure to 0.01 μg L^{-1} of fipronil decreased the membrane-associated progesterone receptor (*MAPR*) expression ($p < 0.05$). Contrarily, 96-h exposure to 0.001 μg L^{-1} of fipronil up-regulated expression of the JH acid methyltransferase (*JHAMT*) gene, and exposure to 0.1 μg L^{-1} up-regulated the mRNA levels of ecdysone receptor (*EcR*), disembodied (*Dis*), and ecdysone-induced protein 93 (*E93*; $p < 0.05$; Figure 2).

Detoxification mechanisms. No alterations occurred in the mRNA levels of the *Cyp9F2*, *GSTt3*, and *ABC* genes in either fipronil conditions or exposure time ($p > 0.05$; Supporting Information S1, Figure S2). After 24-h exposure, no alterations occurred in the expression of detoxification response–related genes, except for down-regulation of the *GSTe2* gene on larvae exposed to 0.001 and 0.1 μg L^{-1} of fipronil ($p < 0.05$; Figure 3). The three tested concentrations down-regulated the expression of *Cyp6d13*, *GSTe2*, and the multidrug resistance–associated protein 1 (*MRP-1*) genes after 96-h exposure. In the same way, a

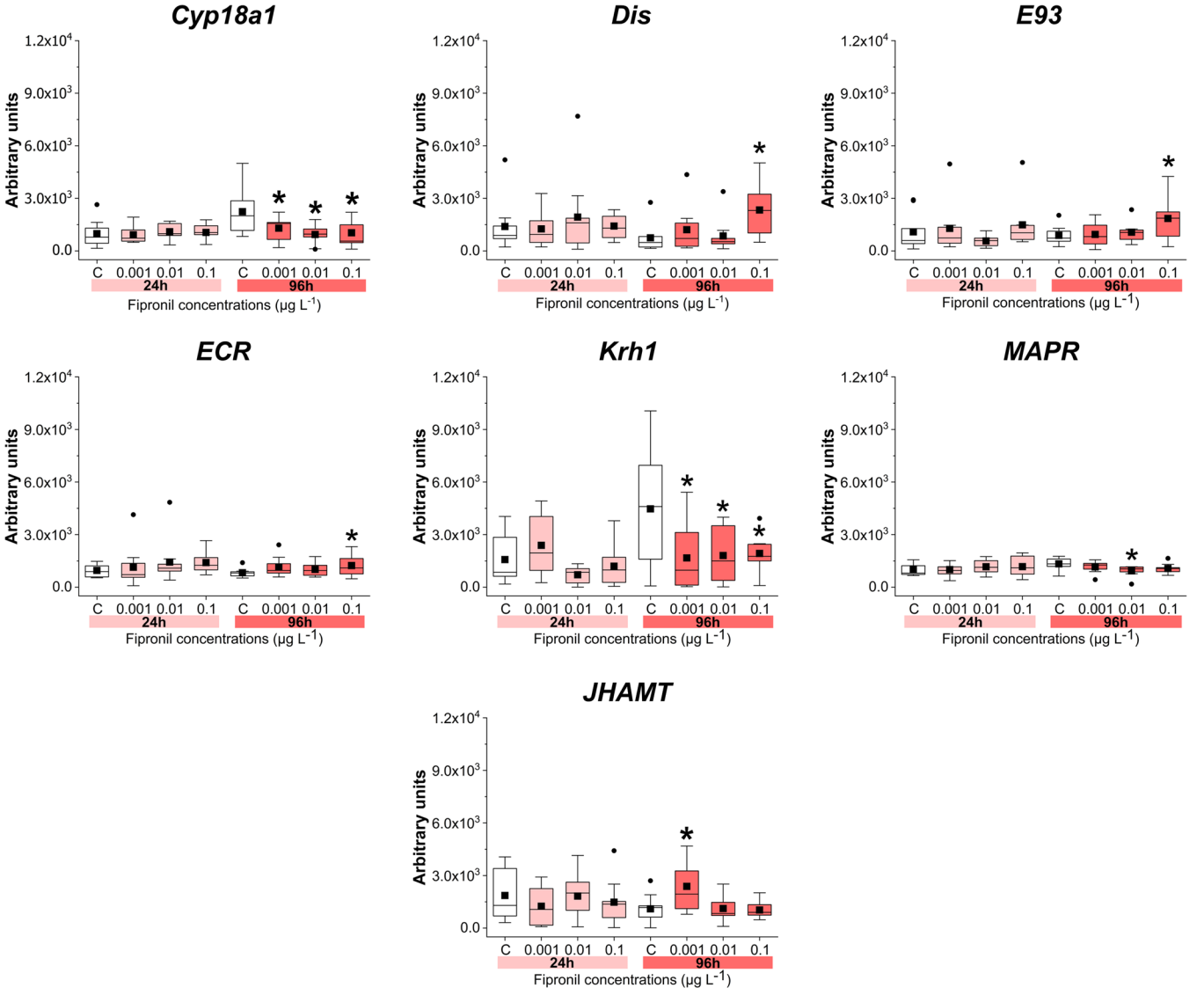

FIGURE 2: Expression of endocrine system–related genes in *Chironomus riparius* larvae ($n = 9$) after 24- and 96-h exposure to the insecticide fipronil. The horizontal line within the box represents the median value, and the black square represents the mean values. The lower and upper boundaries indicate the 25th and 75th percentiles, and black circles are outliers. Asterisks denote differences in gene expression levels from the control ($p < 0.05$) at the respective exposure time. *Cyp18a1* = cytochrome P450 18a1; *Dis* = disembodied; MAPR = membrane-associated progesterone receptor; *E93* = ecdysone-induced protein 93; *ECR* = ecdysone receptor; *Krh1* = Krüppel homolog 1; MAPR = membrane-associated progesterone receptor; *JHAMT* = juvenile hormone acid methyltransferase.

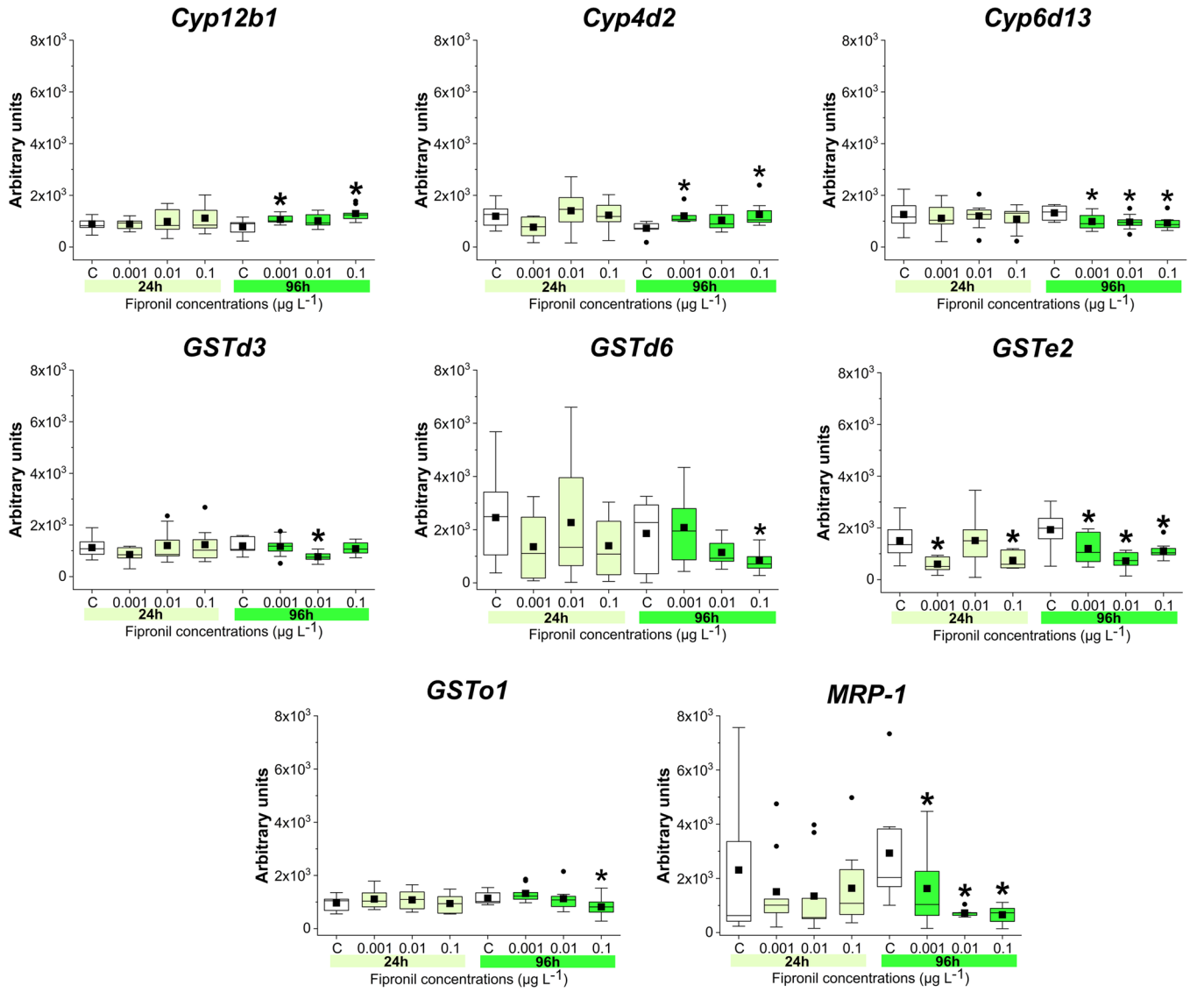

FIGURE 3: Expression of detoxification mechanism–related genes in *Chironomus riparius* larvae ($n = 9$) after 24- and 96-h exposure to the insecticide fipronil. The horizontal line within the box represents the median value, and the black square represents the mean values. The lower and upper boundaries indicate the 25th and 75th percentiles, and black circles are outliers. Asterisks denote differences in gene expression levels from the control ($p < 0.05$) at the respective exposure time. *Cyp* = cytochrome P450; *GST* = glutathione *S*-transferase; *MRP-1* = multidrug resistance-associated protein 1.

down-regulation occurred for *GSTd3* at 0.01 µg L^{-1} and for *GSTd6* and *GSTo1* at 0.1 µg L^{-1} of fipronil in this period ($p < 0.05$). On the contrary, exposure to 0.001 and 0.1 µg L^{-1} of fipronil up-regulated the levels of *Cyp4d2* and *Cyp12b1* genes after 96 h (Figure 3).

Stress response. The stress response was assessed by the expression of genes related to different families of HSPs. No alterations occurred on mRNA levels of the *hsp90*, *hsp70*, *hsp40*, and *shsp24* genes ($p > 0.05$; Supporting Information S1, Figure S3). However, after 24-h exposure, overexpression of *shsp17* and *shsp23* genes occurred at 0.01 µg L^{-1} of fipronil (Figure 4). In the same way, larvae exposed to 0.1 µg L^{-1} presented an up-regulation of *shsp22* after 24 and 96 h and of *hsp60* after 96 h. Conversely, the genes *shsp21*, *hsp10*, and *shsp27* were down-regulated after 96 h in larvae exposed to 0.001, 0.01, and 0.1 µg L^{-1} of fipronil, respectively.

Antioxidant defense. Three antioxidant defense–related genes were assessed. No alterations occurred in the levels of the manganese SOD (*SODMn*; $p > 0.05$; Supporting Information S1, Figure S4) gene. However, after 96-h exposure to 0.01 and 0.1 µg L^{-1}, the expression of the copper and zinc SOD (*SODCuZn*) and *CAT* genes was down-regulated, respectively ($p < 0.05$; Figure 5).

Immune system. Two genes associated with the immune system of *C. riparius* were assessed after exposure to fipronil. No alterations occurred in the mRNA levels of prophenoloxidase (*Proph*) (Supporting Information S1, Figure S5); however,

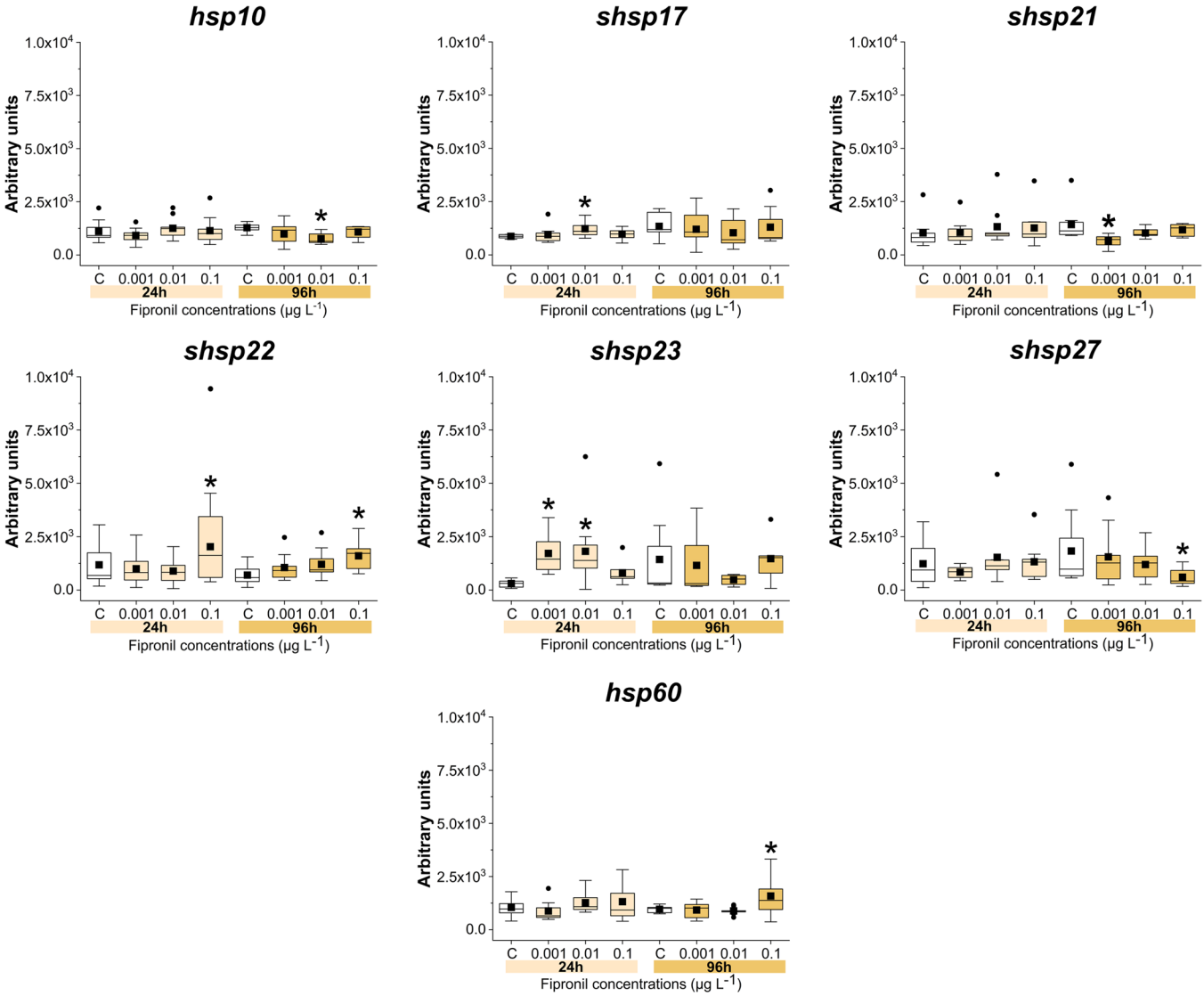

FIGURE 4: Expression of stress response–related genes in *Chironomus riparius* larvae ($n = 9$) after 24- and 96-h exposure to the insecticide fipronil. The horizontal line within the box represents the median value, and the black square represents the mean values. The lower and upper boundaries indicate the 25th and 75th percentiles, and black circles are outliers. Asterisks denote differences in gene expression levels from the control ($p < 0.05$) at the respective exposure time. hsp = heat shock protein; shsp = small heat shock protein.

the gene defensin (*def*) was up-regulated at 0.01 µg L^{-1} after 96 h (Figure 5C).

DNA repair. Regarding the DNA repair–associated genes studied, only the ataxia telangiectasia mutated (*ATM*) gene was up-regulated after 24 h of exposure to 0.001 µg L^{-1} ($p < 0.05$; Figure 5D). No alterations were observed in the levels of the genes Decay, Nemo-like kinase, poly(adenosine diphosphate-ribose) polymerase, and X-ray repair cross-complementing protein 1 ($p > 0.05$; Supporting Information S1, Figure S6).

Enzymatic activity

Figure 6 presents the enzymatic activity measured in *C. riparius* larvae. After 24 h of exposure, no alterations occurred in AChE, CAT, and GST activity ($p > 0.05$). On the contrary, after 96 h, the insecticide provoked increases in AChE activity at 0.01 and 0.1 µg L^{-1} and in GST and CAT activity at 0.01 µg L^{-1} ($p < 0.05$).

DISCUSSION

No lethal effects occurred after exposure to any of the fipronil test concentrations. It was an expected response, given that the 48-h median lethal concentration (LC50) for *C. riparius* was reported to be 1.74 µg L^{-1} (Monteiro et al., 2019). Furthermore, other species belonging to the *Chironomus* genus, such as *C. lebetis* with a 96-h LC50 value of 1.06 µg L^{-1}, already seem to indicate that we would not observe lethality even at the highest of our study concentrations (0.1 µg L^{-1}; Stratman et al., 2013). Besides, even after chronic exposure for 8 days, no significant mortalities were observed in *C. sancticaroli* larvae exposed to 0.4 µg L^{-1} of fipronil (Pinto, Moreina, et al., 2021). These results show that the molecular and

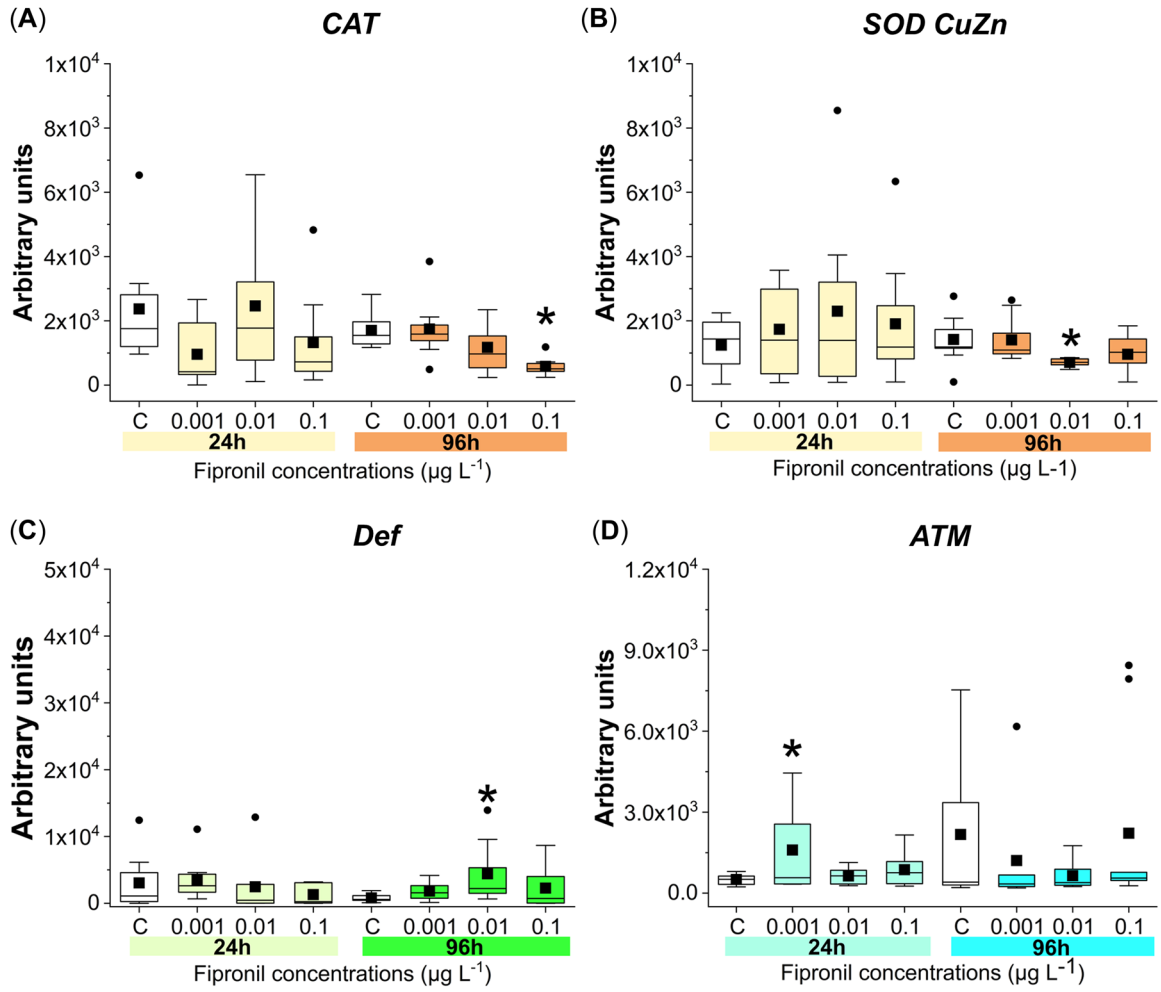

FIGURE 5: Expression of antioxidant defense–(**A**, **B**), immune system–(**C**), and DNA repair–related (**D**) genes in *Chironomus riparius* larvae ($n = 9$) after 24- and 96-h exposure to the insecticide fipronil. The horizontal line within the box represents the median value, and the black square represents the mean values. The lower and upper boundaries indicate the 25th and 75th percentiles, and black circles are outliers. Asterisks denote differences in gene expression levels from the control ($p < 0.05$) at the respective exposure time. CAT = catalase; SOD = superoxide dismutase; *Def* = defensin; *ATM* = ataxia telangiectasia mutated.

biochemical responses described occurred at sublethal concentrations; despite the absence of mortality, some impacts on gene expression were observed. Moreover, it is essential to emphasize that the studied concentrations employed in the present study are in a concentration range already reported in aquatic environments worldwide of 0.006 to 26.2 µg L^{-1} (Fang et al., 2019; Marchesan et al., 2010), reinforcing the relevance of the obtained results.

In the present study, seven of the ten endocrine-associated genes were altered by fipronil after 96 h. At the same time, no alterations occurred in early exposure (24 h). These results reveal a later response in the endocrine-related genes of *C. riparius* after fipronil exposure. Despite fipronil having previously been indicated as a potential endocrine-disrupting chemical (Goff et al., 2017), alterations in the expression of genes associated with the endocrine system were poorly studied. Thus, the present results showed a novel response pathway modulation for aquatic organisms.

Exposure to the higher fipronil concentration up-regulated the mRNA levels of *Dis*, *EcR*, and *E93* and down-regulated the levels of *Cyp18a1* and *Kr-h1*. *Dis* is a Halloween gene encoding a CYP enzyme (CYP302a1) associated with the 20-E synthesis pathway (Gilbert, 2004). On dipterans, a heterodimer composed of the EcR and ultraspiracle (Usp) proteins acts as a 20-E receptor inside the cells. The 20-E–EcR/Usp complex controls a conserved transcription factor network associated with development and metamorphosis (Truman, 2019). This transcription cascade regulated by the 20-E–EcR/Usp complex includes the *E93* gene, which stimulates the molt (Belles, 2020). Repression of *E93* is provoked by *Kr-h1*, which is controlled by the levels of JH and acts as a mediator of the JH antimetamorphic activity on larvae (Belles, 2020; Muñiz-González et al., 2021). Finally, *Cyp18a1* is responsible for encoding the enzyme 26-hydroxylase, responsible for 20-E inactivation by its oxidation into 20-hydroxyecdysonoic acid (Guittard et al., 2011). Considering these mechanisms, the alterations provoked by fipronil at 0.1 µg L^{-1} in *C. riparius* point to possible increases in the activity of 20-E by increasing the mRNA levels of genes associated with 20-E synthesis (*Dis*—3.1 times relative to control), signalling (*ECR*—2.1 times), and response (*E93*—1.5 times) and decreases

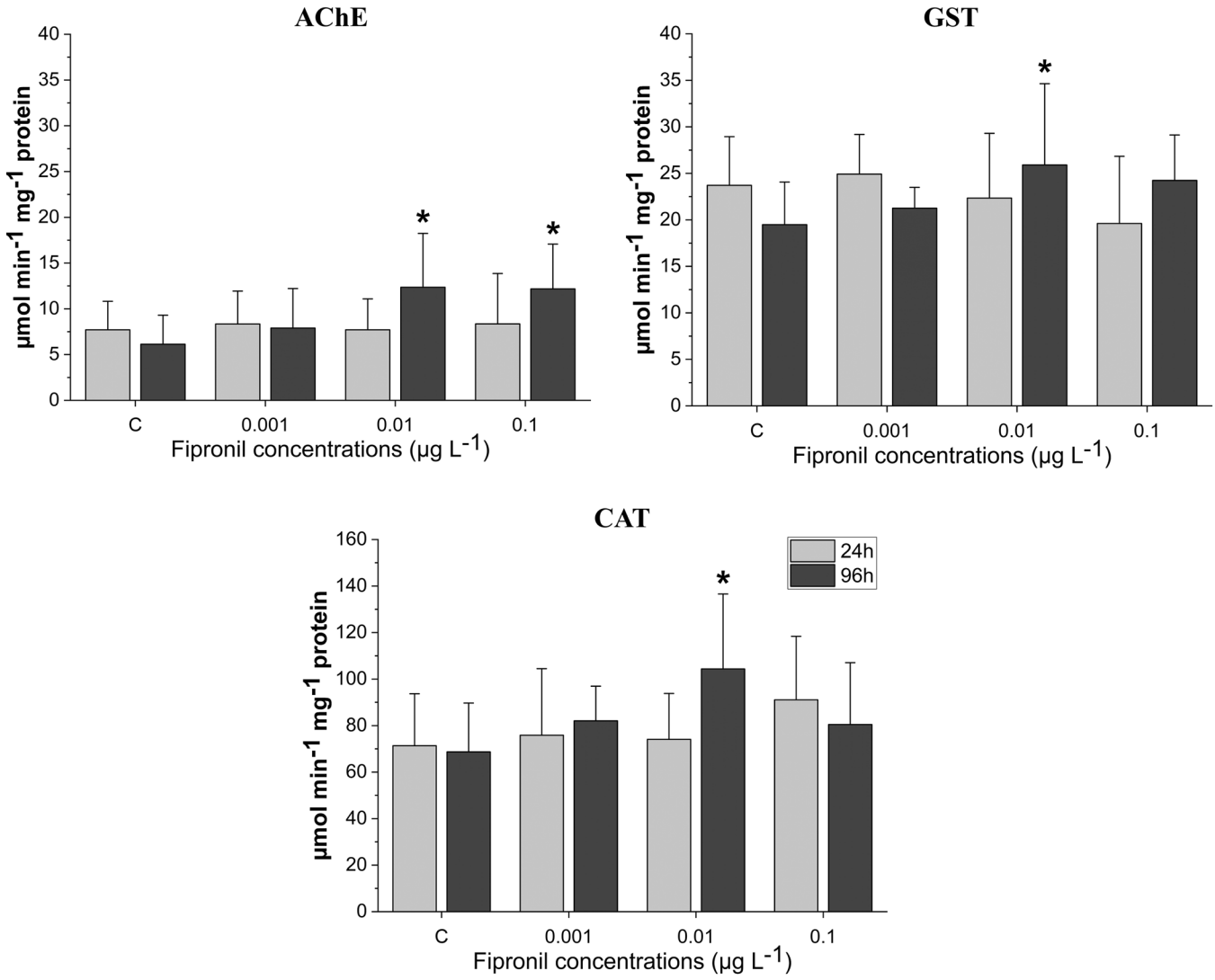

FIGURE 6: Enzymatic activity (mean ± SE) in *Chironomus riparius* larvae ($n = 9$) after 24- and 96-h exposure to the insecticide fipronil. Asterisks denote differences in gene expression levels from the control ($p < 0.05$) at the respective exposure time. AChE = acetylcholinesterase; CAT = catalase; GST = glutathione *S*-transferase.

in genes responsible for 20-E inactivation (*Cyp18a1*—1.7–2.2 times) and metamorphic repression (Kr-h1—2.3–2.7 times), implying delayed development of exposed larvae.

The intermediate concentration provoked down-regulation of the *MAPR* gene, the protein family of which is associated with binding the steroid function (Cahill, 2007). Furthermore, the mRNA levels of *Cyp18a1* and *Kr-h1* were also down-regulated. These genes were already described as having 20-E inactivation and antimetamorphic activity. Finally, the lower concentration of fipronil decreased the *Cyp18a1*, *Kr-h1*, and *JHAMT* mRNA levels. In insects, *JHAMT* is involved in the JH synthesis pathway (Defelipe et al., 2011). In line with the observed results, the alterations observed in the 20-E and JH pathways can trigger developmental delays in *C. riparius* larvae. In a chronic experiment with the same species, no developmental delays occurred after exposure to fipronil at concentrations ranging from 0.005 to 0.081 µg L^{-1}; however, the percentage of viable emerged adults was decreased at 0.04 µg L^{-1}, and no viable adults were produced at 0.081 and 0.162 µg L^{-1} (Monteiro et al., 2019). On the other hand, fipronil delayed the development and increased the pupae lethality of *C. sancticaroli* at concentrations ranging from 0.2 to 1.6 µg L^{-1} (Pinto, Rocha, et al., 2021), more similar to our study conditions. Besides, another study with *C. sancticaroli* reported that fipronil (0.3–3.7 µg L^{-1}) increased mentum deformities, mainly in development-retarded larvae. These results were associated with possible endocrine alterations caused by fipronil (Pinto, Moreina, et al., 2021). Buccal deformities are fully formed in association with physiological disturbances in the molting period (Bisthoven et al., 1992). The present study showed alterations in 20-E pathways, which could influence cuticle formation during molting (Truman, 2019), as previously detected. Thus, our results support, at least partially, the hypothesis described for *C. sancticaroli*. To the best of our

knowledge, no effects on mentum deformities of *C. riparius* associated with fipronil exposure are described in the literature, and this could be an interesting future endpoint to confirm these results in this species.

In the present study, fipronil altered the mRNA levels of genes from the three detoxification phases, mainly after 96-h exposure. Two Cyp-related genes were up-regulated (*Cyp12b1* and *Cyp4d2*), and one was down-regulated (*Cyp6d13*). On the other hand, four GST- (*GSTd3*, *GSTd6*, *GSTe2*, and *GSTo1*) and one ABC transporter–related gene (*MRP-1*) were down-regulated. Lee et al. (2018) assessed the effects of fipronil on Cyp gene expression in two rotifer species. One of them, *Brachionus calyciflorus*, showed down-regulation for seven genes and up-regulation for 12 of the 31 genes studied. The other one, *Brachionus plicatilis*, showed up-regulation of 16 of the 28 studied genes. To both species, the other genes had a variable regulation (up or down) depending on the fipronil doses, varying from 250 to 1000 $\mu g\ L^{-1}$ (Lee et al., 2018). In insects, the activity of the Cyp family is associated with the conversion of lipid-soluble chemicals into water-soluble metabolites. Thus, the detoxification of xenobiotics is directly associated with the overexpression of genes coding Cyp proteins (Lu et al., 2021), as observed in the present study in two of four studied genes.

The down-regulation of four GST-associated genes and *MRP-1* was an unexpected response given that the GST classes and *MRP-1* have been associated with the solubilization of xenobiotics and their transport outside the cells, respectively, reducing their toxicity (Berenbaum & Johnson, 2015). Increases in the expression of GST-related genes in insects were already associated with insects' resistance to fipronil (Gao et al., 2021). On the contrary, decreases in GST activity occurred in *C. riparius* exposed for 48 h to 0.11 and 0.22 $\mu g\ L^{-1}$ of fipronil (Monteiro et al., 2019). These enzymatic results align with the down-regulation of GST-associated genes in our study. However, the enzymatic assays in the present study revealed increases in GST activity at 0.01 $\mu g\ L^{-1}$ after 96-h exposure. We highlight that the enzymatic activity assay analyzes the overall GST group without differentiation between the GST family members (Martínez-Guitarte, 2018). Thus, the increases in GST activity may be associated with other groups not studied in gene expression analyses and other members of the studied group. Besides, the increases observed in GST activity in the present study occurred at concentrations 10-fold lower than the decreases reported by Monteiro et al. (2019) beyond a different exposure time, thus suggesting that these responses were variable and adaptable according to the time and fipronil dose.

Similar to those described for the GST enzyme, Monteiro et al. (2019) also reported decreases in CAT activity in *C. riparius* after 48-h exposure to fipronil at 0.11 and 0.22 $\mu g\ L^{-1}$. However, in the present study, *CAT* mRNA levels declined at 0.1 $\mu g\ L^{-1}$ of fipronil. Thus, our results presented similar responses to those of Monteiro et al. (2019) at the same fipronil levels. Besides, a down-regulation in the *SOD-CuZn* gene occurred at 0.01 $\mu g\ L^{-1}$. The *CAT* and *SOD-CuZn* enzymes are involved in antioxidation defense against free radicals or reactive oxygen species (ROS; Ighodaro & Akinloye, 2018). At the intermediate concentration (0.01 $\mu g\ L^{-1}$), fipronil increased CAT enzymatic activity after 96-h exposure, contrary to the molecular response observed at the higher dose, revealing variable responses according to fipronil concentrations.

Decreases in antioxidant defense provoked by fipronil may imply oxidative stress on cells. The present study assessed the HSP-associated genes as stress responses, including the *hsp90*, *hsp70*, *hsp60*, *hsp40*, and sHSP families. These proteins are used as stress markers considering a direct relationship between increases in HSP expression/synthesis and the generalized stress level (Gupta et al., 2010). Overexpression of *hsp60*, *shsp23*, *shsp22*, and *shsp21* occurred depending on experimental time and fipronil dose, signaling a stress level in exposed larvae. Contrarily, some genes (*shsp27*, *shsp21*, and *shsp10*) were down-regulated at 96-h exposure with potentially harmful effects on organisms because of their essential functions mediated in the organisms. The HSPs are conserved molecular chaperones that play several cellular roles, including the maintenance of the proteins' structure and, consequently, their normal functioning (Liu et al., 2021). The sHSPs, such as *shsp21* and *shsp27* (molecular weight 15–30 kDa), act as molecular chaperones, preventing undesired interactions between proteins and refolding denatured ones (Gupta et al., 2010). The primary function of Hsp60, coupled with Hsp10, is to fold unfolded protein substrates in mitochondria (Zininga et al., 2018), which is essential for correct respiration. In the present study, an inverse pattern in gene expression of *Hsp60* (up-regulated) and *Hsp10* (down-regulated) occurred. Thus, the imbalance between increases and decreases in the expression of these genes may provoke mitochondrial stress, with consequences on maintaining protein homeostasis inside this organelle (Bavisotto et al., 2020).

Besides increasing cellular stress, the formation of ROS in cells may provoke damage in macromolecules, including DNA (Noorimotlagh et al., 2018). In the present study, five DNA repair–associated genes were studied, with early (24 h) modulation observed in mRNA levels of *ATM* at the lower fipronil concentration with up-regulation. The function of ATM kinase on cells is associated with the repair of DNA double-strand breaks (Mota et al., 2019). Thus, our results indicate that short exposure to low doses of fipronil provokes modulation of DNA repair genes that could be related to *C. riparius* DNA damage despite these alterations not persisting until 96 h. A tendency for increases in oxidative defense denoted by the two SOD genes studied was observed at this concentration, helping in DNA damage prevention. The results at the other fipronil concentrations suggest that no DNA damage occurred throughout the exposure time. Therefore, according to these results, the insecticide fipronil seems to have a low capacity as a genotoxic compound.

In insects, the innate immune system plays a vital role in the organism's defense, presenting cellular and humoral responses against pathogens (Sheehan et al., 2018). The humoral response is associated with activating immune genes that encode antimicrobial peptides and an enzymatic cascade related to the hemolymph coagulation and melanization or production of oxygen- and nitrogen-reactive species (Tsakas & Marmaras, 2010). The intermediate fipronil concentration provoked an

overexpression of a defensin peptide encoding-related gene. Contrary to our study, fipronil did not alter defensin-1 gene expression in the honeybee *Apis mellifera* (Zaluski et al., 2015). Increases in defensin gene expression were also reported in *C. riparius* after exposure to the insecticide endosulfan at 10 $\mu g\,L^{-1}$, besides the down-regulation observed at 1 $\mu g\,L^{-1}$ (Muñiz-González et al., 2021). In the same way, the pharmaceutical ibuprofen overexpressed defensin after 96-h exposure at concentrations ranging from 0.1 to 100 $\mu g\,L^{-1}$. The authors associated the activation of the *C. riparius* immune system with the oxidative stress provoked by ibuprofen (Muñiz-González, 2021), which may help to explain the results observed in the present study.

Fipronil is a neurotoxic insecticide in which the active mechanism on insects is associated with alterations in the GABA-gated channels (Gunasekara et al., 2007). However, trying to check alternative toxicity responses, we observed alterations in AChE activity, which is interesting because it reveals another neural response in a non-target insect. In the same way, increases in AChE activity were reported in the dipteran *Musca domestica* (Farooq & Freed, 2018) and the beetle *Leptinotarsa decemlineata* (Nikonorov et al., 2018) after exposure to fipronil. On the contrary, exposure of *A. mellifera* to fipronil decreased AChE activity (Roat et al., 2017), thus indicating a variable response of invertebrates depending on fipronil dose. To our knowledge, no effects associated with fipronil on AChE activity in chironomids are reported in the literature. Thus, our results reveal that, besides the neurotoxicity associated with GABA inhibition, fipronil may increase AChE activity.

The gene expression and enzymatic activity responses reported in the present work occurred after exposure to environmentally relevant concentrations of fipronil. These results demonstrate a concern regarding the exposure of populations of chironomids in freshwater environments, mainly because of the relative persistence of fipronil in aquatic ecosystems that may reach 100 days, depending on the environmental conditions (Singh et al., 2021).

CONCLUSIONS

The present results describe new responses after fipronil exposure on a key organism in the aquatic system. The employment of molecular tools to assess the effects of pesticides on invertebrates is advantageous, given the quick response obtained. Besides, the possibility of investigating multiple response mechanisms simultaneously contributes to filling the gaps in the underlying fipronil toxicity mechanism. Alterations in gene expression and enzymatic activity were detected mainly after 96-h exposure to fipronil, revealing a later response of *C. riparius* larvae. Interestingly, all tested concentrations of fipronil promoted alterations in some 20-E or JH pathway-related genes on *C. riparius*, thus confirming the endocrine-disrupting potential of this insecticide. Fipronil also provoked gene alterations associated with detoxification, stress response, antioxidant activity, DNA repair, and immunity. These results reveal cascade effects on organisms at exposure to sublethal doses, mainly related to increases in oxidative stress in cells that may provoke damage to macromolecules. Fipronil is a neurotoxic insecticide acting on GABA receptors; however, another neurological implication not initially described for fipronil, related to AChE activity, has been detected in the present study. These new mechanisms need to be explored more to expand the discussion about the risks associated with environmental contamination by fipronil, mainly due to these effects that have been reported with realistic concentrations already detected worldwide.

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