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Original Article

Single Acute Sublethal Fipronil Concentration Induces Behavioral Toxicity and Suggests Endocrine-Disrupting Properties in Zebrafish (*Danio rerio*)

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Abstract

Fipronil (FPR), a second-generation phenylpyrazole insecticide, is used in Veterinary Medicine, agriculture, and horticulture against fleas, ticks, ants, cockroaches, and other pests. By selectively inhibiting gamma-aminobutyric acid receptors in vertebrates and invertebrates, studies suggest that acute exposure harms sensory and motor systems, and long-term exposure has an endocrine-disrupting effect. The main objective of this study was to investigate the effects of a sublethal concentration of FPR on the behavior of adult zebrafish, using them as an assessment model to detect early Central Nervous System toxicity. First, to achieve this dose, we exposed the fish to several FRP concentrations and monitored lethality for up to 72 h. Using the FRP sublethal concentration, the behavioral signs of toxicity were observed in the exploratory behavior in the open field, anxiety-like behavior, and social and sexual preferences. The results showed that the sublethal concentration of FRP was 0.5 µg/L. Under this concentration, the fish exhibited significant behavioral changes in the aquarium, including tremors, increased movement frequency and duration, and a longer time to reach the surface. Furthermore, an increase in anxiolytic-like behavior, and a reduction in social and sexual preferences, without interference in the open field exploration, were observed. Thus, our data indicate that acute exposure to sublethal concentrations of FPR induces neurotoxic effects that can be attributed to its GABAergic antagonist action. The FRP endocrine disruptor could, in part, be due to reduced sexual preference, with ecotoxicological importance for fish reproduction. Thus, the present results validate the use of the zebrafish model as a tool in studies related to FPR neurotoxicity.

Keywords: Behavior; fish; insecticide; neurotoxicity; pesticide; phenilpirazol;

INTRODUCTION

Fipronil (FPR), a second-generation phenilpirazol insecticide, is used in agriculture and horticulture against fleas, ticks, ants, and cockroaches (Hamon, Shaw, Yang, 1996; Simon-Delso *et al.*, 2015). In Veterinary Medicine, FPR is used mainly topically to control fleas and ticks. The mechanism of action of FPR occurs through the antagonism of the GABA receptor (γ-aminobutyric acid), blocking chloride channels, interfering with neuromodulation, and leading to death due to hyperexcitation (Santarem *et al.*, 2023). The widespread use of insecticides, such as nicotinoids and FPR, raised concerns about risks to the ecosystem provided by a wide range of species and environments that could be affected by these insecticides (Chagnon *et al.*, 2015). Depending on the substrate and conditions, FPR is slowly degraded in vegetation, soil, and water.

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FPR and degraded products may bioaccumulate, particularly in fish, acting as an endocrine disruptor, modifying rats' thyroid gland function and estrus cycle (Hu *et al*., 2020). In this respect, endocrine disruptors and their mixtures impair the reproductive system of zebrafish and other aquatic organisms, leading to infertility and subfertility in these species (Huang *et al.,* 2015). Fipronil degrades slowly in water and sediment under anaerobic conditions. In aquatic organisms, FRP varies from being highly toxic to very highly toxic when calculated based on exposure to relatively low concentrations under controlled laboratory conditions (Tingle *et al.,* 2003). Thus, FRP may represent an ecological risk to aquatic organisms.

In the aquatic environment, FRP is degraded to fipronil sulfone, a persistent, oxidative FRP metabolite that is commonly detected in aquatic systems and has neurotoxic effects on non-target organisms. Recently, Arruda Leite *et al*. (2025) exposed zebrafish embryos and larvae to environmentally relevant concentrations of fipronil sulfone at different larval developmental stages. Morphological, behavioral, and biochemical evaluations showed that in larvae, high concentrations significantly reduced swim bladder inflation and spinal curvature. Survival decreased progressively with increasing concentrations, reaching 100 % mortality at the highest concentration. At behavioral levels, the larvae exhibited hyperactivity followed by reduced locomotion, attributed to GABAergic receptor blockage and acetylcholinesterase inhibition. In addition, increased oxidative stress was confirmed by increased reactive oxygen species and glutathione S-transferase activity. Thus, FRP water contamination and its metabolites are relevant to their ecotoxicological impacts.

Although mice and rats are commonly used as model organisms for toxicological studies, zebrafish (*Danio rerio*) is a less expensive, rapid, and high-throughput screening system for toxicity evaluation. Thus, embryo, larvae, and adult zebrafish have been used in several toxicity tests since 1970 (Niimi and LaHam, 1976), and comparative studies from 2004 to 2025 suggested that zebrafish could be used as alternatives to mammalian models (Celardo *et al.*, 2025). Nowadays, zebrafish and their larvae have been found in several toxicological studies, including behavioral, intestinal, cardiovascular, hepatic, endocrine toxicity, neurotoxicity, immunotoxicity, genotoxicity, reproductive, and transgenerational tests (Zhao *et al*., 2024). Also, the mechanisms studied include oxidative stress, inflammation, autophagy, and dysbiosis of gut microbiota (Zhao *et al.*, 2024). Zebrafish are valuable complements to rats' models for screening many chemicals and mixtures (Vorhees and Williams, 2021). In this respect, zebrafish help evaluate the aquatic exposure risk to toxicants and neurotoxicological studies of chemicals. Studies on the behavior of adult zebrafish exposed to FPR may reveal the neurotoxicity of this pesticide and serve as a model for the consequences of this exposure in the aquatic environment (Pinheiro-Da-Silva *et al.*, 2020; Pompermaie *et al.*, 2020; Costa *et al*., 2023). Acute exposure to FPR-impaired sensory and motor systems in zebrafish significantly reduced the survival rate. These effects were attributed to damage to lateral hair cells and brain tissue caused by oxidative stress, inflammation, and apoptosis (Wu *et al*., 2021).

FRP acting as a GABAergic antagonist and an endocrine disruptor, could affect zebrafish behavior. Several studies about the FRP neurobehavioral toxicity were performed with toxic doses. Few studies have examined the impact of sublethal concentrations of FPR on adult behavior as a tool to reveal zebrafish toxicity. In this study, we established the FPR acute sublethal concentration by exposing the zebrafish to multiple FPR levels, and investigated the FPR behavioral effects. The results obtained could be important because FRP can be found in the environment, particularly in surface waters, with levels reaching the nanogram per liter (ng/L) range and only occasionally exceeding chronic toxicity limits, posing a risk to aquatic ecosystems.

MATERIALS AND METHODS

Ethical Approval

The experimental protocols followed were approved by the Ethics Committee for Animal Use of Paulista University (protocol number nº 302/15, CEUA-UNIP). All experiments were conducted in accordance with standardized laboratory practice protocols and adherence to quality assurance methods. Every effort was made to minimize animal suffering.

Animals

Zebrafish (*Danio rerio*), aged 8–9 months and with a maximum size of 5 cm, were obtained from a commercial breeder (Izael Ba Hi, Indaiatuba, São Paulo, Brazil) and brought to the laboratory, where they were maintained for 15 days to acclimate. The water distributed by SABESP (SP) was dechlorinated and maintained at 24 ± 2 °C using heaters. The water hardness was 42 mg/L $CaCO_3$, with a pH of 7.0 ± 0.2.

The luminous intensity was 600 lux, with a 12-hour light/12-hour dark photoperiod. Except during the experiments, the aquaria were aerated using air compressors and connected to water filtration systems equipped with acrylic wool and activated charcoal to enhance water quality. The water was changed every seven days, representing 25% of the total water volume. The water was changed every seven days, representing 25% of the total water volume. The fish were fed

Tetramin® as recommended by the manufacturer and in accordance with CETESB 1990 guidelines (CETESB, 2018). The fish did not feed during testing. We analyzed the pH, dissolved oxygen, and conductivity at the beginning and end of each test.

Drugs

The FPR was the commercial product Regent® 800 WG (BASF—Agro Brasil, São Paulo, SP, Brazil) in a powdered form containing 80% FPR. The FPR proportion in the commercial product was corrected when the pesticide was dissolved in aquarium water.

Prospecting the Concentration of FPR at 50% Lethal Concentration and Sublethal Concentration of FPR

Four fish, each at a different concentration, were individually introduced into an aquarium (2,25 L) containing various concentrations of FPR, based on the USEPA maximal concentration in water (6.0, 4.0, 2.4, 2.0, 1.0, and 0.5 µg/L) (USEPA, 1999). The number of dead fish, signs of toxicity, and lethality were observed every 10 minutes over 1 hour. Then, we transferred the fish to an aquarium containing maintenance water, and lethality was recorded at 2, 24, 48, and 72 hours. We considered that the fish died when they did not respond to the tail stimulus. When lethality did not occur at a given concentration, four additional fish were exposed to this concentration to confirm it was a sublethal concentration. The fish's behavior was filmed.

Behavioral Studies

Below are descriptions of the zebrafish behavior, general activity, exploratory behavior in the open field, light/dark preference, and social and sexual preference tests.

General Activity in an Aquarium

Our previous study (Bernardi *et al*., 2018) described zebrafish general activity in an aquarium with a volume of 2.25 L. Because the fish had a maximum dimension of 5 cm, the front wall of the aquarium was divided into six equal sections, each measuring 5 cm. These divisions allow for greater sensitivity in the area covered by the fish. General activity and signs of toxicity were individually observed at 0-10, 11-20, 21-30, 31-40, 41-50, and 51-60 minutes during FPR exposure. Four parameters were recorded as previously described (Bernardi *et al*., 2013; Santos *et al.*, 2018): (A) Tremor frequency, which represents each time the fish started and stopped swimming with progressive full-body contractions from head to tail - scores to tremor intensity were attributed as (1) slight tremor, (2) median tremors, (3) moderate tremors, (4) strong tremors, and (5) powerful tremors; (B) Frequency in which the fish emerges, placing its mouth close to the water surface; (C) Time that the fish remained on the water surface, in seconds; D) Erratic swimming frequency, which represents the frequency the fish sharply changes its swimming direction or speed. Eight zebrafish were individually exposed to a sublethal concentration (0.5 µg/L), filmed for one hour, and then transferred to an aquarium with maintenance water. We observed the lethality up to 72 hours after the exposure. For comparison with fish not exposed to FPR, we used the behavior of only four fish that received no treatment, as recommended by the CEUA-UNIP. All procedures were recorded on video for later analysis.

Exploratory Behavior in the Open Field

We used a round aquarium with a diameter of 25 cm and a height of 9 cm to observe the fish's exploratory behavior (Borba *et al.*, 2025). For this, eighteen zebrafish were divided into a control group (n = 8) and an experimental group (n = 10). The fish in the experimental group were individually exposed to 0.5 µg/L of FPR for 60 minutes in a 2L aquarium and transferred individually to the round aquarium containing the same FPR concentration. The control group was treated similarly to the experimental group without exposure to insecticide. The parameters observed were the locomotion and immobility seconds. All procedures were recorded on video for later analysis.

Light/Dark Preference Test

This procedure was based on the light-dark model by Bernardi *et al.* (2013), described to assess anxiety behavior. We used a 40 cm (6L) aquarium with three equal compartments: one with 15 cm dark walls, another with a 15 cm light side (illuminated by an 835-lux incandescent lamp), and a 10 cm intermediate side, separated from the other two compartments by two glass walls. Previously, we determined that the dark side is preferred by the fish (anxiolytic side), and the light side is the anxiogenic side. We used two equal groups, each consisting of ten fish, for evaluating anxiety-like behavior: one control group and one experimental group. The fish in the experimental group were individually exposed to 0.5 µg/L of FPR for 60 minutes in a 2L aquarium and then placed in the intermediate compartment of the test aquarium containing the same concentration of FPR. After 10 minutes of habituation, we removed the glass wall of the intermediate compartment. The control group underwent the same procedure without exposure to FPR. The behavioral parameters measured for 5 minutes were: 1) the seconds that the fish remained on the clear side, 2) the seconds of immobility in the clear side, 3) the frequency of crossings between the two compartments, and 4) the frequency of attempts to enter the clear side. All procedures were recorded on video for

later analysis.

Social Preference Test

This procedure was developed in our laboratory based on zebrafish behavior, as social behavior is a key aspect of zebrafish ecology, as they naturally form shoals. In this task, the same aquarium used to evaluate the anxiety-like behavior was placed side by side: Aquarium 1 (A) contained six male fish, referred to as the stimulus aquarium. Aquarium (B) was designated as the designated test aquarium in the middle. Aquarium C was the same size as the others, but it contained only aquarium water and was referred to as an unstimulated aquarium (C). The front wall of the test aquarium was divided into three equal parts, with vertical lines indicating the social compartment (closest to aquarium A), the neutral compartment (the central compartment), and the non-social compartment (the compartment on the opposite side of the social compartment).In addition, the test tank had three equidistant horizontal lines, allowing us to observe the fish's swimming behavior. Twenty fish were divided into two equal groups, one control and one experimental. The test fish was exposed to a sublethal concentration of FPR for 60 minutes and then transferred to the test aquarium containing the same concentration of FPR. The fish was allowed 5 minutes to adapt to the new condition, and the behavior was filmed for 5 minutes. The control group was treated the same way, without exposure to FPR. The following parameters were evaluated: 1) time in seconds in each of the divisions of the front vertical wall of the test aquarium; 2) time in seconds of immobility; 3) frequency of running, climbing, and trembling. The 5 minutes of observation spent by the test fish on the side of aquarium A was considered a social preference. All procedures were recorded on video for later analysis.

Male Sexual Preference Test

We developed the present test based on previous observations from the social preference test, in whmale fish interacted preferentially with females, and the study of Lindley *et al*. (2025). The experimental scheme and aquariums were the same in the sexual preference test. Still, one male fish (Aquarium A) and the other female (Aquarium C) were placed in the side aquariums (stimulus fish). The test fish was placed in the central aquarium (B). The test fish was exposed for 1 hour to a sublethal concentration of FPR and then transferred to the central aquarium, where they remained at the same concentration of FPR for 5 minutes. The control group was treated the same way , without exposure to FPR. The sexual preference was observed for 5 minutes. The following parameters were noted: 1) time in seconds that the fish stayed near the male or female side; 2) frequency of interaction with the male or female that corresponded to the number of times that the fish -test placed its mouth on the glass of the aquarium and the other side was a male or female fish; 3) time in seconds of attempts to interact with the male or females which corresponded to the time the fish - test placed its mouth on the aquarium glass. All procedures were recorded on video for later analysis.

Statistical Analysis

Data were presented as means ± SEM or percentages. The Bartlett and Shapiro-Wilk tests were used to evaluate homoscedasticity and normality. The presence of outliers was analyzed by the ROUT method (Q = 5%), and if present, they were removed, and normality recalculated. Then, parametric or non-parametric tests were used to compare means and medians. Repeated-measure ANOVA was used to analyze the surface-emerging frequency and time data. The Student's t-test or the Mann-Whitney test was selected based on the normality tests. In the light-dark aquarium, open-field exploratory behavior, and social and sexual preference tests were conducted. Two-way ANOVA is followed by Sidak's multiple comparisons test, or Kruskal-Wallis, followed by Dunn's multiple comparison test, was used to analyze the erratic movements and tremor intensity scores. A value of $\alpha < 0.05$ was considered the significance level for all tests. All statistical results are shown in Supplementary Table S1.

RESULTS

Prospecting the Concentration of FPR Subletal Lethal Concentration of FPR

In our laboratory, the sublethal concentration was 0.5 µg/L, with no deaths observed within 72 hours (Table 1). At the end of the 72-hour observation period, four fish rin which male fish interacted preferentially with females, and the study remained at the bottom of the aquarium. They presented motor incoordination but responded to the stimulus on the tail.

Table 1. Percentages of zebrafish deaths exposed to different concentrations of fipronil.

Concentration in µg/L/hours of observations	1 h	2 h	24 h	48 h	72 h
6,0	100				
4,0	25	100			
2,4		100			
2,0			75	100	
1,0			25	25	50
0,50			0	0	0

Note: Observations of deaths were made for 1,2, 24,48 and 72 hours after exposure. Data in percentages of 4 samples / concentrations.

FPR Increased Zebrafish General Activity in the Aquarium

The one-way ANOVA revealed differences in the frequency of surface climb between observation sessions ($F(5,42) = 5.39$, $p = 0.0006$, Fig. 1a). The Bonferroni test indicated increased parameter frequency in the 11-20 and 11-30 intervals. The time spent on the surface has differed ($F(5,42) = 8.70$, $p < 0.0001$, Fig. 1b) between the groups, increasing in FPR-treated fishes at 21-30 and 31-40 minutes of observation. The scores for erratic movements ($KW = 44.76$, $p < 0.0001$, Fig. 1c) and tremor intensity ($KW = 44.86$, $p < 0.0001$, Fig. 1d) differed significantly from those of the control group. In both parameters, higher scores were observed at intervals of 31-40 minutes, 41-50 minutes, and 51-60 minutes.

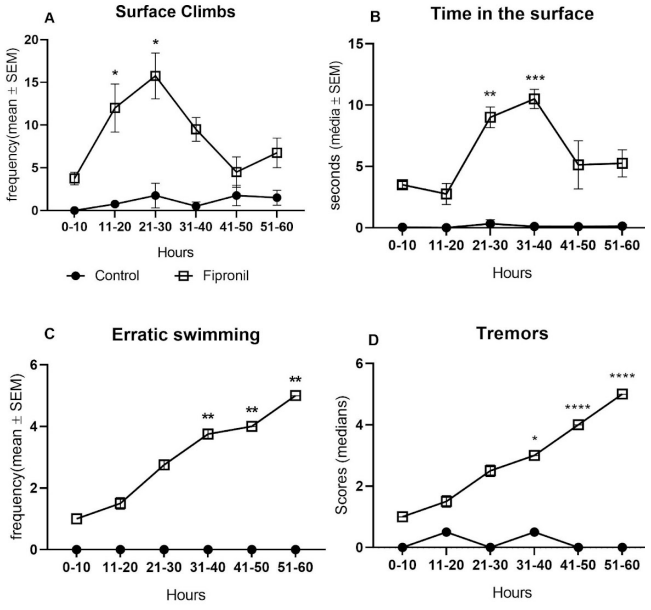

Figure 1. General zebrafish activity was observed for one hour after exposure to a sublethal Fipronil concentration (0.5 µg/L). Two-way ANOVA is followed by Sidak's multiple comparisons test or Kruskal-Wallis followed by Dunn's multiple comparison test. *$p < 0.05$, **< 0.01, ***$p < 0.001$, ****$p < 0.0001$ in relation to the control group. N= 10/group.

FPR Sublethal Concentration Did Not Alter the Zebrafish Exploratory Behavior

Panel I of Figure 2 shows data from the open field test, indicating that the exploratory behavior of zebrafish exposed to FPR was not altered. The locomotion data (M-W $U = 50$; sum of ratings: 105, 105; $p > 0.05$, Fig. 2a) and immobility ($t = 1.42$; $df = 12$; $p = 0.1812$, Fig. 2b) of both groups showed no significant differences between groups.

Sublethal Exposure to FPR Promotes Anxiety-Like Behavior in Zebrafish and Reduces Their Social Behavior

The data obtained from the tests evaluating anxiety behavior are shown in Figure 2, Panel II. Our results show that exposure of zebrafish to a sublethal dose of FPR increased the immobility time of the experimental group [median 8.50; max. 10.00, min. 1.00) was greater than that of animals in control (median 0.00; max 1.00, min 0.00) (MW $U = 0.500$, Sum of Ranks = 55.50, 154.5, $p < 0.0001$, (Fig. 2b)]. Furthermore, significant differences were also observed in the frequency of entry attempts on the light side [(MW $U = 21.50$, Sum of ratings = 133.50, 76.5, $p = 0.023$, Figure 2d) between control (median 1.50; max. 7.00, min 1.00) and experimental groups (median 1.00; max. 6.00, min. 0.00)]. No differences were observed between the data from the control groups (median). 16.00; max 47.0, min 7.00) and experimental (median 10.50; max 82.0, min 2.00) on the light side (MW $U = 45$, Sum of Ranks = 100, 110; $p = 0.72$, Fig. 2a), and in the number of crossings (MW $U = 49.0$, Sum of Ranks = 104, 106, $p > 0.05$, Fig. 2c).

The social preference behavior of zebrafish exposed or not to FPR is depicted in Fig. 2, Panel III. The fish exposed to the insecticide stayed less time in the social compartment ($t = 3.49$, $df = 18$, $p = 0.003$) and a long time in the neutral compartment of the test tank compared to the control group ($t = 3.47$, $df = 18$, $p = 0.003$). Both groups show the same time in the non-social compartment of the aquarium ($t = 1.44$, $df = 18$, $p = 0.17$).

Sublethal Exposure To FPR Impaired Zebrafish's Male Sexual Preference

Figure 2, Panel IV shows the sexual preference of zebrafish exposed or not to FPR. The time the male fish spent in the female compartment (Fig. 2d) was shorter after exposure to the pesticide ($t = 3.03$, $df = 18$, $p = 0.007$), staying longer in the neutral compartment ($t = 3.47$, $df = 18$, $p = 0.003$) compared to the control group. There were no differences in the time spent in the male compartment between groups ($t = 0.58$, $df = 18$, $p = 0.57$, Fig. 2a). The frequency of interaction (Fig. 2b) with females was lower in the FPR-treated group compared to the control group ($t = 2.15$, $df = 18$, $p = 0.046$). In contrast, higher interaction with the male group was observed ($t = 2.33$, $df = 18$, $p = 0.031$). The attempt to interact with females (Fig. 2c) in fish treated with FPR is reduced (median 17.50, max 212.0, min 0.00; MW $U = 21.00$, Sum of Ranks = 134.0, 76.0, $p = 0.023$) when compared to the control group (median 119.0, max 240.0, min 15.0), which did not occur about the attempts to interact with male fish ($t = 0.32$, $df = 18$, $p = 0.75$).

DISCUSSION

The need for pesticides in both agriculture and animal care is indisputable. However, it is estimated that 140 billion kilograms of pesticides find their way into aquatic ecosystems annually, posing a considerable threat

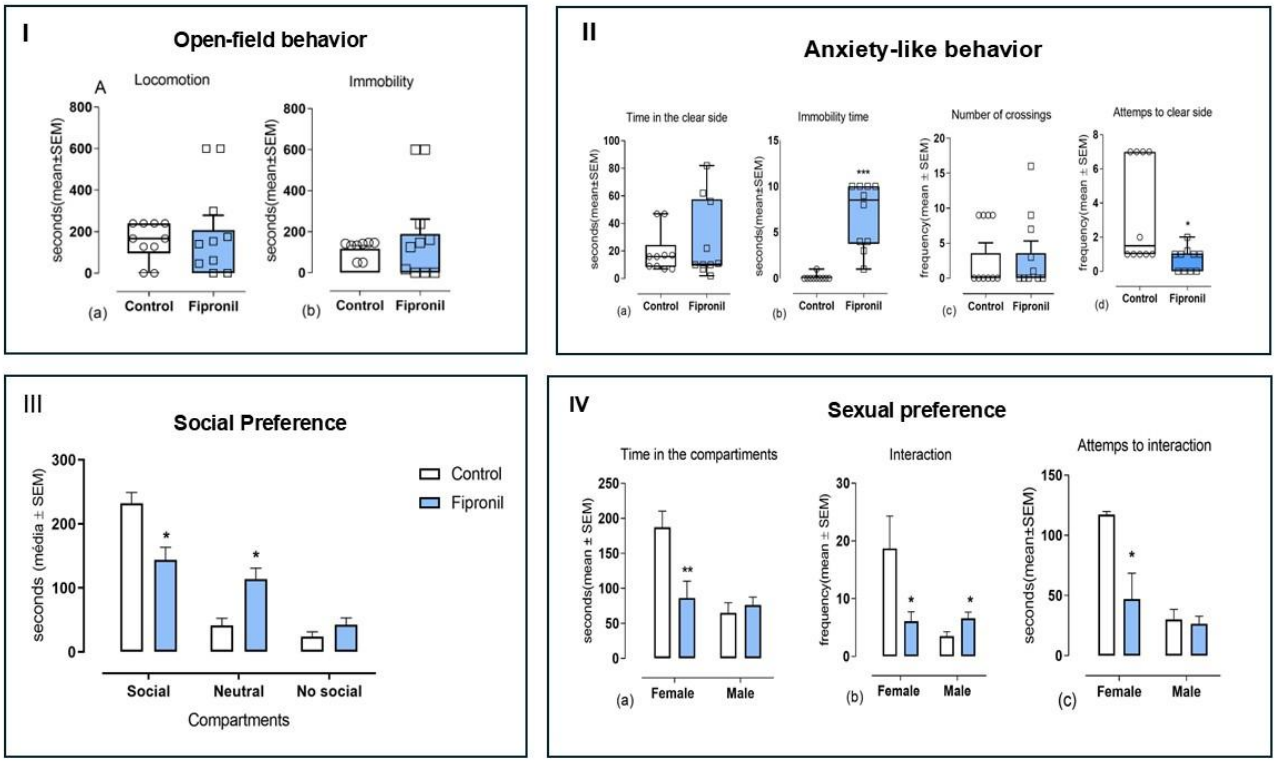

Figure 2. Behavior of zebrafish exposed to a sublethal concentration of FPR. Panel I - open field behavior: a) locomotion; b) immobility; Panel II - anxiety-like behavior: a) time in the dark side; b) time in immobility; c) number of crossings; d) attempts to clear side; Panel III - social preference test; Panel IV - sexual preference test. The zebrafish were exposed to a sublethal concentration of fipronil. Student t-test. *p < 0.05, *** p < 0.001 relative to control group N= 10/group

to public and ecological health (Tudi *et al*., 2021; Mishra *et al.*, 2023). Zhang *et al*. (2018), examined the interactions of FRP within fish and insects, showing similar toxicity to both species and leading to high toxicity.

In our study, we chose to use an FRP subtoxic concentration (0.5 µg/L), since its ecotoxicological risk is already well-documented in the literature, whereas many studies use lethal doses (Ardeshir *et al*., 2017; Al-Badran *et al*., 2018; Smruthi *et al*., 2022). In addition, we used an FRP commercial product to understand better the possible risks at ecotoxicological levels.

The lethal concentration (LC50) values are categorized as follows: minimum (>100), light (11 to 100), moderate (1.1 to 10), high (0.11 to 1.0), extreme (0.01 to 0.1), and superextremal (<0.01) (Helfrich et al., 2009). Our results confirm the high potential risk of the ecological impact of FPR on zebrafish, since at 48h after exposure, 100% of the fish died. In zebrafish embryos, FRP has been reported to have acute sublethal effects on fish development (Stehr *et al*., 2006) endocrine disruption (Sun et al., 2014), and behavioral impairment (Beggel *et al.,* 2012; Wang *et al.,* 2016). FRP increased antioxidant enzymes like superoxide dismutase and catalase activity, and the lipid peroxidation levels in the brain and kidney and triggered neurotoxic effects in the brain of zebrafish embryos, larvae, and adults (Wu *et al*., 2021). Depending on the time and concentration of exposure, the main behavioral effects of FRP were increased anxiety, disturbance of swimming behavior, and locomotor defects (Buzenchi *et al.*, 2024).

Since changes in neurological functions are often expressed through behavior, standardizing behavioral models applied to zebrafish is crucial for establishing toxicity to FPR and other toxicants. Thus, in this study, the behavioral signs were observed at 10-minute intervals during exposure to the sublethal concentration of the pesticide.

The most significant signs of neurotoxicity were tremors, erratic movements, and changes in frequency and time of ascent to the surface. Data related to climbing to the surface and time on the surface represent parameters linked to the respiratory processes of the animals. Therefore, the increased surface climbing and time spent on the surface would be related to the respiratory difficulty presented by the animals during intoxication. In this respect, FRP decreases the superoxide dismutase enzymes, which protect lung function by neutralizing the damaging effects of reactive oxygen species, particularly superoxide anions, abundant in the lungs, and could be responsible for the respiratory difficulty (Tyler, 1995). In addition, sublethal FRP (0.4 ug/L) induced

histopathological changes in the gills of common carp (*Cyprinus carpio* L.), including epithelial uplifting and necrosis of lamellae, lamellar atrophy, disruption of cartilaginous core, fusion and disorganization of lamellae, and telangiectasia. Tremors could reflect an increase in nervous system excitability, either of central or peripheral origin. They appear before seizures and are attributed to FPR action as an antagonist of the GABA and glycine inhibitory neurotransmitters (Mohamed *et al.*, 2004). In our study, the tremor occurred in a time-dependent manner, indicating that this increase is linked to the neurotoxic actions of the pesticide as it accumulates in the fish.

Also, increased erratic movements were observed. Erratic movements in zebrafish were related to anxiety-like behavior. In this sense, Egan *et al.* (2009) observed that stimulant drugs, acute alarm pheromone exposure, and the novel tank diving test produced anxiogenic behavioral responses in zebrafish, including a significantly higher latency to enter the upper half, fewer transitions to the upper half, and more erratic movements, but unaltered freezing behavior. We examined anxious behavior in another paradigm. In this test, the decrease in attempts to enter the light side and the increased immobility time observed express an aversion to light, as previously described (Bernardi *et al.*, 2013).

Studies in the novel tank test, a zebrafish anxiety-like behavior model, can be bi-directionally modulated by drugs affecting the gamma-aminobutyric acid, monoaminergic, cholinergic, glutamatergic, and opioidergic systems (Stewart *et al.*, 2011). Bruce et al. 2025 showed that 8-OH-DPAT, a 5-HT1A agonist, decreased anxiety-like behavior in the novel tank test, but increased it in the phototaxis (light-dark preference) assay, both of which are considered assays for anxiety-like behavior in this species. The same dose decreased social approach in both the social investigation and social novelty phases of the social preference test. Blocking the 5-HT1A receptor shifted the dose-response curve rightward for the novel tank test. These effects suggest participation of the 5-HT1A heteroreceptors in zebrafish anxiety and social preference, modulating anxiety in a test-dependent way and decreasing sociality. Concerning the GABAergic system, Assad *et al*. (2020) studied the pattern of cell activation in the telencephalon of adult zebrafish and the role of the GABAergic system in the modulation of anxiety-like behavior evoked by acute restraint stress. The data support that decreased GABA levels in zebrafish brains have diminished the activation of GABAA receptors, eliciting anxiety-like behavior.

The social behavior of zebrafish can represent a valuable model for aquatic toxicity studies. The rationale for studying social behavior in zebrafish is that it is a highly social species that prefers to swim in groups. Swimming in groups protects against predation and increases the foraging efficiency of the fish, that is, the food search (Paijmans *et al.,* 2020). Exposure to FPR reduced the zebrafish's preference for the stimulus side, causing them to spend more time on the intermediate side of the test aquarium. This reduction in social preference can risk the animal's survival in the wild (Pompermaier *et al.,* 2020). Some signs of neurotoxicity were observed, including tremors, but only during the runs.

GABA inhibition is one of the neurotransmitters that regulates sociability. Decreased GABAergic transmission reduces sociability in the social preference test in mice (Paine *et al*., 2017). In addition, GABAergic agonists such as diazepam facilitate social interaction in knockdown mice of the NMDA receptor (a receptor that reduces GABAergic activity), who have social deficits (Billingslea *et al.*, 2014). In our social model, only the view is privileged for the animal's response to the presence of other fish as a stimulus. Thus, different stimuli, such as olfactory, auditory, and lateral line stimuli, were impossible in our model. The test animal was placed in a separate aquarium between two others: one contained several fish of its kind, and the other was empty, allowing only the visualization of the stimulus. This procedure was chosen because only the test fish was exposed to FPR. Thus, the behavior of the test fish did not reflect what members of a zebrafish school would generally do under natural conditions when exposed to FPR. However, the information obtained in this study may be helpful and predictive of what may occur in the contamination of water bodies by FPR. In this sense, exposure of zebrafish larvae to valproic acid, an anticonvulsant drug, revealed a significant decrease in dopamine and GABA levels in the brain and reduced social behavior, supporting a potential predictive validity of this model for autism spectrum disorder (Ricarte et al., 2024).

Thus, the present results suggest that FRP exposure increased anxiety behavior and decreased social preferences, which also involve the GABAergic system. In teleost fish, sexual behavior is controlled by sex hormones. In males, androgens are essential for exhibiting male sexual behavior (Munakata and Kobayashi, 2010). Endocrine disruptors can interfere with the level of these hormones by stimulating or inhibiting them, causing adverse effects on sexual relations and reproductive behaviors (Huang *et al*., 2015). In our experiment, the male zebrafish remained in a separate aquarium from the females, thus enabling female hormonal interferences on male sexual preferences. In this respect, Bencic et al. (2013) studies in the fathead minnow fish (Pimephales promelas) do not support a hypothesis of adverse outcome linked to sublethal FPR concentration in either male or female reproduction. However, the socio-sexual behaviors of fish shed crucial light on their reproductive tactics,

population dynamics, and general ecological health. Fish activities associated with social interaction and sexual reproduction encompass behaviors including courting, mating, aggressiveness, and interpersonal interactions among group members (Kujur and Parganiha, 2013). Few studies of FRP contamination in fish socio-sexual behaviors are currently limited. Our study, using a sublethal FRP concentration, shows a decreased social and sexual preference in male zebrafish, contributing to understanding the FRP as an endocrine disruptor.

CONCLUSION

Our data allows us to conclude that acute exposure to sublethal concentrations of FPR induces neurotoxic effects that can be attributed to its action as a GABAergic antagonist. The FRP endocrine disruptor could be, in part, due to the reduced sexual preference with ecotoxicological importance on fish reproduction. Thus, the present results validate the use of the zebrafish model as a tool in studies related to FPR neurotoxicity.

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AUTHOR CONTRIBUTIONS

EEB: Data curation, Formal analysis, Investigation, Methodology, Writing original draft preparation. **GKB**: Data curation, Formal analysis, Methodology. **LMDS**: Writing-original draft preparation, Investigation, Visualization, Writing-Reviewing, and Editing. **LBC**: Writing-Reviewing and Editing. **RAFS**: Conceptualization, Supervision, Writing original draft preparation, Writing-Reviewing, and Editing. **MMB**: Conceptualization, Supervision, Writing original draft preparation, Writing-Reviewing, and Editing.

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