

Article

Not peer-reviewed version

---

# Histopathological and Behavioral Impairments in Zebrafish (*Danio rerio*) Chronically Exposed to a Cocktail of Fipronil and Pyriproxyfen

---

[Madalina Andreea Robea](#) , [Adriana Petrovici](#) , [Dorel Ureche](#) <sup>\*</sup> , [Mircea Nicusor Nicoara](#) <sup>\*</sup> , Alin Stelian Ciobica

Posted Date: 6 July 2023

doi: 10.20944/preprints202307.0353.v1

Keywords: pesticide; fipronil; pyriproxyfen; behavior; histopathological changes; zebrafish



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

## Article

# Histopathological and Behavioral Impairments in Zebrafish (*Danio rerio*) Chronically Exposed to a Cocktail of Fipronil and Pyriproxyfen

Madalina-Andreea Robea <sup>1,‡</sup>, Adriana Petrovici <sup>2,3,4,‡</sup>, Dorel Ureche <sup>5,\*</sup>, Mircea Nicoara <sup>6,7,\*</sup> and Alin Stelian Ciobica <sup>6,8,9</sup>

<sup>1</sup> Doctoral School of Biology, Faculty of Biology, "Alexandru Ioan Cuza" University of Iasi, Bd. Carol I, 20A, 700505 Iasi, Romania; madalina.robea11@gmail.com (M.A.R.);

<sup>2</sup> Department of Preclinics, University of Life Sciences Iasi, 3, Mihail Sadoveanu Alley, 700490 Iasi, Romania; p.adriana6@yahoo.com (A.P.);

<sup>3</sup> Regional Center of Advanced Research for Emerging Diseases, Zoonoses and Food Safety, Rovetemerg, 3, Mihail Sadoveanu Alley, 700490 Iasi, Romania;

<sup>4</sup> Department of Molecular Biology, Histology and Embryology, Faculty of Veterinary Medicine, University of Agricultural Science and Veterinary Medicine Ion Ionescu de la Brad, 8, Mihail Sadoveanu Alley, 700489 Iasi, Romania; carmensolcan@yahoo.com (C.S.);

<sup>5</sup> Faculty of Sciences, Department of Biology, Ecology and Environmental Protection, University "Vasile Alecsandri" of Bacau, Calea Marasesti str., no. 157, 600115, Romania dureche@ub.ro (D.U.);

<sup>6</sup> Department of Biology, Faculty of Biology, "Alexandru Ioan Cuza" University of Iasi, Bd. Carol I, 20A, 700505 Iasi, Romania; mirmag@uaic.ro (M.N.); ciobica@uaic.ro (A.S.C.);

<sup>7</sup> Doctoral School of Geosciences, Faculty of Geography and Geology, "Alexandru Ioan Cuza" University of Iasi, Bd. Carol I, 20A, 700505 Iasi, Romania.

<sup>8</sup> Academy of Romanian Scientists, 54, Independence Street, Sector 5, 050094 Bucharest, Romania;

<sup>9</sup> Center of Biomedical Research, Romanian Academy, Bd. Carol I, 8, 700506 Iasi, Romania;

\* Correspondence: mirmag@uaic.ro (M.N.), dureche@ub.ro (D.U.)

‡ These authors have contributed equally.

**Abstract: Background:** Lately, the high incidence of pesticide usage has attracted everyone's interest due to the serious effects produced. Fipronil (FIP) is a phenylpyrazole compound that acts on the insect's GABA neurotransmitter by inhibiting its activity. Moreover, literature reports highlight its implication in neurodevelopmental abnormalities and oxidative stress production in different organisms. Similarly, pyriproxyfen (PYR) is known to affect insects' activity by mimicking the natural hormones involved in the maturation of the young insects. The aim of the present study was to investigate the impact of these pesticides' mixture on zebrafish tissues and behavior. **Methods:** To assess the influence of this cocktail on zebrafish, three groups of animals were randomly selected and exposed to 0, 0.05 and 0.1 mg L<sup>-1</sup> FIP and PYR mixture for 5 days. The fish were evaluated daily by the T-maze tests as locomotor activity and dark-light test and recorded for 4 minutes. The data was quantified using the EthoVision software. **Results:** Our results indicated significant changes in locomotor activity parameters showing increased levels following exposure to the mixture of FIP and PYR. On the other hand, the mixture also triggered anxiety in the zebrafish, which spent more time in the light area than in the dark. In addition, mixture-induced histological changes were observed in the form of numerous hemosiderin deposits found in various zebrafish tissues. **Conclusions:** The current findings indicate that the mixture of FIP and PYR can have considerable consequences on adult zebrafish and may promote or cause functional neurological changes in addition to histological ones.

**Keywords:** pesticide; fipronil; pyriproxyfen; behavior; histopathological changes; zebrafish

## 1. Introduction

Chemicals such as pesticides are frequently used in agricultural production to prevent or manage pests, weeds and other plant pathogens in order to reduce or eliminate yield losses while maintaining good product quality [1–3]. Despite the fact that pesticides are designed to have a low impact on human health and the environment, major concerns have been raised about the health risks associated with occupational exposure and residues found in food and drinking water [2,4].

Environmental exposure affects the human population mainly through eating contaminated food and drinking contaminated water, as well as applying pesticides in the home or living near treated fields. Consequently, risk assessment and prevention of exposure to pesticides is a complex process, especially when several limitations are considered, such as differences in time and level of exposure, chemical structure and specific toxicity of the pesticide, mixtures or cocktails applied and, last but not least, the geographical and climatic characteristics of the areas where they are used [2,4].

In the past few years, fipronil (FIP) has been frequently highlighted in research publications as one of the adopted pest control chemicals whose effects have not been sufficiently investigated. According to the National Pesticide Information Center (NPIC), FIP was first registered in 1996 as a broad-spectrum insecticide against ants, beetles, cockroaches, fleas, ticks, termites, thrips, rootworms, weevils and other sects. Due to its massive use and negative impact on bee populations, FIP has been restricted in several countries. Consequently, in 2013, FIP was banned in Europe for agricultural applications; nowadays, it is only used in several veterinary products [5]. On the other hand, unlike Europe, Asia, and South America, the United States of America continues to use FIP for both agriculture and domestic purposes [6]. For instance, discharge from 12 facilities after a plant treatment reported fiproles concentrations ranging from 0.3 to 112.9 ng L<sup>-1</sup>, exceeding the United States Environmental Protection Agency (USEPA) aquatic invertebrate life threshold established for chronic exposure to FIP (11 ng L<sup>-1</sup>) in over 67% of cases [7].

FIP is a broad-spectrum insecticide from the phenylpyrazole family [8–10]. The mechanism of action is not fully characterized; everything that is known so far is due to the studies carried out on insects in which showed a significant toxicity compared to other organisms. Its main pathway involves binding to GABA<sub>A</sub>-gated chloride channels and further blocking the inhibitory action of GABA<sub>A</sub> in the central nervous system (CNS) [11–13]. FIP has been linked to a variety of negative consequences in humans and other species, such as cytotoxicity, neurotoxicity, hepatotoxicity, and reproductive issues. In rodent studies, FIP has been linked to important alterations in growth and development, genotoxic and cytotoxic disturbances, in addition to those in the reproductive domain [11,14–16]. Dermal exposure of Wistar rats to 70, 140 and 280 mg kg<sup>-1</sup> FIP after 3 hours (h) elicited increases in rearing, freezing and grooming behavior assessed by the open field test [17]. Chronic treatment of male Wistar rats (6 weeks) with 4.85 mg kg<sup>-1</sup> FIP showed impairments in spatial learning and memory performance assessed by the Barnes maze. In addition, decreases in antioxidant system activity and dopamine, elevated levels in malondialdehyde (MDA) and serum corticosterone were obtained [18]. Several of these observed effects, such as reduced food intake and weight gain, were also observed in bobwhite quail (*Colinus virginianus*) after a single dose of 37.5 mg kg<sup>-1</sup> FIP; measured 8, 24, 48, 72 and 96 hours after administration [19].

A recent study on the impact of FIP on freshwater mussels (*Unio delicatus*) showed that exposure to 0.264 and 0.528 mg L<sup>-1</sup> for 48 hours and 7 days can lead to significant alterations in gills and digestive tissues, such as degeneration or even necrosis [20]. Furthermore, the negative impact of FIP exposure was also obtained for liver cells that had major ultrastructural changes characterized by cytoplasmic disorganization, fat accumulation and increased numbers of mitochondria, rough endoplasmic reticulum and Kupffer cells after 15, 25 and 50 mg kg<sup>-1</sup> administered intraperitoneally to female Swiss mice (*Mus musculus*) [21].

In addition, evidence of exposure to FIP in non-target organisms has been reported, particularly when it was used for crop protection. Related to this claim, a 2012 study evaluating the effect of FIP (0.65 mg L<sup>-1</sup>) in rice-field circumstances on common carp (*Cyprinus carpio*) determined that 90 days of exposure to FIP can cause alterations in fish metabolism in terms of toxicological profile. Changes in

superoxide dismutase (SOD), catalase (CAT) activity and levels of lipid peroxidation and protein carbonyl production were recorded, but there was no impact on growth rate or survival [22].

Another chemical introduced to control insects is pyriproxyfen (PYR). The first time it was brought up was in 1991, when experts were trying to fight sweet potato whiteflies (*Bemisia tabaci*) and greenhouse whiteflies (*Trialeurodes vaporariorum*) in cotton and tomato crops. Exposure to different doses of PYR had significant effects on reproductive status: 48 h exposure of female whiteflies to 5 mg L<sup>-1</sup> PYR resulted in suppression of egg viability [23]. A few years later, in 1996, the US introduced PYR for the same reason: to protect cotton crops. PYR is known as an endocrine disruptor that mimics the juvenile hormone needed by insects to molt from the immature stage to the adult stage [24]. Moreover, it has been shown that exposure to PYR in insects can affect the reproductive process [24]. Due to its abilities to persist in the environment (low solubility and hydrophobicity), precautions have often been required [24,25].

Widely used as an insect growth regulator and because it is considered a non-toxic compound, the World Health Organization recommended in 2006 the use of 0.01 mg L<sup>-1</sup> PYR for the disinfection of drinking water, especially for the control of dengue fever [26]. The same recommendation was followed in 2014 by the Brazilian Ministry of Health to fight mosquitoes [27]. Although there are studies that mention the low toxicity of PYR to other species, recent findings argue otherwise. For instance, oral administration of 20, 40, 100, 200, 320, 600, and 1200 mg kg<sup>-1</sup> PYR for 28 days led to a decrease in body weight of mice [28]. This is similar to the study by Mehrnoush (2013) who tested 1000, 2000 and 4000 mg kg<sup>-1</sup> PYR in rats [29]. A reduction in feeding capacity was also recorded for juvenile platy (*Xiphophorus maculatus*) after intake of 2.5 and 5 µg L<sup>-1</sup> PYR [30]. Several findings were made related to the influence of PYR on zebrafish, where the main finding identified was related to neurological changes, namely impairment of acetylcholinesterase activity, oxidative stress products, calcium transport malfunction and mitochondrial damage. Several findings were made related to the influence of PYR on zebrafish, where the main finding identified was related to neurological changes, namely impairment of acetylcholinesterase activity, oxidative stress products, calcium transport malfunction and mitochondrial damage [31].

A large percentage of pesticides in use today are potentially neurotoxic, affecting the nervous systems of target organisms, among other organs and component systems. So, the effect of these compounds can also be observed at the histological level [32–34]. FIP, for example, has been reported to induce multiple tissue changes in various animal models, with a more pronounced effect on the central nervous system (CNS). In rodent models, FIP has been linked to altered kidney, liver and brain anatomy and function following insecticide administration [35–38]. After exposure of common carp (*Cyprinus carpio*) to a dose range between 0.02 and 0.10 mg L<sup>-1</sup> FIP for 12 days, histopathological changes were identified in the gills, liver, kidneys and intestine [39]. Exposure to 1.66 µg ml<sup>-1</sup> PYR for 96 h caused significant alterations in zebrafish heart anatomy, including heart muscle thinning, hyperemia, and pericardial edema [40]. The influence of PYR at the histological level was also demonstrated in female mice after the administration of 30, 100, 300 and 1000 mg kg<sup>-1</sup> which led to an increase in the number of glial cells, vacuolization and degenerated neurons [41].

For our study, we chose one of the most well-known model organisms, *Danio rerio* or zebrafish, which is frequently used in research [42–44]. Its attractiveness for this field is supported by several advantages, including a high genome similarity to humans (about 70%), transparent larval stage, high number of eggs and low cost of care [44]. Along with the advantages mentioned above, the successful adaptation of behavioral tests and the ease of genetic manipulation on them has led to their success compared to rodents [45–47].

Therefore, this experimental protocol aimed to test two doses of FIP and PYR mixture in chronic exposure in zebrafish. The first goal was to evaluate the impact of the mixture on zebrafish locomotor activity parameters and anxious behavior. For the second one, we aimed to investigate the effect of this mixture at the histological level. To our knowledge, the impact of this mixture has not been studied in other works, except for one study belonging to our group [48].

2. Materials and Methods

2.1. Fish Maintenance

The test organisms, adult *Danio rerio* (wild-type zebrafish), were purchased from a local supplier in a total number of 150 with a sex ratio of 1:1 (female:male). The fish were accommodated in the facility for a period of 3 weeks before the start of the experimental study. In addition, it was ensured that all conditions for maintaining a healthy environment were met in accordance with the recommendations of the European Commission regulations for housing and care of animals used for experimental and other scientific purposes, in addition to those of Avdesh et al., and Aleström. et al. [49–51]. To ensure environment-specific conditions, daily water quality measurements of temperature, pH, salinity, conductivity, and ammonia were performed (Table 1). The medium was changed daily in the housing and experimental tanks to avoid accumulation of metabolic wastes such as ammonia. In addition to water quality, the illumination period was also set to a circadian rhythm of 14:10 h (light: dark) by an automatic timer that was started at 8 a.m. and off at 10:00 p.m. The fish were fed twice a day with flakes from TetraMin products.

**Table 1.** Environmental conditions from the housing and experimental tanks.

Type of Tank	Temperature (°C)	pH	Salinity	Conductivity (µS cm <sup>-1</sup> )	Ammonia (mg L <sup>-1</sup> )
Housing tank	26	7.6	0.26	551	0.05
Experimental tank	25 ± 0.5	7.4	0.25	553	0.04

2.2. Chemicals

The mixture of fipronil and pyriproxyfen used in this study was purchased in liquid form from a veterinary store. In addition to these two compounds (67.5 mg FIP + 67.5 mg PYR), the product also contained 0.3 mg butylhydroxyisobutyl, 60 mg benzyl alcohol and 0.15 mg butylhydroxytoluene. For this study, the doses chosen for testing were 0.05 and 0.1 mg L<sup>-1</sup> (based on previous unpublished data). These were obtained by dissolving a certain amount of the prepared stock solution in the zebrafish medium.

2.3. Experimental Design

Fish were randomly transferred from the housing aquarium to the experimental tanks and allowed to habituate to the new space. Zebrafish adults were exposed to the pesticide mixture daily for 5 days. This study had three experimental groups: control (n=10), 0.05 mg L<sup>-1</sup> FIP and PYR (n=10), and 0.1 mg L<sup>-1</sup> FIP and PYR (n=10). When the accommodation period ended, each fish was tested in the T-maze to record its initial behavior. Specific parameters for locomotor activity and anxiety were assessed after a 4-min session (each test is described in the behavioral tests section). For the treatment phase, the pesticide mixture was freshly prepared before exposure and changed every 24 h until the end of the study. To investigate the impact of FIP+PYR on zebrafish behavior, locomotor activity test and light-dark test were performed daily. Finally, fish were killed by immersion in ice-cold water for a minimum of 5 min after opercular motion had stopped and then stored for histological analysis. The study had two more replicates.

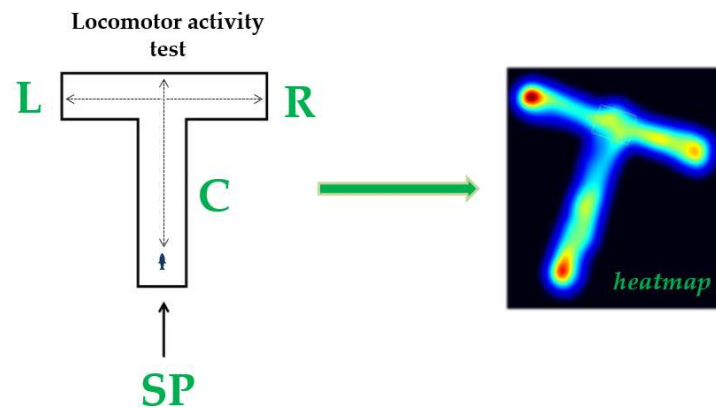
2.4. Behavioral Tests

2.4.1. Locomotor Activity Test

An adapted T-maze was used to measure specific locomotor activity parameters of zebrafish before and after treatment. It was divided into 3 arms (left, right and center) and had a starting point at the end of the central arm (Figure 1). For the analysis of fish locomotion, a series of parameters were quantified: total distance traveled, average velocity, active time and time spent in inactivity. In addition to these, specific parameters were measured to describe the swimming pattern: angular



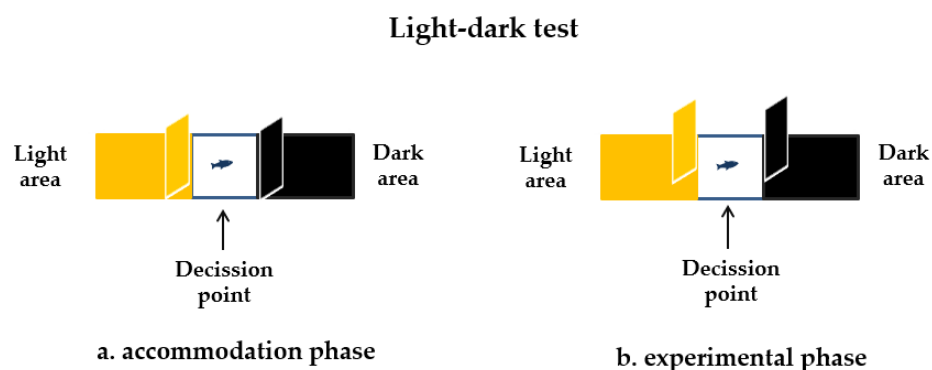
velocity, meander and turning angle. The total distance traveled represents the total distance swam by fish in the T-maze (cm) during the trial. Average velocity refers at how fast the fish is moving (cm/s). Counter-clockwise rotations indicate circular movement of the fish to the left. The angle of turn defines the angle at which the fish's head turns during a turn ( $^{\circ}$ ). Tests were performed every day, and all parameters were quantified for a period of 4 min per sample. Heatmaps are also added to this data.



**Figure 1.** T-maze used to test locomotor activity: organization and heat map (L: left arm, R: right arm, C: center arm, SP: starting point).

#### 2.4.2. The Light-Dark Test

This test aimed to assess the fish's anxiety level when it encounters two different environments: light and dark. To perform this test, the T-maze apparatus was adapted by placing a wall and transforming it into a rectangular shape (10 cm height  $\times$  50 cm length  $\times$  10 cm width) and designed according to the protocol of Araujo [52]. It was further divided by two walls into three areas: light, dark and decision point (Figure 2). Before the session started, each fish was placed at the decision point for 30 s to accommodate (Figure 2 a). After this time, the walls for the light and dark areas were removed and the fish could swim freely between these two areas (Figure 2 b). The session lasted 4 min, and the activity was recorded, like the locomotor activity test, through the camera located above the maze. To validate anxiety behavior, several parameters were recorded such as: distance traveled, average speed and time spent in each zone of the maze.



**Figure 2.** Schematic diagram of the light-dark test adapted to the T-maze and the two steps of behavioral testing (accommodation and experimental phases).

### 2.5. Assessment of Histological Changes

From the control group and from each experimental group, 10 fish were euthanized by immersion in cold water, fixed in 4% PFA, embedded in paraffin, and cut at 12  $\mu\text{m}$ . The entire CNS was cut into consecutive sections for examination of each region. Slides were stained with Luxol Fast-Blue-Cresyl Violet and examined under a light microscope (Leica DM 3000).

### 2.6. Statistical Analysis

Behavioral data were first assessed for normal distribution and confirmed by the Shapiro–Wilk test. To observe the differences between the concentrations of FIP+PYR and the control group, one-way ANOVA was followed by Tukey's post hoc test. Differences were considered significant at  $p < 0.05$ . Data are expressed as mean  $\pm$  standard error of the mean (S.E.M.). Microsoft Package Excel files and OriginPro software were used for data selection, statistical analysis, and graphical presentation.

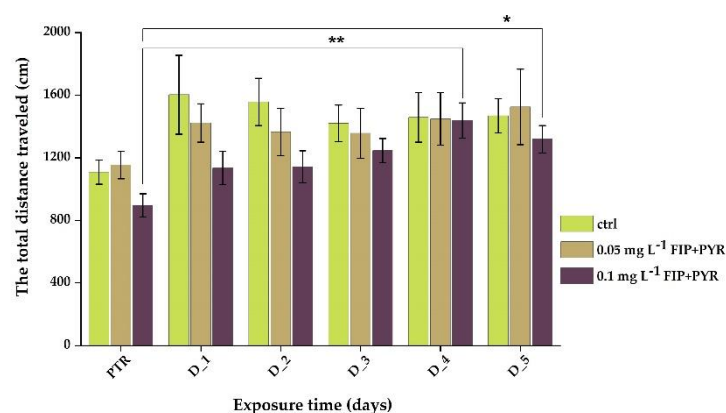
### 2.7. Ethical Approval

Animals were strictly maintained and treated in accordance with the EU Commission Recommendation (2007) on guidelines for the accommodation and care of animals used for experimental and other scientific purposes, Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes [49,53]. This experiment was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Iasi, with registration number 749/04.07.2019.

## 3. Results

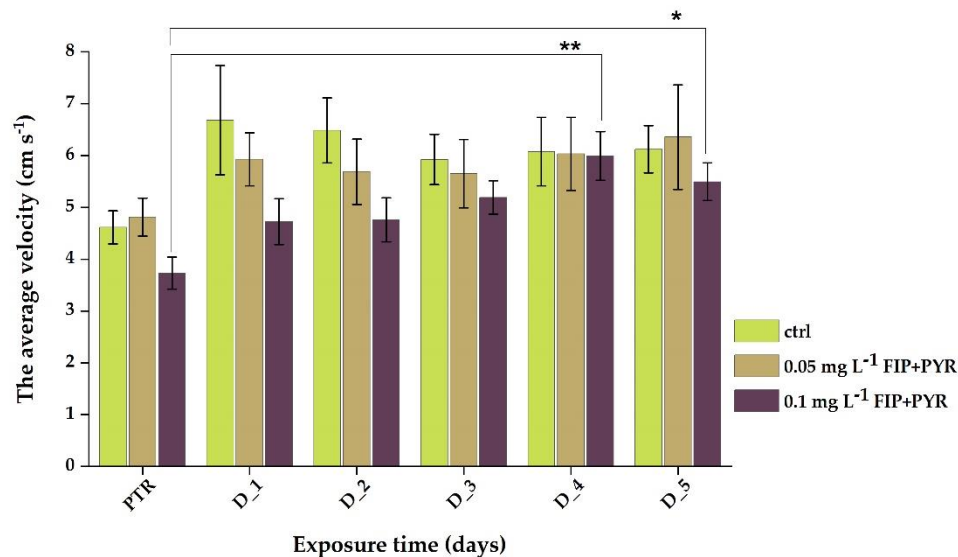
### 3.1. Impaired Locomotor Activity after Chronic Exposure to Pesticide Mixture

There were no significant differences between the control group and the group exposed to the 0.05  $\text{mg L}^{-1}$  mixture ( $p > 0.05$  ANOVA, Tukey) regarding the total distance traveled during the experimental sessions. Thus, the data for this parameter recorded the highest peak on the fourth day of the study for the 0.1  $\text{mg L}^{-1}$  mixture group compared to the initial behavior:  $1437.5 \pm 112.9$  vs  $895.2 \pm 74.3$  cm ( $p = 0.0016$  ANOVA, Tukey). At the end of treatment, its activity was still increased compared to the pretreatment phase:  $1318.3 \pm 87.1$  vs  $895.2 \pm 74.3$  cm ( $p = 0.023$  ANOVA, Tukey) (Figure 3).



**Figure 3.** Total distance traveled parameter recorded for all experimental groups during chronic treatment with the pesticide mixture. All values are presented as mean  $\pm$  S.E.M. ( $n = 10$  per group); \* $p < 0.05$  and \*\* $p < 0.001$  ANOVA, Tukey is significant compared to pretreatment period.

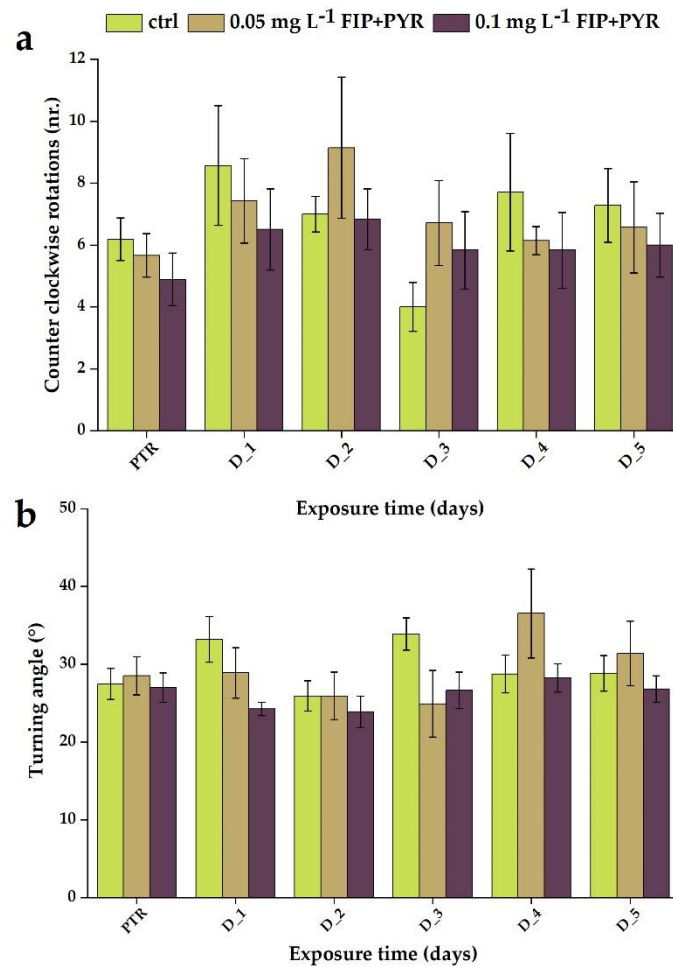
Similar to the above parameter, the average velocity did not show important changes among the individuals from the control and 0.05 mg L<sup>-1</sup> FIP+PYR groups ( $p > 0.05$  ANOVA, Tukey) compared to the 0.1 mg L<sup>-1</sup> FIP+PYR group. It was also indicated that there were elevated averages for the D\_4 ( $5.9 \pm 0.4$  cm s<sup>-1</sup>,  $p = 0.0017$  ANOVA, Tukey) and D\_5 ( $5.4 \pm 0.3$  cm s<sup>-1</sup>,  $p = 0.024$  Tukey) in comparison to the initial behavior ( $3.7 \pm 0.3$  cm s<sup>-1</sup>) (Figure 4).



**Figure 4.** Average velocity parameter recorded for all experimental lots during chronic treatment with the pesticide mixture. All values are presented as mean  $\pm$  S.E.M. ( $n = 10$  per group); \* $p < 0.05$  and \*\* $p < 0.001$  ANOVA, Tukey is significant compared to pretreatment period.

In addition, counterclockwise rotations and turning angle were measured during the locomotor activity test. For both parameters, the control group did not show high variability between baseline behavior and the sham treatment period ( $p > 0.05$  ANOVA, Tukey). Regarding the experimental groups exposed to the pesticide mixture (0.05 and 0.1 mg L<sup>-1</sup> FIP+PYR), a tendency to increase the number of counter-clockwise rotations was observed, but without significant disturbances ( $p > 0.05$  ANOVA, Tukey) (Figure 5 a). Another indicator of possible changes in locomotor activity is the turning rate of the fish. Our results showed that the pesticide mixture did not trigger any significant effect on the turning angle for both tested doses ( $p > 0.05$  ANOVA, Tukey) (Figure 5 b).

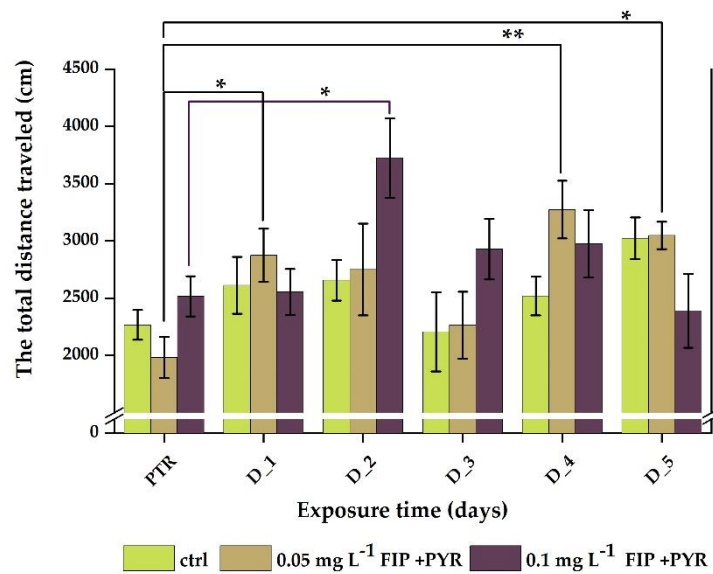




**Figure 5.** Counterclockwise rotations (a) and turning angle (b) parameters recorded for all the experimental groups during the chronic treatment with the pesticide mixture. All values are presented as mean  $\pm$  S.E.M. (n= 10 per group).

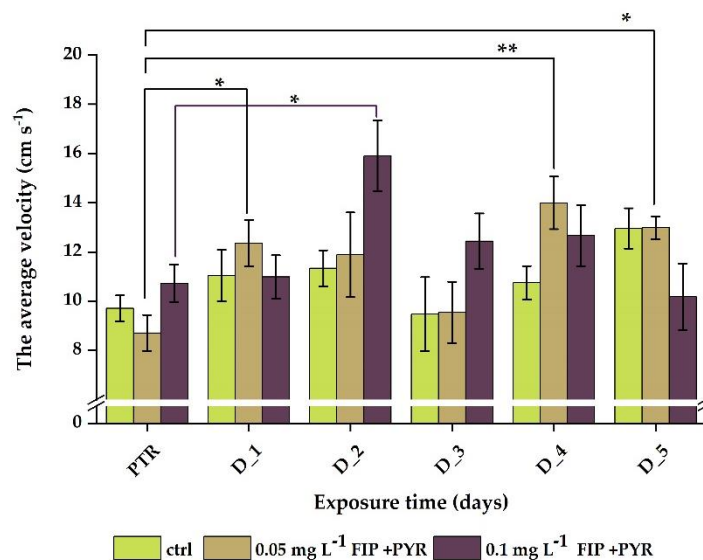
### 3.2. Increased Anxiety Level of Zebrafish

Total distance recorded during the light-dark test showed significant differences between all pesticide groups. No significant changes were observed for the control group ( $p > 0.05$  ANOVA, Tukey). Exposure to 0.05 mg L<sup>-1</sup> FIP+PYR increased the mean distance traveled by fish, with significant data for D<sub>1</sub> ( $2874.6 \pm 231.1$  cm,  $p = 0.01$  ANOVA, Tukey), D<sub>4</sub> ( $3273.1 \pm 252.3$  cm,  $p = 0.003$  ANOVA, Tukey) and D<sub>5</sub> ( $3046.8 \pm 119.6$  cm,  $p = 0.02$  ANOVA, Tukey) compared to baseline behavior ( $1982.6 \pm 180.7$  cm). Compared to the 0.05 mg L<sup>-1</sup> FIP+PYR group, which showed increased activity levels during chronic treatment, the 0.1 mg L<sup>-1</sup> FIP+PYR group had a similar trend, but not as much many changes in distance values except D<sub>2</sub> ( $3723.5 \pm 345.5$  cm vs. pretreatment:  $2515.7 \pm 175.6$  cm,  $p = 0.01$  ANOVA, Tukey) (Figure 6).



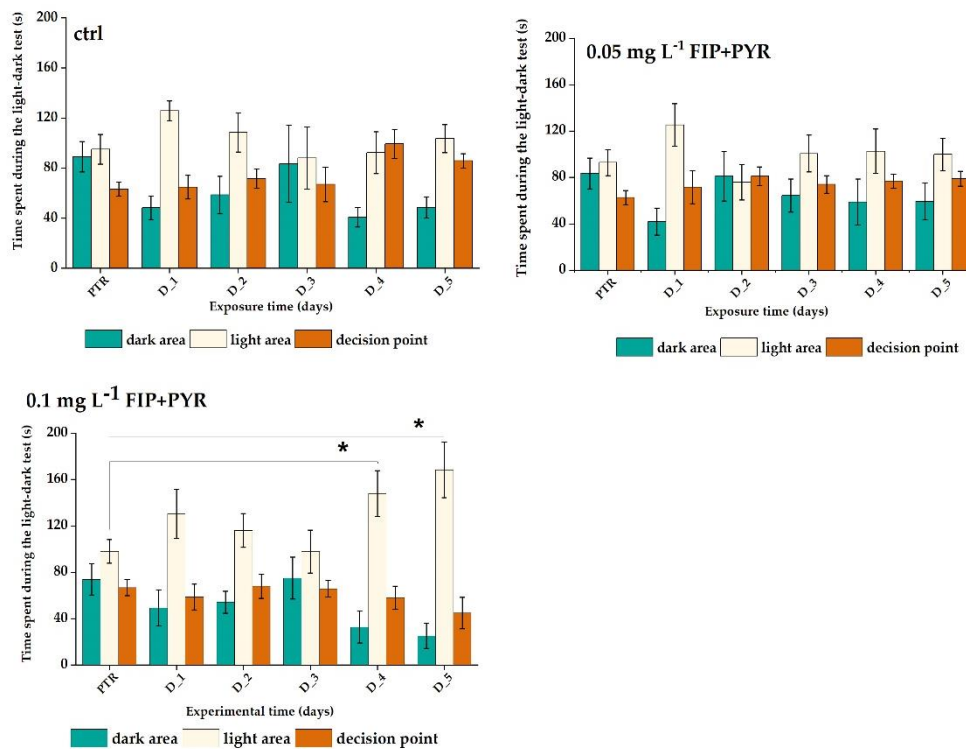
**Figure 6.** Total distance parameter recorded during the light-dark test for all experimental groups. All values are presented as mean  $\pm$  S.E.M. (n= 10 per group); \*p<0.05 and \*\*p<0.001 ANOVA, Tukey is significant compared to pretreatment period.

As shown in Figure 7, both doses of pesticides also affected the average speed of the fish. The control group had ups and downs with no significant changes in their activity. Conversely, the 0.05 mg L<sup>-1</sup> FIP+PYR group showed increased mean velocities in D\_1 ( $12.3 \pm 0.9$  cm s<sup>-1</sup>, p= 0.01 ANOVA, Tukey), D\_4 ( $13.9 \pm 1.1$  cm s<sup>-1</sup>, p= 0.005 ANOVA, Tukey) and D\_5 ( $12.9 \pm 0.4$  cm s<sup>-1</sup>, p= 0.03 ANOVA, Tukey) when compared to the pretreatment phase ( $8.71 \pm 0.7$  cm s<sup>-1</sup>). Regarding the other group exposed to 0.1 mg L<sup>-1</sup> FIP+PYR, the mean velocity showed a high value for D\_2 ( $15.9 \pm 1.4$  cm s<sup>-1</sup>, p= 0.01 ANOVA, Tukey) compared to the pretreatment period ( $10.7 \pm 0.7$  cm s<sup>-1</sup>) (Figure 7).



**Figure 7.** Average speed parameter recorded during the light-dark test for all experimental batches. All values are presented as mean  $\pm$  S.E.M. (n= 10 per group); \*p<0.05 and \*\*p<0.001 ANOVA, Tukey is significant compared to pretreatment period.

The time spent by the groups in a certain area of the experimental apparatus was quantified for the light, dark and decision point compartments, as represented in the Figure 8. There were no significant changes in the activity of the control and 0.05 mg L<sup>-1</sup> FIP+PYR groups for any zone of the maze ( $p > 0.05$  ANOVA, Tukey) (Figure 8). Thus, fish exposed to 0.05 mg L<sup>-1</sup> FIP+PYR showed increased light zone data times for D\_4 ( $147.9 \pm 19.9$  s,  $p = 0.02$  ANOVA, Tukey) and D\_5 ( $168.4 \pm 24.6$  s,  $p = 0.02$  ANOVA, Tukey) compared to baseline behavior ( $98.8 \pm 10.2$  s) (Figure 8).

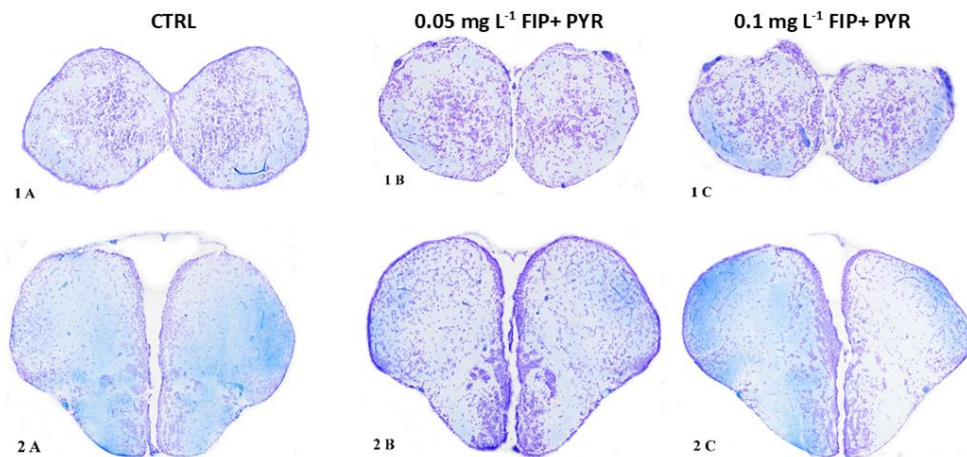


**Figure 8.** The time spent by the experimental groups during the light-dark test. All values are presented as mean  $\pm$  S.E.M. (n= 10 per group); \* $p < 0.05$  ANOVA, Tukey is significant compared to the pretreatment period.

### 3.3. Histological Alterations

The impact of the mixture of FIP and PYR on the zebrafish CNS was analyzed in the following brain regions: olfactory bulbs, telencephalon, diencephalon and mesencephalon, rhombencephalon, and medulla spinalis. In the most rostral region of the adult brain, cell density appeared altered with fewer perikaryon in FIP+PYR-exposed groups. Vasodilatation was evident in both treated groups and infiltrations were visible. No major differences were observed between the 0.05 and 0.1 mg L<sup>-1</sup> FIP+ PYR groups (Figure 9).

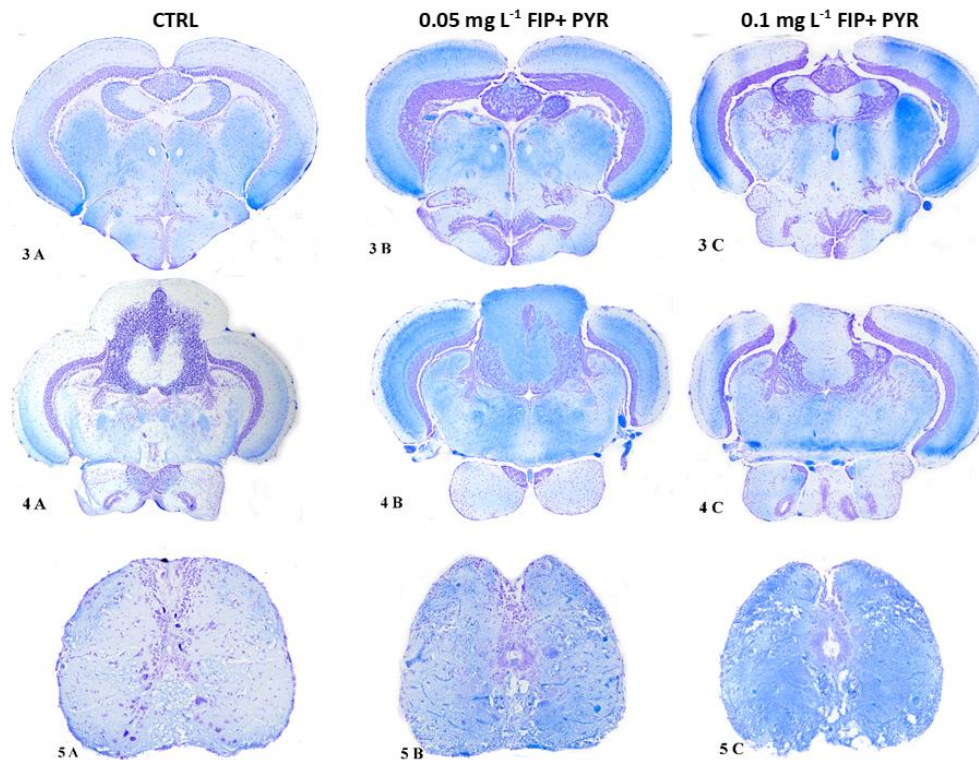
In the telencephalon were found numerous blood vessels, some of which ectatic in both treatment groups, and also blood cell infiltration. In some neurons in the 0.1 mg L<sup>-1</sup> FIP+PYR group, the nucleus was pyknotic and the cytoplasm was more eosinophilic, revealing the apoptotic effect of the mixture (Figure 9).



**Figure 9.** Histological sections from the main regions of the CNS in adult zebrafish. 1 A → 5 A: Sections from the control group. 1 A – Section from olfactory bulbs; 2 A – Section from telencephalon; 1 B – Section from olfactory bulbs; 2 B – Section from telencephalon; 1 C – Section from olfactory bulbs; 2 C – Section from telencephalon; Luxol-Fast Blue – Cresyl Violet staining, x40.

Dilated blood vessels and leucocyte infiltration persisted in treatment groups in the major regions from diencephalon and mesencephalon (Figure 10). The neuropil from 0.1 mg L<sup>-1</sup> FIP+PYR group was inhomogenously stained with Luxol Fast-Blue. The nerve fibers in the torus longitudinalis (TL) of the most exposed group were weakly stained. Central chromatolysis in large neurons in the oculomotor nucleus (NIII) was also distinguished. Dilated blood vessels and leucocyte infiltration persisted across treatment groups in major regions of the diencephalon and midbrain (Figure 10).

Regarding the rhombencephalon, mild infiltration and neuronal damage were observed, especially in the most exposed group of fish. The cerebellum had no obvious changes. Perikaryon swelling in some motoneurons in the group exposed to 0.1 mg L<sup>-1</sup> FIP+PYR was observed in the medulla (Figure 10). Also, an intense vascularization was present in this area.



**Figure 10.** Histological sections from the main regions of the CNS in adult zebrafish. 3 A – Section from diencephalon and mesencephalon; 4 A – Section from rhombencephalon; 5 A – Section from medulla spinalis. 3 B – Section from diencephalon and mesencephalon; 4 B – Section from rhombencephalon; 5 B – Section from medulla spinalis. 3 C – Section from diencephalon and mesencephalon; 4 C – Section from rhombencephalon; 5 C – Section from medulla spinalis. Luxol-Fast Blue – Cresyl Violet staining, x40.

#### 4. Discussion

In this study, the effect of the mixture of FIP and PYR was analyzed on the tissues and behavior of zebrafish after 5 days of exposure. Both treatment groups showed higher values for the total distance parameter during all behavioral testing sessions compared to baseline data. This effect was more pronounced for the 0.1 mg L<sup>-1</sup> FIP and PYR group that recorded significant activities for D\_4 and D\_5. A similar trend was observed for the velocity parameter. These observations could support the hypothesis that FIP can initiate and maintain the body's hyperactivity if the exposure period is prolonged. Our results are in line with the study by Koslowski (2020), where mice treated with FIP at 10 mg kg<sup>-1</sup> for 43 weeks showed that long-term administration can cause changes in locomotor activity. They obtained higher values for distance traveled and time spent mobile after 43 weeks compared to the first session (18 weeks) assessed by the open field test [54]. This may be the result of FIP accumulating in the body with a considerable effect on the neurotransmitter GABA, which triggers these behavioral disturbances that have been observed in most animal studies. For example, Reichel (2015) showed that depletion of GABA-ergic neurons in the hippocampus of C57BL/6 mice can sustain the hyperactivity state [55]. Such disturbances in locomotor performance were not obtained by Gusso (2020), who tested 0.125, 0.675 and 1.75 mg L<sup>-1</sup> PYR for 96 h on adult zebrafish [56].

Reduced levels of the neurotransmitter GABA are linked to attention deficits and hyperactivity in children. Under normal conditions, the binding of GABA to its receptors causes the channels to open, allowing chloride ions to move into the cell. This action decreases the excitability of the cell. When FIP or its metabolites bind to GABA<sub>A</sub> receptors, it prevents the uptake of chloride ions, leading to neuronal hyperexcitability [57,58]. Additionally, the ability of PYR to disrupt the CNS was recently



highlighted in a 2021 study that found inhibitory effects of 0.33 and 92.5  $\mu\text{mol L}^{-1}$  PYR in male zebrafish on acetylcholinesterase activity [31].

The other two parameters assessed for swimming performance were counterclockwise rotations and turning angle. Zebrafish treated with FIP and PYR with 0.05 and 0.1  $\text{mg L}^{-1}$  showed no significant changes. Knowing that counterclockwise rotations are an indicator of abnormal swimming that suggests anxious behavior, in our case, both treated groups had an increased rotation number compared to baseline data. In terms of turning angle, which may support the theory of irregular movement, the mixture groups tended to have lower amplitudes of angles defined by low-angle turns (20 to 40°).

Regarding the anxiety behavior, the experimental groups exposed to the mixture of 0.05 and 0.1  $\text{mg L}^{-1}$  showed an increase in the time spent corresponding to the light zone. This effect was more pronounced at the highest dose of the mixture compared to baseline behavior. In general, zebrafish adults tend to spend more time in the dark area, which is similar to the normal environment, compared to the larval stage, when they prefer the light area. In accordance with our results, the ability of FIP to enhance this type of behavior was validated for zebrafish larvae and adults. Based on the light-dark preference test, it was assessed that early exposure to 200  $\text{mg L}^{-1}$  FIP can promote anxious behavior in zebrafish larvae, where avoidance of the dark area by the organism was considered as an indicator of an anxious state [59]. In contrast to our findings, Gusso's (2020) study demonstrated that exposure to PYR for 96 h could not induce anxiety-like behavior in adult zebrafish [56]. Although PYR may contribute to the onset and development of several cognitive deficits, changes in anxiety are not related to it. Thus, we could assume that FIP is solely responsible for this. Furthermore, dermal exposure to FIP was found to promote changes in emotional, fear and exploratory activity, according to a 2011 study in which Terçariol and his team assessed the behavior of Wistar rats [17].

Histopathological examination revealed congestion, inflammatory changes, and apoptosis in major brain regions in response to FIP+PYR mixtures. Fish in both treatment groups reacted to the substances in a similar way, with slightly more intense congestion and inflammation in the higher dose group. Neuronal impairment was present in all major CNS regions, such as vacuolization, central chromatolysis, and pyknotic nuclei. All of these are well-known signs of impending apoptosis. This may be a consequence of the presence of FIP+PYR that caused the specific oxidative stress disorders. There are plenty of studies that have highlighted the relationship between pesticides and oxidative stress in general [60–62]. Furthermore, oxidative stress as a result of exposure to pesticides has been observed in various fish species such as *Labeo rohita*, *Tor putitora*, *Hoplias malabaricus*, *Lepomis macrochirus*, *Clarias gariepinus*, *Oreochromis niloticus* [2,63–65]. However, oxidative stress is described as an imbalance between oxidants and antioxidants that usually ends with increased levels of ROS [66]. Most of the time, the damage caused by ROS is a side effect.

The occurrence of oxidative stress triggered by the presence of PYR was recently mentioned in the study of Azevedo (2021) who reported that the main targets of PYR action are NADH dehydrogenase and succinate dehydrogenase known to be involved in the mitochondrial respiratory chain [31]. These damages could be reflected in the malfunctioning of the mitochondria and, as a result, the significant increase in the production of reactive oxygen species (ROS). Similarly, Maharajan (2018) obtained increased levels of ROS, lipid peroxidation and nitric oxide in zebrafish larvae after 96 h of exposure to 1.66  $\mu\text{g ml}^{-1}$  PYR. Moreover, at the same dose, the impact of PYR was validated for enzymes recognized as part of the antioxidant system, with reduced levels of SOD, CAT, glutathione peroxidase (GPx) and reduced glutathione (GSH) [40]. Ferreira (2012) demonstrated that the administration of 15, 25 and 50  $\text{mg kg}^{-1}$  FIP in female mice can affect the structure and function of liver cells with changes in the shape and size of hepatocytes and the appearance of steatosis marked by the accumulation of lipid droplets [21]. Similar data were recorded by de Oliveira (2012) on mouse liver cells [35]. FIP can also promote cell death; chromatin composition and distribution in hepatocyte nuclei have been shown to be altered after the presence of FIP, leading to chromatin condensation and marginalization as signals for this process [21].

FIP has also been reported to promote the occurrence of oxidative stress, inflammation and even neuronal apoptosis [36,67–69]. For example, the antioxidant system of the stingless bee (*Partamona helleri*) was triggered by 0.28 ng  $\mu\text{L}^{-1}$  FIP that led to increased SOD, CAT and glutathione S-transferase [68]. Oral administration for 28 days of 2.5, 5 and 10 mg  $\text{kg}^{-1}$  body weight (bw) FIP to mice resulted in decreased SOD and CAT activity and increased levels of lipid peroxidation found in brain and kidney [36]. These aforementioned repercussions were obtained by Wu (2021) in a zebrafish animal model after exposure to 0.5, 1 and 2 mg  $\text{L}^{-1}$  FIP [69].

Several studies suggest that acute exposure to FIP has neurotoxic effects on embryonic, larval, and adult zebrafish brains, including disturbances in sensory and motor systems, altered behavior, and changes in gene expression and enzyme activity. A study by Park (2020) showed that the accumulation of FIP in aquatic environments affects essential processes during the early developmental stage of zebrafish [70]. Stehr (2006) showed in their developmental neurotoxicity study in zebrafish embryos and larvae that FIP affects the development of spinal locomotor pathways in fish by inhibiting a structurally related glycine receptor subtype [71].

Our data revealed leukocyte infiltration in different regions of the zebrafish brain after exposure to FIP + PYR mixtures. This process is known to be correlated with the immune response that occurs and facilitates the entry of several effector cells to act in injury, infection, and cellular stress [72]. In addition, leukocyte infiltration begins as a deleterious complication of oxidative stress [73] and has additionally been linked to non-CNS neurological disorders (e.g., anxiety and cognitive impairment) in peripheral inflammatory diseases [74]. There are several investigations that have found the involvement of proinflammatory cytokines in mediating neurological diseases induced by systemic inflammation [75]. Wu (2021) observed that exposure to FIP caused oxidative stress, inflammation, and apoptosis in the brain tissue of adult zebrafish, leading to impaired sensory and motor systems and reduced survival rates [69].

The ability of FIP to induce oxidative stress was also demonstrated with a fungicide mixture (150  $\mu\text{g L}^{-1}$ ) tested in the zebrafish model for 7 days. This exposure resulted in significant perturbations of locomotor activity by reducing total distance traveled and number of crossings in the novel tank test, as well as increased CAT activity, but no changes in lipid peroxidation [76].

Therefore, the changes observed after histopathological analysis of treated adult zebrafish further support the changes observed for locomotor activity and anxiety state. This may explain some of our behavioral findings. Both compounds act predominantly on the CNS, but the action is general. Important information was obtained regarding the toxicity of the mixture of FIP and PYR.

## 5. Conclusions

The results presented indicate that fipronil and pyriproxyfen at different doses can lead to the disruption of locomotor activity and might support anxious behavior along with histological alterations. Therefore, our study highlights the importance of knowing the possible effects determined by this mixture on organisms. However, there is more to discover about the interaction of these two compounds, which should be explored in future studies, especially at the molecular level.

**Author Contributions:** Conceptualization, M.A.R.; methodology, M.A.R. and A.P.; software, M.A.R.; validation, A.C.; formal analysis, M.A.R. and A.P.; resources, D.U., M.N. and A.C.; data curation, M.A.R. and A.P.; writing—original draft preparation, M.A.R. and A.P.; writing—review and editing, D.U., M.N. and A.C.; supervision, A.C.; project administration, M.N. and A.C.; funding acquisition, M.N. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors are thankful to the Romanian Ministry of Research, Innovation and Digitization, within Program 1—Development of the national RD system, Subprogram 1.2—Institutional Performance—RDI excellence funding projects, Contract no. 11PFE/30.12.2021, for financial support.

**Institutional Review Board Statement:** The animal study protocol was by the Ethical Commission from the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Iasi, with registration number 749/04.07.2019.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Tudi, M.; Daniel Ruan, H.; Wang, L.; Lyu, J.; Sadler, R.; Connell, D.; Chu, C.; Phung, D.T. Agriculture development, pesticide application and its impact on the environment. *Int. J. Environ. Res. Public Health* **2021**, *18*, doi:10.3390/ijerph18031112.
2. Pathak, V.M.; Verma, V.K.; Rawat, B.S.; Kaur, B.; Babu, N.; Sharma, A.; Dewali, S.; Yadav, M.; Kumari, R.; Singh, S.; et al. Current status of pesticide effects on environment, human health and it's eco-friendly management as bioremediation: A comprehensive review. *Front. Microbiol.* **2022**, *13*, 962619, doi:10.3389/fmicb.2022.962619.
3. Serrão, J.E.; Plata-Rueda, A.; Martínez, L.C.; Zancunio, J.C. Side-effects of pesticides on non-target insects in agriculture: a mini-review. *Naturwissenschaften* **2022**, *109*, 17, doi:10.1007/s00114-022-01788-8.
4. Kim, K.-H.; Kabir, E.; Jahan, S.A. Exposure to pesticides and the associated human health effects. *Sci. Total Environ.* **2017**, *575*, 525–535, doi.org/10.1016/j.scitotenv.2016.09.009.
5. Authority, E.F.S. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid. *EFSA J.* **2013**, *11*, 3068, doi.org/10.2903/j.efsa.2013.3068.
6. Donley, N. The USA lags behind other agricultural nations in banning harmful pesticides. *Environ. Heal.* **2019**, *18*, 44, doi:10.1186/s12940-019-0488-0.
7. Sadaria, A.M.; Labban, C.W.; Steele, J.C.; Maurer, M.M.; Halden, R.U. Retrospective nationwide occurrence of fipronil and its degradates in U.S. wastewater and sewage sludge from 2001-2016. *Water Res.* **2019**, *155*, 465–473, doi:10.1016/j.watres.2019.02.045.
8. Hodgson, E. Chapter 9 - Biotransformation of Individual Pesticides: Some Examples. In: Hodgson, E.B.T.-P.B. and D., Ed.; Academic Press: Boston, 2012; pp. 195–208 ISBN 978-0-12-385481-0.
9. Fent, G.M. Fipronil. In: Wexler, P.B.T.-E. of T. (Third E., Ed.; Academic Press: Oxford, 2014; pp. 596–597 ISBN 978-0-12-386455-0.
10. Bhatt, P.; Gangola, S.; Ramola, S.; Bilal, M.; Bhatt, K.; Huang, Y.; Zhou, Z.; Chen, S. Insights into the toxicity and biodegradation of fipronil in contaminated environment. *Microbiol. Res.* **2023**, *266*, 127247, doi:10.1016/j.micres.2022.127247.
11. Tingle, C.C.D.; Rother, J.A.; Dewhurst, C.F.; Lauer, S.; King, W.J. Fipronil: environmental fate, ecotoxicology, and human health concerns. *Rev. Environ. Contam. Toxicol.* **2003**, *176*, 1–66, doi:10.1007/978-1-4899-7283-5\_1.
12. Wang, X.; Martínez, M.A.; Wu, Q.; Ares, I.; Martínez-Larrañaga, M.R.; Anadón, A.; Yuan, Z. Fipronil insecticide toxicology: oxidative stress and metabolism. *Crit. Rev. Toxicol.* **2016**, *46*, 876–899, doi:10.1080/10408444.2016.1223014.
13. Gupta, R.C.; Anadón, A. Chapter 42 - Fipronil. In: Gupta, R.C.B.T.-V.T. (Third E., Ed.; Academic Press, 2018; pp. 533–538 ISBN 978-0-12-811410-0.
14. Ohi, M.; Dalsenter, P.R.; Andrade, A.J.M.; Nascimento, A.J. Reproductive adverse effects of fipronil in Wistar rats. *Toxicol. Lett.* **2004**, *146*, 121–127, doi:10.1016/j.toxlet.2003.08.008.
15. Mossa, A.-T.H.; Swelam, E.S.; Mohafrash, S.M.M. Sub-chronic exposure to fipronil induced oxidative stress, biochemical and histopathological changes in the liver and kidney of male albino rats. *Toxicol. reports* **2015**, *2*, 775–784, doi:10.1016/j.toxrep.2015.02.009.
16. Kartheek, R.M.; David, M.; Mossa, A.-T.H.; Swelam, E.S.; Mohafrash, S.M.M. Assessment of fipronil toxicity on Wistar rats: A hepatotoxic perspective. *Toxicol. Reports* **2018**, *5*, 448–456, doi.org/10.1016/j.toxrep.2018.02.019.
17. Terçariol, P.R.G.; Godinho, A.F. Behavioral effects of acute exposure to the insecticide fipronil. *Pestic. Biochem. Physiol.* **2011**, *99*, 221–225, doi.org/10.1016/j.pestbp.2010.12.007.
18. Mahmoud, Y.K.; Ali, A.A.; Abdelrazek, H.M.A.; Aldayel, T.S.; Abdel-Daim, M.M.; El-Menyawy, M.A.I. Neurotoxic effect of fipronil in male wistar rats: ameliorative effect of l-arginine and l-carnitine. *Biology (Basel)*. **2021**, *10*, doi:10.3390/biology10070682.
19. Kitulagodage, M.; Isanhart, J.; Buttemer, W.A.; Hooper, M.J.; Astheimer, L.B. Fipronil toxicity in northern bobwhite quail *Colinus virginianus*: reduced feeding behaviour and sulfone metabolite formation. *Chemosphere* **2011**, *83*, 524–530, doi:10.1016/j.chemosphere.2010.12.057.
20. Arslan, P.; Günel, A.Ç. Does fipronil affect on aquatic organisms? physiological, biochemical, and histopathological alterations of non-target freshwater mussel species. *Water* **2023**, *15*.
21. Ferreira, M.; De Oliveira, P.R.; Denardi, S.E.; Bechara, G.H.; Mathias, M.I.C. Action of the chemical agent fipronil (active ingredient of acaricide Frontline®) on the liver of mice: an ultrastructural analysis. *Microsc. Res. Tech.* **2012**, *75*, 197–205, doi:10.1002/jemt.21043.
22. Clasen, B.; Loro, V.L.; Cattaneo, R.; Moraes, B.; Lopes, T.; de Avila, L.A.; Zanella, R.; Reimche, G.B.; Baldisserotto, B. Effects of the commercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: implications for rice-fish cultivation. *Ecotoxicol. Environ. Saf.* **2012**, *77*, 45–51, doi:10.1016/j.ecoenv.2011.10.001.
23. Ishaaya, I.; Horowitz, A.R. Pyriproxyfen, a novel insect growth regulator for controlling whiteflies: mechanisms and resistance management. *Pestic. Sci.* **1995**, *43*, 227–232, doi.org/10.1002/ps.2780430308.

24. Sullivan, J.J.; Goh, K.S. Environmental fate and properties of pyriproxyfen. *J. Pestic. Sci.* **2008**, *33*, 339–350.
25. Devillers, J. Fate of Pyriproxyfen in Soils and Plants. *Toxics* **2020**, *8*, doi:10.3390/toxics8010020.
26. World Health Organization - Pyriproxyfen in drinking-water: use for vector control in drinking-water sources and containers. **2008**.
27. Moura, L.; de Nadai, B.L.; Corbi, J.J. One does not simply apply larvicides: *Aedes aegypti* from Araraquara (Brazil) has reduced susceptibility to pyriproxyfen. *Vet. Parasitol. Reg. Stud. Reports* **2023**, *41*, 100875, doi.org/10.1016/j.vprsr.2023.100875.
28. Shahid, A.; Zaidi, S.D.-E.-S.; Akbar, H.; Saeed, S. An investigation on some toxic effects of pyriproxyfen in adult male mice. *Iran. J. Basic Med. Sci.* **2019**, *22*, 997–1003, doi:10.22038/ijbms.2019.33825.8051.
29. Mehrnoush, G.; Mehrdad, S.; Saeid, K.H.A. Effect of pyriproxyfen on function and tissue of testis in adult rat. *Int. J. Curr. Res. Rev.* **2013**, *5*, 66–74.
30. Caixeta, E.S.; Silva, C.F.; Santos, V.S.V.; Olegário de Campos Júnior, E.; Pereira, B.B. Ecotoxicological assessment of pyriproxyfen under environmentally realistic exposure conditions of integrated vector management for *Aedes aegypti* control in Brazil. *J. Toxicol. Environ. Health. A* **2016**, *79*, 799–803, doi:10.1080/15287394.2016.1191400.
31. Azevedo, R.D.S.; Falcão, K.V.G.; Assis, C.R.D.; Martins, R.M.G.; Araújo, M.C.; Yogui, G.T.; Neves, J.L.; Seabra, G.M.; Maia, M.B.S.; Amaral, I.P.G.; et al. Effects of pyriproxyfen on zebrafish brain mitochondria and acetylcholinesterase. *Chemosphere* **2021**, *263*, 128029, doi:10.1016/j.chemosphere.2020.128029.
32. Costa, L.G.; Giordano, G.; Guizzetti, M.; Vitalone, A. Neurotoxicity of pesticides: a brief review. *Front. Biosci.* **2008**, *13*, 1240–1249, doi:10.2741/2758.
33. Abreu-Villaça, Y.; Levin, E.D. Developmental neurotoxicity of succeeding generations of insecticides. *Environ. Int.* **2017**, *99*, 55–77, doi:10.1016/j.envint.2016.11.019.
34. Richardson, J.R.; Fitsanakis, V.; Westerink, R.H.S.; Kanthasamy, A.G. Neurotoxicity of pesticides. *Acta Neuropathol.* **2019**, *138*, 343–362, doi:10.1007/s00401-019-02033-9.
35. De Oliveira, P.R.; Bechara, G.H.; Denardi, S.E.; Oliveira, R.J.; Mathias, M.I.C. Cytotoxicity of fipronil on mice liver cells. *Microsc. Res. Tech.* **2012**, *75*, 28–35, doi:10.1002/jemt.21018.
36. Badgujar, P.C.; Chandratre, G.A.; Pawar, N.N.; Telang, A.G.; Kurade, N.P. Fipronil induced oxidative stress involves alterations in SOD1 and catalase gene expression in male mice liver: Protection by vitamins E and C. *Environ. Toxicol.* **2016**, *31*, 1147–1158, doi:https://doi.org/10.1002/tox.22125.
37. Abdel-Daim, M.M.; Abdeen, A. Protective effects of rosuvastatin and vitamin E against fipronil-mediated oxidative damage and apoptosis in rat liver and kidney. *Food Chem. Toxicol. an Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2018**, *114*, 69–77, doi:10.1016/j.fct.2018.01.055.
38. Awad, M.A.; Ahmed, Z.S.O.; AbuBakr, H.O.; Elbargeesy, G.A.E.-F.H.; Moussa, M.H.G. Fipronil induced oxidative stress in neural tissue of albino rat with subsequent apoptosis and tissue reactivity. *Acta Histochem.* **2021**, *123*, 151764, doi.org/10.1016/j.acthis.2021.151764.
39. Ghaffar, A.; Hussain, R.; Abbas, G.; Kalim, M.; Khan, A.; Ferrando, S.; Gallus, L.; Ahmed, Z. Fipronil (Phenylpyrazole) induces hemato-biochemical, histological and genetic damage at low doses in common carp, *Cyprinus carpio* (Linnaeus, 1758). *Ecotoxicology* **2018**, *27*, 1261–1271, doi:10.1007/s10646-018-1979-4.
40. Maharajan, K.; Muthulakshmi, S.; Nataraj, B.; Ramesh, M.; Kadirvelu, K. Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (*Danio rerio*): A multi biomarker study. *Aquat. Toxicol.* **2018**, *196*, 132–145, doi:10.1016/j.aquatox.2018.01.010.
41. Shahid, A.; Saher, M. Repeated exposure of pyriproxyfen to pregnant female mice causes developmental abnormalities in prenatal pups. *Environ. Sci. Pollut. Res. Int.* **2020**, *27*, 26998–27009, doi:10.1007/s11356-020-08656-w.
42. Spitsbergen, J.M.; Kent, M.L. The state of the art of the zebrafish model for toxicology and toxicologic pathology research advantages and current limitations. *Toxicol. Pathol.* **2003**, *31 Suppl*, 62–87, doi:10.1080/01926230390174959.
43. Parng, C.; Roy, N.M.; Ton, C.; Lin, Y.; McGrath, P. Neurotoxicity assessment using zebrafish. *J. Pharmacol. Toxicol. Methods* **2007**, *55*, 103–112, doi:10.1016/j.vascn.2006.04.004.
44. Choi, T.-Y.; Choi, T.-I.; Lee, Y.-R.; Choe, S.-K.; Kim, C.-H. Zebrafish as an animal model for biomedical research. *Exp. Mol. Med.* **2021**, *53*, 310–317, doi:10.1038/s12276-021-00571-5.
45. Audira, G.; Sampurna, B.P.; Juniardi, S.; Liang, S.-T.; Lai, Y.-H.; Hsiao, C.-D. A Versatile setup for measuring multiple behavior endpoints in zebrafish. *Inventions* **2018**, *3*.
46. Rafferty, S.A.; Quinn, T.A. A beginner's guide to understanding and implementing the genetic modification of zebrafish. *Prog. Biophys. Mol. Biol.* **2018**, *138*, 3–19, doi.org/10.1016/j.pbiomolbio.2018.07.005.
47. Li, Y.; Jia, Z.; Zhang, S.; He, X. Progress in gene-editing technology of zebrafish. *Biomolecules* **2021**, *11*, doi:10.3390/biom11091300.
48. Robea, M.A.; Jijie, R.; Nicoara, M.; Plavan, G.; Ciobica, A.S.; Solcan, C.; Audira, G.; Hsiao, C.-D.; Strungaru, S.-A. Vitamin C attenuates oxidative stress and behavioral abnormalities triggered by fipronil and pyriproxyfen insecticide chronic exposure on zebrafish juvenile. *Antioxidants (Basel, Switzerland)* **2020**, *9*, doi:10.3390/antiox9100944.



49. Commission Recommendation Guidelines for the accommodation and care of animals used for experimental and other scientific purposes (notified under document number C(2007) 2525). *Off. J. Eur. Union* **2007**, 50.
50. Avdesh, A.; Chen, M.; Martin-Iverson, M.T.; Mondal, A.; Ong, D.; Rainey-Smith, S.; Taddei, K.; Lardelli, M.; Groth, D.M.; Verdile, G.; et al. Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *J. Vis. Exp.* **2012**, e4196–e4196, doi:10.3791/4196.
51. Aleström, P.; D'Angelo, L.; Midtlyng, P.J.; Schorderet, D.F.; Schulte-Merker, S.; Sohm, F.; Warner, S. Zebrafish: Housing and husbandry recommendations. *Lab. Anim.* **2019**, 54, 213–224, doi:10.1177/0023677219869037.
52. Araujo, J.; Maximino, C.; de Brito, T.M.; da Silva, A.W.B.; Oliveira, K.R.M.; de Jesus Oliveira Batista, E.; Morato, S.; Herculano, A.M.; Gouveia, A. Behavioral and pharmacological aspects of anxiety in the light/dark preference test bt - Zebrafish Protocols for Neurobehavioral Research. In: Kalueff, A. V, Stewart, A.M., Eds.; Humana Press: Totowa, NJ, 2012; pp. 191–202 ISBN 978-1-61779-597-8.
53. Directive 63 The protection of animals used for scientific purposes. *Off. J. Eur. Union* **2010**, 53.
54. Koslowski, S.; Latapy, C.; Auvray, P.; Blondel, M.; Meijer, L. Long-term fipronil treatment induces hyperactivity in female mice. *Int. J. Environ. Res. Public Health* **2020**, 17, 1579, doi:10.3390/ijerph17051579.
55. Reichel, J.M.; Nissel, S.; Rogel-Salazar, G.; Mederer, A.; Käfer, K.; Bedenk, B.T.; Martens, H.; Anders, R.; Grosche, J.; Michalski, D.; et al. Distinct behavioral consequences of short-term and prolonged GABAergic depletion in prefrontal cortex and dorsal hippocampus. *Front. Behav. Neurosci.* **2015**, 8, 452, doi:10.3389/fnbeh.2014.00452.
56. Gusso, D.; Reolon, G.K.; Gonzalez, J.B.; Altenhofen, S.; Kist, L.W.; Bogo, M.R.; Bonan, C.D. Pyriproxyfen exposure impairs cognitive parameters and alters cortisol levels in zebrafish. *Front. Behav. Neurosci.* **2020**, 14, 103, doi:10.3389/fnbeh.2020.00103.
57. Li, P.; Akk, G. The insecticide fipronil and its metabolite fipronil sulphone inhibit the rat  $\alpha 1\beta 2\gamma 2L$  GABA(A) receptor. *Br. J. Pharmacol.* **2008**, 155, 783–794, doi:10.1038/bjp.2008.309.
58. Ghit, A.; Assal, D.; Al-Shami, A.S.; Hussein, D.E.E. GABAA receptors: structure, function, pharmacology, and related disorders. *J. Genet. Eng. Biotechnol.* **2021**, 19, 123, doi:10.1186/s43141-021-00224-0.
59. Eadie, A.; Vasquez, I.C.; Liang, X.; Wang, X.; Souders, C.L. 2nd; El Chehouri, J.; Hoskote, R.; Feswick, A.; Cowie, A.M.; Loughery, J.R.; et al. Residual molecular and behavioral effects of the phenylpyrazole pesticide fipronil in larval zebrafish (*Danio rerio*) following a pulse embryonic exposure. *Comp. Biochem. Physiol. Part D. Genomics Proteomics* **2020**, 36, 100743, doi:10.1016/j.cbd.2020.100743.
60. Jabłońska-Trypuć, A. Pesticides as inducers of oxidative stress. *React. Oxyg. Species* **2017**, 3, 96–110.
61. Mani, S.; Jain, A.; Gulati, A.; Tyagi, S.; Pal, K.V.; Jaiswal, H.; Singh, M. Oxidative stress: a potential link between pesticide exposure and early-life neurological disorders bt - free radical biology and environmental toxicity. In: Kesari, K.K., Jha, N.K., Eds.; Springer International Publishing: Cham, 2021; pp. 209–251 ISBN 978-3-030-83446-3.
62. Sule, R.O.; Condon, L.; Gomes, A. V. A Common feature of pesticides: oxidative stress-the role of oxidative stress in pesticide-induced toxicity. *Oxid. Med. Cell. Longev.* **2022**, 2022, 5563759, doi:10.1155/2022/5563759.
63. Ullah, R.; Zuberi, A.; Ullah, S.; Ullah, I.; Dawar, F. Cypermethrin induced behavioral and biochemical changes in Mahseer, *Tor putitora*. *J. Toxicol. Sci.* **2014**, 39, 829–836, doi:10.2131/jts.39.829.
64. Nataraj, B.; Hemalatha, D.; Rangasamy, B.; Maharajan, K.; Ramesh, M. Hepatic oxidative stress, genotoxicity and histopathological alteration in fresh water fish *Labeo rohita* exposed to organophosphorus pesticide profenofos. *Biocatal. Agric. Biotechnol.* **2017**, 12, 185–190, doi.org/10.1016/j.bcab.2017.09.006.
65. Amin, K.A.; Hashem, K.S. Deltamethrin-induced oxidative stress and biochemical changes in tissues and blood of catfish (*Clarias gariepinus*): antioxidant defense and role of alpha-tocopherol. *BMC Vet. Res.* **2012**, 8, 45, doi:10.1186/1746-6148-8-45.
66. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.T.D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, 39, 44–84, doi:10.1016/j.biocel.2006.07.001.
67. Wang, C.; Qian, Y.; Zhang, X.; Chen, F.; Zhang, Q.; Li, Z.; Zhao, M. A metabolomic study of fipronil for the anxiety-like behavior in zebrafish larvae at environmentally relevant levels. *Environ. Pollut.* **2016**, 211, 252–258, doi:10.1016/j.envpol.2016.01.016.
68. Farder-Gomes, C.F.; Fernandes, K.M.; Bernardes, R.C.; Bastos, D.S.S.; Martins, G.F.; Serrão, J.E. Acute exposure to fipronil induces oxidative stress, apoptosis and impairs epithelial homeostasis in the midgut of the stingless bee *Partamona helleri* Friese (Hymenoptera: Apidae). *Sci. Total Environ.* **2021**, 774, 145679, doi.org/10.1016/j.scitotenv.2021.145679.
69. Wu, C.-H.; Lu, C.-W.; Hsu, T.-H.; Wu, W.-J.; Wang, S.-E. Neurotoxicity of fipronil affects sensory and motor systems in zebrafish. *Pestic. Biochem. Physiol.* **2021**, 177, 104896, doi:10.1016/j.pestbp.2021.104896.
70. Park, H.; Lee, J.-Y.; Park, S.; Song, G.; Lim, W. Developmental toxicity of fipronil in early development of zebrafish (*Danio rerio*) larvae: Disrupted vascular formation with angiogenic failure and inhibited neurogenesis. *J. Hazard. Mater.* **2020**, 385, 121531, doi.org/10.1016/j.jhazmat.2019.121531.



71. Stehr, C.M.; Linbo, T.L.; Incardona, J.P.; Scholz, N.L. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. *Toxicol. Sci.* **2006**, *92*, 270–278, doi:10.1093/toxsci/kfj185.
72. Nourshargh, S.; Alon, R. Leukocyte migration into inflamed tissues. *Immunity* **2014**, *41*, 694–707, doi.org/10.1016/j.immuni.2014.10.008.
73. Schmitt, C.; Strazielle, N.; Gherzi-Egea, J.-F. Brain leukocyte infiltration initiated by peripheral inflammation or experimental autoimmune encephalomyelitis occurs through pathways connected to the CSF-filled compartments of the forebrain and midbrain. *J. Neuroinflammation* **2012**, *9*, 187, doi:10.1186/1742-2094-9-187.
74. Chen, X.-K.; Kwan, J.S.-K.; Wong, G.T.-C.; Yi, Z.-N.; Ma, A.C.-H.; Chang, R.C.-C. Leukocyte invasion of the brain after peripheral trauma in zebrafish (*Danio rerio*). *Exp. Mol. Med.* **2022**, *54*, 973–987, doi:10.1038/s12276-022-00801-4.
75. Skelly, D.T.; Griffin, É.W.; Murray, C.L.; Harney, S.; O'Boyle, C.; Hennessy, E.; Dansereau, M.-A.; Nazmi, A.; Tortorelli, L.; Rawlins, J.N.; et al. Acute transient cognitive dysfunction and acute brain injury induced by systemic inflammation occur by dissociable IL-1-dependent mechanisms. *Mol. Psychiatry* **2019**, *24*, 1533–1548, doi:10.1038/s41380-018-0075-8.
76. Bevilaqua, F.; Sachett, A.; Chitolina, R.; Garbinato, C.; Gasparetto, H.; Marcon, M.; Mocelin, R.; Dalleggrave, E.; Conterato, G.; Piato, A.; et al. A mixture of fipronil and fungicides induces alterations on behavioral and oxidative stress parameters in zebrafish. *Ecotoxicology* **2020**, *29*, 140–147, doi:10.1007/s10646-019-02146-7.

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.