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Behavioral effects of triadimefon in zebrafish are associated with alterations of the dopaminergic and serotonergic pathways

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ABSTRACT

Triadimefon (TDF) is a triazole fungicide extensively used in agriculture that has been found as a pollutant in numerous water sources. In mammals, it inhibits monoamine uptake through binding to the dopamine transporter, with a mechanism of action similar to cocaine, resulting in higher levels of dopamine at the synapse. Dopamine is a neurotransmitter involved in a broad spectrum of processes such as locomotion, cognition, reward, and mental disorders. In this work we have studied, for the first time, the effects of TDF on behavior of both larval and adult zebrafish and its connection with changes in the dopaminergic and serotonergic systems. We evaluated the acute exposure of 5 dpf larvae to different concentrations of TDF, ranging from 5 mg/L to 35 mg/L. The lowest concentration does not alter neither locomotor activity nor dopamine levels but produced changes in the expression of two genes, *tyrosine hydroxylase 1* (*th1)* and *dopamine transporter* (*dat)*. Besides, it induced a reduction in extracellular serotonin and had an anxiolytic-like effect, supported by a decrease in cortisol production. On the other hand, a high concentration of TDF produced a dose-dependent reduction in locomotion, which was reversed or enhanced by D1 (SCH-23390) or D2 (Haloperidol) dopamine receptor antagonists, respectively. Using *in vivo* electrochemistry, we show that these changes could be associated with higher levels of dopamine in the brain. Thus, in adult zebrafish, though not in larvae, TDF exposure increases locomotor activity, anxiety and aggressiveness, which coincides with the behaviors observed in mammals.

1. Introduction

In order to improve the quality of life, humans have developed numerous synthetic compounds, which often act as sources of contamination. The dissemination of these substances in the environment occurs mainly through domestic effluents or agricultural irrigation. Many of these products have been found in food, surface and waste water, and even drinking water supplies throughout the world (Caliman and Gavrilescu, 2009; Kahle et al., 2008). These compounds, even in minimal concentrations, cause adverse effects on both reproduction and development of various forms of wildlife, and become a potential threat to human health (Colborn, 1993).

Wild fish populations are one of the main sentinels of environmental quality. These organisms are constantly exposed to a wide variety of aquatic pollutants and are sensitive to them at low concentrations (Pereira et al., 2012). Among these contaminants, Triadimefon (TDF; 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-one), is a triazole fungicide commonly used in agricultural crops at concentrations of 200–1000 mg/L (Bayer Crop *Bayer Crop Science,* 2008). Previously, we have demonstrated that TDF affects development and inhibits hatching of zebrafish embryos at sublethal concentrations ranging from 16 to 36 mg/L (De la Paz et al., 2017). Studies performed in rodents have shown that, like many psychostimulants, TDF increases the locomotor and stereotypic activity (Ikaiddi et al., 1997; Walker et al., 1990). By binding to the Dopamine Transporter (DAT), TDF increases dopamine levels at the synapse, resulting in acute stimulatory effects (Gagnaire and Micillino, 2006). In general, TDF appears to act as an indirect dopamine agonist, with a mechanism of action resembling cocaine. Since TDF seems to present some common mechanisms with cocaine, it may also share other behavioral and neurochemical effects.

Over the last decades, zebrafish has become a powerful model organism for behavioral neuroscience, since it provides many benefits

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over the use of mammals, such as rapid and external development, small size and low cost (Kimmel et al., 1995). This organism shares genetic homology with mammals and conserved molecular and cellular mechanisms. Zebrafish also harbors the major neurotransmitter systems such as GABA, glutamate, dopamine (DA) and serotonin (5-HT) among others (Panula et al., 2010; Maximino and Herculano, 2010). The first dopaminergic neurons appear at 18 h post fertilization (hpf) and the final repertoire is found at 5 days post fertilization (dpf) (Schweitzer and Driever, 2009). This maturation is revealed by the presence of Tyrosine Hydroxylase (TH), the enzyme that catalyzes the rate limiting step in the synthesis of catecholamines. In zebrafish there are two paralogous *tyrosine hydroxylase* genes: *th1* and *th2*. On the other hand, serotonin-like immunoreactivity appears at 24 hpf in the habenula and in the posterior tuberculum. By 3 dpf, serotonergic neurons can be found throughout the central nervous system, including the spinal cord, superior and inferior raphe, pretectum, cerebellum and hypothalamus (McLean and Fetcho, 2004). At 5 dpf, all neuronal cell types and projections are functional (Rink and Wullimann, 2002; Tay et al., 2011), which enable zebrafish larvae to perform complex behaviors like swimming, hunting and thigmotaxis, a well-validated index of anxiety in vertebrates (Schnörr et al., 2012).

In this work we exploit established behavioral assays performed both in larval and adult zebrafish to assess the effects of TDF on the nervous system.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) were maintained and raised in our facility according to standard procedures (Westerfield, 2009) and uniform controlled parameters, which reduces the variability among individuals. Adult zebrafish were raised using a 14 h light / 10 h dark cycle and fed twice a day. We used wild type strains (*TAB5*) and the transgenic line *Tg(neurod:TagRFP)*^{w69} (kindly provided by Dr. David Raible). The embryos were collected by natural spawning, staged according to Kimmel et al. (1995) and raised at 28 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, equilibrated to pH 7.0) in Petri dishes until treatment. For experiments, we used 5 dpf larvae and adults over 6 months of age. We took steps to ensure that we used sibling animals within experiments. All the procedures complied with the guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Chile, considering that the number of animals used was the minimum required for proper statistical validation of the experiments.

2.2. Pharmaceuticals

All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of Triadimefon was prepared in E3 medium at 50 mg/L, considering that TDF has a solubility of 71.5 mg/L in water (Wauchope et al., 1992). Dopamine receptor antagonists used in this work were R-(+)-SCH-23390 hydrochloride (D1-like receptors) and haloperidol (D2-like receptors). Stock solutions were prepared in deionized water or dimethyl sulfoxide (DMSO), respectively.

2.3. Locomotor activity

5 dpf larvae were incubated in 96-well multiwell plates with 200 µL of TDF ranging from 5 mg/L to 35 mg/L at room temperature (23–26 °C) for 10 h under constant illumination. Co-incubations of TDF 35 mg/L with SCH-23390 or Haloperidol at 8 µM were also performed. Locomotor activity was measured using the automated Microtracker system (Simonetta and Golombek, 2007).

2.4. Quantitative PCR

Quantitative PCR was performed to determine the effects of TDF on mRNA expression levels of the *tyrosine hydroxylase 1* (*th1*), *tyrosine hydroxylase 2 (th2)* and *dopamine transporter (dat)* genes. 5 dpf larvae (n = 30–50) were incubated with TDF 5 mg/L for 15 min. Animals were euthanized with an overdose of MS-222 (tricaine; A5040; Sigma, Saint Louis, MO, USA) and mRNA was extracted from whole larvae using TRIzol reagent. Primers were obtained from Chen et al. (2012). The methodology and analysis of qPCR were performed following the protocol described in De la Paz et al. (2017).

2.5. Electrochemical measurements

5 dpf larvae were immersed in LMP agarose 1% (v/v) (peqGOLD 35–2099; PEQ-LAB Biotechnologie) with 1% of MS- 222 (tricaine; A5040; Sigma, Saint Louis, MO, USA). Electrochemistry was performed using an extracellular solution containing (in mM): 134 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose (Sigma-Aldrich) equilibrated to pH 7.4, according to Shang et al. (2015). Neurotransmitter levels were recorded in the same specimen 1 min before and after being in contact with the drug. This period was used to avoid the possible oxidation of other molecules (i.e., ascorbate) which could have interfered with the measurement of neurotransmitters, and basal values were constant in this period. Amperometry was accomplished at room temperature with nafion-coated (Nafion, Sigma Aldrich) carbon fiber microelectrodes (CARBOSTAR-1, Kation Scientific, MN, USA) at a constant holding potential of 700 mV for dopamine and 400 mV for serotonin (Njagi et al., 2010), relative to an Ag/AgCl reference electrode. Both recording and reference electrodes were connected to a potentiostat and headstage circuit (AxoPatch 200B Amplifier, Molecular Devices, Sunnyvale, CA, USA), whose output was connected to a digital converter (Dataq 154, Dataq Instruments, Akron, OH, USA) and acquired on a computer at 10 Hz (WinDaq Data Acquisition Software; Dataq Instruments, Akron, OH, USA).

2.6. Thigmotaxis

The setup and timing were performed as described by Schnörr et al. (2012). Briefly, the larvae were placed in a 24-multiwell plate (Nest Biotechnology, Jiangsu, China). In the bottom of every well there were two different areas delimited by an inner circle of 4 mm. 5 mg/L of TDF was added at the beginning of the test. Video recordings started after 5 min of acclimatization and were done during the light phase to record the basal anxiety levels, and after a challenge (turning the lights off), when the highest thigmotaxis should be triggered. We used a 95% light to darkness transition. The videos were captured at a frame rate of 30 frames per second with a resolution of 320*270 pixels. Thigmotaxis was measured as the percentage of total distance moved in the outer zone. Larvae presenting abnormal locomotor activity (immotile) were not considered in the analysis (Bouwknecht and Paylor, 2008; Schnörr et al., 2012). These values were obtained using a custom video tracking program written in Python and based on the open source computer vision library OpenCV (Bradski, 2000). The use of this computer program allows the quantification of thigmotactic behavior without turning to manual tracking and therefore reducing the potential bias.

2.7. Cortisol extraction

In order to determine the levels of cortisol during a stressful stimulus (challenge), we carried out the thigmotaxis test, but removing the larvae after the end of the dark phase to perform the extractions. Around 30 larvae were used per treatment. All the experiments were performed at the same time of the day due to circadian variation in cortisol levels. The larvae were immobilized by placing the Petri dishes on ice and euthanized with MS-222. Cortisol was extracted with ethyl

acetate (Sigma-Aldrich) following the protocol published by Yeh et al. (2013). Cortisol concentrations were quantified using an ELISA human salivary cortisol assay kit (ALPCO, MA, USA) which has been demonstrated to work for zebrafish (Egan et al., 2009). ELISA plates were measured in a plate reader with a 450 nm filter. Whole-body cortisol levels were determined using SOFTmax Pro software. Calculations were performed following the manufacturer's instructions (ALPCO, MA, USA). Cortisol levels were normalized based on the weight of the samples and reported as ng/mL.

2.8. Adult zebrafish behavior

To record and analyze the behavior of adult zebrafish treated with TDF 5 mg/L and 20 mg/L we followed the methods described in Cachat et al. (2011). Experimentally naïve zebrafish of both sexes (50% each) and similar size were used. Since gender could affect the behavioral outcome, we previously analyzed our data observing no statistical differences between males and females (data not shown). All animals belonged to the same strain (TAB5) and were raised and manipulated equally. The acclimatization (10 days) and the test were carried out in a trapezoidal tank made of glass (15 height x top 28 × 22 bottom × 7.5 width, cm, Aquamundo, Santiago, Chile) filled with 1.5 L of aquarium water. On the day of the test, zebrafish were relocated into the test tank which was virtually divided in two horizontal halves by drawing a line in the outside wall. TDF was added to the water and zebrafish were in contact with the drug for 5 min before beginning the recordings. Behavior was recorded for 6 min with a video resolution of 1920*1080 pixels and 30 frames per second. Time spent in the lower and upper halves as well as distance and velocity traveled were obtained using the custom video tracking software described above, which automatically provided the values without the need for manual tracking. Aggression, latency and transitions to upper half were manually counted using blinds. Aggression in a fish was determined by counting the number of bites against its own reflection, according to Norton et al. (2011).

2.9. Statistical analysis

Data are presented as mean values ± SEM (standard error of mean). Statistical analyses were performed using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla, CA, USA). Data distribution was analyzed before performing tests for proper usage of parametric or non-parametric statistics. For two-way ANOVA the value of the F distribution was showed as F(DFn, DFd), where DFn and DFd represent the degrees of freedom of the numerator and denominator, respectively. The sample size was calculated according to the mean and variance of the control group, using an $\alpha = 0.05$ and a power $(1 - \beta) = 85\%$. Thus, all sample sizes were calculated to determine small statistical differences in the measured variables. The probability level for statistical significance was $p < 0.05$. Statistical details are presented in the figure legends.

3. Results

3.1. Triadimefon decreases locomotor activity in a dose-dependent manner

Previous works have reported that TDF gives rise to hyperactivity in rats due an increase in dopamine levels (Ikaiddi et al., 1997; Walker et al., 1990). To compare these results with our model, we decided to expose 5 dpf larvae to different concentrations of TDF dissolved in the water. Fig. 1A shows that, while no differences between the controls and animals treated with 5 mg/L were detected, at the two higher concentrations tested (20 and 35 mg/mL), we found that there was a dose dependent decrease in locomotor activity (Dose effect F(3, 156) = 46.95).

3.2. Triadimefon affects locomotor activity in larvae by increasing dopamine levels

One of the effects of TDF is to increase extracellular dopamine, which leads to hyperactivity in mammals (Ikaiddi et al., 1997). To explore the relationship between the diminished locomotor activity observed in zebrafish (Fig. 1) and dopamine levels in the brain, we decided to perform *in vivo* electrochemistry. For each larva, dopamine was measured before and after adding the TDF to the medium. Dopamine levels in the larvae were largely unaffected by 5 mg/L of TDF (6.19 ± 0.17 nM and 6.51 ± 0.33 nM, before and after treatment, respectively), showing no significant changes ($p > 0.05$; paired *t*-test) in the extracellular dopamine concentration (Fig. 2A). On the other hand, larvae exposed to 35 mg/L of TDF, showed a significant increase ($p < 0.001$; paired t-test) in dopamine levels, from 6.98 ± 1.78 nM to 12.62 ± 2.34 nM before and after the treatment, respectively (Fig. 2B).

3.3. SCH-23390 and Haloperidol have opposite effects on locomotor activity when they are co-incubated with Triadimefon

Since the effects of TDF on locomotor activity are likely related to changes in dopamine levels, we decided to treat larvae with TDF 35 mg/L and either of two different dopamine receptor antagonists: SCH-23390 (a D1-like receptor antagonist) and Haloperidol (D2-like receptor antagonist). Through this approach we wanted to determine whether the effects of a high dose of TDF could be counteracted or potentiated depending on the antagonist, as well as to learn through which of the two types of receptors TDF is exerting its influence on behavior.

We found that 8 μM SCH-23390 *per se* significantly reduced locomotor activity, but reversed the effect of TDF on locomotor activity after 3.0 h of co-incubation ($p < 0.05$; Two-way ANOVA, Tukey's multiple comparisons test, Treatment F(3, 131) = 50.70, Fig. 3A, arrow). On the other hand, 8 μM Haloperidol *per se* also reduced locomotor activity but, conversely to SCH-23390, after 2.5 h it potentiated the depressing locomotor effects when it was co-incubated with TDF ($p < 0.05$; Two-way ANOVA, Tukey's multiple comparisons test, Treatment F(4, 233) = 97.36, Fig. 3B, arrow).

3.4. The expression of the tyrosine hydroxylase 1 and dopamine transporter genes are altered by Triadimefon

To determine if TDF can affect the molecular markers that characterize dopaminergic neurons, we performed qPCR to analyze the mRNA expression of *th1, th2* and *dat* in control and treated animals. To carry out this experiment, we exposed fish for 15 min to 5 mg/L TDF. We selected this condition since longer exposures or higher concentrations significantly affected the levels of the housekeeping genes. We also used *prolactin* (*prl)* as a positive control, since we have previously reported an increase in the expression of this gene by exposure to TDF (De la Paz et al., 2017). We found that the mRNA levels of *th1* increased 1.5 ± 0.14 times after fish are exposed to TDF, whereas *th2* did not show a significant change. In contrast, the expression of *dat* was reduced 0.7 ± 0.11 times after the treatment with TDF (Fig. 4).

3.5. Triadimefon diminishes anxiety-like behavior and cortisol levels in zebrafish larvae

To test the neuroactive properties of drugs, it is common to assess their effects on anxiety-like behavior. To achieve this objective, we measured thigmotaxis in both the light (control) and dark phases (challenge). We used TDF at 5 mg/L since this concentration does not affect locomotor activity (Fig. 1, Fig. S1) and, thus, avoids erroneous interpretations. During the light phase, control animals exhibited a 97.98 ± 0.54% of thigmotaxis while TDF treated ones a 96.32 ± 0.66%, showing no statistical differences (Fig. 5A). In

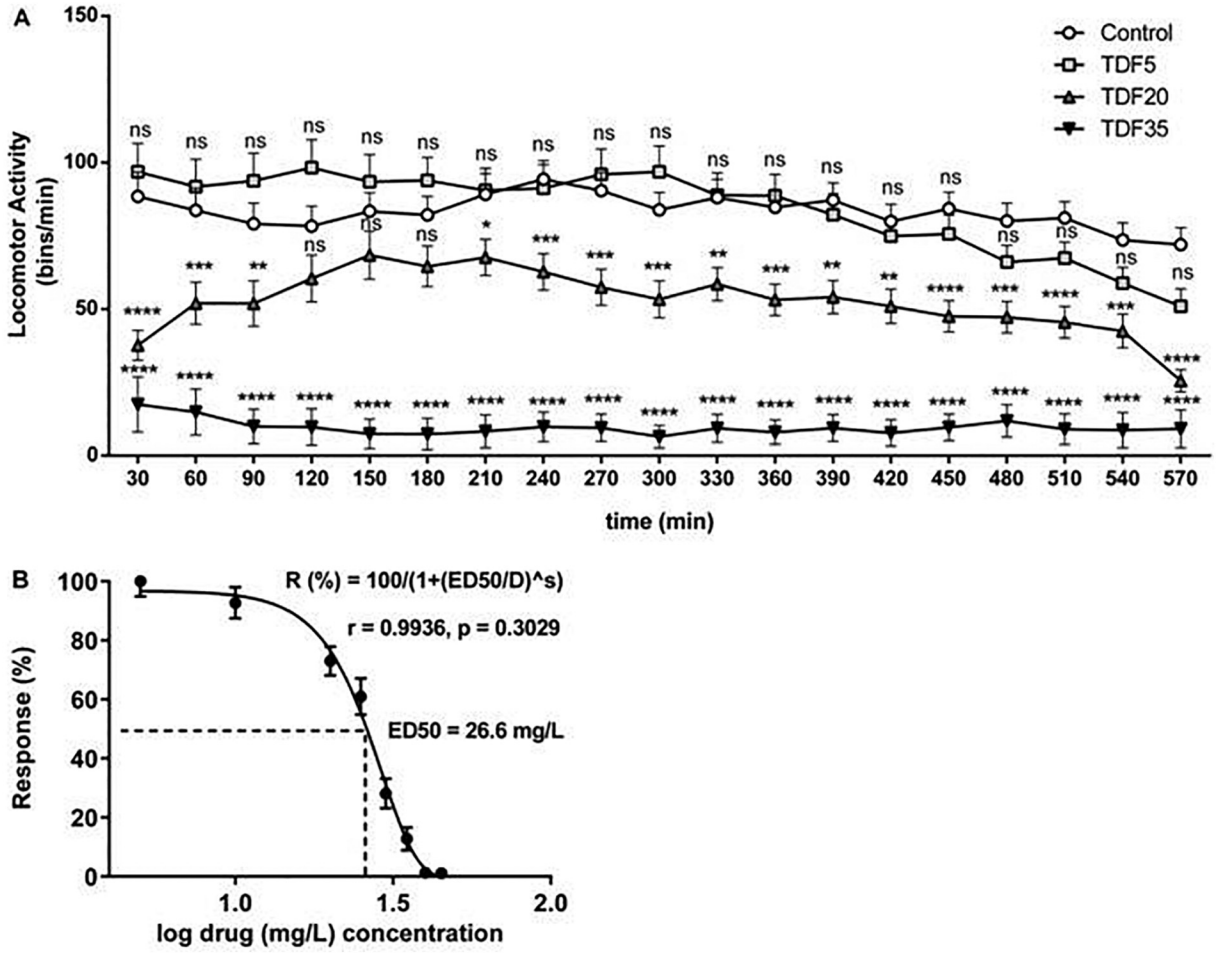

Fig. 1. TDF exposure leads to a concentration dependent decrease in zebrafish larval locomotor activity. (A) Temporal effect of Triadimefon on locomotor activity depends on the applied dose. Data are presented as average ± SEM from three independent experiments (20–25 larvae per condition). Statistical analyses were made by two-way ANOVA with Tukey's correction for multiple comparisons. Error bars = SEM. *p*-values with respect to controls; ****: < 0.0001;*: < 0.05; ns: > 0.05. (B) Dose-response curve shows inhibition of the locomotor activity. Eight doses of TDF, ranging from 5 to 45 mg/L, were used to fit the concentration-response curve. The curve was fitted using a sigmoidal function (R (%) = 1/(1 + (ED_{50}/D)^S), where R(%) represents the percentage responses with respect to control, ED_{50} the half-maximal effect, D the applied dose and S the slope of the curve. ED_{50} is observed at 26.6 mg/L of TDF. Data are presented as average ± SEM from 10 to 12 larvae per condition from three independent experiments. Abscissa in logarithmic units.

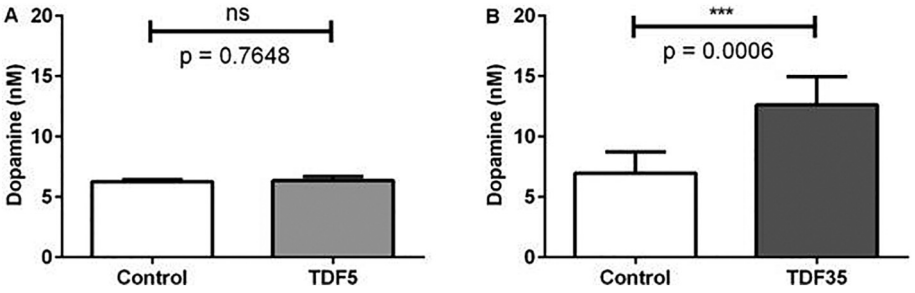

Fig. 2. Extracellular dopamine increases after treatment with 35 mg/L of TDF. Dopamine levels were measured in the zebrafish brain after treating with two different concentrations of TDF. (A) Treatment with 5 mg/L of TDF (TDF5) does not affect dopamine levels. (B) Treatment with 35 mg/L (TDF35) results in a two-fold increase in dopamine levels compared to control fish. Data are presented as average ± SEM from 7 to 8 larvae per condition from three independent experiments. Error bars = SEM. Statistical analyses were made using paired *t*-tests, after performing D'Agostino and Pearson omnibus normality test. ***: *p*-value < 0.001; ns: p-value > 0.05.

contrast, when larvae were stressed with an abrupt change from light to darkness (challenge), the control animals reached a percentage of thigmotaxis of 99.40 ± 0.21%, while the treated ones presented significantly lower levels of anxiety-like behavior (97.11 ± 0.51%) when compared with the control (Fig. 5A; $p < 0.05$, nonparametric one-way ANOVA with Dunn's multiple comparisons test).

Since cortisol is a well known physiological marker of stress and anxiety, we wanted to compare our behavioral results with the levels of this hormone in fish subjected to TDF exposure and a stress challenge. As Fig. 5B shows, cortisol was significantly higher (*p*-value < 0.05, unpaired *t*-test) in non treated larvae (6.8 ± 1.5 ng/mL) compared to TDF treated fish (3.3 ± 0.4 ng/mL).

Therefore, both the thigmotaxis response and the cortisol quantification results, lead us to conclude that TDF has anxiolytic-like effects in zebrafish larvae.

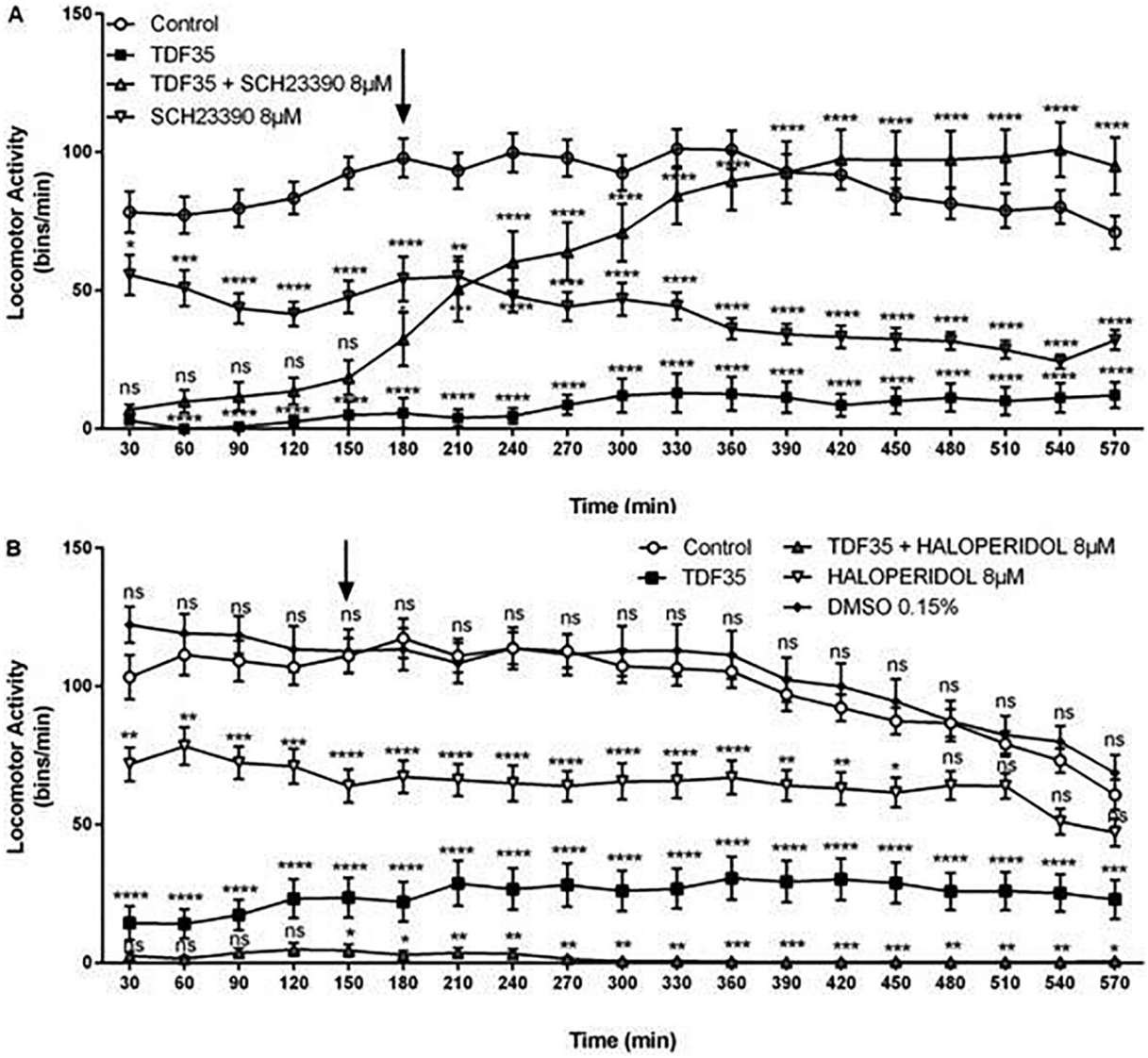

Fig. 3. Dopamine receptor antagonists show opposing effects when they are co-incubated with 35 mg/L of TDF. Effects on locomotor activity of SCH-23390 (A), a D1-like receptor antagonist and Haloperidol (B), a D2-like receptor antagonist. Normal locomotor activity is restored after 180 min (arrow) of co-incubation of TDF35 with 8 μM SCH-23390 (B), whereas the co-incubation of TDF35 with Haloperidol shows complete abolition of locomotor activity after 150 min (arrow). Data are presented as average ± SEM from 15 to 20 larvae per condition from three independent experiments. Statistical significance was calculated between every treatment and the control, with the exception of the co-incubations which were compared with TDF35 alone to detect the time at which the effect of SCH-23390 and Haloperidol were distinguishable. Analyses were made by two-way ANOVA with Tukey's multiple comparisons test. Error bars = SEM, ****: p-value < 0.0001, **: p-value < 0.001, ns: p-value > 0.05.

3.6. Triadimefon reduces extracellular concentration of serotonin

We showed that 5 mg/L of TDF does not affect dopamine levels in exposed fish (Fig. 2). However, this treatment alters their anxiety-like behavior (Fig. 5). To identify a neurotransmitter whose function could explain these findings, we decided to quantify extracellular serotonin, a neurotransmitter related to the control of mood. *In vivo* electrochemistry in larvae showed that the basal levels of serotonin were 82.6 ± 10.2 nM in the brain, and that this value decreased significantly (p-value < 0.05, paired t-test) to 68.5 ± 9.1 nM after exposure to 5 mg/L TDF (Fig. 6A). We summarize our results with both serotonin and dopamine levels by comparing the levels of neurotransmitter before and after adding the drug into the medium and expressing the difference as a fold change (Fig. 6B). A high dose of TDF increases dopamine levels while a lower dose decreases serotonin levels.

3.7. Triadimefon exerts opposing effects on the behavior of larvae and adult zebrafish

An interesting feature that emerged from our results was that TDF has a depressing effect on larval zebrafish behavior while, in assays performed with rats, the authors have seen a hyperactive behavior resembling that induced by cocaine (Reeves et al., 2004; Walker et al., 1990). We wanted to determine whether these differences were species- or developmental stage-specific features. To achieve this objective, we turned to adult zebrafish and used the novel tank diving test (Cachat et al., 2011). Interestingly, when we treated adult fish with 5 mg/L of TDF we observed an increase in anxiety-like behaviors instead of a decrease, as was seen in larvae, (Fig. 7A–B, Fig. S2A–B). In adult fish we also detected an increase in the aggressive behavior (Fig. 7C). Importantly, treatment with 5 mg/L of TDF did not elicit changes neither in the distance traveled nor in velocity, although a higher concentration of TDF (20 mg/L) did induce an increase in these parameters

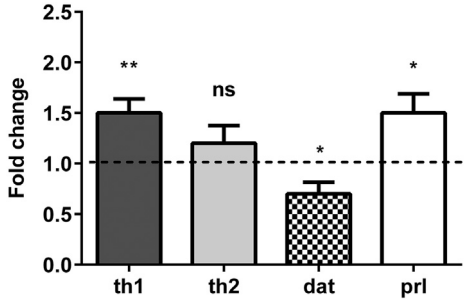

Fig. 4. *tyrosine hydroxylase* (*th1*) mRNA increases and *dopamine transporter* (*dat*) mRNA decreases after larvae are treated for 15 min with 5 mg/L TDF. mRNA levels of *th1*, *th2*, *dat* and prolactin (*prl*) in the zebrafish larvae were assayed by qRT-PCR using *gapdh* mRNA levels for normalization. The gene expression levels were calculated using Pfaffl's method. The data is displayed as a fold difference in experimental animals (TDF exposed) relative to untreated larvae (values in control fish were set at a value of 1.0, dotted line). Statistical analyses were made using an unpaired t-test with Welch's correction. Error bars = SEM, **: p-value = 0.0084, *: 0.0382, ns: p-value > 0.05.

(Fig. 7D–E). These results show a striking contrast to the behaviors observed in larvae, suggesting that significant changes in susceptibility to the effects of TDF occur during development and maturation of the nervous system in the zebrafish.

4. Discussion

Triadimefon is a pesticide widely used in agriculture to control fungal infections. Among its described collateral effects are binding to the dopamine transporter, showing a mechanism of action that resembles that of cocaine (Reeves et al., 2004). In adult mammals, the acute administration of TDF generates a neurotoxic effect characterized by hyperactivity and altered monoamine metabolism (Walker et al., 1990). However, there is little or no information about how exposure to this pesticide could affect behavior and neurochemistry during early stages of development. In this work we used zebrafish to describe the behavioral effects of TDF in the developing animal and its relationship with alterations of the dopaminergic and serotonergic systems. We took advantage of several behavioral tests developed for both larval and adult zebrafish which have been translationally validated.

Since TDF has been described to affect the dopaminergic system, we began by assessing the effects of the fungicide on the movement of the zebrafish larvae. Previous evidence showed that bupropion, a dopamine reuptake blocker, silenced swim episodes in 3 dpf larvae; however it had no effect at 4 and 5 dpf (Thirumalai and Cline, 2008). After 4 dpf, when sustained spontaneous swimming appears, serotonin becomes the major player in the control of the swimming pattern. 5-HT and its agonist quizapine have been shown to increase motility by reducing the intervals of inactivity, while methysergide and ketanserin, both serotonin antagonists, decrease the motor output by increasing the periods of inactivity (Brustein et al., 2003).

The treatment with the dopamine antagonists SCH-23390 (D1 antagonist) and haloperidol (D2 antagonist) demonstrated that both drugs led to a reduction in locomotor activity when they were used separately, as previously reported (Irons et al., 2013). However, co-incubation with TDF produced opposite effects on locomotion depending on the antagonist used. These differences in the action of the antagonists could be explained by the different mechanism of action of the D1 and D2 receptors which mediate excitatory and inhibitory actions, respectively (West and Grace, 2002), as well as the possible binding of the drug to other types of receptors, such as sigma receptors, emulating the effects of cocaine (Aguinaga et al., 2018).

Another behavior that can be measured in larvae as young as 5 dpf is thigmotaxis, a test widely employed in preclinical research for drug screening, since it acts as an index of anxiety (Schnörr et al., 2012). This behavior can be also assessed in adult zebrafish where it has been previously reported that chronic social isolation affects thigmotaxis and whole-brain serotonin levels (Shams et al., 2015). In this work, we have demonstrated that, in the larva, serotonin levels are reduced by TDF exposure, a finding that correlates with the observed changes in thigmotaxis. This makes sense, since the serotonergic system is typically involved in mood regulation and is also the main target of antidepressants and anxiolytics (Morrissette and Stahl, 2014). In addition, we demonstrated that cortisol levels are also reduced by TDF, consistent with the reduction of anxiety-like behaviors in zebrafish larvae.

Even though there were no changes in extracellular dopamine after the treatment with 5 mg/L, we did see an alteration of the expression of genes related to the dopaminergic system like *th1* and *dat*, after 15 min of treatment. It has been shown that the phosphorylation and downregulation of *dat,* as well as the upregulation of *th1,* are mediated by PKC-dependent mechanisms (Cervinski et al., 2005; Tian et al., 2000). To date, Triadimefon has not been described as an activator of the PKC pathway, although propiconazole, a fungicide from the triazole family, is known to affect this pathway (Hester et al., 2006). This result suggests that TDF, even in low concentrations, could affect the dopaminergic system in an indirect way by the activation of PKC.

While the results found in fish larvae are consistent among them, we realize that these findings are markedly different to those observed in rats treated when exposed to TDF (Walker et al., 1990). Given these opposing findings, we decided to determine whether these effects were due to species-specific traits or were related to the developmental stage of the animals. Our observations show that, in adults, TDF affected the same behaviors as in larvae, but in the opposite way. This is not the first time a reversal in behavior has been seen in zebrafish as a consequence of development. For instance, zebrafish larvae have a natural preference for light, or avoidance to darkness (scotophobia), which is replaced by the opposite behavior (scototaxis) in the adult zebrafish

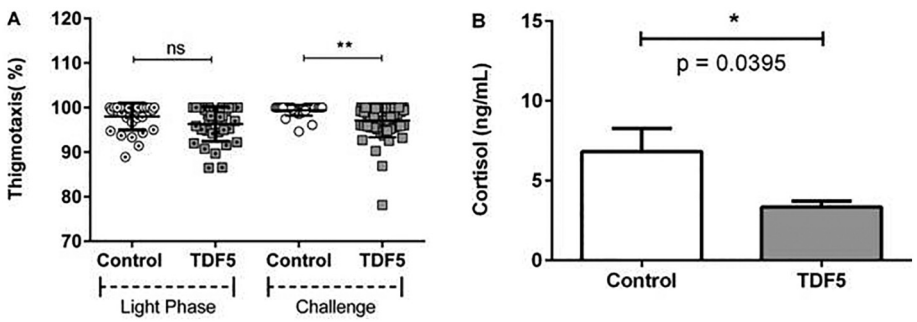

Fig. 5. Assessment of the effects of 5 mg/L of TDF on anxiety-like behavior (A) Treatment with TDF5 during the light phase did not exert neither anxiolytic nor anxiogenic-like behavior when compared with the untreated larvae. After being exposed to a challenge (lights off), 5 mg/L of TDF has an anxiolytic-like effect. Data are presented as average ± SEM from 10 to 16 larvae per condition from three independent experiments. Statistical analyses were made using nonparametric one-way ANOVA (Kruskal-Wallis test) with Dunn's multiple comparisons test. Error bars = SEM, **: p-value 0.0041, ns: p-value > 0.05. (B) The stress-induced endocrine stress response (whole-body cortisol levels) is absent in TDF exposed zebrafish larvae. Cortisol levels were measured by ELISA after acute exposure to 5 mg/L TDF. During the challenge, control larvae respond to the stressful stimulus with increased cortisol levels. Fish treated with TDF fail to show this response. Data are presented as average ± SEM from 30 larvae per condition from seven independent experiments. Statistical analysis was made using unpaired t-test after performing D'Agostino and Pearson omnibus normality test. Error bars = SEM, *: p-value 0.0395, ns: p-value > 0.05.

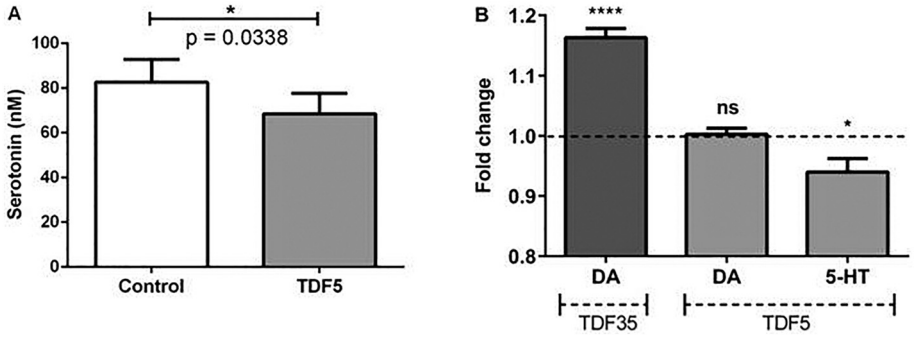

Fig. 6. Extracellular serotonin decreases after treatment with 5 mg/L TDF. Serotonin levels were measured in the zebrafish brain before and after treatment with TDF. (A) Exposure to 5 mg/L of TDF (TDF5) diminished serotonin levels. Data are presented as average ± SEM from 9 larvae per condition from three independent experiments. Paired t-tests were performed to determine statistical significance after performing D'Agostino and Pearson omnibus normality test. Error bars = SEM, *: p-value = 0.0338, ns: p-value > 0.05. (B) Comparison of dopamine and serotonin levels shown as fold change of post-treated relative to pre-treated larvae (values in pre-treated fish were set at an arbitrary value of 1.0, dotted line). Statistical analyses were made using paired t-test. Error bars = SEM, ****: p-value < 0.0001, *: p-value 0.0240, ns: p-value > 0.05.

(Maximino et al., 2010). Scototaxis, as well as thigmotaxis, are used in preclinical studies for assessing the effects of pharmacological compounds on anxiety-like behavior. It is likely that the same pathways involved in light preference are related with the different behaviors we have seen in this work. In the adult zebrafish the endogenous release of 5-HT in the spinal locomotor network decreases the frequency of the fictive swimming induced by NMDA (Gabriel et al., 2009). However, in larval zebrafish, the opposite occurs (Brustein et al., 2003). Thus, it seems that the modulatory effect of 5-HT on the spinal locomotor networks changes from the larval to the adult stages.

5. Conclusion

This work examines some of the behavioral effects of Triadimefon (TDF) in larvae and adult zebrafish, and we provide evidence showing that the effects are mediated through perturbation in monoamine activity. We propose that dopamine is the principal neurotransmitter involved in the locomotor effects exerted by TDF, while serotonin has a pivotal role in anxiety-like behaviors and probably also in locomotion. These results demonstrate the neurotoxic effects that the exposure to TDF causes in aquatic systems even at concentrations as low as 5 mg/L. Further, this work introduces TDF as a molecule useful for studying the nervous system, behavior and addiction.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2018.12.012.

Disclosure of interest

Authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or

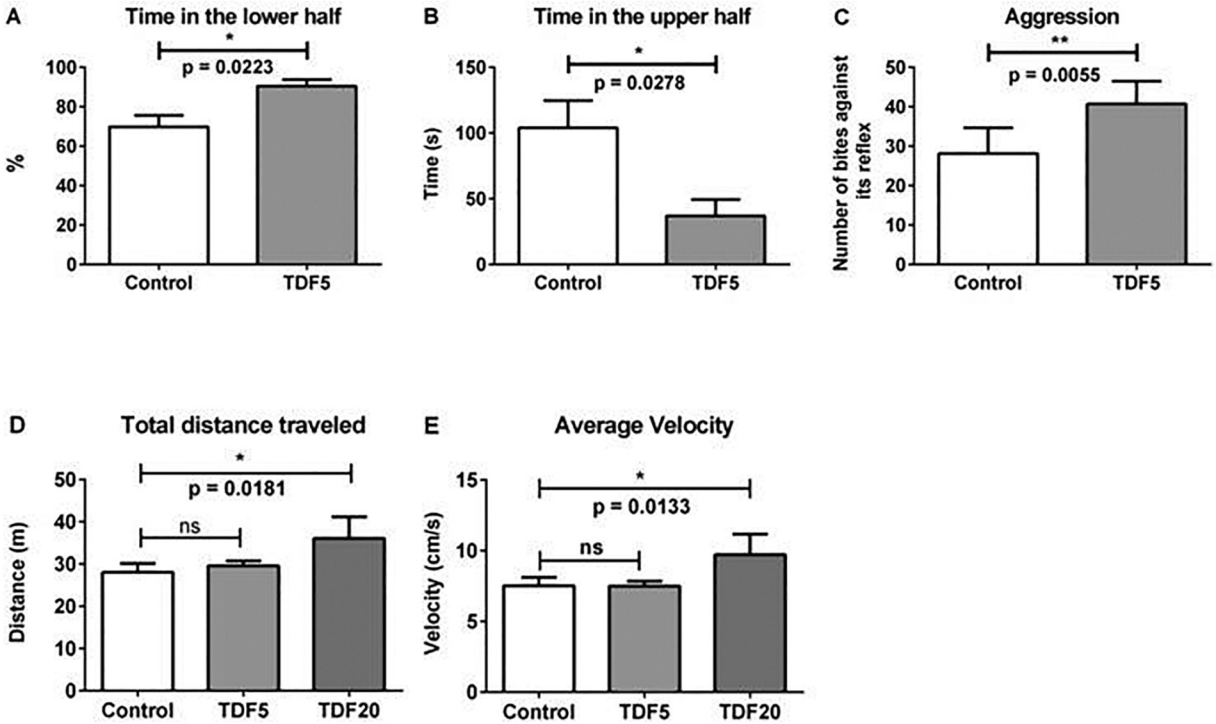

Fig. 7. TDF induces anxiety-like behavior, aggression and locomotion in adult zebrafish. Behavioral effects of acute exposure to TDF on adult zebrafish in the "novel tank diving test". (A–B) Swimming in the bottom of the tank is a well-known indicator of anxiety in fish. (C) The number of bites against its own reflection is used as an indicator of aggressiveness in fish since the image in the mirror is treated as a conspecific. (D, E) Total distance and velocity of swimming in fish exposed to 5 mg/L and 20 mg/L. Data are presented as mean ± SEM, from 7 to 8 fish per condition from three independent experiments. Statistical analyses were made using paired t-test for figs. A-C and ordinary one-way ANOVA with Dunnett correction for multiple comparisons for figs. D-E. *: $p < 0.05$, ***: p-value < 0.001, ns: p-value > 0.05.

interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Author contributions

SP-Z designed and performed the experiments, NT wrote the software for analyzing zebrafish behavior and participated in the data analysis, JDP contributed to experimental design of locomotor activity assays, JA participated in the *in vivo* electrochemical approach, discussions and reviewed the draft and final versions of the manuscript. SP-Z wrote the paper and performed most of the data analysis. MLA contributed with resources and the general direction of this research and did the final editing and proofing of the manuscript.

Statement

For the above mentioned manuscript, submitted to *Progress in Neuro-Psychopharmacology & Biological Psychiatry,* I declare that all of the procedures complied with the guidelines and were approved by the Animal Ethics Committee of the University of Chile (CICUA Certificate #18141-FCS-UCH).

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