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Tebuconazole alters morphological, behavioral and neurochemical parameters in larvae and adult zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Tebuconazole reduced exploratory behavior in larvae.
- Tebuconazole showed decreased distance travelled in adults.
- Tebuconazole inhibited AChE activity in larvae and in zebrafish adult brain.
- Tebuconazole increased AChE gene expression in larvae, but not in zebrafish adults.

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ABSTRACT

In this study, we evaluated the effects of tebuconazole on morphology and exploratory larvae behavior and adult locomotion. Furthermore, we analyzed the effects of this fungicide on AChE activity and gene expression in zebrafish larvae and in the adult zebrafish brain. Tebuconazole (4 mg/L) increased the ocular distance in larvae and reduced the distance travelled, absolute turn angle, line crossing and time outside area in exposed larvae. Moreover, adult zebrafish that were exposed to this fungicide (4 and 6 mg/L) showed a decrease in distance travelled and mean speed when compared to the control group. However, tebuconazole did not alter the number of line crossings or time spent in the upper zone. Tebuconazole inhibited AChE activity at concentrations of 4 mg/L for larvae and 4 and 6 mg/L in the adult zebrafish brain. However, this fungicide did not alter AChE gene expression in the adult zebrafish brain but increased AChE mRNA transcript levels in larvae. These findings demonstrated that tebuconazole could modulate the cholinergic system by altering AChE activity and that this change may be associated with the reduced locomotion of these animals.

1. Introduction

The growth of crop pests has exponentially increased the use of pesticides for agricultural maintenance. Given the toxicity of pesticides to non-target organisms, these agents pose a threat to different ecosystems. Environmental contamination may occur in two ways, either by draining the contaminant or by incorrect handling of the application equipment (De Wilde et al., 2007; Karanasios et al., 2012). Organisms may accumulate these chemicals through any route, including inhalation, ingestion or direct contact (El-Amrani et al., 2012; Toni et al., 2011). Pesticides in aquatic ecosystems may be transferred through phytoplankton to fish and finally to humans (Toni et al., 2011). There are numerous classes of pesticides, among them fungicides, such as benzimidazoles, phenylamides, dicarboximides, quinone and carboxylic amides. Within the main classes of fungicides, the triazoles represent approximately 20% of the global market for systemic fungicides. In the United Kingdom, prothioconazole, tebuconazole and

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epoxiconazole are the three most commonly used fungicides (Price et al., 2015).

Tebuconazole is used to control various fungal diseases in cereals, fruits and vegetables (Li et al., 2012; Sahoo et al., 2012; Wang et al., 2015). It is a systemic follicular fungicide that is rapidly absorbed in the vegetative parts of the plant and inhibits the enzyme lanosterol 14α-desmetilase and promotes a reduction in the biosynthesis of ergosterol, which is necessary for the proper functioning of fungal membranes in pathogens (Konwick et al., 2006). Tebuconazole has been classified by the United States Environmental Protection Agency as a possible human carcinogen, and it has high toxicity to aquatic organisms, causing long-term adverse effects (Hu et al., 2007; Yu et al., 2013). A study performed in the west of France showed that the concentration of tebuconazole was 81 μg/L in runoff events (Lefrancq et al., 2017). The presence of tebuconazole in stream water has been detected and its concentration in surface waters has reached 175–200 μg/L (Montuelle et al., 2010). A study reported that the bioconcentration factor of tebuconazole in zebrafish is 38.8 L/Kg, which is a hazardous concentration for both fish and humans (Andreu-Sánchez et al., 2012). In addition, a previous study determined that lipid and carbohydrate metabolism and some enzymatic activities in zebrafish were affected by exposure to tebuconazole, requiring greater attention to this fungicide in relation to environmental safety (Sancho et al., 2010).

The zebrafish has emerged as an important model for toxicology studies since it is an animal that is susceptible to intoxication by toxic agents. Studies done with the zebrafish have demonstrated that different pesticides can affect neurotransmission systems, including the cholinergic system (Jin et al., 2016; Pereira et al., 2012; Schmidel et al., 2014; Senger et al., 2005). The cholinergic system plays an important role in many essential functions, with acetylcholine (ACh) being the neurotransmitter of this system (Descarries et al., 1997; Geffard et al., 1985; Mesulam et al., 2002). ACh plays a role in the CNS and in the peripheral nervous system and is related to modulation of the neuronal response by sensory stimuli (Murphy and Sillito, 1991) and behavior, while participating in neural circuits related to sleep control, learning and memory (Shaked et al., 2008). ACh is considered a classical neurotransmitter, and its synthesis is performed by the enzyme choline acetyltransferase and depends on the availability of acetyl-CoA and choline, which are important products of lipid metabolism in the intracellular environment. After synthesis, ACh is transported within vesicles to the terminals of cholinergic axons, where it is stored. Its release depends on variations in the electrical potential of the nerve terminal membranes, and this process is dependent on the concentration of intracellular calcium. Upon release, ACh interacts with specific nicotinic and muscarinic receptors (nAChRs and mAChR, respectively), causing depolarization and propagation of the action potential in the postsynaptic cell (Edwards et al., 2007; Oda, 1999; Park et al., 2008; Schröder et al., 1989; van der Zee et al., 1989).

The ACh in the synaptic cleft is degraded by cholinesterases, which cleave it into choline and acetate, eliminating the effects triggered by the ACh molecule. There are two different types of cholinesterases, which are classified according to their catalytic properties, inhibitor specificity and tissue distribution: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8). Both cholinesterases are widely distributed throughout the body. The AChE gene has already been cloned and sequenced, and its enzyme activity has already been detected in zebrafish brains (Bertrand et al., 2001; Rico et al., 2007). This teleost presents AChE that is encoded by a single gene, but several molecular forms are observed (monomers, dimers, trimers and tetramers) as a result of the occurrence of alternative splicing in the exons of the C-terminal region (Massoulié et al., 2008). In addition, subunits of nAChRs and mAChR are also expressed in zebrafish (Zirger et al., 2003).

Since ACh is a neurotransmitter critically involved in the movement-psychomotor control and an important modulator of cognitive functions, such as learning and memory (Capiotti et al., 2014; Hasselmo, 2006; Janeczek et al., 2017), and numerous pesticides have shown toxicological action on this system, leading also to behavioral changes (Jin et al., 2016; Pereira et al., 2012; Schmidel et al., 2014), it is important to evaluate whether the intoxication by tebuconazole may affect behavior and cholinergic system in zebrafish. The objective of this study was to evaluate the toxicological effects of this fungicide on zebrafish larvae and adults. Thus, the effects of tebuconazole on larvae morphology, exploratory larvae behavior and adult locomotion were assessed. We also analyzed the activity and gene expression of AChE in larvae and zebrafish adult brains after 96-h tebuconazole exposure.

2. Materials and methods

2.1. Animals

Embryo and larval (0–5 days post fertilization) and adult stage (6–8 months, 0.2–0.4 g) wild-type *Danio rerio* from the *Tübingen* background were used. Animals were obtained from our breeding colony, which was maintained in recirculating systems (Zebtec, Tecniplast, Italy) with reverse osmosis filtered water equilibrated to reach the species standard temperature (28 °C ± 2 °C), pH (7.0 and 7.5), and ammonia, nitrite, nitrate and chloride levels. Animals were subjected to a light/dark cycle of 14/10 h, respectively. Animals received paramecium between 6 and 14 days post fertilization (dpf), and after 14 dpf, they received commercial flakes (TetraMin Tropical Flake Fish®) three times a day that were supplemented with brine shrimp (Westerfield, 2000).

For breeding, females and males (1:2) were placed in breeding tanks overnight that were separated by a transparent barrier, which was removed after the lights went on the following morning. The fertilized eggs that were retained in the fitted tank bottom were used for the experiments. For the experiments with larvae, the embryos were collected, sanitized and immediately subjected to the treatment. For the experiments with adult animals, the embryos were collected and maintained for up to 7 dpf at a density of one larva per 7 mL in Petri dishes in a biochemical oxygen demand (BOD) incubator. They were immediately transferred to a tank with a density of one larva per 60 mL. When the animals reached 30 dpf, they were maintained at a density of one animal per 200 mL until adulthood.

Water used in the experiments was obtained from a reverse osmosis apparatus (18 MOhm/cm) and was reconstituted with marine salt (Crystal Sea™, Marinemix, Baltimore, USA) at 0.4 ppt. The total organic carbon concentration was 0.33 mg/L. The total alkalinity (as CO_3^{2-}) was 0.030 mEq/L. During fish maintenance, water parameters were monitored daily and maintained in the following ranges: pH: 6.5 to 7.5; conductivity: 400 to 600 μS; ammonium concentration: < 0.004 ppm; and temperature: 25–28 °C. The animals were euthanized by hypothermal shock. All protocols were approved by the Animal Care Committee of Pontifícia Universidade Católica do Rio Grande do Sul (13/00354-CEUA-PUCRS).

2.2. Tebuconazole exposure

For larvae treatment, embryos were placed in Petri dishes (30 embryos per dish), and subjected to tebuconazole (1-(4-Chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)-3-

pentanol; PESTANAL®, Sigma-Aldrich, St. Louis, MO) treatment at concentrations of 1, 2 and 4 mg/L (Yu et al., 2013) for 120 h (1 h post fertilization (hpf) to 5 dpf). Two control groups were utilized, one in which the animals were exposed only to water and the another that contained the vehicle (0.1% DMSO). Animals were monitored daily for survival as determined by presence of a heartbeat. We performed the assays at least in three different experimental days.

During adult treatment, animals of both sexes, with ages ranging from 6 to 8 months, were placed in a 2 L aquarium (8 animals per tank) and exposed to tebuconazole at concentrations of 1, 4 and 6 mg/L (Sancho et al., 2010) for 96 h. Two control groups were utilized, one in which the animals were exposed only to water and another that contained the vehicle (0.1% DMSO). We performed the assays at least in three different experimental days.

2.3. Morphological measures

Potential tebuconazole teratogenicity was estimated by monitoring morphological defects in 5 dpf larvae under a stereomicroscopy. Body length (μm), ocular distance (μm), and surface area of the eyes (μm^2) were evaluated (n = 30) using NIS-Elements D software for Windows 3.2 (Nikon Instruments Inc., Melville, USA). Body length was estimated using the method described by Capiotti et al. (2011) with modifications; the distance from the larval mouth to the pigmented tip of the tail was measured. The ocular distance was evaluated using the distance between the inner edge of the two eyes (similar to the inner intercantal distance in humans), and the size of the eyes was determined by measuring the surface area of the eyes (Lutte et al., 2015).

2.4. Behavioral analyses

2.4.1. Larvae exploratory behavior

The exploratory behavior of the larvae was based on Colwill and Creton (2011) and was evaluated at 5 dpf. The experiments were performed in a temperature-controlled room (27 ± 2 °C) between 1 pm and 5 pm. Each larva was individually placed in a cell culture 24-well plate containing 2 mL of water per well, and the total distance travelled, absolute turn angles, and time outside area of each animal were evaluated. After a 60 s acclimatization period, the sessions were filmed and recorded for 5 min and were later analyzed using ANY-Maze® software (Stoelting Co., Wood Dale, IL, USA), which is able to track the swimming activity of the animals at a rate of 30 positions per second. The video-tracking data were then used to determine the relevant measures through detection of animals that was done by looking at the contrast between the animals and the background of the apparatus. The data of the time spent outside area indicated a behavior related to anxiety/fear, when the larva enters a new environment it tends to swim against the walls of the well, this behavior is related to the anxiety in this animal (Colwill and Creton, 2011).

2.4.2. Adult behavior

Adult exploration was evaluated after 96 h of treatment. The experiments were performed in a temperature-controlled room (27 ± 1 °C) between 9 am and 1 pm. Animals were placed individually in experimental tanks (30 cm long x 15 cm high x 10 cm wide), and after 60 s of acclimatization, their locomotor behavior was recorded for 5 min. The videos were analyzed using the ANY-Maze® software (Stoelting Co. Wood Dale, IL, USA), with the experimental tank divided into equal parts by three digital vertical lines and one horizontal line. The behavioral parameters analyzed were distance travelled, mean speed, line crossings, and time spent in the upper zone (Gerlai et al., 2000). The time spent in the upper zone indicated an anxiolytic-like behavior index because normal exploratory behavior of the zebrafish, when introduced to a new environment, is to spend more time at the bottom of the tank and then gradually move to the upper zone after a few minutes (Levin et al., 2007). The behavioral analysis was performed on a computer using ANY-Maze® software (Stoelting CO, USA) to track the swimming activity of the animals at a rate of 30 positions per second. The video-tracking data were then used to determine the relevant measures through the detection of animals, which was done by looking at the contrast between the animals and the background of the apparatus.

2.5. Determination of acetylcholinesterase activity

The experiments were conducted using samples containing a pool of two adult brains and 10 larval brains. The animals were euthanized by hypothermal shock (Matthews and Varga, 2012; Wilson et al., 2009), and the samples were homogenized on ice with an Ultra-Turrax (T10 basic IKA®) in 60 vol (v/w) of 50 mM Tris-HCl, pH 8.0. The protein was measured by the Coomassie blue method (Bradford, 1976), and bovine serum albumin was used as a standard. AChE activity was determined according to the method proposed by Ellman et al. (1961) with minor modifications. The activity in the homogenate was briefly measured by determining the rate of hydrolysis of acetylthiocholine iodide (ACSCh, 0.88 mM) in a final volume of 300 μL, with 33 μL of 100 mM phosphate buffer, pH 7.5, mixed with 33 μL of 2.0 mM DTNB (5,50-dithionitrobis2-nitrobenzoic acid). In this solution, 5 μg of protein from each sample were added and preincubated at 25 °C for 10 min. The reaction was started with the addition of the substrate acetylthiocholine, and as soon as the substrate was added, the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nm for 3 min (in intervals of 30 s) using a microplate reader. AChE activity was expressed as micromoles of thiocholine (SCh) that were released per hour per milligram of protein. All enzyme assays were performed in at least four different experiments, and each one was performed in triplicate.

2.6. Gene expression analysis by RT-qPCR

AChE gene expression was evaluated in both adult and larval samples, using pools of 20 larvae and five zebrafish brains per sample and following MIQE Guidelines (Bustin et al., 2009, 2013). Total RNA was isolated using *Trizol® reagent* (Invitrogen, USA) and was quantified by spectrophotometry using NanoDrop Lite (ThermoScientific, USA) after DNase treatment (*DNase I Amplification Grade*, Sigma-Aldrich, EUA). The cDNA species were synthesized using the ImProm-II™ Reverse Transcription System (Promega, USA) and 1 μg of the total RNA as a template. Quantitative PCR reactions were performed in 25 μL containing a final concentration of 0.2 × SYBR Green I, 100 mM dNTP, 1 × PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase and 200 nM each of forward and reverse primers (F: 5′-GCTAATGAGCAAAAGCATGTGGGCTTG-3′; R: 5′-TATCTGTGATGTTAAGCAGACGAGGCAGG-3′) (Pereira et al., 2012) plus 12.5 μl of diluted cDNA (1:50). PCR cycling conditions followed an initial 5 min at 95 °C polymerase activation step, plus 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. Finally, a melting curve analysis was included with fluorescence measures from 60 °C to 99 °C. The threshold cycle (Cq) values were obtained with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosys-tems). Relative expression levels were determined using *ef1α* and *rlp13α* as reference genes (Tang et al., 2007) by the $2^{-\Delta\Delta Cq}$ method, using raw fluorescence data to calculate PCR efficiency per sample in *LinRegPCR Software* v2016.1 (http://LinRegPCR.nl).

2.7. Statistical analysis

Results from the behavioral analysis, morphological measures and acetylcholinesterase activity and expression are expressed as the mean ± S.E.M. The data were analyzed using one-way ANOVA, followed by post-hoc Tukey's test. For all comparisons, the significance level was set at $p < 0.05$. A Pearson's correlation was performed to morphological measures, considering as factors body length, ocular distance and surface area. Positive correlation was considered when $r^2 > 0.850$, and negative correlation when $r^2 > -0.850$.

3. Results

First, it was observed that the water-control group did not differ in comparison to the 0.1% DMSO-vehicle group in all parameters analyzed in this study.

The effect of tebuconazole exposure on larva-morphological parameters was verified. Tebuconazole was able to increase the ocular distance (μm) in larvae that were exposed to 4 mg/L ($F_{(4,145)} = 5.379$; n = 30; $p = 0.0005$; Fig. 1b) when compared to the 0.1% DMSO group. However, there were no differences in body length ($F_{(4,145)} = 3.131$; n = 30; $p = 0.1404$) or surface area ($F_{(4,145)} = 1.738$; n = 30; $p = 0.1448$) of the eyes between the 0.1% DMSO group and the tebuconazole-exposed groups (Fig. 1a and c, respectively). In addition, the Pearson's correlation was performed considering as factor the body length, ocular distance and surface area. The data showed no correlation (r2 = 0.640, which was the highest value obtained in our analysis) between the groups when analyzed these factors (data not shown).

The exploratory behavior of the larvae was examined at 5 dpf to determine whether tebuconazole exposure could alter larvae locomotion and orientation. Animals that were exposed to tebuconazole at concentrations of 4 mg/L displayed reduced locomotor behavior when compared to the 0.1% DMSO group. The parameter of distance travelled ($F_{(4,105)} = 5.567$; n = 22; $p = 0.0004$; Fig. 2a), absolute turn angle ($F_{(4,105)} = 3.132$; n = 22; $p = 0.0178$; Fig. 2b) and time outside area ($F_{(4,105)} = 6.541$; n = 22; $p < 0.0001$; Fig. 2c) were also affected, showing decreases after exposure to this fungicide. Moreover, it was observed that adult animals that were exposed to

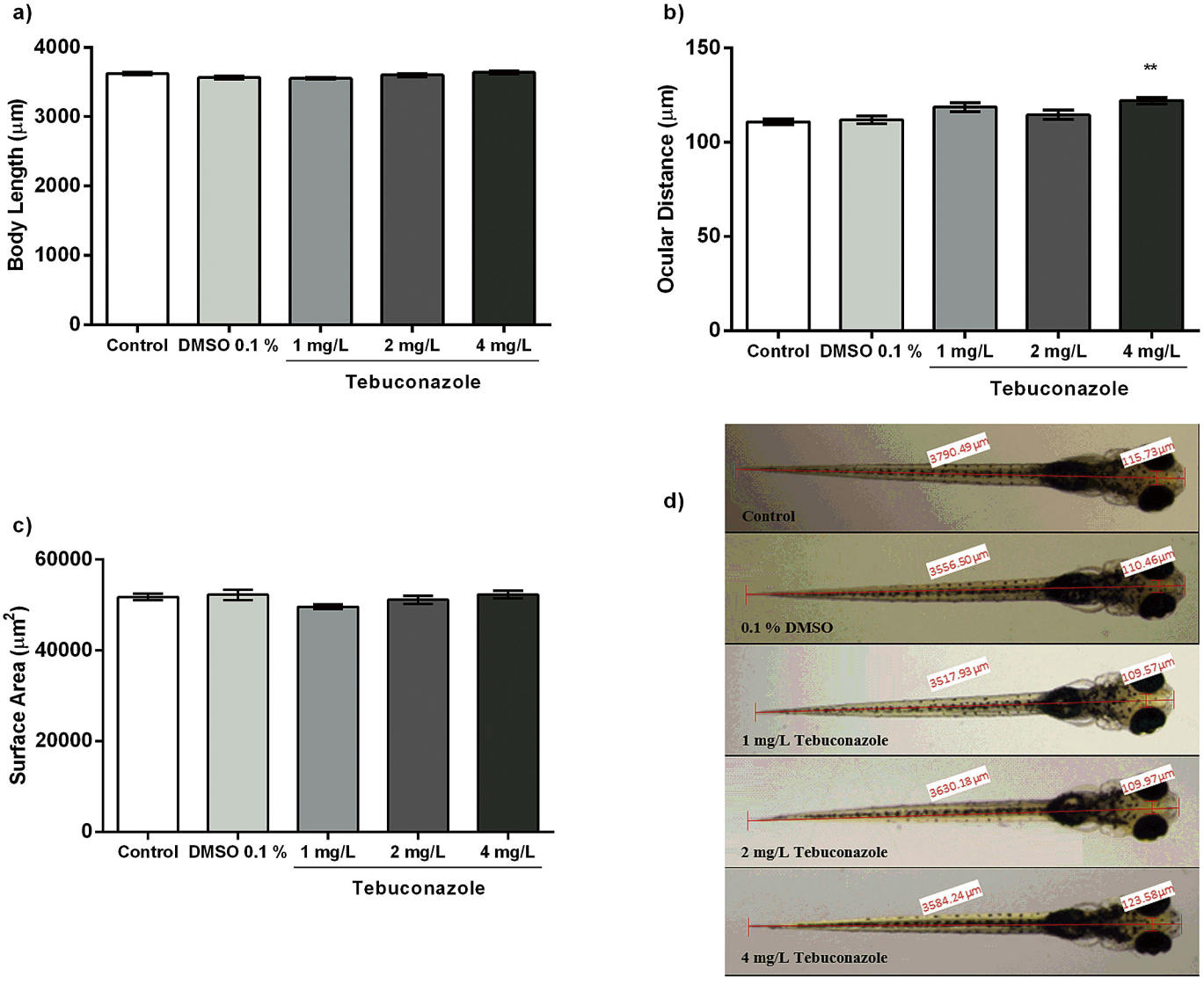

Fig. 1. Effects of 120-h tebuconazole exposure. Body length (a), ocular distance (b) and surface area of the eyes (c) were evaluated in zebrafish larvae at 5 dpf. Data are expressed as the mean ± S.E.M. of 30 animals analyzed individually for each group using one-way ANOVA followed by Tukey's *post-hoc* test. $^{**}p < 0.01$. (d) Pictures represent morphological parameters at 5 dpf larvae, with scale bar showing the body length and ocular distance of the larvae exposed to water, vehicle and tebuconazole concentrations.

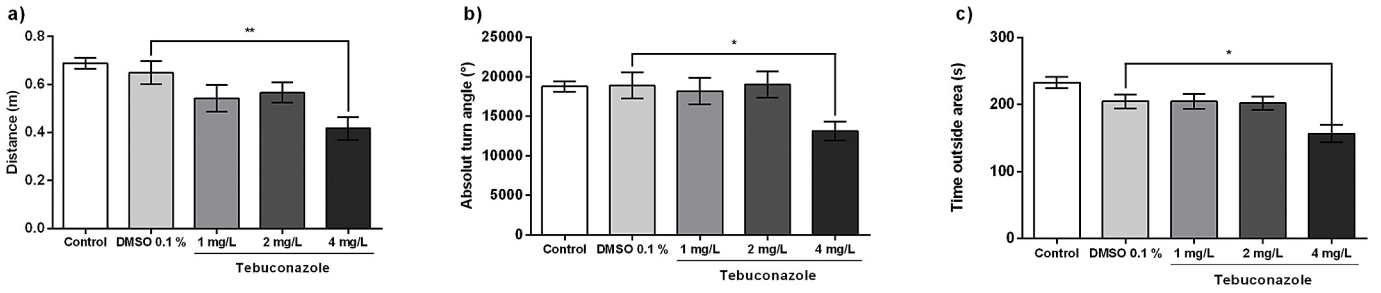

Fig. 2. Effects of 120-h tebuconazole exposure in exploratory behavior of zebrafish larvae. Distance travelled (a), absolute turn angle (b) and time outside area (c) were evaluated at 5 dpf. Data are expressed as the mean ± S.E.M. of 22 animals analyzed individually for each group using one-way ANOVA followed by Tukey's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$.

tebuconazole at concentrations of 4 and 6 mg/L had reduced travelled distances ($F_{(4,120)} = 8.761$; n = 25; $p < 0.0001$; Fig. 3a) compared with the 0.1% DMSO group. However, this fungicide did not alter the parameters of line crossing ($F_{(4,120)} = 1.410$; n = 25; $p = 0.2347$; Fig. 3b) or time spent in the upper zone ($F_{(4,120)} = 0.2755$; n = 25; $p = 0.8933$; Fig. 3c), when 0.1% DMSO group were compared with tebuconazole-exposed groups.

To verify whether the behavioral changes promoted by tebuconazole could be related to cholinergic signaling, AChE activity was performed in both larvae and adult zebrafish brains. Tebuconazole did not alter the ACh catabolism in larvae treated with 1 and 2 mg/L when compared to the 0.1% DMSO group (Fig. 4a). However, this fungicide reduced the AChE activity in zebrafish larvae that were exposed to 4 mg/L for 120 h ($F_{(4,15)} = 6.770$; n = 4; $p = 0.0025$; Fig. 4a). Similarly, when the adult animal brain was analyzed, this fungicide was found to reduce AChE activity in animals that were treated with 4 and 6 mg/L for 96 h, when compared to the 0.1% DMSO group ($F_{(4,25)} = 13.34$; n = 6; $p < 0.0001$; Fig. 4b). On the other hand, the results demonstrate that tebuconazole did not alter the ACh cascade in the brains of animals exposed to 1 mg/L when compared to the 0.1% DMSO group (Fig. 4b).

To determine whether the changes in enzyme activity promoted by tebuconazole exposure could be a consequence of transcriptional control, a RT-qPCR analysis was carried out to assess the enzyme expression. The analysis of the relative expression of AChE demonstrated that the amount of mRNA transcripts in zebrafish larvae increased significantly after tebuconazole exposure at concentrations of 4 mg/L ($F_{(2,20)} = 3.781$; n = 4; p 0.0405; Fig. 5a) when compared to the 0.1% DMSO group. However, the results did not show significant differences in mRNA transcripts of AChE in adult zebrafish brains, after 96-h fungicide exposure at concentrations of 4 and 6 mg/L ($F_{(3,28)} = 0.3211$; n = 4; $p = 0.8100$; Fig. 5b) when compared to the 0.1% DMSO group.

4. Discussion

In the present study, we evaluated the effects of different concentrations of tebuconazole in both the larval and adult stages of zebrafish development. This study has shown that 96-h exposure to tebuconazole causes small morphological alterations in zebrafish larvae. In addition, this fungicide causes behavioral and neurochemical changes in animals that are submitted to treatment in the larval or adult stage.

This study found that larvae exposed to the highest concentration of tebuconazole for 120 h showed an increase in ocular distance, which is a parameter verified at 5 dpf. However, this fungicide did not cause changes in the body length or ocular surface area of this animal. Studies have shown that others neurotoxic agents can cause numerous morphological changes in zebrafish larvae. Richendrfer et al. (2012) and Kienle et al. (2009) observed that chlorpyrifos, exposed at 7 and 11 dpf, respectively, had spinal deformity, cardiac edema and reduction of body size. In addition, one study demonstrated that exposure of over 144 hpf to the fungicide thifluzamide also caused cardiac edema and deformation in the spine (Yang et al., 2016). Morphological changes were also observed in larvae obtained from adults that were exposed to sublethal concentrations of endosulfan over 96 h (Velasco-Santamaría et al., 2011).

Studies have shown that pesticides can alter exploratory parameters in zebrafish larvae (Andrade et al., 2016; Jin et al., 2016; Liu et al., 2016; Pérez et al., 2013). In this study, we demonstrated that exposure to tebuconazole for 120 h was able to reduce the exploratory capacity of zebrafish larvae, when analyzed at 5 dpf. Exposure to a 4 mg/L concentration also caused alterations in all parameters analyzed. The reductions in distance travelled, absolute turn angle and time outside the area of the well indicate that the animal that has been exposed to tebuconazole has impaired

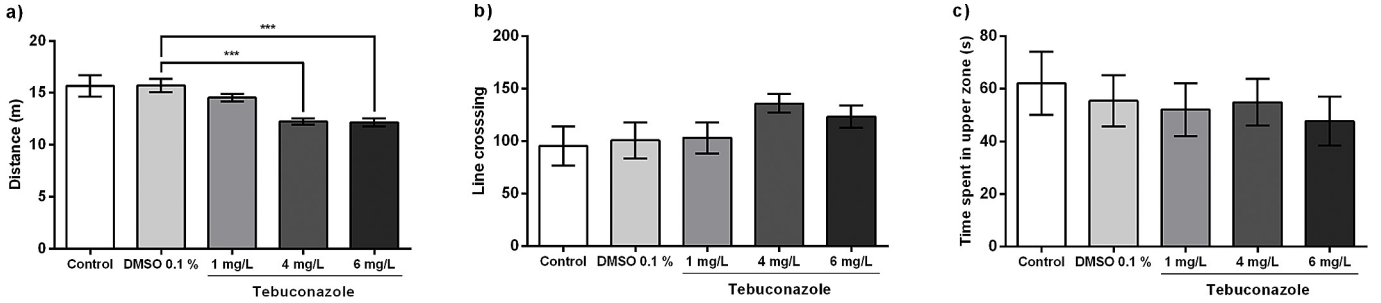

Fig. 3. Effects of 96-h tebuconazole exposure in locomotor behavior of zebrafish larvae. Distance travelled (a), line crossing (b) and time spent in the tank upper zone (c) were evaluated 96 h after treatment. Data are expressed as the mean ± S.E.M. of 25 animals analyzed individually for each group using one-way ANOVA followed by Tukey's *post-hoc* test. $^{***}p < 0.001$.

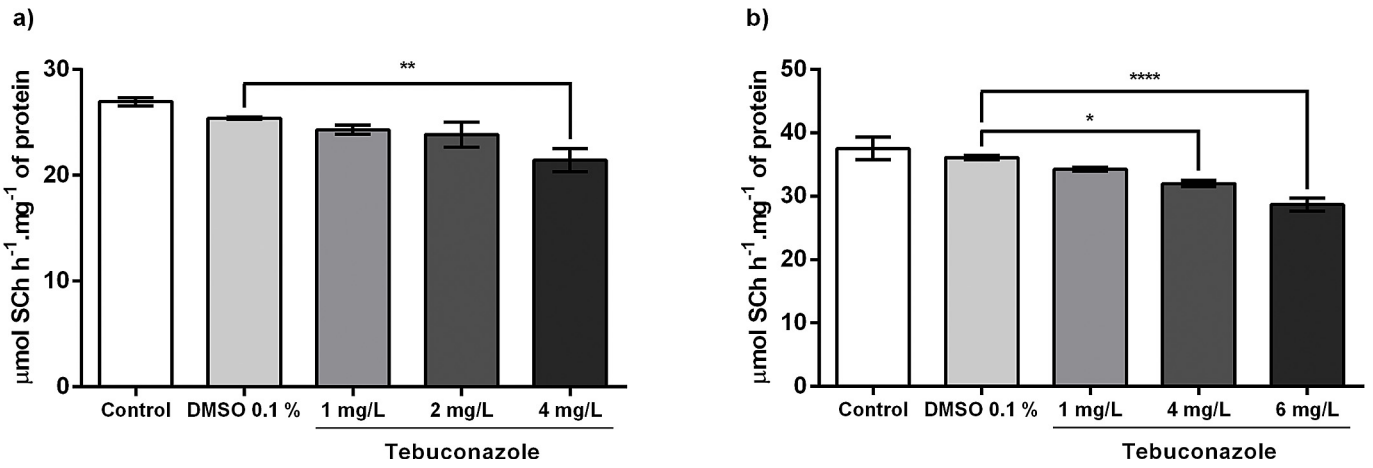

Fig. 4. Effects of 120 and 96-h tebuconazole exposure in zebrafish at the larvae stage (a) and in the adult zebrafish brain (b), respectively, on AChE activity at different concentrations. Data are expressed as the mean ± S.E.M. of four and six independent experiments, respectively. Results were analyzed using one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

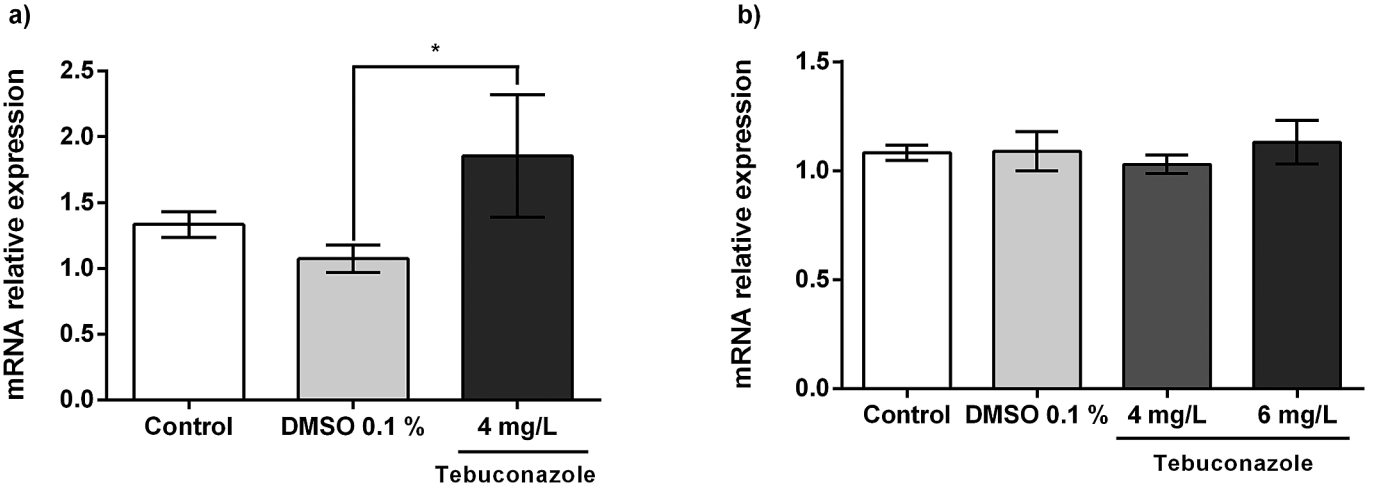

Fig. 5. Effects of 120 and 96-h tebuconazole exposure in zebrafish at the larvae stage (a) and in the adult zebrafish brain (b), respectively, on AChE gene expression at different concentrations. Data are expressed as the mean ± S.E.M. of four independent experiments. Results were analyzed using one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$.

movements. Other studies have shown that some pesticides are also capable of altering the exploratory capacity of the zebrafish larva. Carbendazim and, atrazine and their subsequent metabolites, caused a significant reduction in distance travelled in larvae analyzed at 5 dpf and exposed for 120 h (Andrade et al., 2016; Liu et al., 2016). This decrease in exploratory behavior shows that zebrafish larvae that are exposed to tebuconazole could be more susceptible to predation since their locomotor state was altered after this fungicide exposure.

Behavioral changes related to locomotion have also been seen in adult animals. The results of this study showed that tebuconazole exposure for 96 h reduced zebrafish locomotion. Data on the distance travelled were lower for two of the tested concentrations, 4 and 6 mg/L, when compared with the control groups. Data with other pesticides also show a reduction in the swimming capacity of the adult zebrafish. Tilton et al. (2011) observed that chlorpyrifos significantly reduced the treated adult animal's swimming rate for 24 h. Likewise, Pereira et al. (2012) demonstrated that the 96-h exposure to endosulfan reduces the distance travelled and mean speed, which agrees with our study. The analysis of line crossings and time spent in the upper zone of the aquarium was not altered by tebuconazole exposure. Although this fungicide reduced locomotor behavior, becoming the treated larvae slower than the control group, the ability to explore the environment remained similar in both groups. The behavioral differences observed in adult and larvae may be related to the effect of the exposure to this fungicide in different developmental stages.

The analysis of AChE activity was done to verify whether tebuconazole could be interfering in the behavior through the cholinergic system since ACh is linked to movement. The analysis performed on larvae showed that the same concentration needed to alter the behavior (4 mg/L) caused a reduction in AChE activity, which is responsible for the acetylcholine catabolism. Similarly, AChE activity in the adult zebrafish brain was decreased in concentrations (4 and 6 mg/L) that altered zebrafish behavior. Therefore, this fungicide is capable of altering AChE activity and may promote behavioral changes in animals, which suggests the involvement of cholinergic signaling in these effects. To verify that this fungicide was able to promote transcriptional control, RT-qPCR analysis was carried out for the treatment concentrations that altered AChE activity. We determined that tebuconazole increased AChE mRNA transcripts at a concentration of 4 mg/L in zebrafish

larvae. However, this fungicide did not alter transcript levels of a gene related to AChE in any of the concentrations tested (4 and 6 mg/L).

Studies of larvae showed that chlorpyrifos and atrazine were also able to reduce AChE activity (Richendrfer and Creton, 2015; Sun et al., 2016). In contrast to our results, which showed increased AChE expression in zebrafish larvae that were exposed to tebuconazole, studies have shown that organophosphates and atrazine metabolites reduced AChE activity and also decreased the gene expression of this enzyme (Liu et al., 2016; Sun et al., 2016). Roex et al. (2003) verified a reduction of AChE activity in adult zebrafish brains after exposure to parathion over a period of 142 and 250 days. Endosulfan, an insecticide from coffee crop pests, also reduced AChE activity, and similar to our study results, it did not alter the gene expression of this enzyme in adult zebrafish brain that were exposed for 96 h (Pereira et al., 2012). AChE can be used as a marker of cholinergic function since its levels are controlled by the interaction of ACh with its receptors, and when this interaction is accentuated, AChE levels increase (Fernandez and Hodges-Savola, 1992). Thus, our findings suggest that the inhibition of AChE may induce an increase of the availability of ACh in the synaptic cleft. The inhibitory effect on AChE is not directly related to transcriptional control at this tebuconazole treatment, since there was an increase of mRNA AChE transcript in larvae changes on AChE activity and there were no changes on AChE gene expression in adults. Therefore, it is possible to suggest that these changes on AChE activity involves a posttranscriptional or post-translational modulation. Another possibility to explain our results is that tebuconazole exposure may be causing destruction to cholinergic neurons, and therefore resulting in loss of AChE activity.

Previous studies have demonstrated that inhibition of AChE activity may be involved in locomotor changes in zebrafish larvae. Richendrfer and Creton (2015) verified that a reduced AChE activity promoted by exposure to chorpyrifos induced a decrease on locomotor parameters in 5 dpf zebrafish larvae. Jin et al. (2015) also observed a decrease in locomotion in zebrafish larvae at 96 hpf, which is also related to AChE inhibition by clorpyrifos. For adult zebrafish, Pereira et al. (2012) observed that endosulfan decreased AChE activity and this exposure also decreased distance travelled, but did not alter line crossings. Therefore, it is possible to suggest that AChE inhibition induced by tebuconazole may be involved in the changes in locomotion observed in zebrafish larvae and adults.

In summary, our findings indicate that tebuconazole can affect morphological parameters and cholinergic signaling by inhibiting AChE, and these changes could be related to the behavioral deficit presented by the zebrafish in both larval and adult stages.

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References

Andrade, T.S., Henriques, J.F., Almeida, A.R., Machado, A.L., Koba, O., Giang, P.T., Soares, A.M., Domingues, I., 2016. Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos. Aquat. Toxicol. 170, 390–399. http://dx.doi.org/10.1016/j.aquatox.2015.11.017. Erratum in: Aquat Toxicol. 173, 228.

Andreu-Sánchez, O., Paraíba, L.C., Jonsson, C.M., Carrasco, J.M., 2012. Acute toxicity and bioconcentration of fungicide tebuconazole in zebrafish (Danio rerio). Environ. Toxicol. 27, 109–116. http://dx.doi.org/10.1002/tox.20618.

Bertrand, C., Chatonnet, A., Takke, C., Yan, Y.L., Postlethwait, J., Toutant, J.P., Cousin, X., 2001. Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7. Gene structure and polymorphism; molecular forms and expression pattern during development. J. Biol. Chem. 276, 464–474. http://dx.doi.org/10.1074/jbc.M006308200.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 218–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. http://dx.doi.org/10.1373/clinchem.2008.112797.

Bustin, S.A., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G., Wittwer, C.T., Schjerling, P., Day, P.J., Abreu, M., Aguado, B., Beaulieu, J.F., Beckers, A., Bogaert, S., Browne, J.A., Carrasco-Ramiro, F., Ceelen, L., Ciborowski, K., Cornillie, P., Coulon, S., Cuypers, A., De Brouwer, S., De Ceuninck, L., De Craene, J., De Naeyer, H., De Spiegelaere, W., Deckers, K., Dheedene, A., Durinck, K., Ferreira-Teixeira, M., Fieuw, A., Gallup, J.M., Gonzalo-Flores, S., Goossens, K., Heindryckx, F., Herring, E., Hoenicka, H., Icardi, L., Jaggi, R., Javad, F., Karampelias, M., Kibenge, F., Kibenge, M., Kumps, C., Lambertz, I., Lammens, T., Markey, A., Messiaen, P., Mets, E., Morais, S., Mudarra-Rubio, A., Nakiwala, J., Nelis, H., Olsvik, P.A., Pérez-Novo, C., Plusquin, M., Remans, T., Rihani, A., Rodrigues-Santos, P., Rondou, P., Sanders, R., Schmidt-Bleek, K., Skovgaard, K., Smeets, K., Tabera, L., Toegel, S., Van Acker, T., Van den Broeck, W., Van der Meulen, J., Van Gele, M., Van Peer, G., Van Poucke, M., Van Roy, N., Vergult, S., Wauman, J., Tshuikina-Wiklander, M., Willems, E., Zaccara, S., Zeka, F., Vandesompele, J., 2013. The need for transparency and good practices in the qPCR literature. Nat. Methods 10, 1063–1067. http://dx.doi.org/10.1038/nmeth.2697.

Capiotti, K.M., De Moraes, D.A., Menezes, F.P., Kist, L.W., Bogo, M.R., Da Silva, R.S., 2014. Hyperglycemia induces memory impairment linked to increased acetylcholinesterase activity in zebrafish (Danio rerio). Behav. Brain Res. 274, 319–325. http://dx.doi.org/10.1016/j.bbr.2014.08.033.

Capiotti, K.M., Menezes, F.P., Nazario, L.R., Pohlmann, J.B., de Oliveira, G.M., Fazenda, L., Bogo, M.R., Bonan, C.D., Da Silva, R.S., 2011. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (Danio rerio). Neurotoxicol. Teratol. 33, 680–685. http://dx.doi.org/10.1016/j.ntt.2011.08.010.

Colwill, R.M., Creton, R., 2011. Locomotor behaviors in zebrafish (Danio rerio) larvae. Behav. Process. 86, 222–229. http://dx.doi.org/10.1016/j.beproc.2010.12.003.

Descarries, L., Gisiger, V., Steriade, M., 1997. Diffuse transmission by acetylcholine in the CNS. Prog. Neurobiol. 53 http://dx.doi.org/10.1016/S0301-0082(97)00050-6, 603–325.

De Wilde, T., Spanoghe, P., Debaer, C., Ryckeboer, J., Springael, D., Jaeken, P., 2007. Overview of on-farm bioremediation systems to reduce the occurrence of point source contamination. Pest Manag. Sci. 63, 111–128. http://dx.doi.org/10.1002/ps.1323.

Edwards, J.G., Greig, A., Sakata, Y., Elkin, D., Michel, W.C., 2007. Cholinergic innervation of the zebrafish olfactory bulb. J. Comp. Neurol. 504, 631–645. http://dx.doi.org/10.1002/cne.21480.

El-Amrani, S., Pena-Abaurrea, M., Sanz-Landaluze, J., Ramos, L., Guinea, J., Cámara, C., 2012. Bioconcentration of pesticides in zebrafish eleutheroembryos (Danio rerio). Sci. Total Environ. 425, 184–190. http://dx.doi.org/10.1016/j.scitotenv.2012.02.065.

Ellman, G.L., Courtney, K.D., Andrés, J.V., Fearthertone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. http://dx.doi.org/10.1016/0006-2952(61)90145-9.

Fernandez, H.L., Hodges-Savola, C.A., 1992. Trophic regulation of acetylcholinesterase isoenzymes in adult mammalian skeletal muscles. Neurochem. Res. 17, 115–124. http://dx.doi.org/10.1007/BF00966872.

Geffard, M., McRae-Degueurce, A., Souan, M.L., 1985. Immunocytochemical detection of acetylcholine in the rat central nervous system. Science 229, 77–79. http://dx.doi.org/10.1126/science.3892687.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782. http://dx.doi.org/10.1016/S0091-3057(00)00422-6.

Hasselmo, M.E., 2006. The role of acetylcholine in learning and memory. Curr. Opin. Neurobiol. 16, 710–715. http://dx.doi.org/10.1016/j.conb.2006.09.002.

Hu, M.L., Jiang, M., Wang, P., Mei, S.R., Lin, Y.F., Hu, X.Z., Shi, Y., Lu, B., Dai, K., 2007. Selective solid-phase extraction of tebuconazole in biological and environmental samples using molecularly imprinted polymers. Anal. Bioanal. Chem. 387, 1007–1016. http://dx.doi.org/10.1007/s00216-006-1004-2.

Janeczek, M., Gefen, T., Samimi, M., Kim, G., Weintraub, S., Bigio, E., Rogalski, E., Mesulam, M.M., Geula, C., 2017. Variations in acetylcholinesterase activity within human cortical pyramidal neurons across age and cognitive trajectories. Cereb. Cortex 1, 1–9. http://dx.doi.org/10.1093/cercor/bhx047.

Jin, Y., Liu, Z., Peng, T., Fu, Z., 2015. The toxicity of chlorpyrifos on the early life stage of zebrafish: a survey on the endpoints at development, locomotor behavior, oxidative stress and immunotoxicity. Fish. Shellfish Immunol. 43, 405–414. http://dx.doi.org/10.1016/j.fsi.2015.01.010.

Jin, Y., Zhu, Z., Wang, Y., Yang, E., Feng, X., Fu, Z., 2016. The fungicide imazalil induces developmental abnormalities and alters locomotor activity during early developmental stages in zebrafish. Chemosphere 153, 455–461. http://dx.doi.org/10.1016/j.chemosphere.2016.03.08.

Karanasios, E., Tsiropoulos, N.G., Karpouzas, D.G., 2012. On-farm biopurification systems for the depuration of pesticide wastewaters: recent biotechnological advances and future perspectives. Biodegradation 23, 787–802. http://dx.doi.org/10.1007/s10532-012-9571-8.

Kienle, C., Köhler, H.R., Gerhardt, A., 2009. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. Ecotoxicol. Environ. Saf. 72, 1740–1747. http://dx.doi.org/10.1016/j.ecoenv.2009.04.014.

Konwick, B.J., Garrison, A.W., Avants, J.K., Fisk, A.T., 2006. Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 80, 372–381. http://dx.doi.org/10.1016/j.aquatox.2006.10.003.

Lefrancq, M., Jadas-Hécart, A., La Jeunesse, I., Landry, D., Payraudeau, S., 2017. High frequency monitoring of pesticides in runoff water to improve understanding of their transport and environmental impacts. Sci. Total Environ. 587–588, 75–86. http://dx.doi.org/10.1016/j.scitotenv.2017.02.022.

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiol. Behav. 90, 54–58. http://dx.doi.org/10.1016/j.physbeh.2006.08.026.

Li, Y., Dong, F., Liu, X., Xu, J., Li, J., Kong, Z., Chen, X., Liang, X., Zheng, Y., 2012. Simultaneous enantioselective determination of triazole fungicides in soil and water by chiral liquid chromatography/tandem mass spectrometry. J. Chromatogr. A 1224, 51–60. http://dx.doi.org/10.1016/j.chroma.2011.12.044.

Liu, Z., Wang, Y., Zhu, Z., Yang, E., Feng, X., Fu, Z., Jin, Y., 2016. Atrazine and its main metabolites alter the locomotor activity of larval zebrafish (Danio rerio). Chemosphere 148, 163–170. http://dx.doi.org/10.1016/j.chemosphere.2016.01.007.

Lutte, A.H., Capiotti, K.M., da Silva, N.L., da Silva, C.S., Kist, L.W., Bogo, M.R., Da Silva, R.S., 2015. Contributions from extracellular sources of adenosine to the ethanol toxicity in zebrafish larvae. Reprod. Toxicol. 53, 82–91. http://dx.doi.org/10.1016/j.reprotox.2015.04.001.

Massoulié, J., Perrier, N., Noureddine, H., Liang, D., Bon, S., 2008. Old and new questions about cholinesterases. Chem. Biol. Interact. 175, 30–44. http://dx.doi.org/10.1016/j.cbi.2008.04.039.

Matthews, M., Varga, Z.M., 2012. Anesthesia and euthanasia in zebrafish. ILAR J. 53, 192–204. http://dx.doi.org/10.1093/ilar.53.2.192.

Mesulam, M.M., Guillozet, A., Shaw, P., Levey, A., Duysen, E.G., Lockridge, O., 2002. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 110, 627–639. http://dx.doi.org/10.1016/j.cbi.2008.04.039.

Montuelle, B., Dorigo, U., Bérard, A., Volat, B., Bouchez, A., Tlili, A., Gouy, V., Pesce, S., 2010. The periphyton as a multimetric bioindicator for assessing the impact of land use on rivers: an overview of the Ardières-Morcille experimental watershed (France). Hydrobiologia 657, 123–141. http://dx.doi.org/10.1007/s10750-010-0105-2.

Murphy, P.C., Sillito, A.M., 1991. Cholinergic enhancement of direction selectivity in the visual cortex of the cat. Neuroscience 40, 13–20. http://dx.doi.org/10.1016/0306-4522(91)90170-S.

Oda, Y., 1999. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. Pathol. Int. 49, 921–937. http://dx.doi.org/10.1046/j.1440-1827.1999.00977.x.

Park, E.J., Lee, Y.K., Kim, Y.J., Lee, C.J., 2008. Cholinergic modulation of neural activity in the telencephalon of the zebrafish. Neurosci. Lett. 439, 79–83. http://dx.doi.org/10.1016/j.neulet.2008.04.064.

Pereira, V.M., Bortolotto, J.W., Kist, L.W., Azevedo, M.B., Fritsch, R.S., Oliveira, R.da L., Pereira, T.C., Bonan, C.D., Vianna, M.R., Bogo, M.R., 2012. Endosulfan exposure inhibits brain AChE activity and impairs swimming per-formance in adult zebrafish (Danio rerio). Neurotoxicology 33, 469–475. http://dx.doi.org/10.1016/j.neuro.2012.03.005.

Pérez, J., Domingues, I., Monteiro, M., Soares, A.M., Loureiro, S., 2013. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. Environ. Sci. Pollut. Res. Int. 20, 4671–4680. http://dx.doi.org/10.1007/s11356-012-1443-6.

Price, C.L., Parker, J.E., Warrilow, A.G., Kelly, D.E., Kelly, S.L., 2015. Azole fungicides - understanding resistance mechanisms in agricultural fungal pathogens. Pest Manag. Sci. 71, 1054–1058. http://dx.doi.org/10.1002/ps.402.

Richendrfer, H., Pelkowski, S.D., Colwill, R.M., Créton, R., 2012. Developmental subchronic exposure to chlorpyrifos reduces anxiety-related behavior in zebrafish larvae. Neurotoxicol. Teratol. 34, 458–465. http://dx.doi.org/10.1016/j.ntt.2012.04.010.

Richendrfer, H., Creton, R., 2015. Chlorpyrifos and malathion have opposite effects on behaviors and brain size that are not correlated to changes in AChE activity. Neurotoxicology 49, 50–58. http://dx.doi.org/10.1016/j.neuro.2015.05.002.

Rico, E.P., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol. Lett. 174, 25–30. http://dx.doi.org/10.1016/j.toxlet.2007.08.005.

Roex, E.W., Keijzers, R., van Gestel, C.A., 2003. Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, Danio rerio, after chronic exposure to parathion. Aquat. Toxicol. 64, 451–460. http://dx.doi.org/10.1016/S0166-445X(03)00100-0.

Sahoo, S.K., Jyot, G., Battu, R.S., Singh, B., 2012. Dissipation kinetics of trifloxystrobin and tebuconazole on chili and soil. Bull. Environ. Contam. Toxicol. 88, 368–371. http://dx.doi.org/10.1007/s00128-011-0464-z.

Sancho, E., Villarroel, M.J., Fernández, C., Andreu, E., Ferrando, M.D., 2010. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (Danio rerio). Ecotoxicol. Environ. Saf. 73, 370–376. http://dx.doi.org/10.1016/j.ecoenv.2009.09.020.

Schmidel, A.J., Assmann, K.L., Werlang, C.C., Bertoncello, K.T., Francescon, F., Rambo, C.L., Beltrame, G.M., Calegari, D., Batista, C.B., Blaser, R.E., Roman Júnior, W.A., Conterato, G.M., Piato, A.L., Zanatta, L., Magro, J.D., Rosemberg, D.B., 2014. Subchronic atrazine exposure changes defensive behaviour profile and disrupts brain acetylcholinesterase activity of zebrafish. Neurotoxicol. Teratol. 44, 62–69. http://dx.doi.org/10.1016/j.ntt.2014.05.006.

Schröder, H., Zilles, K., Maelicke, A., Hajós, F., 1989. Immunohisto- and cytochemical localization of cortical nicotinic cholinoceptors in rat and man. Brain Res. 502, 287–295. http://dx.doi.org/10.1016/0006-8993(89)90624-0.

Senger, M.R., Rico, E.P., de Bem Arizi, M., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2005. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (Danio rerio) brain membranes. Toxicology 212, 107–115. http://dx.doi.org/10.1016/j.tox.2005.04.007.

Shaked, I., Zimmermann, G., Soreq, H., 2008. Stress-induced alternative splicing modulations in brain and periphery: acetylcholinesterase as a case study. Ann. N. Y. Acad. Sci. 1148, 269–281. http://dx.doi.org/10.1196/annals.1410.001.

Sun, L., Xu, W., Peng, T., Chen, H., Ren, L., Tan, H., Xiao, D., Qian, H., Fu, Z., 2016. Developmental exposure of zebrafish larvae to organophosphate flame retardants causes neurotoxicity. Neurotoxicol. Teratol. 55, 16–22. http://dx.doi.org/10.1016/j.ntt.2016.03.003.

Tang, R., Dodd, A., Lai, D., McNabb, W.C., Love, D.R., 2007. Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim. Biophys. Sin. (Shanghai) 39, 384–390. http://dx.doi.org/10.1111/j.1745-7270.2007.00283.x.

Tilton, F.A., Bammler, T.K., Gallagher, E.P., 2011. Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 9–16. http://dx.doi.org/10.1016/j.cbpc.2010.07.008.

Toni, C., Loro, V.L., Santi, A., de Menezes, C.C., Cattaneo, R., Clasen, B.E., Zanella, R., 2011. Exposure to tebuconazol in rice field and laboratory conditions induces oxidative stress in carp (Cyprinus carpio). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 128–132. http://dx.doi.org/10.1016/j.cbpc.2010.09.008.

van der Zee, E.A., Matsuyama, T., Strosberg, A.D., Traber, J., Luiten, P.G., 1989. Demonstration of muscarinic acetylcholine receptor-like immunoreactivity in the rat forebrain and upper brainstem. Histochemistry 92, 475–485. http://dx.doi.org/10.1007/BF00524759.

Velasco-Santamaría, Y.M., Handy, R.D., Sloman, K.A., 2011. Endosulfan affects health variables in adult zebrafish (Danio rerio) and induces alterations in larvae development. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 372–380. http://dx.doi.org/10.1016/j.cbpc.2011.01.001.

Wang, Y., Wang, C., Gao, J., Liu, C., Cui, L., Li, A., 2015. Dissipation, residues, and safety evaluation of trifloxystrobin and tebuconazole on ginseng and soil. Environ. Monit. Assess. 187, 344. http://dx.doi.org/10.1007/s10661-015-4591-6.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press, Eugene.

Wilson, J.M., Bunte, R.M., Carty, A.J., 2009. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). J. Am. Assoc. Lab. Anim. Sci. 48, 785–789.

Yang, Y., Qi, S., Wang, D., Wang, K., Zhu, L., Chai, T., Wang, C., 2016. Toxic effects of thifluzamide on zebrafish (Danio rerio). J. Hazard Mater 307, 127–136. http://dx.doi.org/10.1016/j.jhazmat.2015.12.055.

Yu, L., Chen, M., Liu, Y., Gui, W., Zhu, G., 2013. Thyroid endocrine disruption in zebrafish larvae following exposure to hexaconazole and tebuconazole. Aquat. Toxicol. 139C, 35–42. http://dx.doi.org/10.1016/j.aquatox.2013.04.001.

Zirger, J.M., Beattie, C.E., McKay, D.B., Boyd, R.T., 2003. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expr. Patterns 3, 747–754. http://dx.doi.org/10.1016/S1567-133X(03)00126-1.