Developmental toxicity of the fungicide ziram in zebrafish (Danio rerio)

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ABSTRACT

Ziram is a broad spectrum pesticide that belongs to the class of dimethyl-dithiocarbamate (DTC) fungicides. The objectives of this study were to assess the effects of ziram in developing zebrafish. Ziram was highly toxic to zebrafish embryos, with a 96-h LC50 value of 1082.54 nM (~0.33 mg/L). Zebrafish embryos at 6 h post-fertilization (hpf) were exposed to solvent control (0.1% DMSO), or one dose of 1, 10, 100, and 1000 nM ziram for 96 h. Ziram induced lethality in a dose-dependent manner, decreased hatching rate and heartbeat, and caused wavy deformities at 72 and 96 hpf at 100 and 1000 nM. Basal respiration of embryos was decreased following 24 h exposure to 1000 nM Ziram.

Keywords: Ziram; Zebrafish embryos; Mitochondrial bioenergetics; Dark/light preference

Abbreviations: ALDH, aldehyde dehydrogenase; DA, dopaminergic; dpf, days post-fertilization; DMSO, dimethyl sulfoxide; DTC, dithiocarbamate; hpf, hours post-fertilization; OCR, oxygen consumption rate.

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1. Introduction

Ziram [Zinc bis (dimethyldithiocarbamate)] is a broad spectrum pesticide that belongs to the class of dimethyl-dithiocarbamate (DTCs) fungicides (Houeto et al., 1995; Kanchi et al., 2014). It was first registered in the United States in 1960 for controlling the scab in apples and pears, leaf curl in peaches, as well as anthracnose and early blight in tomatoes (US EPA, 2004), and estimates suggest that there are ~2 million pounds of ziram used annually (US-EPA, 2015). Ziram is still widely applied in the United States, Italy, Japan, India, and China today (PPDB, 2018). Recommended minimum amounts of ziram for aerial application is 28.77 kg per acre, and that for ground applications is 57.61 kg of spray per acre, with at least 7-day intervals (Joyner, 2015). Ziram remains a concern for its environmental and human health risks (Kanemoto-Kataoka et al., 2017, 2018; Martin et al., 2016) and this warrants further investigation into its toxicity and mechanisms of action in non-target organisms and humans. For example, epidemiological studies have suggested that environmental exposures to ziram alone or combined with other pesticides are linked to neurodegenerative diseases such as Parkinson’s disease (Fitzmaurice et al., 2014; Rhodes et al., 2013; Wang et al., 2011). Quantifying adverse effects associated with ziram-induced neurotoxicity may therefore shed some light on the relationship between this pesticide and neurodegeneration.

Zebrafish (Danio rerio) are useful for assessing neurotoxicity and neurobehavioral toxicity because they are rapidly developing vertebrates with well-characterized neurotransmitter systems (Panula et al., 2010), well described dopamine neuronal network that shows some conservation to that of mammals (Milanese et al., 2012; Wen et al., 2008), and because they have a number of optimized behavioral assays that can be used to evaluate neurotoxicity (Ek et al., 2016; Tierney, 2011). Moreover, zebrafish in early life stages are susceptible and sensitive to environmental contaminants (Jin et al., 2009; Schulz et al., 2010), and are an exquisitely model organism to study adverse effects of chemicals in intact organisms. To the best of our knowledge, there is a single study investigating mechanisms of ziram-induced toxicity in zebrafish embryos and larvae. The study characterized behavioral responses in a light-dark assay (dark photokinesis assay) and determined that exposure to ziram induced neurotoxicity to dopamine neurons (Lulla et al., 2016). Specifically, the study reported that ziram at 50 nM induced damage in dopaminergic neurons through overexpressing the \(\gamma\)-synuclein 1 \((\gamma 1)\) gene in zebrafish embryos after 5-day exposure (Lulla et al., 2016). Therefore, there may be a molecular link between PD and ziram toxicity, prompting further studies to better discern the mechanisms of ziram.

The mechanisms underlying neurotoxicity can be many, but some important themes include the loss of dopaminergic neurons, mitochondrial dysfunction, and oxidative damage. Indeed ziram-mediated neurotoxicity can also occur through the inhibition of the mitochondrial enzyme aldehyde dehydrogenase (ALDH) through its metal complex, which is needed to degrade oxidative metabolites in dopaminergic neurons (Fitzmaurice et al., 2014). Therefore, measuring endpoints related to the dopamine system, mitochondrial bioenergetics, and behavior are meaningful and may provide insight into the mechanisms underlying ziram toxicity.

In this study, we assessed morphological and physiological endpoints including survival, hatching rate, heartbeat and the presence of deformities in response to ziram exposure. Oxygen consumption rates were also measured for the first time to clarify the effects of ziram on mitochondrial bioenergetics. This is important as other recent studies demonstrate that pesticides can adversely affect mitochondrial bioenergetics in developing zebrafish embryos (Wang et al., 2018a,b; Wang et al., 2018c). As ziram was found to result in oxidative stress (Kanemoto-Kataoka et al., 2018; Martin et al., 2016), transcriptional responses related to oxidative damage response were measured. Additionally, transcripts involved in dopamine signaling were assessed. The rationale was based on the study of Lulla et al. (2016) which showed dopaminergic cell depletion in zebrafish in response to ziram. Lastly, we conducted a dark-light preference test, a different test from that conducted in Lulla et al. (2016) (i.e. dark photokinesis), to determine if ziram caused neurotoxicity based on the presence of anxiety-related behavioral responses. These data generate new knowledge and understanding as to the physiological responses underlying ziram toxicity.

2. Materials and methods

2.1. Chemicals and reagents

Ziram (CAS: 137-30-4, purity > 97%) was obtained from Sigma-Aldrich Co. LLC (USA). Stock solutions of 1, 10, 100 and 1000 \(\mu\)M ziram were prepared by dissolving ziram in dimethyl sulfoxide (DMSO). Exposure solutions of 1, 10, 100 and 1000 nM ziram were prepared by adding 10 \(\mu\)L of each stock solution into 10 mL of embryo rearing medium (ERM; 0.8 g/L NaCl, 0.04 g/L KCl, 0.00358 g/L NaHCO3, pH 7.2) and 1 h later, eggs were collected and rinsed using ERM.

2.2. Maintenance and egg production of zebrafish

Parental adult zebrafish (wild-type AB/Tu strain, Danio rerio) were raised in the Cancer Genetics Research Center at the University of Florida. Parental zebrafish were maintained in a flow-through equipment with a pH value of 7.5 ± 0.5, a conductivity value of 600 ± 10 \(\mu\)S cm\(^{-1}\), a light/dark cycle of 14:10 h, dissolved oxygen concentration of up to 80% of air saturation, and the temperature of 26 ± 1 °C.

Breeding was induced in the morning when the light was turned on, and 1 h later, eggs were collected and rinsed using ERM. Fertilized and normally developed eggs were sorted and selected (occurring over ~6 h post-fertilization, hpf) using a dissecting microscope for all toxicity experiments. Embryos were staged using the criteria outlined by a previous study (Kimmel et al., 1995). Experimental procedures were approved by the guidelines of Institutional Animal Care and Use Committee of University of Florida.

2.3. Developmental toxicity of ziram on zebrafish

Zebrafish embryo toxicity assays were performed according to
OECD guidelines for Fish Embryo Toxicity (FET) Test (OECD 236, 2013) to obtain LC50 values of ziram in zebrafish embryos. Approximately 300 zebrafish embryos at 6 hpf were randomly assigned into groups and exposed to solvent control (0.1% DMSO, v/v), or one dose of 1, 10, 100 and 1000 nM ziram for 96 h. Each beaker contained 10 zebrafish embryos and 10 mL of exposure solutions. Five replicates were set aside for each treatment (N = 5 per treatment). All beakers were placed into an incubator, which was maintained at a light/dark cycle of 14:10 h and the temperature of 26 ± 1 °C. Approximately 90% of exposure solutions were renewed in a timely fashion. Mortality was recorded daily and dead embryos were removed from the beakers daily.

To evaluate the developmental toxicity of ziram on zebrafish embryos, approximately 250 zebrafish embryos at 6 hpf were placed randomly in groups and exposed to solvent control (0.1% DMSO, v/v), or one dose of 1, 10, 100 and 1000 nM ziram for 96 h. Each beaker contained 10 zebrafish embryos and 10 mL of exposure solutions. Each treatment contained five replicates (N = 5 per treatment). All beakers were placed into an incubator, which was maintained at a light/dark cycle of 14:10 h and the temperature of 26 ± 1 °C. Mortality, malformation rate and hatching rate of zebrafish embryos were recorded daily, and heartbeat in 20s intervals was counted from 48 hpf using a dissecting microscope. Morphological deformations were photographed using an EVOS FL Auto Imaging System (Life Technologies). Dead individuals were photographed using an EVOS FL Auto Imaging System (Life Technologies). Dead individuals were removed in a timely fashion. Since ziram was stable in water with a hydrolysis half-life of 2–18 days (HSDB, 2003), approximately 90% of exposure solutions were renewed daily. At 24 hpf, one zebrafish embryo (normally developed) was selected from each replicate beaker for the 10, 100 and 1000 nM ziram treatments to measure oxygen consumption rates of zebrafish embryos.

2.4. Mitochondrial respiration measurement

To determine whether ziram caused adverse effects on mitochondrial bioenergetics at an earlier stage of development, oxygen consumption rate (OCR) of zebrafish embryos was measured at 24 hpf using the Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, USA). We selected embryos at 24 hpf to test OCR, as it is difficult to measure OCR of hatched larval fish once they are free swimming. One zebrafish embryo from each beaker was placed into one well of 24-well microplate (N = 5 per treatment). Four background controls were included, which only contained ERM solutions. Each treatment contained one dose of 1, 10, or 100 nM ziram for gene expression analysis. Each beaker contained 10 zebrafish embryos and 10 mL of exposure solutions. Exposure conditions were maintained as per conditions used in the study of Lulla et al. (2016). Zebrafish larvae at 6 hpf were randomly assigned to groups and exposure to either a solvent control (0.1% DMSO, v/v), or one dose of 1, 10, or 100 nM ziram for gene expression analysis. Each beaker contained 20 zebrafish embryos and 10 mL of exposure solutions. Exposure conditions were maintained as per conditions used in the developmental toxicity assay. After 96 hpf, zebrafish larvae from each beaker were pooled, frozen in liquid nitrogen and stored at −80 °C for RNA extraction (N = 8 biological replicates per treatment).

Total RNA of samples was extracted using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. The quality of RNA for all samples was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples had an average RNA integrity number (RIN) of 9.26 ± 0.10 and were purified using RNeasy Mini Kit columns (Qiagen, Valencia, CA, USA). The concentration of RNA samples was measured by Qubit Fluorometric Quantitation (Thermo Scientific, USA). Total RNA (2 μg) per sample was reverse-transcribed to cDNA using iScript (BioRad, USA) and stored at −20 °C for further analysis.

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed by the CFX Connect System (BioRad, USA), and each treatment was prepared with 8 biological replicates in duplicate. Each plate included three no reverse transcriptase control (NRT) in duplicate and one no template control (NTC) in duplicate. Negative controls were used to assess for genomic DNA contamination. The procedure of RT-qPCR was as follows: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and primer annealing at 58 °C for 5 s, followed by a dissociation curve of starting at 65.0°C to 95.0°C with increments of 0.5°C every 5 s.

Gene-specific primers were obtained from published sequences of zebrafish (Table S1). The mRNA levels of genes encoding superoxide dismutase 1 (sod1 and sod2) in the oxidative stress pathway due to evidence for ziram caused oxidative stress in rat cells by Matei and Trombetta (2016) and Kanemoto-Kataoka et al. (2018), as well as dopamine synthesis (tyrosine hydroxylase 1, th1), dopamine transport (dat) and dopamine receptors (drd1a, drd2a, drd3 and drd4b) in the dopamine system as ziram was toxic to dopaminergic neurons of zebrafish embryos in the study of Lulla et al. (2016). Ribosomal subunit 18S (rps18) and ribosomal protein L13a (rpl13a) were selected as housekeeping genes, which were used to normalize all target gene expression. The target stability function was determined by the CFX Manager™ software (v3.1) and the combined M-value for rps18 and rpl13a was 0.81 (CV = 0.28). Each primer was tested for linearity (>0.97) and efficiency (90–120%) using a 4-point standard curve generated by a dilution series (5 times) from a cDNA pool of all samples. Normalized gene expression values were extracted using CFX Manager™ software (baseline subtracted) and were analyzed by using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

2.6. Dark/light preference assay

To determine if ziram affects the activity of zebrafish larvae at sublethal concentrations, two trials of dark/light preference assay were conducted with the same doses using different batches of embryos for rigor and reproducibility. For each trial, approximately 150 zebrafish embryos at 6 hpf were randomly selected to be exposed to either the solvent control (0.1% DMSO, v/v), or one dose of either 1 or 10 nM ziram for 7 days post-fertilization (dpf). Each beaker contained 10 zebrafish embryos and 10 mL of exposure solutions. Each treatment contained five replicates (N = 5 per treatment). Zebrafish larvae were not fed prior to behavioral assays.
Exposure conditions were maintained the same as that outlined in the developmental toxicity assay. After 7 dpf, 5 to 6 normally developed zebrafish larvae were selected from each beaker at random and placed into a 96-well plate with 200 μL ERM per well (N = 32 individuals per treatment). The 96-well plate was placed into DanioVision™ instrument (Noldus Information Technology, Leesburg, VA); the 96-well plate had a left side that was dark and a right side that was not dark due to a cover placed over the bottom of the plate. After a delay of 10 min, the activities of zebrafish larvae were tracked using an infrared analog camera (25 frames/second) that was installed in the DanioVision™ Observation Chamber for the typical 60-min white light routine, beginning at 3:00 p.m. of the 7 dpf. Data on total activity, total velocity, distance moved in light and dark zone, and velocity in light and dark zone over 60 min was generated in EthoVision software (v12) and was exported to determine if ziram affected larval behavior. In addition, total distance moved, mean time in dark zone, frequency in dark zone, and cumulative duration in dark zone per 15 min were also exported and analyzed anxiety-related behaviors in zebrafish larval using EthoVision software.

### 2.7. Statistical analysis

SPSS 16.0 software (SPSS Inc., USA) was applied to calculate the LC₅₀ values of ziram on zebrafish embryos. Statistical analysis was performed by using Graph-Pad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The normality of distribution was tested by Shapiro-Wilk’s test, and the homogeneity of variances was tested by Levene’s test prior to ANOVA. Significant differences between the control and treatment groups were analyzed using

### Table 1

The LC₅₀ values of ziram in zebrafish embryos following 96 h exposure.

| Times | LC₅₀ (nM) | 95% confidence limit (nM) | Regression equation | R² |
|-------|-----------|---------------------------|---------------------|----|
| 48 h  | 2355.93   | 1513.37–5364.08           | y = 1.27x – 4.27    | 0.978 |
| 72 h  | 1613.21   | 1090.23–3166.25           | y = 1.19x – 3.81    | 0.988 |
| 96 h  | 1082.54   | 774.76–1783.60            | y = 1.21x – 3.66    | 0.983 |

Fig. 1. Lethal effects and developmental deficits in embryos after 96 h of exposure to ziram. A. Cumulative mortality at 24, 48, 72 and 96 hpf. B. Cumulative hatching rate at 72 and 96 hpf. C. Heartbeat in 20 s at 48, 72, and 96 hpf. D. Notochord distortion rate at 48, 72, and 96 hpf. Values are shown as mean ± standard error of mean (SEM) (N = 5). For simplification, mortality of the control group (<5%) was set at 0% and mortality of all treatments was adjusted according to that of the control group. Different letters denote significant differences between the control and treatments (p < 0.05 determined by Tukey’s post-hoc test).
one-way ANOVA followed by Tukey’s post-hoc test. Values are shown as mean ± standard error of mean (SEM). Significant differences occurred when $p < 0.05$.

3. Results

3.1. LC50 values of ziram

The LC50 values of ziram in zebra fish embryos are presented in Table 1. Ziram displayed a relatively high toxicity to zebra fish embryos, with a 96 h-LC50 of 1082.54 nM (equal to 0.33 mg/L).

3.2. Developmental toxicity of ziram

There was no difference in mortality, hatching rate, heartbeat and notochord deformity rate between the control group and 1 nM ziram nor 10 nM ziram after 96 h exposure (Fig. 1). Conversely, the difference in these endpoints was detected among the control group, 100 nM ziram and 1000 nM ziram. Differences in cumulative mortality were observed between the control group and 100 nM at 72 and 96 hpf ($F(4, 20) = 38.30$, $p < 0.001$; $F(4, 20) = 21.79$, $p < 0.001$) (Fig. 1A); cumulative mortality was also different between the 1000 nM ziram group and the control group at 24 hpf ($F(4, 20) = 7.54$, $p = 0.003$) (Fig. 1A).

Compared to the control group, there was a significant decrease in cumulative hatching rate with 100 and 1000 nM ziram exposure at 72 hpf ($F(4, 20) = 26.80$, $p = 0.045$ and $p < 0.001$), and with 100 and 1000 nM ziram exposure at 96 hpf ($F(4, 20) = 40.43$, $p < 0.001$ and $p < 0.001$) (Fig. 1B). At the highest dose of 1000 nM, the cumulative hatching rate was approximately 4-fold less than controls. We also assessed heartbeat in zebra fish at 48, 72 and 96 hpf (Fig. 1C). Heartbeat was significantly decreased after exposure to 100 and 1000 nM ziram at 48, 72 and 96 hpf ($F(4, 20) = 89.55$, $p < 0.001$; $F(4, 20) = 34.71$, $p < 0.001$; $F(4, 20) = 138.8$, $p < 0.001$) in comparison with the control group (Fig. 1C). Serious notochord distortion was observed in zebra fish larvae in 100 and 1000 nM ziram treatments at 72 and at 96 hpf (Fig. 2). As shown in Fig. 3 (magnified Fig. 2), the notochord is severely twisted in deformed larvae treated with 100 and 1000 nM ziram. Ziram increased notochord distortion rates in 100 and 1000 nM treatments in a time-dependent manner, with deformity rates of 9.6 ± 2.5% and 22.7 ± 2.7% ($F(4, 20) = 36.21$, $p = 0.005$ and $p < 0.001$) at 48 hpf,

Fig. 2. Representative pictures of zebra fish following exposure to 0.1% DMSO (control), 100 and 1000 nM ziram at 72 and 96 hpf. A and D indicate larvae in control group at 72 and 96 hpf. B and E indicate larvae with notochord distortion in 100 nM ziram group at 72 and 96 hpf. C and F indicate larvae with notochord distortion in 1000 nM ziram group at 72 and 96 hpf. Red arrows indicate the distorted site. The scale bar in each picture is 2000 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Magnified pictures of Fig. 2 of zebra fish after exposure to 0.1% DMSO (control), 100 and 1000 nM ziram at 72 and 96 hpf. A and D indicate zebrafish larvae in control group at 72 and 96 hpf. B and E indicate zebrafish larvae with notochord distortion in 100 nM ziram group at 72 and 96 hpf. C and F indicate zebrafish larvae with notochord distortion in 1000 nM ziram group at 72 and 96 hpf. Red arrows indicate the distorted site. The scale bar in each picture is 400 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 4. Oxygen consumption rate (OCR) of mitochondrial bioenergetics in zebrafish embryos exposed to 0.1% DMSO (Control), 10, 100, and 1000 nM ziram at 24 hpf. A. OCR per embryo throughout the assay. B. Basal respiration. C. ATP-linked respiration. D. Maximal respiration. E. Non-mitochondrial respiration. Values are shown as mean ± standard error of mean (SEM) (N = 5). Different letters denote significant differences between the control and treatments (determined by Tukey’s post-hoc test, \( p < 0.05 \)).
respectively, and $56.8 \pm 7.4\%$ and $69.1 \pm 3.6\%$ ($F_{(4, 20)} = 88.98$, $p < 0.001$ and $p < 0.001$) at 96 hpf, respectively (Fig. 1D).

3.3. Effects of ziram on mitochondrial bioenergetics of zebrafish embryos

The effects of ziram on mitochondrial bioenergetics in zebrafish embryos were assessed following a 24 h exposure to 10, 100 and 1000 nM ziram. The basal respiration in zebrafish embryos was significantly decreased at 1000 nM ziram compared to the controls ($F_{(3, 16)} = 7.03$, $p = 0.007$) (Fig. 4B). However, ATP-linked respiration, maximal respiration, and non-mitochondrial respiration in zebrafish embryos were not altered after 24 h exposure with the doses of 10, 100 and 1000 nM ziram in comparison with the control (Fig. 4C, D, and E). Thus, ziram negatively impacts basal respiration in embryos at 1000 nM.

3.4. Effects of ziram on gene expression related to oxidative stress

Since ziram was reported to cause oxidative stress and here it affected mitochondrial bioenergetics, relative mRNA levels of sod1 and sod2 were measured following 96 h exposure to determine if ziram affects transcriptional response related to oxidative damage in larval zebrafish (Fig. 5). There was no significant difference in mRNA levels of sod1 and sod2 in zebrafish larvae treated with 1, 10 and 100 nM for 96 h compared with the control group (Fig. 5).

Given that ziram induced behavioral changes and dopaminergic cell depletion in zebrafish larvae, we hypothesized that DA signaling may be disrupted as this neurotransmitter system plays a predominant role in the behavior. Transcriptional levels of genes involved in DA synthesis (th1), transport (dat), and receptors (drd1, drd2a, drd3, and drd4b) were measured (Fig. 6). Following 96 h exposure to ziram, no difference in expression levels of th1, dat, drd1, drd2a, drd3, and drd4b were detected in any tested concentration of ziram compared to the control (Fig. 6).

3.5. Effects of ziram on behavioral response of zebrafish larvae

We ran two trials with the same doses using different batches of embryos. In the first trial, total activity of zebrafish larvae was significantly increased compared with the control group following a 7 dpf exposure to 10 nM ziram ($F_{(2, 86)} = 3.4$, $p = 0.031$) (Fig. 7A).

Exposure to 10 nM ziram significantly increased the velocity in light zone of zebrafish larvae at 7 dpf when compared to the control group ($F_{(2, 85)} = 84.11$, $p < 0.001$) (Fig. 7D). The total distance moved of the second 15-min was significantly increased at 10 nM ziram ($F_{(2, 86)} = 29.82$, $p = 0.024$) (Fig. 7 E); conversely, exposure to 1 and 10 nM ziram resulted in a significant decrease in mean time in dark zone of the second 15-min compared to the control group ($F_{(2, 78)} = 71.51$, $p < 0.001$ and $p < 0.001$) (Fig. 7 F). However, there was no alteration in total velocity, distance moved in light and dark zone, velocity in dark zone, frequency in dark zone, and cumulative duration in dark zone in zebrafish larvae at 1 and 10 nM ziram (Fig. 7 B, C, D, G and H). In the second trial, there was no change in the tested endpoints of larval behavior at 1 and 10 nM examined (Fig. S1). Taken together, there was some evidence for behavioral modification in zebrafish but this appeared to be clutch specific.

4. Discussion

In the present study, the calculated 96 h-LC50 of ziram in zebrafish embryos was 1082.54 nM (equal to 0.33 mg/L). Significant mortality, decreased hatching rate and heartbeat, and increased notochord deformity rate was observed following 96 h exposure to 100 and 1000 nM ziram; conversely, no significant degree of lethality or developmental deficits were observed in zebrafish embryos treated with doses of ziram at lower than 10 nM. Our results are in good agreement with Lulla et al. (2016), who reported that 50 nM ziram was the highest dose at which there was no significant mortality observed, but was sufficient to induce a shorter body axis, pericardial edema, and notochord distortion in zebrafish embryos at 5 hpf.

We propose that reduced hatching rate and heartbeat may be associated with dysfunction in mitochondrial bioenergetics caused by ziram, as the mitochondria are major producers of energy and defects would affect health and development of the fish (DiMauro and Schon, 2008; Zhang et al., 2017). By assessing oxygen consumption rates, we found that basal respiration was significantly decreased in zebrafish embryos after 24 h exposure to 1000 nM ziram. Thus, the impairment in mitochondrial bioenergetics by ziram is proposed to induce developmental defects and hatching delays in fish. Other pesticides have also been shown to affect mitochondrial bioenergetics and developmental defects in fish. For example, the herbicide paraquat decreased maximal respiration and induced premature hatching in zebrafish treated with 100 μM

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**Fig. 5.** Effects of ziram on relative mRNA levels of (A) sod1 and (B) sod2 genes related to oxidative stress in zebrafish larvae exposed to 0.1% DMSO (Control), 1, 10, and 100 nM ziram for 96 h. Each point represents the normalized expression of each biological replicate, and the line represents the median value of the data. Values are shown as mean ± standard error of mean (SEM) (N = 6–8).
Another study reported that zebrafish exposed to 0.5 μM fluazinam, a pyridinamine fungicide, displayed mortality, gross malformations (axial malformation, pericardial edema and yolk sac edema), hatch delay and respiration inhibition following 96 h exposure (Wang et al., 2018c). Other compounds such as tributyltin, triclosan, and quercetin have also been shown to affect the metabolic respiration of early staged zebrafish (Liang et al., 2017; Shim et al., 2016; Zhang et al., 2017).

Our data support the hypothesis that ziram impairs oxidative phosphorylation and this could lead to mitochondrial dysfunction. This is relevant for human diseases as mitochondrial dysfunction is one mechanism that is associated with neurodegeneration and PD.

Fig. 6. Effects of ziram on relative mRNA levels of (A) th1, (B) dat, (C) drd1, (D) drd2a, (E) drd3, and (F) drd4b genes related to DA synthesis, transport, and receptors in zebrafish larvae exposed to 0.1% DMSO (Control), 1, 10, and 100 nM ziram for 96 h. Each point represents the normalized expression of each biological replicate, and the line represents the median value of the data. Values are shown as mean ± standard error of mean (SEM) (N = 6–8).
Fig. 7. Behavioral response of zebrafish larvae exposed to 0.1% DMSO (control), 1 and 10 μM ziram at 7 dpf of the first trial. A. Total activity within 60 min. B. Total velocity within 60 min. C. Distance moved in light and dark zone within 60 min. D. Velocity in light and dark zone within 60 min. E. Total distance moved per 15 min bin. F. Mean time in dark zone per 15 min bin. G. Frequency in dark zone per 15 min bin. H. Cumulative duration in dark zone per 15 min bin. Values are shown as mean ± standard error of mean (SEM) (N = 24). Different letters denote significant differences between the control and treatments (p < 0.05 determined by Tukey’s post-hoc test).
For example, studies report that ziram can affect proteins in the mitochondria. Fitzmaurice et al. (2014) reported that ziram inhibited the mitochondrial enzyme activity of ALDH in the neurons derived from the substantia nigra of new-born rats exposed to 10 μM ziram. In addition, a recent study showed that ziram decreased the mitochondrial activity of rat thymocytes treated with 0.1 and 0.3 μM for 2 h (Kenmoto-Kataoka et al., 2018).

Oxidative damage is proposed to be another reason for the developmental defects induced by ziram exposure, as teratogenic toxicity of many chemicals to fish early life stages are highly correlated with oxidative stress (Jin et al., 2013; Mu et al., 2015; Zhu et al., 2015). Ziram has been reported to induce oxidative stress in rat hippocampal astrocytes when the cells are treated with 1.0 μM for 1 h (Matei and Trombetta, 2016) and in rat thymocytes treated with 0.1 and 0.3 μM for 2 h (Kenmoto-Kataoka et al., 2018). Based on this knowledge, we assessed the expression levels of sod1 and sod2 in zebrafish larvae as one endpoint to determine if ziram induced oxidative stress responses at the molecular level. We did not detect a transcriptional response of sod1 and sod2 in zebrafish larvae treated with 100 nM ziram for 96 h, despite the fact that 100 nM ziram induced pronounced developmental deficits; no significant difference in the expression levels of these two genes were detected in zebrafish larvae following 96 h exposure to 1 and 10 nM ziram. Other fungicides that directly affect the mitochondria have been shown to alter sod1 and sod2, along with developmental defects in early staged zebrafish. For example, azoxystrobin, known as an inhibitor of mitochondrial complex III, was reported to up-regulate expression levels of sod1 and sod2, which was one of the mechanisms for developmental defects in zebrafish embryos (Cao et al., 2018). Exposure to 0.5 μM fluazinam, a pyridinamine fungicide and a potent uncoupler of mitochondrial oxidative phosphorylation, delayed hatching time, caused malformations and decreased the expression level of sod1 in zebrafish larvae (Wang et al., 2018c). Thus, there is evidence that other mitochondrial toxicants affect sod1 and sod2 expression in larval zebrafish; however ziram did not affect sod1 nor sod2 expression at the doses and time point examined.

Dopaminergic signaling is crucial for neurodevelopment and directly regulates behavior in larval zebrafish (Kacprzak et al., 2017; Souza and Tropepe, 2011). A study demonstrated that 0.5 μM ziram damaged dopaminergic neurons in rat primary ventral mesencephalic cells after a 10-day treatment (Chou et al., 2008). Lulla et al. (2016) reported that 50 nM ziram caused the loss of DA neurons and decreased tyrosine hydroxylase 1 (TH1) protein in transgenic zebrafish larvae (VMAT2: GFP) at 5 dpf. Thus, we hypothesized that ziram would affect the expression of the dopamine system, and that this would also be associated with altered behavior. Indeed, a previous study with the dopamine receptor antagonist domperidone has been associated with hyperactivity in larval zebrafish (Shontz et al., 2018). There was no difference in the expression levels of genes associated with dopamine synthesis (thl), dopaminergic receptors, or dopamine transporter (dat) were detected in larvae treated with 1, 10 and 100 nM for 96 hpf. One limitation of the analysis was that the expression levels of the genes in dopaminergic signaling were measured in the whole homogenized body of larvae and transcriptional responses might not completely reflect the transcriptional changes of these genes in specific neurons of the central nervous system (CNS). Nevertheless, the modulation of the dopamine system via altered mRNA levels may not be a mechanism underlying ziram toxicity.

Behavioral responses of zebrafish during early life stages have been proven sensitive endpoints for detecting neurological impairments caused by environmental contaminants (Selderslags et al., 2010; Tierney, 2011). Lulla et al. (2016) found that 50 nM ziram significantly decreased distance traveled in dark zone of zebrafish larvae at 7 dpf, but there was no difference in activity in the light zone. Our first trial of dark/light preference assay showed that there was no difference in distance moved in both dark and light zone following 7 dpf exposure to 1 and 10 nM ziram; whereas the velocity in the light zone was significantly increased with 10 nM ziram. We also found that 10 nM significantly increased the total activity of zebrafish larvae. However, no change in behavioral endpoints was detected in the second trial of dark/light preference assay. These data suggest that ziram affects the behavior of zebrafish larvae at sublethal concentrations, but the effects may depend on the genetic background derived from a specific clutch.

Zebrafish larvae have a natural preference for a light environment (Lau et al., 2011), and the dark/light preference test assessing anxiety-related behavioral responses of fish is widely used to measure anxiolytic properties of chemicals (Blaser et al., 2010; Maximino et al., 2010a). An increase in dark zone activity should reflect anxiety-promoting behavior, whereas an increase in white zone activity (duration and/or entries) should reflect anti-anxiety behavior in zebrafish larvae (Maximino et al., 2010a, 2010b). Here, we analyzed the mean time in dark zone, the frequency that zebrafish larvae visit dark zone, and the cumulative duration in dark zone per 15 min to determine whether ziram can cause an anxiety-related behavioral response in the developing zebrafish. We did not detect significant differences in the frequency in dark zone and the duration in the dark zone, suggesting that ziram did not induce anxiety-related behavioral changes.

In conclusion, these data demonstrate that ziram negatively impacts embryonic development (i.e. survival, hatching, heartbeat, and notochord development) of zebrafish and decreases basal respiration of embryos at ≥ 100 nM, and can alter behavioral responses in larvae at sub-lethal concentrations in some cases. Determining the biological effects of DTCs fungicides is significant because these chemicals are currently used and are linked to human diseases such as such as Parkinson’s disease (Fitzmaurice et al., 2014; Rhodes et al., 2013; Wang et al., 2011). Moreover, we show significant developmental consequences to exposure, and literature is currently scarce on whether epigenetic changes can occur and persist across generations. Improved understanding as to the scope of adverse biological effects in vertebrates at early stages of development will aid in determining safer limits for pesticide applications and chemical design.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.09.105.

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