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Sub-lethal effects of the triazole fungicide propiconazole on zebrafish (*Danio rerio*) development, oxidative respiration, and larval locomotor activity

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ABSTRACT

Propiconazole is a triazole fungicide used in agriculture. Via run-off, it can enter the aquatic environment, and can adversely affect organisms. However, data are scarce on how propiconazole may affect early developmental life stages of fish. The objectives of this study were to evaluate the potential sub-lethal effects of propiconazole during zebrafish development. Wildtype zebrafish (ABTu strain) embryos and larvae were exposed to propiconazole (0.1–100 μM) for up to 150 hours post fertilization (hpf) depending upon the endpoint measured. Propiconazole decreased survival and induced hypopigmentation in fish at 100 μM compared to the water and solvent controls. Pericardial edema was also noted in embryos and larvae (beginning at 2–3 dpf) exposed to 100 μM propiconazole. To visualize the effects of propiconazole on the circulatory system in more detail, we exposed transgenic zebrafish (globin-LCR:eGFP) to the fungicide. Hematopoietic changes were observed within 48 h of exposure to 100 μM, and localization of blood cells in the cardic region became diffuse, indicating pooling of blood in the pericardial region. We measured oxidative respiration in embryos as sufficient ATP is needed for development. Exposure to 100 μM propiconazole (~6–30 hpf) reduced basal respiration (~50%), oligomycin-induced ATP linked respiration (~70%), proton leak (~30%), and non-mitochondrial respiration (~50%), indicating compromised mitochondrial bioenergetics. A Visual Motor Response (VMR) test was used to measure dark photokinesis behavior in larval fish exposed to propiconazole for a 6-day period. Larval fish exposed to the highest concentration in the assay (10 μM) showed evidence of hypoactivity. This study demonstrates that propiconazole can induce hypopigmentation in zebrafish, disrupt mitochondrial bioenergetics, and can alter locomotor activity. However, these sub-lethal responses were observed at concentrations above what is typically detected in the environment.

1. Introduction

Fungicides are widely used to control fungi and molds problematic for agriculture; however, the widespread use of any chemical can lead to unintentional exposures in non-target aquatic organisms via runoff into water systems. As such, defining the scope of adverse biological effects induced by fungicides remains a priority for environmental toxicology, and it is necessary for developing regulations for fungicide use in agricultural settings. Azole fungicides (triazoles) are used for crop protection (Cools et al., 2006; Jung et al., 1987; Shahinasi et al., 2017) and this class includes > 30 different chemicals (e.g. cyproconazole, flusilazole, and propiconazole among others). These compounds are heterocyclic (general molecular formula of $C_2H_3N_3$) and contain a five-membered ring of two carbon atoms and three nitrogen atoms; they are closely related to imidazole antifungals, chemicals that are used in medicine to control dermal fungal and yeast infections (e.g. athlete's foot, ring worm). Global application of triazoles in agriculture has been on the rise since 1990, and production estimates for the triazole (and diazole) fungicides is 20,977.24 (tonnes of active ingredients) globally in 2020 (Zhang, 2018). These production levels place triazole fungicides in a position as some of the most widely used and ubiquitous fungicides in agriculture.

Propiconazole was first registered in 1981 by the United States Environmental Protection Agency (EPA) in applications to treat a broad

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range of fungal diseases for cool and warm season grasses and turf, ornamental flowers, and shrubs. The fungicide effectively treats plant and crop-related issues that include rust fungi, Summer Patch, Leaf Blight, Brown Patch, and Powdery Mildew in addition to other mold infestations. Propiconazole shows fungistatic activity, slowing the growth of fungus which prevents further infection of the host. The fungicide acts via demethylation of the C14 in ergosterol biosynthesis, an event that disrupts fungal cell wall integrity, effectively impeding further fungal growth (Johansen et al., 2007; Waterfield and Sisler, 1989).

Propiconazole has been detected in the environment in the effluent from waste water treatment plants (WWTP) and in surface water. In Belgium, propiconazole was detected in surface water samples (16 locations) and ranged from 1.9 to 178.3 ng/L in effluent from waste water treatment plants (Van De Steene et al., 2010). Noteworthy in the study was that propiconazole was detected at comparable levels in both waste water and surface water, suggesting direct seepage from rural areas applying the pesticide. In Switzerland, agricultural fungicides and four azole pharmaceuticals used to treat fungal infections were measured in WWTP water. Results showed that azole chemicals were detected in the range of 1–30 ng/L in influents in WWTP samples (Kahle et al., 2008). In another study, Mottes et al. (2017) reported that high amounts of propiconazole and fosthiazate applied at low frequencies caused river pollution peaks for weeks following only a single application. Pesticide use in the area was monitored for 67 weeks with catchment of outlet pollution. The study detected 16 pesticides: 4 forbidden-use pesticides, 2 metabolites of known pesticides, and 10 authorized chemicals. The authors reported that these pesticides (e.g. fosthiazate, propiconazole and dithiocarbamates) may increase the risk of chronic pollution in the area (Mottes et al., 2017). Furthermore, in a study addressing the fate and transport of propiconazole in agricultural practices, Edwards et al. (2016) reported that propiconazole showed a DT50 value (time taken for 50% of the parent compound to dissipate from a given area) of 99–116 days. The authors went on to conclude that the horizontal movement of the pesticide after rain events as agricultural runoff increased the likelihood of exposure to non-target aquatic species. Taken together, reports suggest that propiconazole is detected in aquatic environments and can remain in soil for months after first application, posing a risk for aquatic organisms.

Conazole fungicides can elicit a range of biological effects in non-target organisms. These toxicity mechanisms can be endocrine-related (e.g. aromatase inhibition) or non-endocrine related (e.g. oxidative stress). Similar to the related chemical ketoconazole (Perkins et al., 2008), propiconazole can directly inhibit cytochrome P450 family 19 subfamily A member 1 (CYP 19) (Laville et al., 2006), the enzyme that converts androgens into estrogens (testosterone into 17beta-estradiol). This can lead to determinantal reproductive effects in fish. In one study, adult female fathead minnows (*Pimephales promelas*) were exposed to one dose of 0, 5, 50, 500, or 1000 μg propiconazole/L in a 21 day reproductive test. Females exposed to 500 μg/L showed decreased vitellogenin (the egg yolk precursor protein), reduced plasma 17beta-estradiol, and altered fecundity (Skolness et al., 2013). Importantly, propiconazole is predicted to have multiple modes of action (MOA), based on in vitro cell-based assays (Mihaich et al., 2017a; Mihaich et al., 2017b). These MOAs include aryl-hydrocarbon receptor activation (Knebel et al., 2018), oxidative stress and metabolic disruption (Toni et al., 2011) and mitochondrial dysfunction (Mihaich et al., 2017a). However, while there is evidence that propiconazole induces adverse effects in adult fish (Egaas et al., 1999; Li et al., 2010a; Li et al., 2010b), data are lacking for early developmental stages. Kumar and colleagues exposed zebrafish to different azole fungicides at environmentally relevant concentrations (0.3, 1.0 and 1000 μg/L) and observed altered expression of genes associated with oxidative and cell damage (e.g. *cyp51*, *gst*, and *p53*) (Kumar et al., 2019). The authors also reported that lipid peroxidation occurred with concentrations of 1 and 1000 μg/L, and there was an increase in caspase 3/7-dependent apoptosis following propiconazole exposure. Impacts on lipid metabolism have also been noted in zebrafish larvae following treatments to propiconazole in the 2.5–4.5 mg/L range (Teng et al., 2019). Thus, data support the notion that propiconazole affects different biological systems in fish.

This study aimed to quantify lethal and sub-lethal effects of propiconazole to developing zebrafish. We hypothesized that exposure to propiconazole induces developmental abnormalities in a dose-dependent manner. We also hypothesized that propiconazole impairs oxygen consumption rate due to reports that the fungicide causes oxidative stress in zebrafish (Kumar et al., 2019). Lastly, we hypothesized that propiconazole would decrease locomotor activity, based on our previous work with conazole fungicides (Perez-Rodriguez et al., 2018). To test these hypotheses, zebrafish embryos and larvae were exposed to a concentration gradient of propiconazole (0.1, 1, 10, and 100 μM). Deformities and mortality were recorded daily. As adequate ATP is required for development, oxygen consumption rates were measured to assess mitochondrial bioenergetics, as other conazole fungicides have been shown to affect mitochondrial function in fish embryos (Cao et al., 2019a; Perez-Rodriguez et al., 2018). Moreover, locomotor activity was assessed in this study because other azole fungicides such as tebuconazole have been shown to affect locomotor activity in zebrafish (Altenhofen et al., 2017; Perez-Rodriguez et al., 2018).

2. Methods

2.1. Chemicals

Propiconazole (CAS Number 60207-90-1, analytical standard) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nominal stock solutions of 0.1, 1, 10, and 100 μM propiconazole were prepared by dilution in dimethyl sulfoxide (DMSO) (CAS no. 67-68-5, purity ≥ 99.9%, Sigma-Aldrich, USA). Oligomycin A (≥99%, CAS Number 579-13-5), carbonyl cyanide-4-phenylhydrazone (≥98%, CAS Number: 370-86-5), sodium azide (≥99%, CAS Number 26628-22-8) were purchased from Sigma. Embryo rearing media (ERM stock: 8 g NaCl, 0.4 g KCl, 0.358 g Na_2HPO_4, 0.72 g $CaCl_2$, 0.6 g KH_2PO_4, 1.23 g $MgSO_4$, 0.35 g $NaHCO_3$, 1.92 L distilled water, pH ~ 7.2) was filtered and sterilized prior to use. Recipes can be found in the Zebrafish book (Westerfield, 2000) (https://zfin.org/zf_info/zfbook/chapt1/1.3.html).

2.2. Fish husbandry

Experiments with embryos and larvae were conducted at the Center for Environmental and Human Toxicology (CEHT, University of Florida). Adult zebrafish (~4-12 months of age, ABTu strain, *Danio rerio*) were bred in the Cancer Genetics Research Center, UF and moved to the CEHT for experiments. Zebrafish were maintained in an automated recirculating Pentair Aquatic Eco-Systems with the following parameters; mean water pH of ~7.2 ± 1, mean temperature of ~28 ± 1 °C, conductivity value of 600 ± 100 $\mu S \cdot cm^{-1}$, dissolved oxygen up to 80% air saturation, salinity < 5 ppt, non-detectable nitrate, and photoperiod cycle of 14 h of light and 10 h of dark. In the morning, the embryos were collected according to the criteria of Kimmel et al. (1995). Multiple breeding pairs of fish were used for the generation of eggs for all experiments over a two year period. Experimental procedures for collecting eggs followed that outlined previously (Cao et al., 2018; Wang et al., 2018). Experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

2.3. Experimental design for exposures to propiconazole

Exposure to propiconazole commenced when the zebrafish embryos were ~6 h post fertilization (hpf) (Cao et al., 2018; Cao et al., 2019a). At this stage, embryos are easily discerned as being fertilized and

viable, and equal animals are used in biological replicates. Ten embryos were assigned randomly to the control (ERM alone or 0.1% DMSO solvent) or one of four treatments of propiconazole (e.g. 0.1, 1, 10, or 100 μM) (34.2 μg/L, 342 μg/L, 3.42 mg/L, and 34.2 mg/L respectively). These concentrations were selected to assess the upper limit of environmental levels as well as supraphysiological concentrations to discern the potential for MOAs of propiconazole in fish (i.e. mitochondrial dysfunction). However, we point out that propiconazole at environmental or supraphysiological levels may exert effects on the fish differently (i.e. mode of action may differ with concentration). The DMSO solvent control had a final concentration of 0.1%, as this level of DMSO has been shown to have negligible effects for development, deformity, and proteins involved in the heat shock response (Hallare et al., 2006). This final concentration of DMSO was used throughout the study for all endpoints assessed.

For developmental exposures, ten embryos from each breeding event were first determined fertilized and viable, and then randomly selected and placed into sterile glass beakers. Each experimental group was represented by 4–6 beakers of 10 fish, which varied based on the number of embryos collected and available for the developmental assays. The beaker was considered the biological replicate. Each beaker contained 10 ml of ERM. Five exposures in total were conducted, and the total sample size for all experiments combined was as follows (ERM = 19, DMSO = 33, 0.1 μM = 24, 1 μM = 29, 10 μM = 35, and 100 μM = 10). Embryos and larvae were exposed at 26 ± 1 °C for different lengths of time depending on the endpoint measured. Fresh chemical in ERM was added in a 90% ERM change every 24 h.

For deformities, hatch percent, and survival analyses, zebrafish were assessed over 96 hpf. Cumulative mortality was recorded over the exposure period. Five independent experiments in wildtype zebrafish were conducted over a two-year period. For each experiment, zebrafish were scored under a microscope daily for deformities (pericardial edema, scoliosis), pigmentation, hatch time, and survival. Deformities, mortality, and hatch rate were scored individually on an Evos® FL Auto Cell Imaging Microscope, version 1.6 (Revision 31201, ThermoFisher Scientific) from time-lapse picture files for onset as well as severity of edema. Edema was measured as an ellipse size using the first clear picture from the end of the recordings (91.5–98.5 hpf) in the Evos® FL Auto software. Experiments indicated a high incidence of pericardial edema in zebrafish, and to explore this effect in more detail, we used a transgenic zebrafish line (globin-LCR:eGFP) to better visualize the patterns of blood circulation in the fish during exposure. Experiments for globin-LCR:eGFP zebrafish proceeded as that outlined above for 96 hpf and was conducted once.

2.4. Mitochondrial respiration measurement

For mitochondrial bioenergetics, embryos were exposed for 24 h, starting at ~6 hpf. Measurement of oxygen consumption rate followed our protocols for fungicides (Cao et al., 2019b; Cao et al., 2018). The XFe24 Extracellular Flux Analyzer (Agilent) was used to measure oxygen consumption rates (OCR) in zebrafish. A Utility Plate was filled with 1 mL of XFe Calibrant fluid in each well and incubated with the sensor cartridge overnight at 28 °C. Following plate calibration, one embryo was placed in each well of an Islet Capture Microplate with 525 μL of ERM as per our methods (Souders et al., 2018). The four experimental groups were as follows: ERM, 0.1% DMSO, 10, or 100 μM propiconazole (N = 5 embryos/treatment, each well containing a single embryo).

The following pharmacological agents were added sequentially to assess oxidative phosphorylation. The final concentrations per well were 9.4 μM oligomycin, 6 μM FCCP, and 20 mM sodium azide (Liang et al., 2017). The basal OCR of embryos was first measured with 12 measurement cycles. Oligomycin, an inhibitor of ATP production, was then added to measure oligomycin-induced OCR for 18 measurement cycles. FCCP was then added to determine FCCP-induced OCR, and 8 measurements were taken. Lastly, NaN_3 was added to inhibit mitochondrial oxidative respiration to measure NaN_3 inhibition of OCR with 24 measurement cycles. All cycles consisted of 2 min to mix, 1 min to wait, and 1 min to measure.

The assay was conducted at 26 ± 1 °C. OCR data were exported to PRISM (V6) using Wave Desktop 2.6 Software (Agilent Technologies, USA). Calculations for mitochondrial endpoints were conducted as follows: Basal respiration [defined as mean basal OCR measurement], oligomycin induced ATP-linked respiration [defined as (mean basal OCR − mean OCR following oligomycin injection)], FCCP-induced maximum respiration [mean maximum OCR measurement − final NaN_3 OCR measurement], spare capacity [difference between maximum respiration and (basal respiration − non-mitochondrial respiration)], proton leak [defined as difference between basal respiration and oligomycin-induced ATP-linked respiration], and non-mitochondrial respiration [defined as final plateaued NaN_3 OCR] were calculated as per Seahorse XF Cell Mito Stress Test Kit User Guide (User Guide Kit 103015-100, Agilent).

2.5. Visual Motor Response (VMR) Test

To assess behavioral responses in zebrafish larvae, we utilized a Visual Motor Response Test (alternating light-dark cycle) and measured total distance travelled (mm) over 1 min bins (i.e. measure of larval locomotor activity). Studies have used the Visual Motor Response Test to capture behavioral responses in zebrafish to neuroactive chemicals and pesticides (Cao et al., 2018; Cao et al., 2019a; Crosby et al., 2015). The most pronounced response in the test typically occurs in the last two cycles of dark (20–30 min and 40–50 min), as the fish are habituated to the first dark environment at the start of the assay and typically show reduced movement during this time compared to the later dark cycles (Fernandes et al., 2012; MacPhail et al., 2009). Solvents have been shown to affect zebrafish larval behavior (Chen et al., 2011), increasing activity in the VMP test. Thus, we include both an ERM and DMSO solvent control for behavioral assays and interpret results based on both controls.

To test locomotion, zebrafish larvae were treated with propiconazole from ~6 hpf to 150 hpf (~6 dpf). Three concentrations of propiconazole were assessed (0.1, 1.0 and 10 μM) in the VMR test. At 150 ± 1 hpf, zebrafish were randomly selected and placed into a 96-well culture plate that contained ERM. Any malformed or dead larvae were excluded from the assay. Treatments were randomized within the 96-well plate to reduce location-based test artifact. The 96-well plate was transferred to a DanioVision™ Observation Chamber (Noldus, Leesburg, VA). The plate was situated in a warm, circulating water bath (26 ± 1 °C). Zebrafish were allowed to acclimate and habituate for 20 min in the dark in the instrument.

At ~150 ± 1 hpf (6 dpf), the movement of fish were simultaneously and individually tracked using an infrared analog camera installed in the DanioVision™ Observation Chamber. All individuals were confirmed to be alive during the assay as movement data for each individual was individually inspected for appropriate tracking. The camera was connected to a USB port on the EthoVision® XT (Noldus Information Technology, Leesburg, VA) computer to digitize the analog signal. Larvae were tracked following a standard 50-minute light routine: 10 min dark, 10 min white light, 10 min dark, 10 min white light, and 10 min dark and data for total distance moved was collected.

Four behavioral trials were performed with N = 16–24 fish per group from different breeding pairs of fish. All experiments were performed in late afternoon (3:00–6:00 p.m.) to minimize variability due to circadian rhythms throughout the day. Data over time were graphed as distance moved (mean distance moved (mm) / minute). Data were binned into a mean for all fish every minute. In each of the 5 intervals, there were 10 data points (mean distance) collected for each minute of the 10 min period of light and dark.

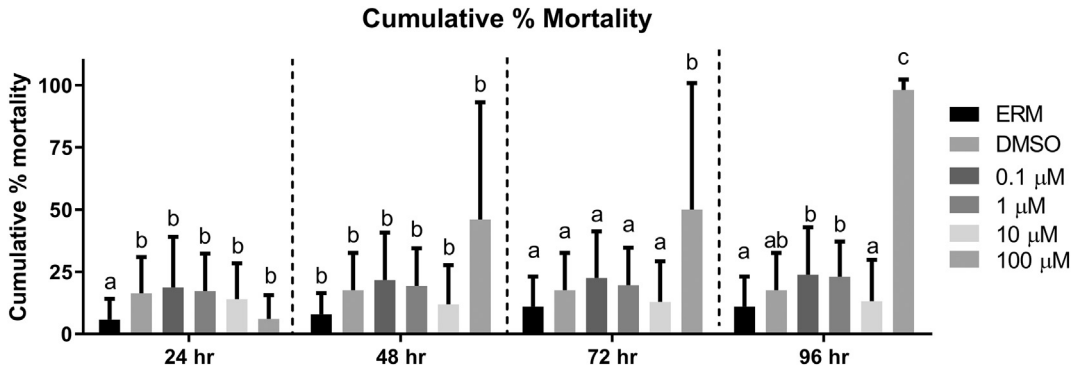

Fig. 1. Percent mortality of zebrafish embryos exposed to the fungicide propiconazole from 6 hpf until 96 hpf. Columns represent mean ± SD ($p < 0.05$, one-way ANOVA). Groups that do not share a letter are significantly different.

2.6. Statistical analysis

An ordinary one-way ANOVA along with Tukey's multiple comparison test was used to analyze differences among treatments for % cumulative mortality, hatch time, onset of melanization, area of pericardial space, and mitochondrial endpoints. For behavior, an ANOVA was used to analyze differences among treatments and a Holm-Sidak's multiple comparisons test (alpha = 0.05) was used for *post-hoc* correction. Alpha level was set at 0.05 for all statistical tests and p-value < 0.05 indicated significant difference between groups. Data are expressed as mean ± standard deviation for the majority of assays except the behavioral assays in Fig. 6. Data are expressed as mean ± SEM to better portray the patterns in behavior.

3. Results

3.1. Mortality, time of hatch, and melanization

Exposure to propiconazole resulted in significant mortality at 100 μM (Fig. 1) compared to the ERM and solvent control groups ($p < 0.05$). Zebrafish embryos exposed to 100 μM did not hatch but some remained viable for 3–4 dpf. Environmentally relevant concentrations of propiconazole (0.1 μM) did not affect mortality, and mortality among groups did not differ up to 10 μM propiconazole. By 96 h, there was close to 100% mortality for zebrafish exposed to 100 μM propiconazole. This was a consistent finding across assays and the data are compiled into one figure.

There was no difference among groups in hatch time (Fig. 2A) ([$F_{(DFn,\ DFd)}$, p value]; $F_{(3,\ 66)} = 0.18$, $p = 0.91$). The highest dose of 100 μM propiconazole is not shown in the figure because no larvae hatched alive in this treatment. However, there were a small subset that were photographed for pigmentation that were dead just after hatching. Noteworthy was that in the 100 μM propiconazole treatment, approximately 33% of fish showed a loss of melanin (hypopigmentation) early on as embryos, and some zebrafish had a complete absence of the pigment after exposure to 100 μM (see Fig. 3E for example). We also measured the onset of pigmentation and there was a significant difference among treatment groups ($F_{(4,\ 83)} = 3.7$, $p = 0.009$) (Fig. 2B). Zebrafish treated with 10 μM propiconazole showed a 2–3 hour delay in first pigmentation compared to the ERM control but not the DMSO control. No other group differed in the onset of pigmentation. Noteworthy was that zebrafish in the 100 μM exposure showed higher variability in the onset of pigmentation compared to the control groups. The signs of first pigmentation occurred around 20–40 hpf.

3.2. Pericardial edema

The most prevalent deformity was pericardial edema following exposure to propiconazole. Some fish in the control groups (ERM and DMSO) presented with pericardial edema (~10%) in our assay, however there was a dramatic increase in the prevalence of pericardial edema with higher concentrations of propiconazole; 55% in the 1 μM treatment, and > 95% in the 10 μM and 100 μM experimental groups. We measured the area around the cardiac tissue and found that fish exposed to 10 μM showed a trend towards increased pericardial area compared to fish in the ERM groups ($F_{(3,\ 62)} = 5.18$, $p = 0.0029$) (Fig. 2C) but this was not different from the DMSO control. Supplemental Figs. S1 and S2 depict the severity of pericardial edema in both embryonic and larval zebrafish. Due to the presence of pericardial edema, we further investigated the pattern of blood flow during development using a transgenic zebrafish line (globin-LCR:eGFP). At 2 dpf, fish exposed to 100 μM propiconazole appeared to have hemoglobin that predominantly remained in the tail, the site where hematopoiesis typically occurs in developing zebrafish embryos. Melanin

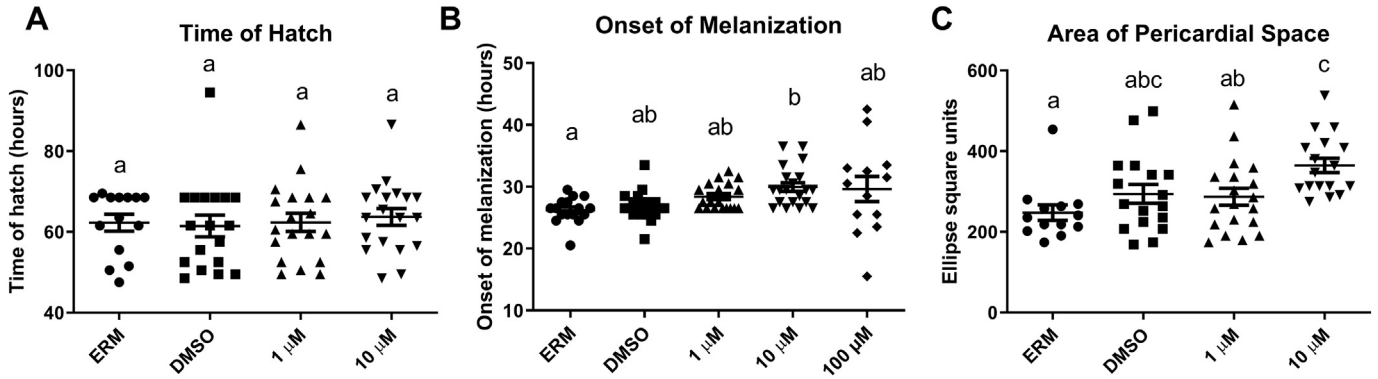

Fig. 2. (A) Time of hatch, (B) onset of melanization, (C) area of pericardial space. Each point represents an individual. The mean ± SD is shown within the data points. Groups that do not share a letter are significantly different ($p < 0.05$, one-way ANOVA).

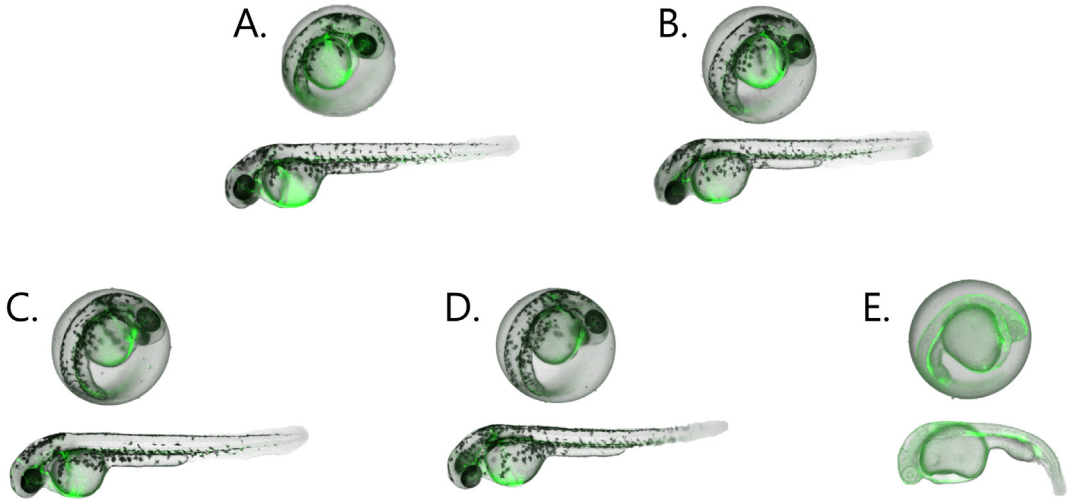

Fig. 3. Transgenic zebrafish (globin-LCR:eGFP) exposed to propiconazole. Photos were taken at approximately 50 hpf (embryos) –as part of a time lapse using an EVOS digital microscope. A) ERM only. B) 0.1% DMSO (solvent control). C). 1 μM propiconazole. D) 10 μM propiconazole. E) 100 μM propiconazole (representative fish is dead but one can see the absence of pigment).

was also significantly reduced or lost in these embryonic fish (Fig. 3A–E). At 3 dpf, zebrafish exposed to 10 μM appeared to have significant pooling of blood cells in in the pericardial region. Hemoglobin and red blood cells appeared to have fallen out of circulation, as indicated by the diffuse GFP signal within and outside the cardiac tissue (Supplemental Fig. 3A–D).

3.3. Oxidative consumption rates

Mitochondrial respiration was measured in 30-hour zebrafish embryos treated with either 10 or 100 μM propiconazole for 24 h (Fig. 4). Basal respiration significantly varied across treatments ($F_{(3, 16)} = 11.06$, $p = 0.0004$). Basal respiration of zebrafish exposed to 100 μM showed a 50–60% mean decrease when compared to either control (ERM and DMSO) ($p < 0.05$ and $p < 0.01$ resp.) (Fig. 5A). There was no difference in mean basal respiration between fish in the control groups versus those in the 10 μM treatment. There was also a significant difference among groups for oligomycin-induced ATP-linked respiration ($F_{(3, 16)} = 10.71$, $p = 0.0004$) (Fig. 5B). ATP-linked respiration in embryos treated with 100 μM for 24 h were decreased ~70% compared to both the ERM and DMSO group ($p < 0.05$ and $p < 0.01$ resp.). There was no difference in oligomycin-induced ATP-linked respiration between fish in the control group versus those in the 10 μM treatment. FCCP-induced maximal respiration was affected in zebrafish embryos after 24 h ($F_{(3, 16)} = 5.47$, $p = 0.0088$) (Fig. 5C). There was a difference between fish in the DMSO solvent compared to fish exposed to 100 μM but no difference from the ERM control. Groups did not differ in regards to spare capacity ($F_{(3, 16)} = 1.86$, $p = 0.18$) (Fig. 5D). Proton leak varied significantly across treatment groups ($F_{(3, 16)} = 7.93$, $p = 0.0018$) (Fig. 5E) and this endpoint was different in zebrafish exposed to 100 μM compared to both ERM and DMSO control ($p < 0.01$). Proton leak was decreased by ~30%. Lastly, non-mitochondrial respiration was different among groups ($F_{(3, 16)} = 21.12$, $p < 0.001$) (Fig. 5F), and zebrafish treated with 100 μM propiconazole were different that zebrafish in the ERM, DMSO, and 10 μM treatments by approximately 50%.

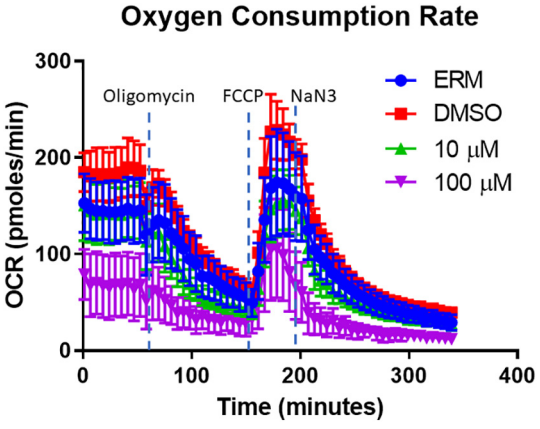

Fig. 4. Oxygen consumption rates for control (ERM), solvent control (0.1% DMSO), 10 μM, and 100 μM propiconazole over time in zebrafish (mean ± SD). Injection periods are indicated with the dotted line for reach chemical stressor for mitochondrial function.

3.4. Visual Motor Response Test

Exposure to propiconazole induced changes in the activity of zebrafish larvae based upon the Visual Motor Response Test. Four independent trials were conducted using fish from different parents (Figs. 6, 7). During the dark cycle (20–30 min), larvae exposed to 10 μM propiconazole became hypoactive in the last 5 min of the assay [Experiment 1, $F_{(3, 16)} = 15.99$, $p < 0.0001$; Experiment 2, $F_{(3, 16)} = 6.55$, $p = 0.0042$; Experiment 3, $F_{(4, 20)} = 6.67$, $p = 0.0014$; Experiment 4, $F_{(4, 20)} = 8.72$, $p = 0.0003$]. A decrease in activity was also observed in the last dark cycle of the assay (45–50 min) [Experiment 1, $F_{(3, 16)} = 4.83$, $p = 0.014$; Experiment 2, $F_{(3, 16)} = 17.17$, $p < 0.0001$; Experiment 3, $F_{(4, 20)} = 6.67$, $p = 0.0014$; Experiment 4, $F_{(4, 20)} = 9.51$, $p = 0.0002$].

Taken together, the data suggest that behavior at 10 μM is different when compared to the control groups.

4. Discussion

Triazoles are azole-based fungicides that are used in agriculture, and some are also used in human and veterinary medicine for the treatment of fungal infections (Kjærstad et al., 2010). We conducted experiments to address the potential for developmental toxicity in developing zebrafish. We observed significant mortality with 100 μM propiconazole and fish did not hatch at this concentration. There were no differences in hatch time for lower, environmentally relevant concentrations. Pericardial edema was prevalent in embryos prior to hatch with 10 μM exposure (48 h), and in larval fish later in development. We

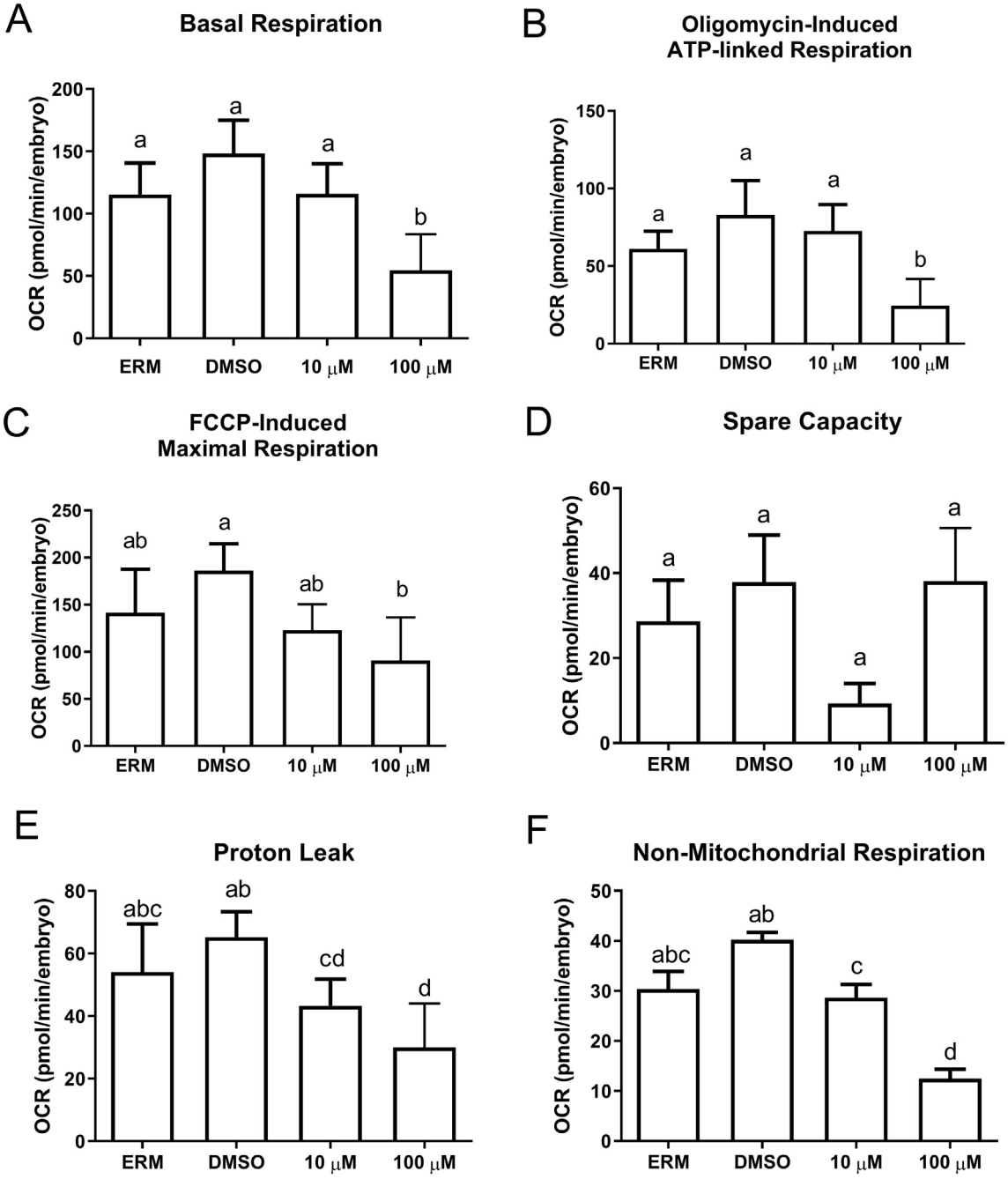

Fig. 5. (A) Basal respiration, (B) oligomycin-induced ATP-linked respiration, (C) FCCP-induced maximal respiration (D) spare capacity, (E) proton leak, and (F) Non-mitochondrial respiration. Columns represent mean ± SD ($p < 0.05$, one-way ANOVA). Groups that do not share a letter are significantly different.

also observed significant pooling of hemoglobin in the heart region and in the tail. Other exposures conducted in carp and rainbow trout have linked propiconazole to significant decreases in hemoglobin concentrations and red blood cell counts, as well as other hematological parameters (Hemalatha et al., 2016; Li et al., 2010b).

The literature reports that other triazole fungicides cause deformities such as yolk sac edema, pericardial edema and spine deformation. Mu et al. (2016) reported that zebrafish embryos exposed to difenoconazole for 4 dpf at 0.5 to 2.0 mg/L showed hatching regression, decreases in heart rate, growth inhibition, and teratogenic effects (yolk sac edema, pericardial edema and spine deformation). Another study by Liu et al. (2010) found that triadimefon caused a dose-specific effect on malformations such as bent spine, uninflated swim bladder, inability to stay righted, pericardial edema, and yolk sac edema in zebrafish 120 hpf. A study by Şişman and Türkez (2010) showed that zebrafish embryos exposed to imazalil (i.e. enilconazole) at 5, 10, 20, 50 and 100 uM for 144 hpf showed a reduction in melanin pigmentation, as well as a wavy notochord, crooked trunk, tail defect and pericardial edema. In another study, zebrafish embryos/larvae exposed to concentrations of cyproconazole > 50 μM showed decreased spontaneous movement, impaired hatching percent, and reduced heartbeat (Cao et al., 2019a). In the study, which tested concentrations ranging 1–500 μM, malformations (i.e., pericardial edema, yolk sac edema, tail deformation, and spine deformation) were observed with exposure to ≥ 50 μM cyproconazole. Significant increases in cumulative deformity rate were also observed at 48, 72, and 96 hpf. In a recently published study exposing zebrafish to 2.5–4.5 mg/L propiconazole over the first 5 days of development, fish exhibited spinal deformities and tail

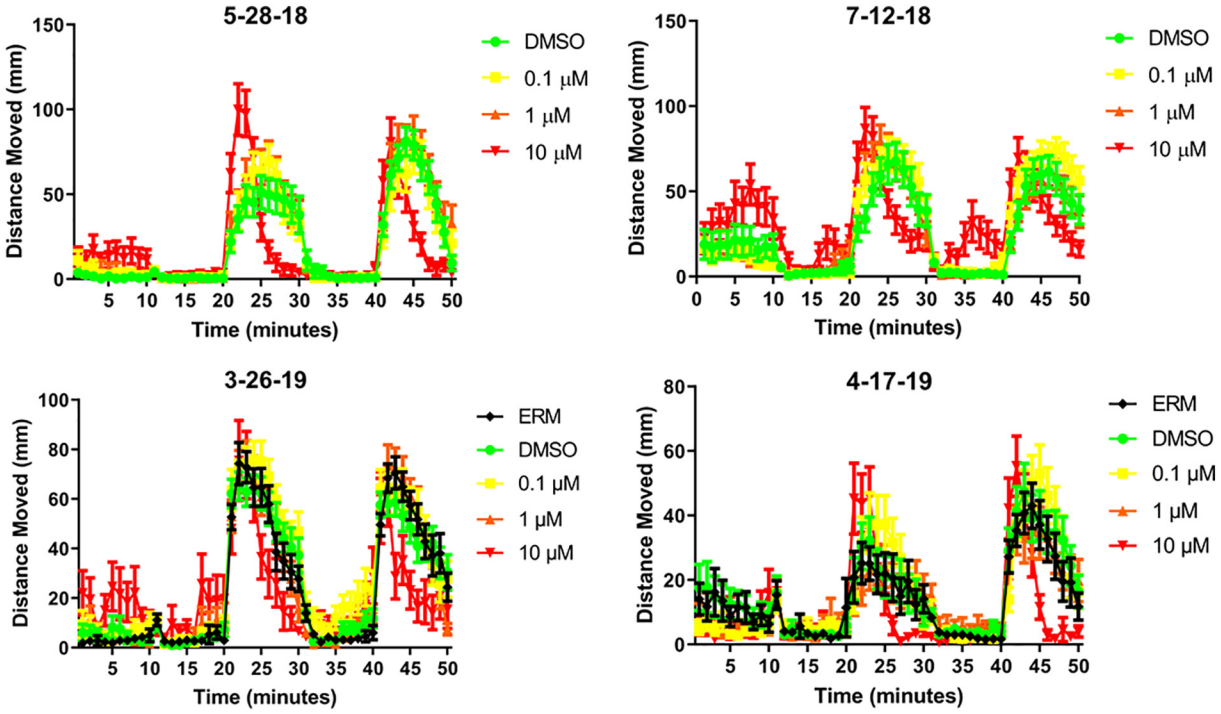

Fig. 6. Distance moved over the 50 min Visual Motor Response Test. For improved visualization of the graphs, the mean ± SEM is shown rather than SD. SD is used in Fig. 7. Four tests were conducted, indicated by the date.

curvatures (Teng et al., 2019). Data with other azole fungicides are consistent with our findings in that we observed significant hypopigmentation and pericardial edema in embryos exposed to propiconazole.

Hypopigmentation was observed in fish exposed to 100 μM propiconazole, and zebrafish exposed to higher concentrations of the fungicide took longer to develop their pigment, if they were able to do so. Melanin is a pervasive pigments found in nature, which apart from UV-absorption, serves important protective roles in the organism (Solano, 2014). In many pathogenic fungi, melanin expression has been shown to be a virulence factor (Eisenman and Casadevall, 2012). In zebrafish, melanin is involved in background adaptation and predator avoidance, and as such, pigment dispersal has been shown to respond to norepinephrine and epinephrine and might be an important rapid indicator of fear and anxiety. Melanogenesis is regulated through a variety of receptors that activate adenyl cyclase and cAMP signaling, including α and β estrogen receptors and G protein-coupled estrogen receptors (Natale et al., 2016). Azole fungicides have been shown to affect a variety of heme functional groups, including those in hemoglobin and cytochrome P450 monooxygenases (CYPs) (Hemalatha et al., 2016). Propiconazole is a low potency estrogen receptor and androgen receptor antagonist, which can reduce levels of testosterone and estrogen (Kjærstad et al., 2010). This loss of steroid regulation may explain the loss of melanogenesis following exposure to the fungicide as steroids are intimately involved in the process of melanogenesis (Colanesi et al., 2012). Interestingly, the suppression of melanin has also been reported in fungi exposed to high doses of propiconazole (Kaverinathan et al., 2017; Lendenmann et al., 2014) underscoring the high evolutionary conservation of the pathways underlying propiconazole toxicity.

Imbalance in oxidative homeostasis can occur following fungicide exposure. Propiconazole decreased OCR in embryos at 100 μM compared to fish exposed to the solvent or the ERM control following the 24-hour exposure. The fungicide at 100 μM educed oligomycin-induced ATP linked respiration and proton leak in embryos, suggesting mitochondrial dysfunction. However, it is important to note that these responses occurred at levels that are well above those detected in surface waters. Previous studies demonstrate that exposure to propiconazole leads to altered metabolism and induces oxidative damage responses in fish. For example, *Channa punctate* with an average weight of 80–100 g were assigned into one of three groups (control, 0.5 ppm

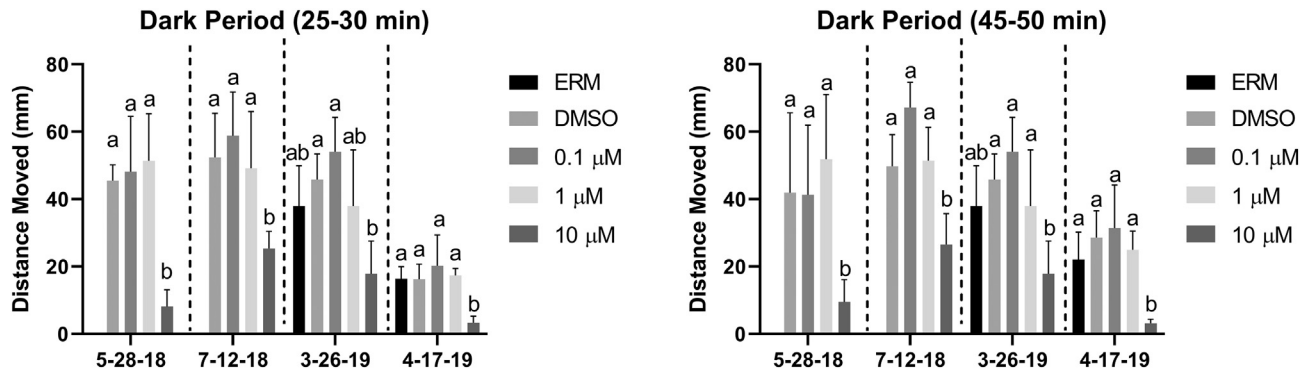

Fig. 7. Distance moved in the second and third dark period. Recorded are the last 5 min of the period. The mean ± SD is shown for each group. Groups that do not share a letter are significantly different ($p < 0.05$, one-way ANOVA).

propiconazole, or 5 ppm propiconazole) and exposed to the fungicide for 96 h (Tabassum et al., 2016). There were clear responses for biochemical and enzymatic biomarkers, such as catalase activity, glutathione peroxidase, glutathione S-transferase, and change in thiobarbituric acid reactive substances. Kumar et al. recently exposed zebrafish embryos to different azole fungicides and reported a decrease in spare capacity in 48-hour embryos after exposure to 1 mg propiconazole/L. There was no change in basal respiration, maximum respiration, and ATP-linked respiration. Our results differ in that we observed no effect on spare capacity in embryos exposed to propiconazole for 24 h. However, we observed that OCR did not differ between propiconazole-treated fish and controls (both ERM and DMSO) until doses reached 34 mg/L (100 μM). Thus, differences may be due to the concentrations used in each study. An important point to make is that oxygen consumption can vary based on the stage of the embryo due to mitochondrial biogenesis (Stackley et al., 2011) and it is expected that chemical will affect OCR measurements in a temporal fashion as demonstrated for other contaminants, such as that described for tributyltin (Liang et al., 2017).

Conazole fungicides have been shown to alter OCR in zebrafish embryos. Tebuconazole, another triazole fungicide used on vegetables and grains, also decreased basal OCR in zebrafish embryos at 100 μM, and similar to propiconazole, did not have effects on OCR in embryos at environmentally relevant levels. In another study, the azole fungicide cyproconazole impaired mitochondrial bioenergetics in zebrafish embryos, however this chemical required concentrations in upwards of 500 μM, well above environmentally relevant concentrations (Cao et al., 2019a). This fungicide affected both basal respiration and oligomycin-induced ATP production, but not FCCP-induced maximum respiration nor non-mitochondrial respiration. Thus, for the azole fungicides currently tested in mitochondrial bioenergetics assays, both tebuconazole and propiconazole appear to affect mitochondrial bioenergetics at lower doses compared to cyproconazole. Conazole fungicides, such as propiconazole, are predicted to have multiple mechanisms of action based on high-throughput cell-based (e.g. mitochondrial dysfunction and oxidative damage) (Mihaich et al., 2017a). Differences in structural moieties among conazole fungicides may explain the broad range of potencies when assessing OCRs in the mitochondrial stress test. As mentioned above, propiconazole is reported to induce oxidative stress responses in fish, thus altered mitochondrial bioenergetics may be a precursor to this redox imbalance and oxidative stress. Conversely, changes in mitochondrial bioenergetics may also reflect a secondary response to increased oxidative damage. Additional research is needed to determine whether the mechanism is specific among triazole fungicides on the mitochondria, or whether this is a general indicator of toxicity.

Propiconazole induced hypoactivity in zebrafish larvae in the Visual Motor Response Test. Disruptions in zebrafish larval behavior have been reported for other conazole fungicides. In a recent study, exposure to tebuconazole decreased larval activity at 10 μM (3.08 mg/L), while disruptions in activity at lower doses were less consistent among clutches of fish (Perez-Rodriguez et al., 2018). In this study, when distance travelled was binned for tebuconazole, there was no effect on the mean distance travelled within the 10-minute intervals from light to dark. This suggests that tebuconazole may have more of a pronounced effect on behavior. However, noteworthy was the pattern for propiconazole. We observed hypoactivity, but only over the second half of the dark period (i.e. we posit the fish may have reduced energy stores compared to controls for maintaining prolonged swimming over the entire period). This type of behavioral response was not observed for tebuconazole and may reflect a unique mechanism of toxicity yet to be explained for propiconazole.

Two recent studies also report on zebrafish locomotor behavior following exposure to propiconazole. Teng et al. (2019) exposed zebrafish to 2.5–4.5 mg/L continuously with daily water changes and assessed locomotor activity in larvae at 120 hpf. The authors reported that propiconazole decreased average velocity, total distance moved, and average acceleration compared to the control group at concentrations of 2.5 mg/L and above. These data correspond to our results, and we observed a significant decrease in locomotor activity in the dark period at 10 μM or 3.42 mg/L. Conversely, Kumar et al. (2019) reported that there were no discernable effects of propiconazole on locomotor activity in zebrafish at 5 dpf at doses up to 1 mg/L. Thus, based on these studies, and data generated here, propiconazole may not affect locomotor activity until ~2.5–3.5 mg/L in early staged zebrafish larvae.

Other studies have reported on the behavioral effects of conazole fungicides. Altenhofen et al. demonstrated that zebrafish showed hypoactivity during larval exploratory behavior with exposure to 1, 2 and 4 mg/L tebuconazole for 5 days (Altenhofen et al., 2017). In addition, exposure to 10–300 μg/L imazalil, another conazole fungicide and one similar to tebuconazole, also decreased locomotor activity in zebrafish the light-dark assay (Jin et al., 2016). Moreover, a sub-lethal range of carbendazim (0.16, 0.8, 4, 20, 100 and 500 μg/L), a broad-spectrum benzimidazole fungicide, was used to assess locomotor activity in zebrafish (Andrade et al., 2016). In the study, the total distance moved decreased with increasing concentrations of carbendazim in the light cycles. There is also some evidence for hypoactivity induced by another azole fungicide, cyproconazole. The activity of zebrafish in the dark period of the Visual Motor Response Test was significantly reduced in individuals exposed to concentrations ranging from 1 to 25 μM cyproconazole (Cao et al., 2019a). Thus, data support the hypothesis that conazole fungicides induce hypoactivity in larval zebrafish, however the response of larvae to propiconazole appears somewhat unique in that it the hypoactive state in the dark periods does not appear until the last 5 min of the assay. Additional studies will be required to clarify the neurotoxic mechanisms underlying propiconazole exposure in zebrafish.

In summary, we demonstrate that propiconazole can affect development, mitochondrial respiration, and behavior in early staged zebrafish. Propiconazole is suspected to have a MOA that includes mitochondrial dysfunction (Mihaich et al., 2017a), and more sensitive measures of mitochondrial dysfunction may be required to elucidate these effects (e.g. mitophagy, altered membrane potential). We report on a unique pattern not observed previously with other conazole fungicides, and this requires additional work with propiconazole to determine the mechanisms of neurotoxicity.

Transparency document

The Transparency document associated this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ntt.2019.106809.

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