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# The organophosphate insecticide diazinon and aging: Neurobehavioral and mitochondrial effects in zebrafish exposed as embryos or during aging

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ABSTRACT

Organophosphate (OP) compounds comprise one of the most widely used classes of insecticides worldwide. OPs have been shown to have negative human health impacts, particularly developmental neurotoxicity. However, neurotoxic impacts in later adulthood and during the aging process are relatively uncharacterized. The present study examined diazinon (DZN), an OP, to determine the neurobehavioral consequences, in addition to mitochondrial dysfunction on a macroscale (whole organism basal respiration) and on a microscale (whole organ mitochondrial respiration), using zebrafish (ZF) as a model. One group of 14-month-old adult ZF were exposed acutely as adults (0.4, 1.25, and 4.0 μM) for five days and tested as adults, and another group was exposed developmentally 5–120 h post-fertilization (70, 210, and 700 nM) and tested at larval, adolescent, adult, and aging life stages. ZF exposed acutely as adults did not display many significant neurobehavioral impacts or mitochondrial dysfunction. Conversely, the embryonically exposed ZF showed altered behavioral functions at each stage of life which emerged and attenuated as fish transitioned from each developmental stage to the next. Mitochondrial oxygen consumptions measurement results for developmentally DZN exposed ZF showed significant increases in the low and middle dose groups in organs such as the brain and testes. Overall, there is an indication that early developmental exposure to DZN had continuing adverse neurobehavioral and cellular consequences throughout their lives well into adulthood and aging periods.

## 1. Introduction

Organophosphate (OP) compounds are esters of phosphoric acid widely used as insecticides. Their oxon metabolic products inhibit acetylcholinesterase, which slows the breakdown of acetylcholine and stimulates cholinergic systems in the brain, sympathetic and parasympathetic nervous systems. Neurotoxic effects of low dose OP exposure are seen in mammals particularly after a developmental exposure. Adverse neurotoxicity is seen even at doses lower than those that inhibit acetylcholinesterase (Slotkin et al., 2006). Very potent OPs have been used as nerve gases (e.g. sarin, soman, tabun, and VX). More modestly potent OPs are widely used as insecticides. Worldwide, there have been nearly 300,000 deaths from OP poisoning with millions more people exposed to lower sub-lethal levels of the numerous OP insecticides in agricultural settings and in the household through products such as ant and roach sprays (Robb and Baker, 2019). Widely-used OP insecticides have included parathion, methyl parathion, malathion, dichlorvos, and chlorpyrifos (CPF). Parathion and methyl parathion are completely banned in the U.S., but the rest are still broadly used in homes, agriculture, and other landscapes, such as golf courses. Another common OP insecticide is diazinon, which currently is only allowed for use on commercial farms, not in residences, after a buy-back program started in 2004 when literature indicated it was a human health risk (Rush et al., 2010).

Diazinon (DZN), O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, is a thiophosphoric acid ester in the OP insecticide family. DZN has a variety of adverse impacts on human health, primarily deriving from its neurotoxicity. Its principal mechanism of action is through its oxon increasing acetylcholine concentrations, via acetylcholinesterase inhibition, that leads to overstimulation of cholinergic receptors causing seizure activity and excitotoxic neuronal death (Lallement et al., 1998). Such cell death following OP exposure is associated with oxidative stress and perturbations of mitochondrial function (Farkhondeh et al., 2020). Non-cholinergic

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perturbations are also likely, and may be particularly important for understanding the risk posed by lower doses with minimal cholinesterase inhibition, including effects on g-protein related mechanisms such as M2 muscarinic receptors, CB1 cannabinoid receptors and adenylyl cyclase, as well as effects on serine hydrolases and proteases, and proteins required for intracellular transport (see review, Terry Jr, 2012). The net effect of these effects would be disruption of various key cellular processes and acute neural dysfunction.

Epidemiological studies have examined the neurological impacts of DZN in humans who were residentially and occupationally exposed to the chemical, either developmentally or in adulthood. For example, preschool-aged children living near agricultural areas have been reported to perform poorly on memory and attention tests compared to their counterparts that did not live near farms (Rohlman et al., 2005). A range of similar studies suggest that in utero or childhood exposures to OPs can result in psychiatric issues in childhood, including increased odds of ADHD diagnosis and deficits in working memory, processing speed, executive function, and other cognitive skills (see review, Sapbamrer and Hongsibsong, 2019). Adult exposure to OPs can also result in behavioral impairments. For example, adult sheep farmers were reported to have higher rates of anxiety and depression and perform poorly on tests that examined cognitive function and mental health (Sanne et al., 2003; Stephens et al., 1995). The effects of chronic organophosphate exposure in adulhood have been more broadly described as a syndrome called chronic organophosphate–induced neuropsychiatric disorder (COPIND), characterized by peripheral motor effects, sensory alterations, mood instability and cognitive impairments such as attentional, memory and executive functioning problems (see review, Stallones and Beseler, 2016).

In addition to short-term effects, aging is a high-risk factor for some of the most prevalent human diseases. Dementia and neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and Amyotrophic Lateral Sclerosis become more prevalent with aging, and these adverse outcomes are associated with chronic exposures to pesticides in adulthood (Mostafalou and Abdollahi, 2018; Sánchez-Santed et al., 2016). The exact mechanisms of this increased risk is still unclear for adult exposures, and it is not yet established whether early developmental exposures to organophsophates carry similar risks. Of the various potential mechanisms which could be targetted by OPs in the long term, cholinergic functions are obvious targets for higher doses, but other vulnerable systems should also be considered. Mitochondria are thought to play a role in risk for diseases in aging since they often interact with proteins associated with these diseases and regulate cell survival and death. As mitochondrial DNA mutations increase over time, the net production of reactive oxygen species increases and causes oxidative stress (Lin and Beal, 2006). Oxidative stress leads to free radical attack on neural cells that causes neurodegeneration (Chen et al., 2012). These diseases are most often diagnosed in older adults and contributing factors, including the role of exposure to environmental toxicants, are still not well understood.

Studies using model organisms to examine neurological function after exposures to DZN, and other OP insecticides, during critical periods of development support the epidemiological evidence that exposure leads to adverse behaviors. Seven-day old zebrafish larvae embryonically exposed to CPF, an OP like DZN, had decreased larval activity (Cao et al., 2018), while fish at five months old has disruption in behaviors such as choice accuracy and motor function (Levin et al., 2003). Little data are currently available on DZN using zebrafish, although existing studies suggest that early exposures may still lead to lifelong risks (Bailey et al., 2014; Yen et al., 2011). More data are presently available from rodent studies. In rats, early postnatal exposure to DZN did not impact learning and locomotor tests at six weeks of age, but at eleven and twelve weeks of age, substantial effects of DZN were revealed, including behavioral abnormalities in adolescence and adulthood (Timofeeva et al., 2008; Roegge et al., 2008). Neuronal cell number and size were also measured in rats at 30, 60, and 100 days old after a postnatal exposure to DZN and increased cell-packing density was found, indicative of neuronal loss, which could explain some aspects of adverse behaviors (Slotkin et al., 2008). Early life exposures to DZN were also associated with altered neurotransmission within serotonergic, cholinergic, and noradrenergic systems (Slotkin et al., 2019; Slotkin et al., 2017). Of importance to work on aging outcomes, our own rodent work has also shown that early exposure to another OP, parathion, leads to cognitive and serotonergic/cholinergic alterations which emerge in late adulthood, rather than being persistent until aging (Levin et al., 2010). Work with these animal models generally suggests that developmental exposures to OPs may have lasting impacts on neurobehavioral functions and neural function. However, the developmental context and regulation of OP effects remains unclear in many cases, and a more thorough analysis of trajectories of impairment is still needed.

While persistent symptoms following exposure to OPs and future risk for neurodegeneration could be attributed to losses in neuronal cells and perturbations in neurotransmission, the potential for persistent changes in intracellular function should also be considered. Mitochondrial function is sensitive to environmental disruption, and this system is known to go through aging-related alterations (Chistiakov et al., 2014). Any lasting impacts of a prior exposure on these systems may then contribute to unique outcomes in both typical senescence and risk for neurodegeneration. However, little data are presently available to evaluate the potential connections between age-related changes in behavioral outcomes following OP exposure and bioenergetic status.

The goal of this experiment was to determine the interactions of maturation, aging and neurotoxicant effects through two studies, with one study examining early developmental toxicant exposure, and a second study examining later toxicant exposure effects on neurobehavioral and mitochondrial function in the zebrafish (*Danio rerio*) model. In the first study (referred to as study 1), a group of larval zebrafish were embryonically exposed to DZN (e.g. a critical developmental period). In the second study (referred to as study 2), an adult group of zebrafish was acutely exposed (14 months, pre-scenescent/prior to age-related increases in spontaneous death and behavioral dysfunction). Study 1 sought to answer how fish behaviors change over the course of their lifespan after they were embryonically exposed to DZN and to determine whether these early life exposures lead to altered cellular respiration in late adulthood. Study 2 sought to compare these findings with fish exposed as late adults, to provide an indication as to whether behavioral and mitochondrial functions will be similarly or differentially disrupted by DZN exposures at these two different developmental stages.

The zebrafish was the model organism in this research project because of several notable advantages. Zebrafish are an established model for early developmental and are increasingly being adapted for use in aging research, based on a strong degree of sequence and functional homology with humans (Bailey et al., 2014). Zebrafish share 71.4% of their genes with humans and 84% of those genes known to be associated with human disease have a zebrafish counterpart (Howe et al., 2013). In addition, zebrafish tissues and organs such as the brain, heart, muscles, kidney, and liver, have anatomical, physiological, and molecular similarities to humans (Dubińska-Magiera et al., 2016). These qualities make the zebrafish a suitable model to study various disorders based on conserved cellular mechanisms across vertebrates. Of particular value, these studies can leverage a cost- and space-effective, higher throughput in vivo model to examine lifespan development and aging outcomes. This model is also relatively novel for lifespan-length toxicity studies and the current work offers evidence of their value for this understudied area of toxicology. Although it is not an epidemiological study, following zebrafish throughout their life from 6 days old (human infant age) to 14 months old, and comparing data from a battery of behavior tests that they have experienced multiple times, can give an indication of the impacts or risk that similar exposures could have on humans.

## 2. Materials and methods

### 2.1. Fish care

Laboratory-reared wild-type zebrafish (AB*) were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) with a 14:10 h light/dark cycle. The water temperature was sustained between 27 and 29 °C with a pH of 6.8–7.0 during the rearing and behavioral testing period, and a pH of 7.0–7.4 during the metabolic phase of testing (shifted to new housing in between). Alkaline and neutral regulators (Seachem, Madison, GA, USA) and/or sodium bicarbonate were used to return the pH to these ranges if weekly checks found them to be outside those target ranges. This system water was supplemented with commercial salts (0.065% salinity). Fish were fed two times a day, once with brine shrimp (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and once with Zeigler's Adult Zebrafish Complete Diet (Zeilger Bros Inc., Gardners, PA, USA). All zebrafish care and husbandry procedures were approved by the Duke University Institutional Animal Care and Use Committee (Protocol Numbers A253–19-11 and A109–19-05). Cohorts of tested fish contained equal numbers of male and female zebrafish, as available within offspring bred for this study. Behavioral testing was conducted in fresh system water at room temperature.

### 2.2. Exposure and study design

Doses within each study were selected based on prior tolerability studies, which identified the lowest overtly toxic dose of DZN at each age and set out three doses, separated by ½ log units, decending from a high, sub-lethal/dysmorphogenic dose. In study 1, zebrafish were embryonically exposed to DZN from 5 to 120 hpf at three dose groups (70 nM, 210 nM, and 700 nM, diluted in 0.1% DMSO) and a vehicle control group (0.1% DMSO). Breeder tanks containing multiple male and female fish (10–20 adults per tank) were bred to create a heterogenous sample of fertilized eggs. These eggs were rinsed with a 0.001% bleach solution once and with clean system water twice, after which fertilized eggs were sorted out and placed into glass petri dishes at a density of 40 embryos per 40 ml of system water. DZN exposure began at 5 h post-fertilization (hpf), when system water was drained and replaced with a vehicle of 0.1% DMSO in system water containing 0-700 nM DZN. Exposure solutions were then replaced every 24 h until 122 hpf (5 days post-fertilization (dpf)), when the exposure ended. Each group was made up of fish from 2 replicate dishes. Testing was conducted at 6 dpf (larval effects), 2 months (adolescent effects), 8 months (full adult effects), and 14 months (late adulthood effects) which began with final behavioral testing and was followed by mitochondrial and respiratory testing. N's were set for 48 per group (24 per replicate) for the larval motility test. Sample sizes of available fish for further testing varied across the treatment groups, as only a percentage of viable fish embryos reach maturity, however these differences did not suggest reduced viability due to DZN exposure. At adolescence, survival was as follows: DMSO control 27, 70 nM DZN 43, 210 nM DZN 34, and 700 nM 42. All fish available were tested and final data was limited to an N of up to 30 (balanced between replicates) to reduce between-group variability in sample size. Sample sizes of analyzed data were between 21 and 30 for each group for any given test, based on survival to late adulthood and adequate motion tracking of collected videos (subject identified in >96% of images within video).

Novel tank test was the only test performed at the periadolescent time point, as prior pilot work has indicated that under current parameters of testing, the small size of the fish leads to poor automated motion tracking in the apparatus' used in the tap, shoaling, and predator test. The overall time windows of larval (infant), 2 months (adolescent), 8 months (full adult) and 14 months (late adult) were selected to observe differences in the expression of neurobehavioral toxicity during/after maturation and prior to the onset of senescence. The aging time point of 14 months was selected based on observations of increased spontaneous mortality at or after the age of 18 months in non-exposed breeder fish, and recently published increases in locomotor behavior by this time point relative to full adulthood (8 months) (Hawkey et al., 2020; Hawkey et al., 2021). This profile suggests that the 14 month time point provides meaningful developmental changes without deterioration in performance or increased mortality. Mitochondrial and respiratory testing followed the completion of the 14 month behavioral battery. In study 2, 14-month-old fish were subchronically exposed to DZN to produce three dosage groups (0.4 μM, 1.25 μM, and 4.0 μM, diluted in 100% DMSO to make stocks) and a control (0.1% DMSO) ($n = 15$ for each group) in static tanks containing 5 l of water. This exposure spanned five days and each day, between 12 and 1 pm, there was a water change with a fresh dose of DZN added. Behavioral testing on the battery began one week following adult exposures, and this was followed by mitochondrial and respiratory testing. A graphical summary of the exposure and testing sequence for Study 1 and Study 2 is provided in Fig. 1.

### 2.3. Light/dark larval motility test

The light-dark locomotion test (adapted from MacPhail et al., 2009) was performed on zebrafish larvae from study 1 at 6 dpf, which was 24 h following the end of the exposure period and following development of a swim bladder. This ensured that any effects detected on larval locomotion were due to toxic effects of DZN, rather than acute effects of exposure. Individual larvae were placed into single wells on a 96 well plate inside the DanioVision Observation Chamber (Noldus Information Technology, Wageningen, The Netherlands). The test duration was 50 min with a period of 10 min acclimation in the dark, and two alternating 10-min cycles of light and dark (100% and 0% illumination respectively from LED light pad underneath the 96 well plate). Fish run in this test came from two replicate exposure groups for each treatment, totalling 48 total fish tested per treatment group.

### 2.4. Behavioral assessment

All zebrafish surviving from study 1 and study 2 were tested on a battery of four behavioral tests, including the novel tank dive test, startle tap test, shoaling test, and predator avoidance test. All fish participated in each test, and at each age range within study 1 (2 months, 8 months, and 14 months). Only the novel tank test was performed at 2 months of age, due to previously detected difficulties in reliably tracking the movements of small fish in the tap and shoaling/predator apparatus'. Each test was conducted on a different day between 10 AM and 4 PM, after the fish were fed and acclimated to the testing room for 1 h before testing. An HD camcorder was used for recording the assays and EthoVision XT software analyzed the videos for fish tracking and activity analysis. All tests are well-established and commonly used to evaluate cognitive and sensorimotor tasks (Glazer et al., 2018a/2018b; Bailey et al., 2014).

#### 2.4.1. Novel tank dive test

The novel tank dive test (Levin et al., 2007) is a test with adult fish for anxiety vs. risk taking-like behavior and measures exploration in a new, unfamiliar environment for 5 min. Diving to the bottom of the tank constitutes anxiety-like behavior. Two adjacent 1.5-l plastic tanks were filled with 10 cm of colony tank water from the housing system. Each tank was shaped like a trapezoid— 22.9 cm long on the bottom, 27.9 cm × 6 cm at the top, 15.2 cm tall, 15.9 cm along the diagonal side, and 5.1 cm wide. Measurements collected via EthoVision software (Noldus Information Technology, Wageningen, The Netherlands) were locomotor speed (cm/min) and vertical position in the tank (cm from the floor) for each min. Control fish in this test show adaptation of exploratory behavior across the 5-min session, leading to significant (non-zero), positive slopes for activity and/or distance from the bottom of the tank across the 5-min session.

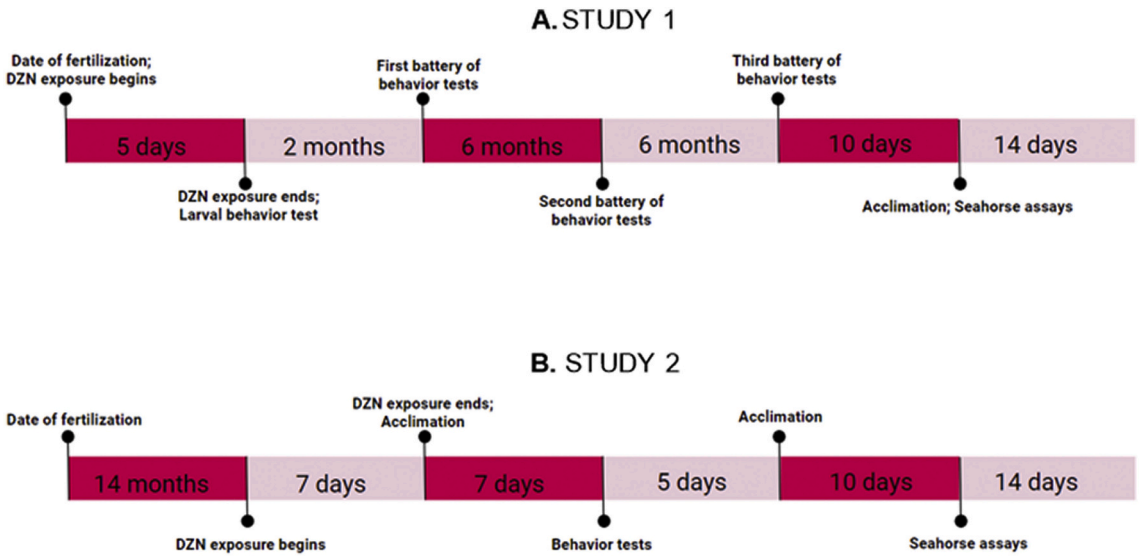


**Fig. 1.** Experimental timelines for A) study 1 and B) study 2.

### 2.4.2. *Tap startle test*

The tap test (Eddins et al., 2010) assesses sensorimotor startle and its habituation in adult fish. A 24-V DC push solenoid provides 10 sudden taps at 1-min intervals under each arena, containing a single fish and 40 ml of colony water. The testing apparatus consists of a flat white 23 × 29 cm surface with white, 23 × 27 cm barriers on the front and back. Eight clear, acrylic cylindrical arenas, 5.7 cm in diameter, wer attached onto the flat surface in two rows and each arena has slightly angled sides to enable visibility to the camera fixed overhead. Opaque screens separate the arenas and isolate fish from each other during trials. After netting and placement into each well, the tapping program was initiated and an acclimation delay of 30 s began. After 30 s, the first tap was delivered, with each successive tap being delivered at 1-min intervals. The total length of the test was 10 min. The total distance traveled in the 5 s before and after each tap was measured by EthoVision software (Noldus Information Technology, Wageningen, The Netherlands). Locomotor responses due to the tap (startle magnitude) were calculated by subtracting the pre-tap activity (5 s) from the post-tap activity. Control fish readily show adapatation of the startle response over successive taps, as indicated by a significant (non-zero), negative slope across the 10 startle responses.

### 2.4.3. *Shoaling test*

The shoaling test (adapted from Fernandes and Gerlai, 2009) is a proxy for peer recognition, where two 49.35 cm LCD monitors on either end of a test tank display either a still, control screen or a video of zebrafish swimming in a shoal. Adult fish were isolated singly for thirty min in 1.5-l tanks surrounded by blue opaque dividers, and then the fish were placed into the middle of the test tank. The test tank contained two lanes of equal size, separated by solid black plexiglas. Each lane was 9.5 cm wide, 30.5 cm long, and filled to a depth of 10 cm. The test was seven min long and after a two-min acclimation period, a video of shoaling fish appeared on one of the screens. Control shapes (ovals of equal size and striping as the projected zebrafish) were shown on the opposing screen during the video and on both screens prior to the video. Measurements gathered were swimming speed (cm per min) and the mean distance (cm) from the side of the tank on which the shoaling video is displayed for each min, as measured by EthoVision software (Noldus Information Technology, Wageningen, The Netherlands). Social approach responses were calculated by substracting the distance from the screen-adjacent side of the tank during the video presentation (test) from the distance from that side during the acclimation period (baseline). Control fish show a reduction in distance from the screen during the presentation of the video.

### 2.4.4. *Predator avoidance test*

The predator avoidance test (adapted from Luca and Gerlai, 2012) tests the avoidance response to an aversive stimulus, which is an enlarging circle with the appearance that it is approaching. This test is conducted in the same set arena as the shoaling test, with two monitors on either side of the test tank. Individual adult fish were placed in the lanes of the tank and after a 1-min acclimation, 8 min of alternating stimulus/no stimulus events were provided. Even numbered min (min 2, 4, 6 and 8) coincided with the presentation of a predator cue on one of the two screens, while odd numbered min (3, 5, 7 and 9) showed only blank white screens. The stimuli were either a blue, slow-growing dot or a red, fast-growing dot appearing in the first two (min 2 and 4) and the last two stimulus events (min 6 and 8), respectively. Stimuli were delivered via a PowerPoint presentation on one of the two screens. The opposing screen was blank throughout the testing session. Measurements as measured by EthoVision software (Noldus Information Technology, Wageningen, The Netherlands) were the swimming speed (cm/min) and the mean distance (cm) to the side of the tank on which the stimulus was displayed. Fleeing responses were represented as a pattern where fish remain further from the screen during predator cue presentations than during the intervening periods where no cue was presented. Control fish show elevated distances from the predator-adjacent side of the tank during the stimulus presentations and significant fleeing responses.

## 2.5. *Respirometry measurements*

Organismal routine metabolism ($RMO_2$) is the oxygen consumption rate for fish in a resting state and was measured using two mini 170 ml swim tunnel respirometers (Loligo Systems, Tjele, Denmark) that contain fiber optic oxygen transmitters (PreSens-Precision Sensing GmbH, Regensburg, Germany). Fish were isolated and restricted from food for 24 h before each measurement, which were obtained between 9 am and 5 pm. On the day of testing, fish acclimated for 1 h in the tunnel with a black tarp over the system and then for another hour, four oxygen consumption measurements (mg $l^{-1}$) were taken at 15-min intervals. Measurements lasted for 5 min and oxygen was replenished for 10 min, equating to four measurements in the hour. The highest number prior to turning the oxygen off and then turning it back on were subtracted from each other and an average was taken to obtain one value representing oxygen consummation rate (OCR) (mg $O_2$ $h^{-1}$). The OCR value was

divided by the mass, in grams, of the fish to obtain the $RMO_2$ measurement (μmol/l/g/5 min) (Jayasundara et al., 2015).

### 2.6. Tissue-specific OCR using XFe24 extracellular flux analyzer

Following the procedure outlined in Jayasundara et al. (2015), fish were anesthetized in ice water, weighed, measured, and euthanized by cervical dislocation. The heart, liver, brain, and sex specific gonads were removed from each fish, weighed, and if they exceeded 10 mg in mass, a portion was removed. Each tissue was placed in its own well of a XF24 islet capture plate (Seahorse Bioscience, Billerica, MA, USA) and after being held to the bottom with an islet plate capture screen, each was washed in 525 μl of Ringer's solution, (8.4 mmol l-1 sodium pyruvate, 5.6 mmol $l-1$ $NaHCO_3$, 0.97 mmol $l-1$ HEPES, and 3.2 mmol $l-1$ HEPES sodium salt was added fresh to a stock of 115 mmol $l-1$ NaCl, 2.7 mmol $l-1$ KCl, 1.2 mmol $l-1$ $MgCl_2$, 0.64 mmol $l-1$ $NaH_2PO_4$, and 2.1 mmol $l-1$ $CaCl_2$, pH 7.0 at 28 °C), as adapted from Little and Seebacher (2014).

While the Extracellular Flux Analyzer instrument calibrated, the plate incubated at 28 °C for 30–45 min, and 250 μl of Ringer's solution was replaced before being inserted into the analyzer. Once in the analyzer, 8 OCR measurements (basal OCR) were taken and FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) was injected into the wells to uncouple mitochondria, allowing for measurement of maximal respiration, followed by 8 more measurements. Lastly, a mixture Antimycin A and rotenone was injected to inhibit cytochrome *c* oxidase function, allowing for measurement of non-mitochondrial respiration, followed by twenty-five measurement cycles.

The total basal OCR was calculated by averaging the three lowest values from basal measurements. Total maximal respiration was calculated by averaging the three highest values for the FCCP treatment measurements. Non-mitochondrial respiration was calculated by averaging the three lowest values for the antimycin A/Rotenone mixture. Other OCR measurements, such as basal mitochondrial, maximal mitochondrial, and mitochondrial spare capacity were calculated from these measurements.

### 2.7. Statistical analysis

All analyses were conducted using Graph Pad Prism 8.0 (San Diego, CA, USA). For behavioral analyses, two-way ANOVA with treatment and cohort factors with a Tukey's multiple comparisons test were used to test for effects. For swim tunnel and Seahorse assay analyses, one-way ANOVA tests were used and if the data did not meet the normality assumptions, the Kruskal-Wallis test was employed. Outliers were removed from the tissue-specific OCR assay data using the GraphPad Prism 8.0 QuickCalcs Outlier Calculator at alpha $p < 0.05$.

## 3. Results

### 3.1. Behavioral assessment of study 1

In the following section, results from behavioral assessments performed on zebrafish in study 1 (embryonically exposed group) are reported by test.

### 3.2. Light/dark larval motility test

Six dpf, zebrafish were tested in the light/dark larval motility test to examine larval locomotor activity (Fig. 2). Data are shown min-by-min across the 50-min session (Fig. 2A) and as total locomotor activity per 10-min light or dark phase of the test (Fig. 2B). The 210 nM and 700 nM groups were signficantly hypoactive compared to the control groups in both the light and dark phases of the test ($p < 0.05$, respectively) (Fig. 2A/B). There was a significant DZN main effect ($F(3,213) = 13.05$, $p < 0.05$), a significant time-period effect (indicating differences in activity between dark/lit conditions) ($F(5.240, 1116) = 155.9$, $p < 0.05$), and an interaction ($F(147, 10{,}437) = 2.468$, $p < 0.05$) (Fig. 2A). Although a light phase by treatment interaction was found, the 210 and 700 nM DZN groups differed from controls during all light and dark phases ($p < 0.05$).

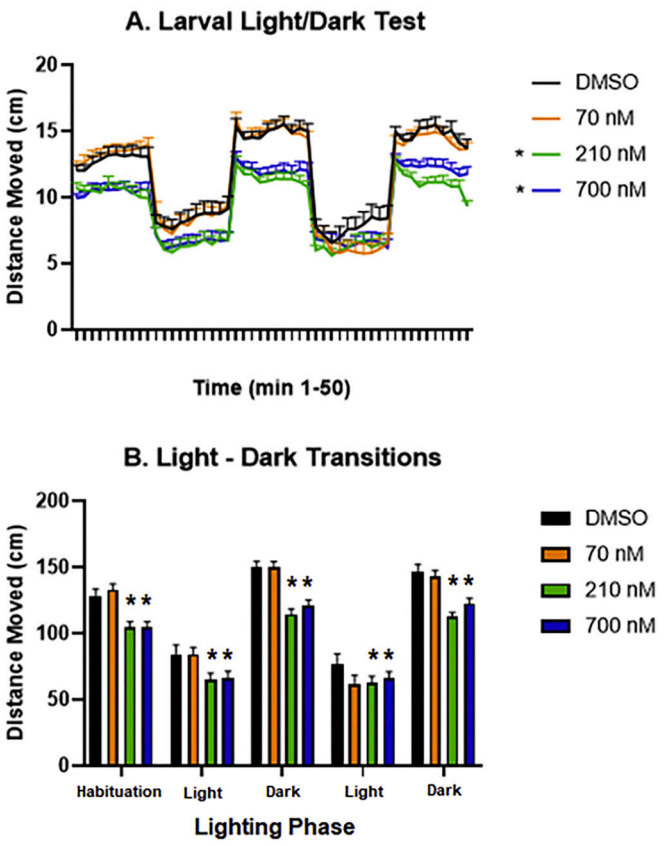


**Fig. 2.** Larval test performed on zebrafish embryonically exposed to diazinon at 6 dpf. Locomotor activity is presented as group mean (± SEM). An asterisk (*) indicates significance at $\alpha = 0.05$ ($p < 0.05$) and SEM bars are displayed. A) Distance moved from min one to fifty. B) Average locomotor activity across light/dark phases and transitions. Overall, concentrations of 210-700 nM reduced larval locomotion, regardless of light phase.

#### 3.2.1. Novel tank diving test

At two, 8, and 14 months of age, the same group of zebrafish experienced the novel tank diving test. Data are shown across 5, 1-min time blocks, and with locomotor activity (Fig. 3) and diving response (Fig. 4) as separate analyses. Two month-old fish in the 210 nM group moved signifcantly more than the control group ($p < 0.05$) (Fig. 3A). Eight month-old fish showed no treatment effects of DZN (Fig. 3B). Fourteen month-old fish in every DZN-treated group traveled significantly less compared to the control group ($p < 0.05$) (Fig. 3C). When tested at two months of age the fish showed a significant DZN main effect ($F(3, 109) = 2.907$, $p < 0.05$), and a significant time-period effect ($F(2.933, 319.7) = 58.61$, $p < 0.05$) for the total distance moved (Fig. 3A). In the 8 month of age test, there was no significant DZN main effect ($F(3, 104) = 1.895$, $p = 0.1351$) and a time-period effect ($F(2.498, 259.7) = 38.57$, $p < 0.05$). At 14 months of age, there was a significant DZN main effect ($F(3,100) = 3.639$, $p < 0.05$) and a significant time-period effect ($F(2.798, 279.8) = 33.99$, $p < 0.05$).

Another parameter measured in the novel tank test is the distance that the fish travel from the bottom of the tank and controls often show a pattern where distance from the bottom increases throughout the duration of the test. Two month-old fish (Fig. 4A) and 8 month-old fish (Fig. 4B) showed no changes in swimming depth relative to controls. All

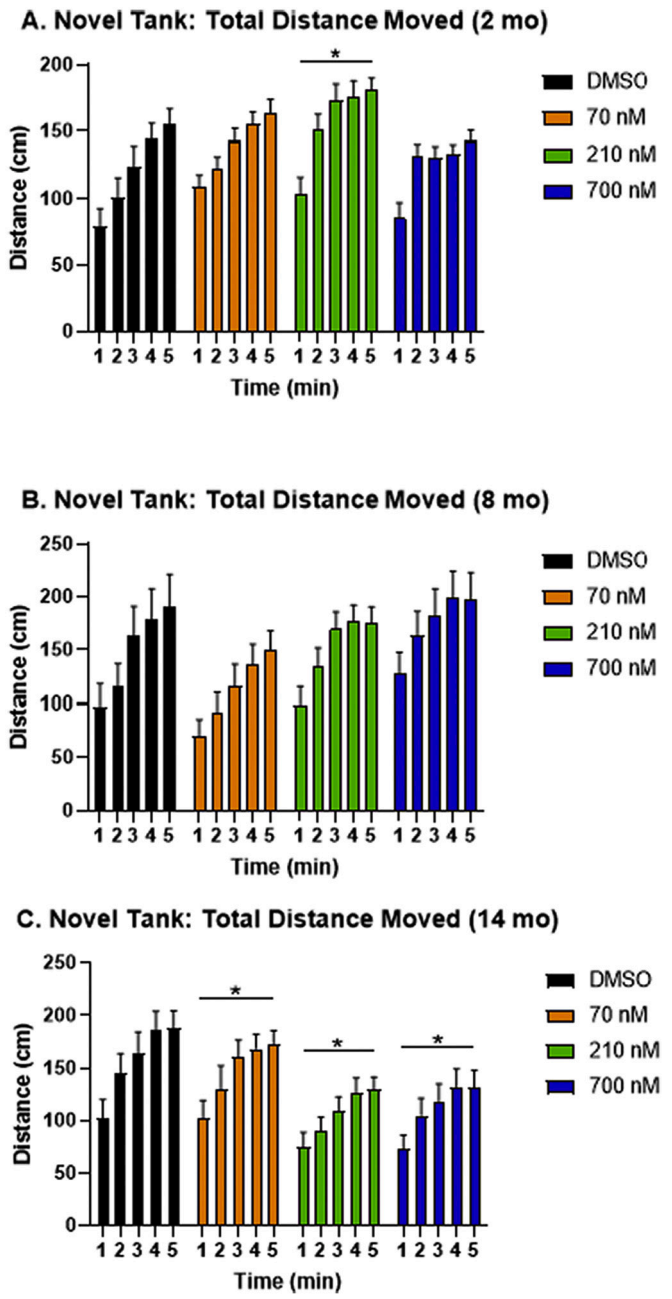


**Fig. 3.** Novel tank test, total distance traveled, with bars displaying standard error measurements for study 1 zebrafish, embryonically exposed to DZN. Locomotor activity is presented as group mean (± SEM). A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Depicts total distance traveled for the five-min period at 2 months of age. Locomotor activity was increased in the 210 nM group. B) 8 months of age. No treatment effects were detected. C) 14 months of age. Locomotor activity was reduced among all DZN-exposed fish.

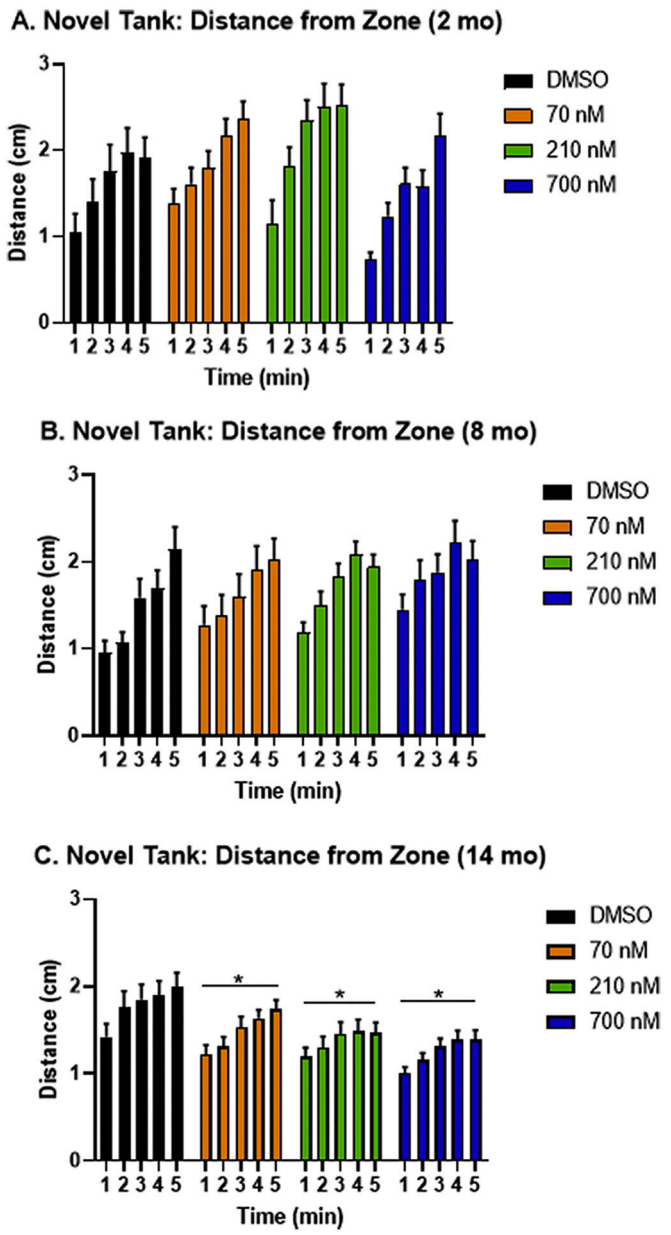


**Fig. 4.** Novel tank test, distance from the zone (bottom of the tank), with bars displaying standard error measurements for study 1 zebrafish, embryonically exposed to DZN. Distance from the floor is presented as group mean (± SEM). A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Depicts distance from the bottom for the five-min period at: 2 months of age. Increased distance from the bottom among 70 nM fish. B) 8 months of age. Increased distance from the bottom among 700 nM fish. C) 14 months of age. Reduced distance from the bottom among all DZN-treated fish.

groups traveled significantly closer to the bottom of the tank compared to the control group (70 nM, $p < 0.0001$; 210 nM, $p = 0.0488$; 700 nM, $p < 0.05$) (Fig. 4C). ANOVA analysis of the distance from the zone measurements found that for the two-month test, there was a time-period effect ($F(2.731, 297.7) = 42.28$, $p < 0.05$). For the 8-month test, there was a time-period effect ($F(2.577, 268.0) = 37.71$, $p < 0.05$) (Fig. 4B). The 14-month test had a DZN main effect ($F(3, 100) = 3.794$, $p < 0.05$) and a time effect ($F(2.649, 264.9) = 34.69$, $p < 0.05$) (Fig. 4C).

### 3.2.2. *Tap startle test*

The second behavior assay in the adult battery was the tap test, which evaluates the magnitude and persistence of startle responses over successive repetitions of a loud tapping stimulus. Only 8 and 14 month-old fish were tested. Control fish show a reduction in startle magnitude across the taps. At 8 months, the 70 nM and 700 nM groups showed a significantly greater average startle magnitude across the 10 taps than the control group ($p < 0.05$) (Fig. 5A). At 14 months old, no treatment differences were detected relative to the control group ($p < 0.05$) (Fig. 5B). For the 8 month test there was a significant DZN treatment effect ($F(3, 82) = 2.78$, $p < 0.05$) and a time effect ($F(4.088, 335.2) = 6.665$, $p < 0.05$). At 14 months old, there was a significant time effect (F

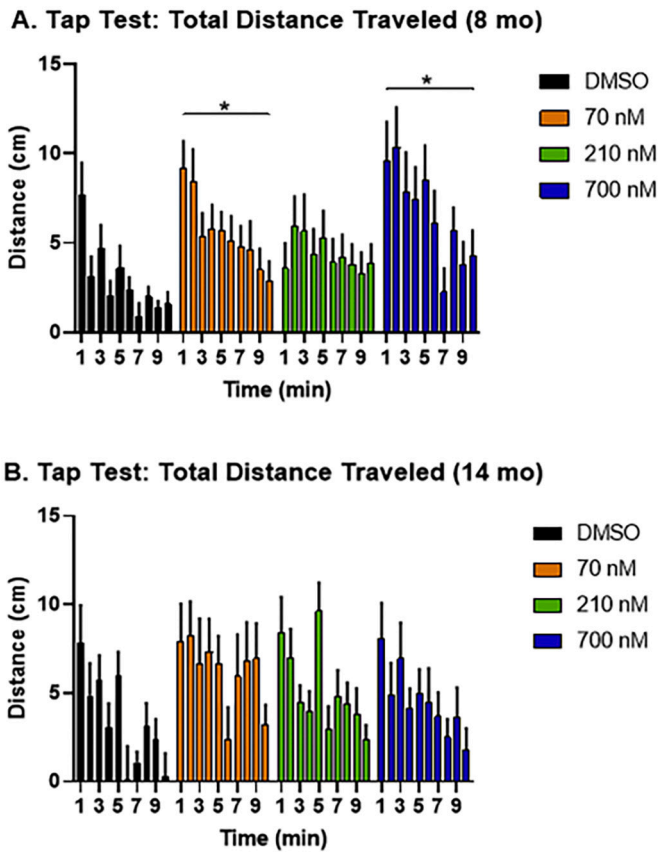


**Fig. 5.** Tap test for study 1 zebrafish embryonically exposed to DZN. Locomotor activity is presented as group mean (± SEM). The total distance traveled measurement indicates how much a fish moves around the arena after a stimulus event. A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Fish tested at 8 months old. Increased startle response among 70 nM and 700 nM fish. B) Fish tested at 14 months old. No effect of treatment.

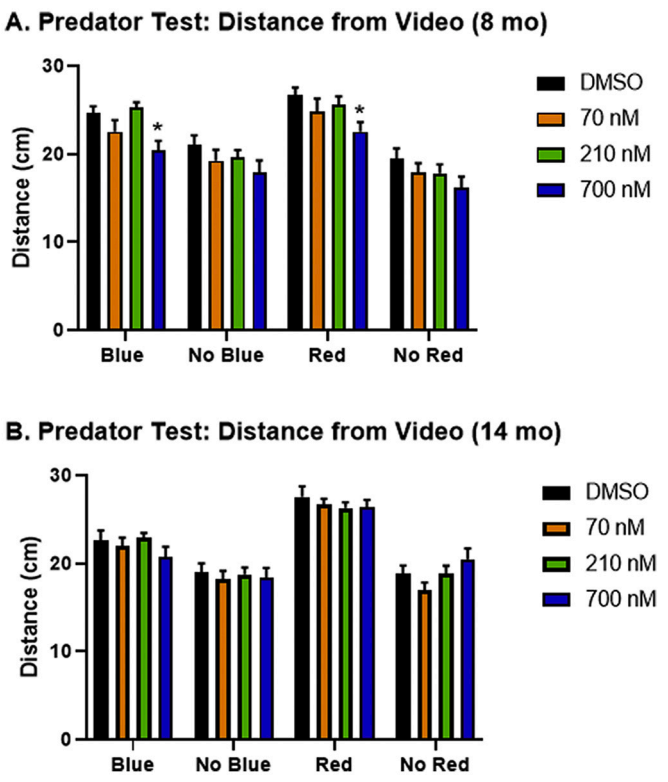


**Fig. 6.** Predator avoidance test for study 1 zebrafish embryonically exposed to DZN. Exploration relative to a presented video is presented as group mean for the distance from the screen-adjacent side of the tank (± SEM). Bars indicate standard error measurements and a single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Predator avoidance test is divided into two stimulus events and removals (blue/slow displayed/not displayed and red/fast displayed/not displayed. Fish exposed to embryonic 700 nM DZN remained closer to the screen than controls during predator cue presentations. B) No treatment effects were detected among 14-month-old fish with embryonic DZN exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(5.722, 349.0) = 6.953, $p < 0.05$) (Fig. 5B) but no treatment effect.

### 3.2.3. Shoaling test

The shoaling test is the third test of the battery and it was only performed at 8 and 14 months of age. Control fish showed a reduction in distance from the video-adjacent side of the screen during the video presentation. There were no significant DZN effects for either age (*not shown*). There was a time effect (F (1, 82) = 87.12, $p < 0.05$) for the 8-month test (Supplemental Fig. 2A) and the 14-month test (F (1, 109) = 42.81, $p < 0.05$) (Supplemental Fig. 2B), indicating that, overall, the distance of fish from the screen was reduced once the video was presented.

### 3.2.4. Predator avoidance test

The last test conducted was the predator test and graphs display the distance away from the video when the blue (slow) and red (fast) stimuli were displayed and when they were not (Fig. 6). Eight month-old fish in the 700 nM group remained significantly closer to the screen ($p < 0.05$) when the red and blue stimuli were displayed, compared to the control (Fig. 6A). There was a significant time effect (F (2.566, 241.2) = 75.25, $p < 0.05$) in the 8-month-old test (Fig. 6A), and in the 14-month-old test (F (4.648, 483.4) = 54.39, $p < 0.05$) (Fig. 6B), indicating that distance from the screen varied based on the presentations of predator cues, but no effect of DZN treatment at 14 months of age (Fig. 6b).

## 3.3. Behavioral assessment of study 2

In the following section, results from behavioral assessments performed on zebrafish in study 2 (adult exposed group) are reported by test.

### 3.3.1. Novel tank dive test

Adult, 14 month-old fish were tested on the novel tank test 7 days after the DZN exposure ended. The average distance moved in each exposed group was significantly less than the control (0.4 μM, $p = 0.0020$; 1.25 μM, $p < 0.0001$; 4.0 μM, $p = 0.0002$) (Fig. 7A). The 0.4 μM also explored significantly further from the bottom of tank compared to controls ($p < 0.05$) (Fig. 7B). There was a significant time effect for the total distance moved (F (4,12) = 23.26, $p < 0.05$) (Fig. 7A). The average distance from the floor measurement also had a significant DZN effect (F (3,12) = 9.562, $p < 0.05$) and a time effect (F (4,12) = 4.203, $p < 0.05$) (Fig. 7B).

### 3.3.2. Tap startle test

The tap test graph shows the average distance moved after all taps and before all taps (Supplemental Fig. 3). There were no significant results between the distance moved in the exposed groups compared to the control before and after taps for both the 8 and 14 month-old (Supplemental Fig. 3).

### 3.3.3. Shoaling test

The shoaling test graph displays the measured distance that the fish are from the video of shoaling fish when the video started playing. Fish in each exposure group and in the control group moved closer to the

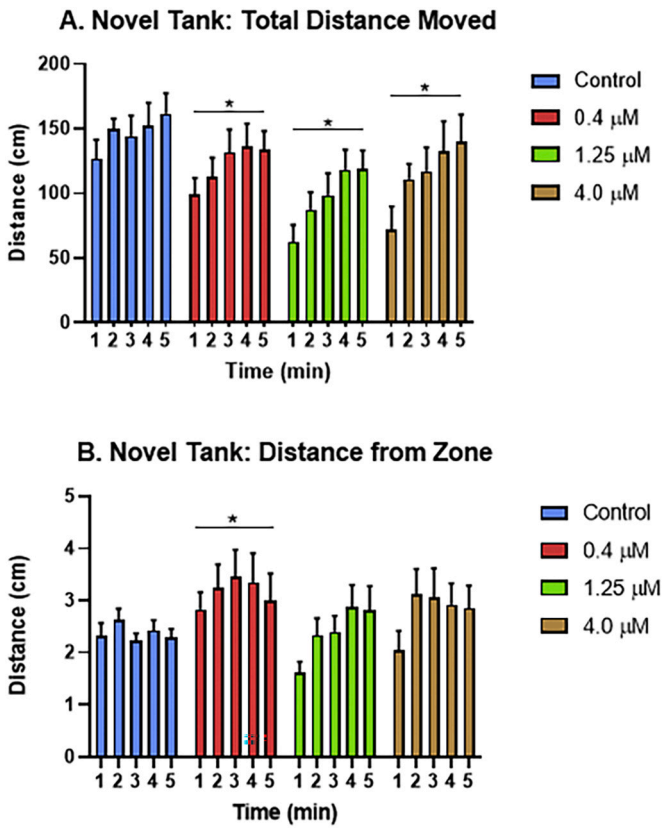


**Fig. 7.** Novel tank test for Study 2 zebrafish acutely exposed to DZN at 14 months old. Locomotor activity is presented as group mean (± SEM). A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Depicts total distance traveled for the five-min period. All DZN-treated groups showed reduced activity relative to controls. B) Depicts the average vertical distance fish traveled from the bottom of the tank (zone). Fish exposed to the lowest dose (0.4 uM) explored further from the bottom of the tank compared to controls.

screen but differences based on treatment were not statistically significant (Supplemental Fig. 4A).

### 3.3.4. *Predator avoidance test*

The predator avoidance test graph shows a normal reaction to the red and blue aversive stimuli where the fish in each group moved away from the screen when the blue and red stimuli appeared and there were no significant differences based on treatment (Supplemental Fig. 4B). Following this test at 14 months of age, behavior analysis was complete, and all fish were transferred to a second protocol to complete whole organism and tissue specific respiration analyses, discussed below.

### 3.4. *Whole organism basal respiration: Study 1 and study 2*

After dividing each average OCR measurement by the corresponding fish's weight, no significant effects were found for either study 1 or study 2 zebrafish when both groups were tested at 14 months of age (Supplemental Fig. 6).

### 3.5. *Tissue-specific oxygen consumption: Study 1*

The results from study 1 fish tissues showed significant results in a few of the OCR parameters and in multiple tissues.

Total basal respiration in the brain was significantly higher ($p < 0.05$) in the 70 nM and 210 nM groups compared to the control (Fig. 8A). Mitochondrial basal respiration was significantly higher ($p < 0.05$) in the brains of the 70 nM group, in addition to maximal mitochondrial respiration being significantly higher ($p < 0.01$) in the 210 nM group

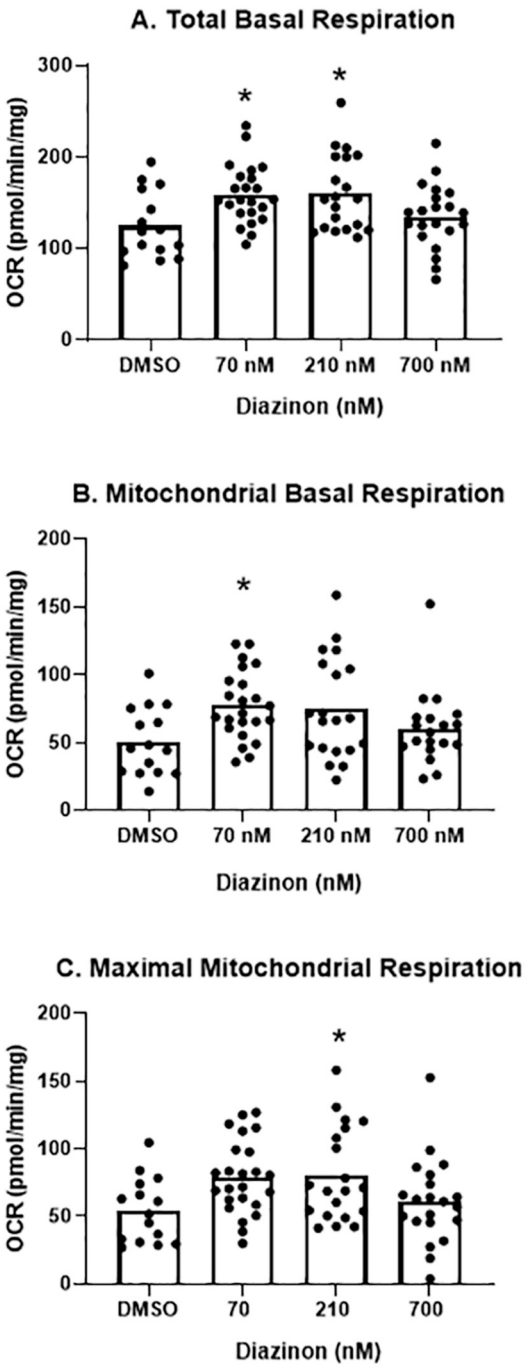


**Fig. 8.** Oxygen consumption measurements in the brains of study 1 zebrafish embryonically exposed to DZN, with each dot representing an individual sample. Bars represent group means (± SEM). A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Total basal respiration measurements show significantly increased OCR in the low and middle dose groups. B) Mitochondrial basal respiration is significantly increased in the low dose group. C) Maximal mitochondrial respiration is significantly increased in the middle dose group.

(Fig. 8B/C).

A similar dose response was seen in the testes (Fig. 9). Total basal respiration was significantly higher ($p < 0.05$) in the 70 nM and 210 nM groups, in addition to mitochondrial basal respiration in the same groups ($p < 0.001$ and $p < 0.01$, respectively) (Fig. 9A/B). Maximal mitochondrial respiration was significantly elevated in the 70 nM and 210 nM groups ($p < 0.05$) (Fig. 9C). A similar trend was seen in the

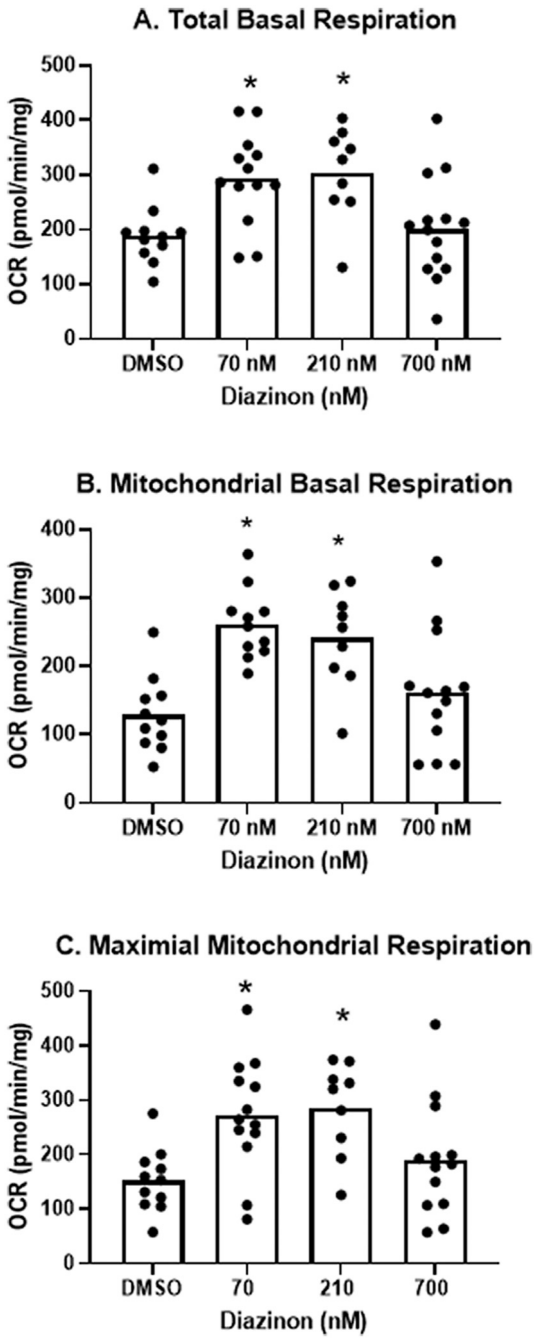


**Fig. 9.** Oxygen consumption measurements in the testes of study 1 male zebrafish embryonically exposed to DZN, with each dot representing an individual sample. Bars represent group means (± SEM). A single asterisk indicates significance at $\alpha = 0.05$ ($p < 0.05$). A) Total basal respiration measurements show significantly increased OCR in the low and middle dose groups. B) Mitochondrial basal respiration is significantly increased in the low and middle dose groups. C) Maximal mitochondrial respiration is increased in the low and middle dose groups.

ovaries, where total basal respiration and mitochondrial basal respiration were slightly increased in the 70 nM and 210 nM groups (Supplemental Fig. 6A/B).

There were sex differences seen in the livers of males and females (Supplemental Fig. 7). Males have significantly increased total basal respiration compared to females in the control, 70 nM, and 210 nM groups ($p < 0.05$; $p < 0.001$; $p < 0.05$) (Supplemental Fig. 7A). There were also trends that mitochondrial basal respiration was increased in males compared to females (Supplemental Fig. 7B).

Sex differences were found in the brain when measuring maximal mitochondrial respiration and maximal respiration, but not for total basal respiration (Fig. 10A). The females exposed to 210 nM of DZN had a significantly higher mitochondrial basal respiration ($p < 0.01$) than males in the 210 nM exposure group and in the 700 nM group ($p < 0.05$) (Fig. 10B). For maximal respiration, the 210 nM female dosage group

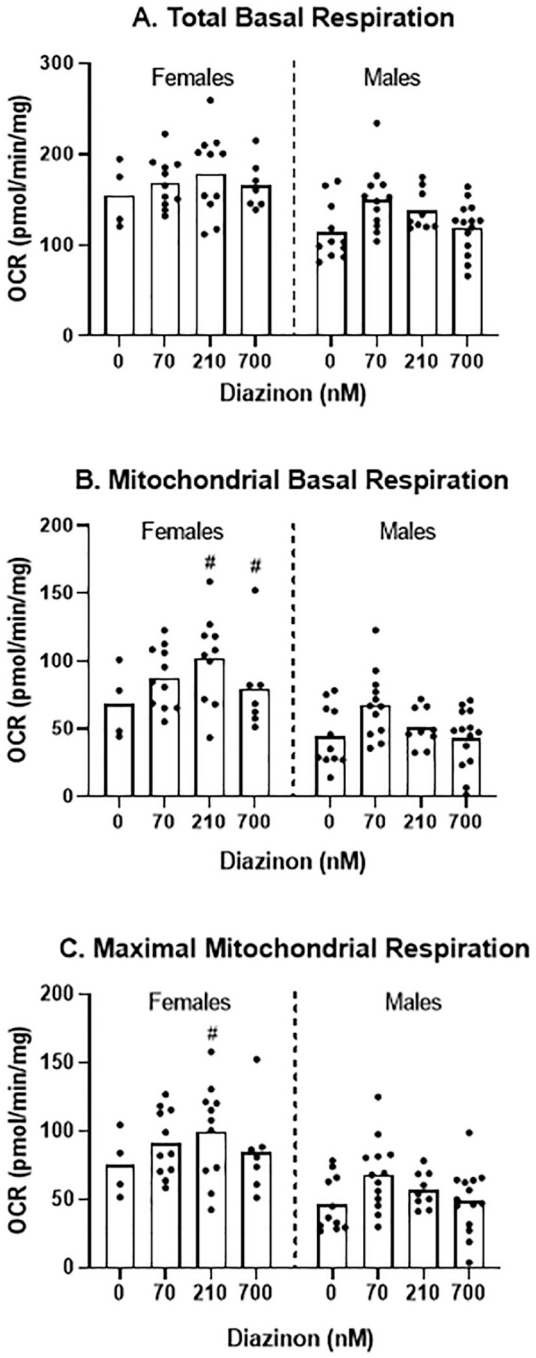


**Fig. 10.** Trends of sex differences were seen in brain tissue of study 1 males and females embryonically exposed to DZN, and each dot represents an individual sample. Bars represent group means (± SEM). A single pound symbol indicates significance at $\alpha = 0.05$ ($p < 0.05$) between individuals of different sexes in the same dosage group. A) Total basal respiration looked to be higher in females than males in all groups. B) Mitochondrial basal respiration was significantly higher in the middle and high groups of females compared to males in those groups. C) Maximal mitochondrial respiration was significantly higher in females of the middle dose group compared to males.

values were significantly higher ($p < 0.01$) than the male group (Fig. 10C).

### 3.6. Tissue-specific oxygen consumption: Study 2

Results for Seahorse assay analyses for the zebrafish in study 2 were insignificant for nearly every parameter measured for every tissue. However, there were trends in the brains and livers of males and females. While total basal respiration, maximal mitochondrial respiration, and maximal respiration in livers of control males and females were similar, there seemed to be a dose-dependent decrease in the OCR of females and an inverted-U shaped dose response in the low (0.4 μM) and middle (1.25 μM) dose concentrations of males (Supplemental Fig. 7). There was a significantly increased level of OCR for the maximal mitochondrial respiration parameter in males of the 1.25 μM group compared to their female counter parts of the same group (Supplemental Fig. 7A). Also, maximal mitochondrial respiration was significantly higher in males of the 0.4 μM group compared to 0.4 μM females (Supplemental Fig. 7B).

In the heart tissue of acutely exposed fish, there was increased OCR in the low and middle dosage groups of female's non-mitochondrial basal respiration, but a decreasing OCR trend in males (Supplemental Fig. 8A). Female fish maximal respiration showed a trend for an inverted U-shape dose response, but male OCR for the same parameter was the same in every group (Supplemental Fig. 8B).

## 4. Discussion

Aging zebrafish showed significant neurobehavioral and physiological effects of diazinon exposure either during early development or in adulthood. Evidence is growing that early exposure to diazinon could lead to persistent symptoms in humans, particularly with respect to mental health. Links between OP insecticide exposure and neurodegenerative diseases such as AD, PD, and ALS are unclear, but it is important for research to evaluate this potential relationship. Cognitive impairments that manifest themselves in neurological diseases can be studied in zebrafish via multiple tests. For instance, humans that are diagnosed with PD or AD have cognitive or motor impairment and changes in mood, and certain zebrafish behavior tests can be proxies for these symptoms (Bailey et al., 2014; Best and Alderton, 2008). By understanding how zebrafish exhibit behaviors that are comparable to those of patients, it will be clearer how to translate abnormal behaviors of zebrafish that occur as they age and as they are exposed to environmental chemicals, to humans.

The present study provides three important comparisons related to vulnerability and symptomology following DZN exposures. Study 1 used a longitudinal design to evaluate the presence and persistence of behavioral symptoms following embryonic exposure to DZN. Study 2 provided an indication of the relative similarity or disparity between fish of an equivalent age (late adulthood) when exposed to this compound during embryonic development or in late adulthood. Finally, the respiration analyses across the two studies provided an indication of whether expected impacts of a DZN exposure of mitochondrial function lead to lasting changes which can be observed during aging.

The initial study indicated that while behavioral alterations were certainly present at all stages of life, the nature of those effects were substantially modulated by the stage of development when assessment took place. Larval motility and novel tank diving test analyses suggested a bell shaped trajectory of impairment across the age windows, with larval and aged fish showing hypoactivity (mid-high doses in larvae, all doses in aging), adolescent fish showing hyperactivity (specifically the middle dose), and adult fish showing no effect. Diving responses, by contrast, showed no effects of DZN treatment until the aged time point, at which point all DZN groups remained closer to the bottom than controls. Enhanced diving is generally associated with anxiety-like functions in zebrafish. Tap test and predator avoidance tests showed enhanced startle responses (low and high dose) and reduced predator avoidance (high dose) in younger adulthood, but not in aging. Across these findings, there are indications of developmental modulation of behavioral symptoms, with outcomes like locomotor activity showing differences in the dosimetry and nature of the deficits at different ages, while tap and predator responses showed the potential for deficits to attenuate across adulthood.

The second study provided an additional comparison, whereby aged fish with recent vs developmental DZN exposures completed the same tests. The locomotor symptoms noted at this late age were remarkably similar between the two studies, as was the apparent sparing of startle responses, shoaling, and predator avoidance at this age. In both cases, all aged fish with DZN exposure were hypoactive compared to their respective controls, despite the differing recency of exposure (14 month embryonic, 1 week adult) and lack of repeated testing in the study 2 animals. These effects did diverge with respect to anxiety-like diving behavior. Embryonically-exposed fish showed a combination of reduced exploration and greater bias towards the bottom of the tank. Fish exposed to low doses of DZN in late adulthood showed a reduced bias towards the bottom of the tank, rather than an enhancement of this pattern, and mid-high doses failed to alter diving, despite overall changes in locomotor behavior. These data indicate that individuals exposed to DZN at different points in the lifespan may have overlapping, but not identical, symptom risks when assessed in late adulthood.

Results from the mitochondrial bioenergetics assays showed increased mitochondrial OCR in brain and gonadal tissues of fish in the low and middle dose groups, primarily those that were embryonically exposed to DZN. The inverted U-shaped curve is a dose response that is typically associated with “hormesis,” which describes a biphasic dose response where a low level exposure to a stressor has beneficial outcomes, while a high-level exposure has deleterious outcomes (Meyer et al., 2018). “Mitohormesis” is hormesis in mitochondria, where external stressors induce mitochondrial responses that can improve health and viability within a cell (Yun and Finkel, 2014). In the low and middle dose groups, OCR in the brain and testes was increased for multiple parameters (e.g. total basal respiration, mitochondrial basal respiration, and maximal mitochondrial respiration). This may be adventagous in providing more energy to these tissues or alternatively may be disadventagous with increased free radical formation. However, it cannot be concluded that these results were a hormetic response, because hormesis conveys the idea that lower doses of DZN are beneficial to organismal health. It would be more advantageous to examine a hormetic response on a larger scale of physiological function, such as through the basal respiration measurements via the swim tunnel, which in this project, resulted in no significant differences between any of the groups. What is clear from this analysis is that embryonic toxicant exposure can alter mitochondrial respiration long-term, and that the potential for compensatory enhancements, rather than detriments, of respiration may occur at low and moderate dose levels.

Considering that the low and middle dose groups of embryonically exposed zebrafish had increased OCR rates for a few of the parameters, but that the high dose OCR was similar to the control group, it is hypothesized that there would be a dose-dependent decrease in OCR above 700 nM, especially in the brain and testes. Males and females had a significantly different OCR in the liver, which is not an uncommon finding because it is a dimorphic organ, with 70% of the genes expressing at different levels in both genders (Yang et al., 2006). Females may be more susceptible to liver damage after exposure to DZN under these circumstances. There was also a trend that females had higher OCR in the brain compared to males, but in the behavioral assays, males did not perform differently than females, indicating that both sexes are likely to experience neurotoxicity.

Embryonically exposed zebrafish larvae did not have many significant impacts in tissue-specific mitochondrial respiration, but did lead to notable compensatory changes in respiration in brain and gonadal tissue. Fourteen months passed from DZN exposure to tissue analysis,

allowing for physiological processes to occur that could have mediated any mitochondrial damage incurred after exposure and potentially led to overcorrections that increased mitochondrial respiration in certain tissues. It is highly likely that DZN exposure caused oxidative stress in mitochondria, and there is evidence that reactive oxygen species (ROS) could signal pathways that facilitate beneficial adaptations after exposure (Sena and Chandel, 2012). In *C. elegans*, another model organism for mitochondria and aging, ROS is generated and can act as a protective signal that changes gene expression and slows the effect of aging (Yang and Hekimi, 2010). While those mutations in subunits of mitochondrial respiratory complexes can lead to increased life span in *C. elegans*, it could be possible that similar pathways were employed in the zebrafish. As fish exposed to DZN during late adulthood failed to show similar effects relative to controls, and principally showed dose-dependent sex differences (male vs female, within group), it is concluded that the stage of development may be an important mediator of compensatory respiratory changes following organophosphate exposure.

The present findings provide additional support for the potential neurotoxicity of DZN and other OPs and expand upon the developmental consequences of such exposures. Prior work on DZN with zebrafish has been relatively limited with respect to long term development, although a number of studies have examined larval behavior. The results of these studies have been mixed within the dose ranges studied, with prior studies either showing comparable hypoactivity effects (Schmitt et al., 2019), effects at higher doses (e.g. 10uM) (Yen et al., 2011; Cao et al., 2018), or no effects (Velki et al., 2019). Directly comparing these prior studies is complicated by the variety of behavioral methods to make these assessments and dates of testing selected. Additional concerns for dosimetry would include the use of glass or plastic exposure dishes, as plastic can absorb hydrophobic contaminants from the water and reduce the internal dose of the chemical. The present data does fit nicely with, and expand upon, prior studies examing the long term impacts of organophosphate exposures during embryonic development. A comparable dose range (66-660 nM) of DZN was previously found to lead to measurable behavioral deficits in early adulthood, specifically in the novel tank task, (Bailey et al., 2014). Additionally, a number of studies have shown that exposure to other OPs alter behavior at later points in the lifespan (Eddins et al., 2010, Glazer et al., 2018a/b, Levin et al., 2003, Oliveri et al., 2015, Sledge et al., 2011). The major advancement with the present work is to begin to put such adult deficits in a developmental context, showing at what stage of life they emerge, and whether they persist into later stages.

The presence of both behavioral and mitochondrial changes in late adulthood is notable, although there was a disparate dosimetry for those effects, where locomotor and diving effects were usually more prominent as the dose increased while brain tissue respiration was uniquely elevated among lower dose groups. Taken together, this suggests that the presence of enhanced mitochondrial respiration in brain tissue is not necessary for behavioral deficits to be observed. So, it may be best to interpret these results as suggesting that these outcomes exist in parallel rather than in a causative relationship.

One notable limitation within the current study is the need for repeated testing within the behavioral portions of study 1. This study was designed as a longitudinal analysis, which highlights the presence of behavioral symptoms across multiple developmental windows. With this comes a necessary confound, where subjects experience a given test up to three times over the course of their livespan. Latter tests are then understood to demonstrate persistence or attenuation of effects, but these outcomes coincide with increasing familiarity with the test equipment/stimuli and the potential for learning. As fish cannot be reasonably identified across these tests, a typical repeated-measures analysis was not possible, so it is assumed that these tests, spaced apart by six months, operate relatively independently from one another. However, it is possible that repeated testing may influence the outcome of such tests. Future research in this area may need to directly compare cohorts of behaviorally naïve fish at varying ages to evaluate the possible influence of experience on toxicant-induced behavioral deficits.

A second limitation relates to the mechanistic interpretation of the dosimetry of the effects discussed here. This analysis did not include measurement of AchE function and inhibition across the dose ranges used. The doses for adults were generated based on tolerability, identifying the minimum overtly toxic dose (increased incidence of death) and using 3 tolerable doses which descended by ½ log units from a high, sub-lethal dose. This is a method we have used repeatedly when determining dose ranges when a chemical is used in our system for the first time. Doses for embryos were generated based on previous work from our lab (Bailey et al., 2014; Yen et al., 2011) and this same tolerability approach was used as a part of that work. Yen et al. (2011) showed that 300 nM DZN produced an 80% reduction in AchE activity, which places the doses selected in a range expected to produce variable and substantial levels of measurable AchE inhibition. However, those levels were not measured directly, so it is unknown how the degree of AchE inhibition varies across the doses and pertains to risk for future neurobehavioral impairment. Additionally, no information is available for the dosing of adult fish. Given that mitochondrial functions appear to have limited relevance for observed effects based on aging data, additional mechanistic studies are certainly needed to establish a threshold for acute AChE inhibition capable of producing lasting behavioral effects. Additional replications of these data may also be informative, as study 1 was completed using two replicate exposures, but study 2 was completed with only a single exposure per treatment group. These replications may serve to demonstrate the relevance of observed trends and the nature of dosimetry functions, particularly with respect to oxygen consumption data, and particularly for study 2 where only a single replicate exposure was performed.

Diazinon, like all OP insecticides is a neurotoxicant, and zebrafish exposed to it as embryos and as older adults both experienced abnormal behaviors. Future research should further examine the impact that the aging process has on mitochondrial function, by using zebrafish of older ages. While 14-month-old zebrafish are considered “old,” they do not show substanial losses in behavioral performance or enhanced mortality, so they are more representative of a middle-aged human. Eighteen-month-old fish, for example, would be closer to a geriatric age, where mitochondrial diseases occur more frequently. Ultimately, results found in this study, along with others, provide evidence that DZN can be detrimental to human health through neurotoxicity which impacts nervous system function across the lifespan. This new data suggests that impacts may also exist on a more cellular level by causing mitochondrial stress that can modulate cellular and physiological functions long-term.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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