Comparative analyses of the neurobehavioral, molecular, and enzymatic effects of organophosphates on embryo-larval zebrafish (*Danio rerio*)

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**Keywords:**
Organophosphates insecticides (OPs) are common surface water contaminants in both urban and agricultural landscapes. Neurobehavioral effects on larval fish are known to occur at concentrations higher than those reported in the environment. The aim of this study was to perform a comparative analysis of neurobehavioral, molecular, and biochemical responses of four OPs (diazinon, dichlorvos, malathion, methyl-parathion) via the following endpoint measurements: distance traveled, velocity, gene expression (AChE, c-Fos, LINGO-1B, GRIN-1B), enzymatic acetylcholinesterase (AChE) activity, and carboxylesterase (CES) activity. OP exposures (5 hpf - 120 dpf) on embryo-larval zebrafish (*Danio rerio*) were assessed using a larval zebrafish behavior assay at concentrations: 0.01, 0.1, 10, and 100 μg/L. Individual OPs had varying degrees of neurotoxicity. Significant hypoactivity was observed in the 100 μg/L treatments for diazinon and malathion (p < 0.05) as compared to the controls. Diazinon-exposed larvae exhibited a 26% locomotor decrease, and hypoactivity was observed in malathion-exposed larvae at a reduction of 22% and 29% for distance traveled and velocity, respectively. Gene regulation and enzymatic activity changes were measured for both 0.1 and 100 μg/L exposures across OP treatments. Increased CES activity was observed for the 0.1 μg/L treatments of diazinon and methyl-parathion as well as the 100 μg/L treatment of dichlorvos; meanwhile, decreased CES activity was observed for 100 μg/L treatments of diazinon and malathion. Relative enzymatic activity of AChE was inhibited as compared to the control for the 0.1 μg/L diazinon. No other treatment group exhibited a significant effect on biochemical AChE activity; however, AChE upregulation was observed in the 0.1 μg/L exposure for diazinon, dichlorvos, and malathion. Methyl-parathion was observed to downregulate c-Fos at 0.1 μg/L exposure. Malathion upregulated LINGO-1B at 100 μg/L, a gene associated with neuronal regeneration; meanwhile, downregulation of LINGO-1B was observed for 0.1 μg/L exposure of methyl-parathion. Additional downregulation was observed for GRIN-1B in the 100 μg/L diazinon, 100 μg/L dichlorvos, and 0.1 μg/L methyl-parathion treatments. Exposure of ZF embryos to independent concentrations of 100 μg/L concentrations of diazinon and malathion resulted in hypoactivity and decreased CES activity at 5 dpf. No changes in swimming behavior were observed for either the 0.1 μg/L or 100 μg/L dichlorvos or methyl-parathion treatments. Observations from this study indicate that AChE inhibition may not be the most sensitive biomarker of OP pesticide exposure in zebrafish. Rather, the enzyme CES demonstrated higher sensitivity as a biomarker of OP toxicity.

1. Introduction

Organophosphates insecticides (OPs) are a common class of insecticides which contaminate surface waters globally due to their extensive applications in both urban and agricultural landscapes (Stehle et al., 2018; Stone et al., 2014). The use of these insecticides has led to increased crop production (Oerke, 2006); nevertheless, leaching of pesticides into surrounding aquatic ecosystems can present a significant hazard to non-target organisms including humans and fish (Jin et al., 2013; Malaj et al., 2014; Stehle et al., 2018). For instance, diazinon and malathion concentrations have been reported as “exceeding aquatic life benchmarks” in urban surface waters across the United States (Stone et al., 2014). OPs are known to cause neurobehavioral effects on larval fish at high concentrations (Rodriguez-Fuentes et al., 2015; Watson et al., 2014), but limited studies have performed comparative analyses of multiple OPs under standardized laboratory procedures.

OP exposure results in acetylcholine esterase (AChE) inhibition, the key molecular event leading to interminable neuronal firing and acute mortality (Russom et al., 2014). AChE inhibition is the accepted mode of action for OP insecticides across a wide range of invertebrates and...
vertebrates. Progressive symptoms of exposure include impaired neuronal growth and signaling at the cellular level, physiological responses via excitation of the nervous system, subsequent behavior alterations, and ultimate mortality of the exposed individual or population (Russom et al., 2014; Volz et al., 2011).

OP pesticides are activated through the oxidation of their thiophosphate group by cytochrome p450 (Neal, 1967; Yen et al., 2011) The subsequent oxon metabolites have a strong binding affinity with the AChE enzyme (O’Brien, 1963). AChE normally functions by cleaving ACh from cholinergic receptors; however, OPs primary mechanism of action is enzymatic inhibition of AChE, which obstructs AChE from cleaving ACh-synapse bonds (Fukuto, 1990). This disruption leads to hyperstimulation of neurons due to an accumulation of ACh in the synaptic cleft. AChE inhibition and ACh accumulation has been confirmed in both mammalian and aquatic vertebrates models (Colović et al., 2013; Koenig et al., 2016; Namba et al., 1971). Highly conserved across species, OPs increase adverse risk to non-target species through the inhibition of normal AChE function within receptors. Although ample literature confirms that OP exposure alters behavior, the mechanisms of action are not entirely clear.

Mammalian studies have shown OP exposure can lead to neuronal degeneration and detrimental modifications to behavior and motor function (Adeara et al., 2018; Wani et al., 2017). Many researchers have studied the use of AChE as a biomarker of exposure to agrochemicals, mostly OPs, but some work has focused on carbamates as well (Fulton and Key, 2001; Galloway et al., 2002; Sachana et al., 2018; Sturm et al., 2000). That said, AChE activity may be a less sensitive marker for OP exposure in fish. A study by Wheelock et al. (2005) demonstrated the potential of CES inhibition as a more sensitive biomarker of OP exposure than AChE inhibition, because fish metabolize xenobiotics at slower rates than mammals.

Xenobiotic metabolism in fish species is often different from that in mammals, due to differences in bioactivation of the OP parent compound in the liver and contrasts in sensitivity of the AChE in the brain across species of fish and developmental stages. Rainbow trout exhibited greater sensitive to OP exposure than carp (Lavado and Schlenk, 2011), with zebrafish (ZF) exhibiting pronounced insensitivity to bioactivation of OPs (Keizer et al., 1995). Environmental parameters, such as salinity, may also affect OP toxicity (Lavado et al., 2011), resulting in varied inhibitory response to OPs across species. Therefore, neuronal developmental biomarkers could prove an important endpoint of OP toxicity, particularly when the gene regulation changes coincide with behavioral alterations.

OP toxicity is characterized as the oversimulation of cholinergic receptors via AChE inhibition. Prior research has shown that AChE may not be the most sensitive indicator of OP toxicity in zebrafish (Velki et al., 2017) and as compared to other fish (Wheelock et al., 2005); therefore, this study included genes associated with neurodegeneration (c-FOS, LINGO-1B, and GRIN-1B) and biochemical biomarkers (AChE and CES). C-Fos activity is widely accepted as a general indicator of neurotoxicity and is positively correlated with changes in AChE activity during treatment (Keizer et al., 2019). Subsequent toxic effects of OP exposure include N-methyl-D-aspartate (NMDA) receptor activation due to an influx of excitatory amino acids such as glutamate (Shih and McDonough, 1997), potentially resulting in neuronal inflammation from “accumulation of intracellular CA2+” (Faria et al., 2017). A subunit of NMDA is glutamate ionotropic receptor NMDA type 1 (GRIN-1B). Meanwhile, acute OP exposure has been linked to neuronal degeneration and apoptosis in the central nervous system (CNS) of humans (Lotti and Moretto, 2005) and rats (Caughlan et al., 2004). LINGO-1B shares similar expression to LINGO-1A (Yin and Hu, 2014) and is located primarily in the CNS. One gene transcript was selected for each signaling pathway based on the toxic potential of OPs investigated. The pathways of interest to this study include: 1. cholinergic (AChE), 2. NMDA (GRIN-1B), 3. Oligodendrocyte differentiation/ myelination (LINGO-1B), and 4. general neuronal activation (c-Fos). The combination of c-Fos, GRIN-1B, and LINGO-1B were evaluated in a prior study conducted by (Zheng et al., 2017).”

ZF embryo-larvae toxicity studies offer researchers the ability to compare multiple molecular and behavioral endpoints under expedited and controlled exposure conditions. ZF exposed to OPs during larval-embryo development exhibit a variety of significant adverse effects. Developmental exposure to 0.1 μM (~35 μg/L) chlorpyrifos resulted in significant decreases in swim speed and AChE activity; meanwhile, malathion investigations revealed increased swim speed and no significant decrease in AChE activity (Richendrfer and Creton, 2015). Similar studies have examined behavioral endpoints and multiple molecular biomarkers for diazinon (Diaz) (Velki et al., 2017), dichlorvos (Dich) (Watson et al., 2014), malathion (Mala) (Cook et al., 2005), and parathion (Para) (Yen et al., 2011). Despite reported insensitivity of ZF to OP-related AChE inhibition, there remain behavioral alterations and acute toxic effects associated with other key events such as oxidative stress (Rodriguez-Fuentes et al., 2015). A comparative study examining behavior, AChE and carboxylesterase inhibition, and other neurodevelopmental gene expression across OPs would be useful in determining the toxicity of these compounds.

The goal of this study was to compare the toxic response of four organophosphorus compounds (diazinon, dichlorvos, malathion, and methyl-parathion) via the measurement of behavioral, molecular, and enzymatic responses post-exposure. Each compound was assessed individually at an environmentally relevant low concentration (0.1 μg/L) and a high laboratory concentration (100 μg/L). A comprehensive review of surface water contaminants conducted by Bradley et al. (2017) reports maximum concentrations sampled in the environmental for diazinon (0.28 μg/L) and malathion (0.06 μg/L). These compounds are major sources of nonpoint surface water runoff during rain events, stemming from both agricultural and residential use (Pedersen et al., 2006). As the accepted mode of action for OPs is AChE inhibition, it is reasonable to expect the tested compounds will induce similar adverse effects. Careful examination of OPs, utilizing the same ZF population throughout the duration of the study, provides vital information into the impact of insecticide applications. This present study compared the effects of select OP insecticides on embryo-larvae ZF post 5-day exposure. Results from which will inform regulators and assist with mitigating future impacts of OP exposures with reliable data and improved understanding of molecular biomarkers.

Although we hypothesize an inverse correlation between gene expression and enzymatic activity, a relational absence between the two biological endpoints has been increasingly observed in the literature (Craig et al., 2007; Glanemann et al., 2003; Velki et al., 2017). Direct correlation is difficult to ascertain due to the complexity of interactions between transcriptomic RNA processes and enzymatic activity (Velki et al., 2017). For instance, physiological and morphological abnormalities can occur without significant changes in enzymatic activity, such as decreased brain size upon exposure to malathion at treatments higher than this study investigated (Richendrfer and Creton, 2015). We further anticipate hypoactivity for 100 μg/L exposures of OP insecticides. The addition of behavioral assessment may assist with accurately assessing how biochemical and molecular alterations affect aquatic organisms in the environment.

2. Materials and methods

2.1. Chemicals

Diazinon (CAS Number: 333-41-5), malathion (CAS Number: 121-75-5), and dichlorvos (CAS Number: 62-73-7) were purchased from Sigma Aldrich (St. Louis, MO, USA), while methyl-parathion (CAS Number: 298-00-0) was purchased from Chem Service (West Chester, PA, USA). All other chemicals used in this study were analytical grade.
2.2. Test organism

ZF husbandry, housing, and experimentation were performed in accordance with institutional animal care and use committee (IACUC) standardized protocol (#16086-09). All experimentation was conducted using wild type 5D embryo-larval ZF. Fish were purchased in mating pairs from the Sinnhuber Aquatic Research Laboratory at Oregon State University, and subsequently housed in separate (size) tanks on a recirculating system at 28 ± 1 °C with a light-dark cycle of 14:10 at the Texas Tech University in Lubbock, Texas. The system was monitored twice weekly to ensure maintenance of pH 7.5 ± 0.5, conductivity 800 ± 200 µS/cm, 80% dissolved oxygen, and negligible nitritification and ammonification. Fish were fed commercial dry food (Skretting® Gemma Micro, Westbrook, ME) twice daily, ad libitum. For breeding, male and female 5D ZF were combined in a net lined tank (5 L) on the recirculating system at a ratio of 2:1 near the end of a light cycle, and group spawning was naturally induced by the following light cycle. Eggs were collected 1 h post fertilization (hpf), rinsed with clean water, and aerated in a beaker with fresh egg water (1 L distilled water and 60 mg sea salt) in an incubator at 28 °C. At 4 hpf, fertilized eggs were sorted from non-viable eggs through visual determination of sphere-dome stage uniformity under a stereomicroscope (Steremaster®, Fisher Scientific, Pittsburg, USA).

2.3. Exposures

2.3.1. Behavioral exposure

Stock solutions of each OP were initially dissolved in ethanol then serially diluted in egg water for experimental treatments. The final ethanol concentrations were below < 0.1% for all treatments. Fertilized ZF eggs were exposed at 5 hpf to individual OPs at the following concentrations: 0.01, 0.1, 10, 100 µg/L. Each treatment exposure consisted of 16 ZF (n = 16) and 4 to 6 controls per 24-well plate. Embryo-larvae were exposed for a duration of 5 h post fertilization (hpf) to 120 hpf.

2.3.2. Gene expression exposure

ZF were exposed to two separate concentrations (0.1 and 100 µg/L). Eggs (5 hpf) were exposed to individual OP concentrations in 5 sterilized petri dishes with a density of 25 per dish (n = 5) then incubated in the dark at 27 ± 1 °C. At 5 dpf, larvae were euthanized using MS-222 (Ethyl 3-aminobenzoate methanesulfonate) and aggregate larvae in each petri dish were collected as individual samples, flash frozen and stored at −80 °C until further use.

2.3.3. Enzymatic assay exposure

ZF were exposed to the same two concentrations (0.1 and 100 µg/L) as the gene expression samples from 5 hpf to 5 dpf at a density of 40 eggs per sterilized petri dish (n = 3). Petri dishes were incubated in the dark at 27 ± 1 °C. At 5 dpf, larvae were euthanized using MS-222 and aggregate larvae in each petri dish were collected as individual samples, flash frozen and stored at −80 °C until further use.

2.4. Behavioral assay

Individual eggs were randomly selected and placed in each well of a 24 well plate with 2 mL of treatment solution. The top 6 wells of each treatment plate were egg water controls, and the remaining 18 wells were a single treatment concentration; therefore, each organophosphate tested required (4) 24 well plates. The plates were incubated 5 hpf–120 hpf in the dark at 28 ± 1 °C. At 120 hpf, larvae were visually inspected for gross malformation or death. Unviable eggs or malformed embryos were not included in statistical analyses. Quality control standards required a minimum of 50% living to malformed/dead controls for the plate to be included in statistical evaluation. Controls were compared for inter-plate variability. Exploratory behavior of larvae was recorded for 50 min using DanioVision™ on Standard White Light. The trial included a 10 min acclimation period followed by two 10 min light/dark cycles. The neurobehavioral endpoints (distance traveled and velocity) were calculated using Ethovision™XT (Noldus, VA, USA). The Lowess method for smoothing was utilized for regression analysis. Results from this assay informed the selection of the 100 µg/L dose exposure in the gene expression and enzymatic assays.

2.5. Gene expression

Total RNA (~25 ZF embryos/sample) was extracted using 500 µL TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA, USA) per the method outlined in (Awoyemi et al., 2019). Ultra-pure™ RNase/DNase-free distilled water was added to dissolve total RNA (30 µL), and samples were stored at −80 °C until further analyses. Total RNA concentrations (ng/µL) and quality were assessed using Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, MA, USA) ratios (A260/A280 and A260/A230) as previously described in (Kim et al., 2015; Kumar et al., 2019; Robinson et al., 2016). High Capacity cDNA preparation kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize complementary DNA on Veriti 96 well Fast Thermal Cycler (Applied Biosystems).

Gene specific primer sequences (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA, USA). The expression of mRNA transcripts (AChE, c-Fos, LINGO-1B, and GRIN-1B) was determined using Real-time PCR (RT-PCR). RT-PCR was performed using 96-well plates consisting of cDNA samples, Fast SYBR Green I kit on the QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) to obtain the cycle threshold values. Samples were analyzed in technical duplicates. All The RT-PCR cycling conditions included a 10 s polymerase activation cycle at 95 °C, 40 denaturation cycles of 1 s at 95 °C, a 20 s annealing cycle at 60 °C. Melt curve analyses were performed at 95 °C and 60 °C. Double peaks were not detected, indicating single product amplification and specificity. Two negative controls were obtained for each plate consisting of individual ultra-pure water and primers/master mix. No amplification or peaks were detected in the melting curve of negative controls. A preliminary study was conducted of three reference genes: β-Actin, Ribosomal Protein L8 (RPL8), and Elongation transcription factor-1 (ELF1). β-Actin was selected as the reference gene for all samples based on its uniformity of expression. Comparative cycle threshold (Ct) method was used to quantify the relative fold change 2ΔΔCt expression (Schmittgen and Livak, 2008) of genes of interest and normalized to the reference gene β-actin.

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<th>Table 1: Genes, primer sequences, and accession numbers used in qPCR reactions.</th>
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Genes, primer sequences, and accession numbers used in qPCR reactions.
2.6.2. Carboxylesterase (CES) activity measurement

The enzymatic activity was quantified using an adaptation of the method developed by (Velki et al., 2017) as nmol of 4-nitrophenol formed per min per mg of protein.

2.7. Statistical analysis

The behavioral assay locomotor observation results of ZF larvae post 5 dpf to OPs are presented in (Fig. 1A-B). ZF with discernible morphological malformation, as determined by visual examination, were omitted from statistical analysis. A significant decrease in distance traveled (Fig. 1A) resulted from treatments of Diaz (100 μg/L) (One-way ANOVA: p = 0.020, df = 4, F = 3.12; Tukey-Kramer HSD: p = 0.031) and Mala (100 μg/L) (One-way ANOVA: p = 0.002, df = 4, F = 4.51; Tukey HSD: p = 0.043). Likewise, velocity decreased (Fig. 1B) in direct correlation to the distance traveled for treatments of Diaz (100 μg/L) (One-Way ANOVA: p = 0.020, df = 4, F = 3.12; Tukey-Kramer HSD: p = 0.031) and Mala (100 μg/L) (One-Way ANOVA: p = 0.002, df = 4, F = 4.57; Tukey-Kramer HSD: p = 0.004). A dose-dependent decrease was observed for distance traveled and velocity in Mala (100 μg/L) exposed ZF larvae (Jonckheere-Terpstra: p = 0.003, JT = 670.; p = 0.005, JT = 664, for distance and velocity, respectively). No differences were observed in distance traveled or velocity of dichlorvos or methyl-parathion ZF treatments as compared to control.

3.2. Gene expression

The results of RT-PCR gene expression of AChE in ZF larvae post 120 h exposure to individual OPs compared to the controls are...
presented in (Fig. 2A-D). The treatment groups are as follows: egg water (control), environmentally relevant exposure (0.1 μg/L), and laboratory exposure (100 μg/L). Significant changes in RNA were measured for all genes of interest.

Upregulation of AChE RNA (Fig. 2A) as compared to control was observed in ZF larvae to Diaz (0.1 μg/L) (One-Way ANOVA: \( p = 0.014, \ df = 2, \ F = 5.18; \) Tukey HSD: \( p = 0.010 \)), Dich (0.1 μg/L) (One-Way ANOVA: \( p = 0.035, \ df = 2, \ F = 3.91; \) Tukey HSD: \( p = 0.027 \)), and Mala (0.1 μg/L) (One-Way ANOVA: \( p = 0.002, \ df = 2, \ F = 7.76; \) Tukey HSD: \( p = 0.012 \)). C-Fos (Fig. 2B) downregulation was only observed in the Para (0.1 μg/L) treatment (One-way ANOVA: \( p < 0.001, \ df = 2, \ F = 30.1; \) Tukey HSD: \( p < 0.001 \)). Significant alterations in gene regulation were observed across multiple genes for both LINGO-1B (Fig. 2C) and GRIN1B (Fig. 2D).

3.3. Acetylcholine esterase activity

AChE activity are measured as nmol per min per μg protein and presented in (Fig. 3). Significant inhibition of AChE enzymatic activity was observed in ZF larvae exposed to Diaz (0.1 μg/L) (One-way ANOVA: \( p = 0.005, \ df = 2, \ F = 7.80; \) Tukey-HSD: \( p = 0.019 \)). No other differences in AChE activity were observed for ZF exposed to Diaz (100 μg/L), dichlorvos, malathion, or methyl-parathion as compared to the control.

3.4. Carboxylesterase activity

CES activity, measured as nmol per min per μg protein, for all OPs at 100 μg/L concentrations are presented in (Fig. 4). The greatest significant inhibition of CES was observed in ZF exposed to the Diaz (100 μg/L) (one-way ANOVA: \( p < 0.001, \ df = 2, \ F = 120; \) Games-Howell: \( p < 0.001 \)) and Mala (100 μg/L) (Welch’s ANOVA: \( p = 0.005, \ df = 2, \ F = 4.36; \) Games-Howell: \( p < 0.001 \)) as compared to the control. Exposure resulted in CES activity increases for treatments of Diaz (0.1 μg/L) (one-way ANOVA: \( p < 0.001, \ df = 2, \ F = 120; \) Tukey HSD: \( p < 0.001 \)), Dich (100 μg/L) (Welch’s ANOVA: \( p = 0.042, \ df = 2, \ F = 4.11; \) Games-Howell: \( p = 0.035 \)), and Para (0.1 μg/L) (Kruskal-Wallis: \( p = 0.036, \ df = 2, \) ChiSquared = 6.60; Dunn’s: \( p = 0.033 \)).

Fig. 2. Average normalized gene expression of AChE, c-Fos, LINGO-1B, and GRIN-1B as compared to β-actin reference gene in ZF larvae post exposure to independent OPs at 0.1 and 100 μg/L concentrations from 5 hpf–120 hpf. Each compound was independently analyzed as compared to controls. Bars represent ± SEM (N = 5). Letters (a,b,c) denote differences between treatments.

Fig. 3. 120 hpf larval enzymatic AChE activity per nmol per min per mg protein following OP exposure (5 hpf–120 hpf) at 0.1 and 100 μg/L concentrations. Bars represent ± SEM (N = 3). Letters (a,b,c) denote differences between treatments.

Fig. 4. 120 hpf larval enzymatic CES activity per nmol per min per mg protein following OP exposure (5 hpf–120 hpf) at 0.1 and 100 μg/L concentrations. Bars represent ± SEM (N = 3). Letters (a,b,c) denote differences between treatments.
Exposure to organophosphate insecticide during embryo-larval development resulted in significant changes of carboxylesterase enzymatic activity across more treatment groups than other biomarkers measured in this study. The ranking of toxicity potential across multiple OPs presents complications; however, some endpoints provide a better indication of biological impairment than others. This study included investigation of the neurodevelopmental genes LINGO-1B and GRIN-1B. Foremost, alterations in behavior indicated overall changes in biochemical and enzymatic processes. The current study is novel in its exploration of general neurodevelopment to evaluate efficient biomarkers of OP exposure. As ZF are increasingly accepted as a model risk assessment species, determination of CES as a more sensitive biomarker than AChE increases our ability to assess risk to more sensitive species.

Diazinon and malathion induced decreased locomotor response in ZF larvae exposed to 100 μg/L, with upregulation of AChE in both 0.1 μg/L treatments. Meanwhile, a study by Yan et al. (2011) reported no observed behavioral effect for diazinon at 300 nM exposure (~91 μg/L), and a study by Richendrfer and Creton (2015) reported decreased swimming activity in ZF larvae exposed to malathion at concentrations as low as 0.01 nM (~3.3 μg/L). No behavioral effects were observed for the dichlorvos exposed ZF larvae at either treatment, but decreased survivability and anatomical abnormalities have been previously reported at similar concentrations (Watson et al., 2014). These organophosphate behavior studies vary in their exposure time - from initial hour exposed to day observed (Cao et al., 2018; Steele et al., 2018). Variability in hatching time (48–72 h), diet, and time exposed can drastically alter the resulting effect (Dametto et al., 2018; Penglase et al., 2012). One of the aims of this present study is to emphasize the importance of utilizing standardized comparative analyses of compounds in future investigations.

Other significant changes were observed for diazinon and malathion treatments across the genes tested, but the resulting effects were less consistent than the neurobehavioral data. The 100 μg/L treatments of dichlorvos resulted in increased CES activity and downregulation of GRIN-1B, yet upregulation of AChE in the 0.1 μg/L treatment was the only other change observed for dichlorvos-exposed ZF embryos. Meanwhile, methyl-parathion exhibited increased CES activity and downregulation of c-Fos, LINGO-1B, and GRIN-1B at the 0.1 μg/L treatment.

AChE gene expression RT-PCR results included upregulation for 0.1 μg/L treatments of diazinon (3.49 fold), dichlorvos (2.85 fold), and malathion (3.28 fold). Albeit higher than this study’s low concentration, Velki et al. (2017) reported similar significant upregulation of AChE at their lower dose treatment of 0.66 μM (~20 μg/L) of diazinon with no significant change at 100 μg/L exposures.

The simplest models suggest that enzymatic assays should mirror their gene expression assay counterparts, with upregulation of one resulting in the inhibition result of the other (Glanemann et al., 2003). This was true for the 0.1 μg/L diazinon treatment group as significant inhibition of AChE enzymatic activity was observed with corresponding AChE upregulation. However, AChE enzymatic inhibition was not observed for diaz (100 μg/L), dichlorvos, malathion, methyl-parathion treatment groups. Yen et al. (2011) observed that the lethality rate rapidly outpaced the likelihood of inducing an AChE inhibition response as concentrations of diazinon and methyl-parathion increased. Disruption of AChE may be less pronounced in fish due to slower metabolic processes as compared to mammalian species (Wheelock et al., 2005). A prior toxicity study reported no observed AChE inhibition in diazinon exposed 96 hpf ZF at 3.30 μM (~101 μg/L), while CES activity was inhibited (Velki et al., 2017). Wheelock et al., (2005) further suggests CES activity be utilized as an additional endpoint for OP exposure due to the greater preferential binding affinity of some OPs to carboxylesterase than AChE.

C-Fos expression informs the overall neuronal activity post exposure to stimuli (Krukoff, 1999). The transcription factor and proto-oncogene protein, c-Fos, has been used to measure general neuronal activity of responsive neurons within a few hours after an initiating event (Bullitt, 1990; Dragunow and Faull, 1989). The persistent effects of OPs on ZF are presently unknown. Downregulation of the multifunctional proto-oncogene protein, c-Fos, has been attributed to abnormal physiology, behavior, apoptosis, differentiation, and cellular growth in the form of tumor development in mice (Velázquez et al., 2015; Verma and Graham, 1987). In mammalian species, c-Fos RNA expression has a well characterized mode of action integral to overall biological function; however, the role of c-Fos in ZF is not well understood (Özdemir et al., 2018). Genes associated with c-Fos have been shown to be important for cell differentiation and proliferation, especially as it relates to neurodevelopment. The magnitude of c-Fos upregulation has been correlated to increased action potentials due to exposure to intense stimulus (Labiner et al., 1993).

Downregulation of c-Fos has been observed young neonatal mice exposed to the pyrethroid insecticide, while older neonatal mice exhibited the conventional upregulation of c-Fos (Imamura et al., 2002). These findings suggest that age alters c-Fos interactions and expression within the organism tested. Strong excitation of processes within cholinergic receptors are associated with induction of c-Fos (Kauf er et al., 1998). The current study observed upregulation of multiple OP treatments, but AChE enzymatic activity was only observed in the 0.1 μg/L concentration diazinon exposure. Cholinergic activity may have proven insufficient for the induction of c-Fos in the OP exposed ZF larvae. Transcription and enzymatic activity are not 1:1 (Velki et al., 2017). The expression of genes is regulated by a myriad of factors including RNA stability and complex systems of translation (Macdonald, 2001; McCarthy and Gualeri, 1990).

LINGO-1B in ZF is a leucine-rich mammalian homolog that functions as a neuronal fitness promotor via regulation of oligodendrocyte differentiation and myelination (Yin and Hu, 2014). Although humans have one set of the gene LINGO-1, ZF have two copies consisting of LINGO-1A and LINGO-1B due to genome divergence (Postlethwait et al., 1998). Reduced expression of LINGO-1B in developing ZF has been observed to result in aberrant behavior modifications and morphological abnormalities (Ekker, 2004). Glutamate ionotropic receptor NMDA (GRIN) is a subunit which encodes for the glutamate activated ion channel of N-methyl-D-aspartate (NMDA) receptors (Horzmann and Freeman, 2016). Grin-1B has been attributed to critical long term memory and learning processes in zebrafish (Braida et al., 2014).

OP exposure presented disparate effects dependent on the compound administered. Significant upregulation of LINGO-1B was measured in the Mala (100 μg/L) (5.14 fold). GRIN-1B was downregulated for Diaz (100 μg/L) (0.007 fold), Dich (100 μg/L) (0.590 fold), and Para.
(0.1 μg/L) (0.004 fold) treatments. Although statistically significant gene alterations for c-Fos, LINGO-1B, and GRIN-1B were observed in the 0.1 μg/L of methyl-parathion, high variance attenuated confidence in the treatment results. No differences in locomotor activity were observed despite increased CES activity for 0.1 μg/L of methyl-parathion. Upregulation of both LINGO-1B, increased CES activity, and hyperactivity were observed for Mala (100 μg/L).

ZF exposed to 100 μg/L diazinon and malathion treatments resulted in reduced swimming behavior and CES activity without observed AChE inhibition. Malathion has been reported to decrease the brain mass of developing embryo-larval ZF despite lack of perceptible AChE inhibition (Richendrfer and Creton, 2015). While no behavioral changes were observed within the dichlorvos groups, there was a significant increase of CES activity for ZF larvae exposure at the 100 μg/L concentration. CES acts as a ubiquitous metabolic mechanism for many pesticides, including organic OP insecticide compounds. Active OP metabolites target CES, but the dual nature of CES as a potentially “therapeutic scavenger of OP reactive metabolites” provides an asymmetric complication to the ultimate toxicity of OPs to biological systems (Ross et al., 2010). The increased CES activity of Dich (100 μg/L) may be eliciting a detoxification response, which explains the lack of observed behavioral or neurodevelopmental gene expression changes (Velki et al., 2017).

Of the four OPs tested, dichlorvos is the solitary active parent. Most OPs require metabolic activation of the P = S bond to P = O by cytochrome p450s to induce toxic effect (Fukuto, 1990; Stegeman et al., 2015). Diaz (100 μg/L) and Mala (100 μg/L) treatments exhibited both decreased CES activity and decreased locomotor activity. Velki et al. (2017) confirmed CES inhibition in ZF at exposures as low as 0.066 μM (~20 μg/L) for diazinon and reported a relationship between CYP1A upregulation and increased CES activity. Although CYP1A was not tested in this study, increased CES activity observed which may have induced detoxification. These findings indicate a potential correlation between CES activity and OP toxicity. Decreased locomotor activity and CES activity were observed for Diaz (100 μg/L) and Mala (100 μg/L). Larval ZF behavior upon exposure to OPs is determined by factors beyond AChE inhibition. Therefore, CES may be a more sensitive indicator of OP exposure than AChE in ZF.

The octanol water partition coefficients (log KOW) of the OPs included in this study indicate the moderate to high lipophilicity typical of OP compounds (Hansch et al., 1995). Lipid affinity ranking of the OPs included in this study (Diaz > Mala > Para > Dich) corresponds with the effects observed. Nevertheless, further investigation is required to determine actual pre-hatching exposures and uptake due to confounding factors. Variance in the half-life of compounds, oxon-formulation efficiency, and investigative choice to include/remove the chorion barrier present common difficulties for determining true relationships between variables (Jacobson et al., 2010; Yen et al., 2011).

Each structurally unique OP varies in response within biological systems. CES activity was the most responsive biomarker of those tested and literature review confirms CES utility as an indicator of OP exposure in addition to AChE measurements. Although ample literature exists for individual OPs and their primary mode of action, this study is unique in its comparative exploration of the complex inter-workings of OPs in aquatic vertebrates. Through the inclusion of multiple biomarker measurements including neurodevelopmental genes (c-Fos, LINGO-1B, and GRIN-1B) and enzymatic endpoints, studies such as these potentially illuminate the greater mechanisms of OPs not explained by measurement of the classical mode of action alone.

In conclusion, diazinon and malathion caused the greatest neurobehavioral swimming and enzymatic activity changes in ZF larvae at the high concentration (100 μg/L). Enzymatic CES inhibition and a dose dependence decrease in swimming behavior was observed in Malathion, yet biochemical AChE inhibition was only observed for the para μg/L/L treatment. CES activity was notably increased in the 100 μg/L dichlorvos treatment while exhibiting the least observable behavioral or gene regulatory effect. Corroborating prior findings, OPs caused a greater enzymatic response in CES than AChE (Velki et al., 2017). Future assessments would benefit from continued investigation into multiple endpoints to further understand the relationships between behavioral, biochemical, and molecular response to developmental OP exposure.

Conflicts of interest statement

The authors declare no conflicts of interest.

Transparency document

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

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