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RESEARCH ARTICLE

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Assessment of zebrafish embryo photomotor response sensitivity and phase-specific patterns following acute- and long-duration exposure to neurotoxic chemicals and chemical weapon precursors

Chance M. Carbaugh[1,4] | Mark W. Widder[1] | Christopher S. Phillips[2] | David A. Jackson[1] | Valerie T. DiVito[3] | William H. van der Schalie[1,4] | Kyle P. Glover[2]

[1]U.S. Army Center for Environmental Health Research, Fort Detrick, Frederick, Maryland

[2]U.S. Army Combat Capabilities Development Command Chemical Biological Center, Aberdeen Proving Ground, Maryland

[3]U.S. Army Medical Research and Development Command, Military Operational Medicine Research Program, Fort Detrick, Frederick, Maryland

[4]Oak Ridge Institute for Science Education, Oak Ridge, Tennessee

Correspondence
Chance M. Carbaugh, U.S. Army Center for Environmental Health Research, Fort Detrick, Frederick, MD 21702, US.
Email: chance.m.carbaugh.ctr@mail.mil

Abstract

Zebrafish are an attractive model for chemical screening due to their adaptability to high-throughput platforms and ability to display complex phenotypes in response to chemical exposure. The photomotor response (PMR) is an established and reproducible phenotype of the zebrafish embryo, observed 24 h post-fertilization in response to a predefined sequence of light stimuli. In an effort to evaluate the sensitivity and effectiveness of the zebrafish embryo PMR assay for toxicity screening, we analyzed chemicals known to cause both neurological effects and developmental abnormalities, following both short (1 h) and long (16 h+) duration exposures. These include chemicals that inhibit aerobic respiration (eg, cyanide), acetyl cholinesterase inhibitors (organophosphates pesticides) and several chemical weapon precursor compounds with variable toxicity profiles and poorly understood mechanisms of toxicity. We observed notable concentration-responsive, phase-specific effects in the PMR after exposure to chemicals with a known mechanism of action. Chemicals with a more general toxicity profile (toxic chemical weapon precursors) appeared to reduce all phases of the PMR without a notable phase-specific effect. Overall, 10 of 20 chemicals evaluated elicited an effect on the PMR response and eight of those 10 chemicals were picked up in both the short- and long-duration assays. In addition, the patterns of response uniquely differentiated chemical weapon precursor effects from those elicited by inhibitors of aerobic respiration and organophosphates. By providing a rapid screening test for neurobehavioral effects, the zebrafish PMR test could help identify potential mechanisms of action and target compounds for more detailed follow-on toxicological evaluations.

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1 | INTRODUCTION

Zebrafish are increasingly being used as surrogate models for a variety of human diseases and pre-clinical toxicity evaluations due to their high degree of genetic homology with humans and conserved organ and nervous system attributes (Barbazuk et al., 2000; Cornet et al., 2017; Howe, Clark, Torroja, et al., 2013; Lieschke & Currie, 2007). The evolutionary conservation of the fish and mammal neurological systems make zebrafish an attractive substitute for neurological disease modeling and toxicity screening (Horzmann & Freeman, 2016; Kalueff, Stewart, & Gerlai, 2014, Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). Even as early as 24 h post-fertilization (hpf), zebrafish embryos have been shown to be suitable for screening potentially hazardous substances (Hagstrom, Truong, Zhang, Tanguay, & Collins, 2019).

Given their short life-span, high fecundity and modest laboratory footprint, zebrafish are one of the few in vivo systems amenable to high-throughput test schemes. Thus, large numbers of exposure conditions and experimental permutations can be achieved in a whole organism test system in a short amount of time. This enables more ambitious experimental designs, more substances to be screened, and more mechanistic evaluations of new substances (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Kokel & Peterson, 2008; Lieschke & Currie, 2007; MacRae & Peterson, 2015). An increased throughput also allows for dramatic increase in available data for modeling and advanced analytics needed for behavioral profiling and target prediction based on phenotypic outcomes (Wagner, Pan, Sinha, & Zhao, 2016).

Behavior based assays, such as the photomotor response (PMR) assay in zebrafish embryos, can be adapted to high throughput screening and can elicit reproducible behavioral signatures that are representative of chemical mechanisms of action (Kokel et al., 2010). The PMR, conducted at 24- to 32 hpf, is a non-visual behavioral response to high intensity light through the activation of light sensitive neurons located in the hindbrain of the developing zebrafish embryo (Kokel et al., 2013). The standard PMR assay is based on detecting changes in movement in response to a specific pattern of light stimuli, which is defined by three phases: 1) background phase (spontaneous movement), 2) the PMR, and 3) a refractory phase. Previous studies have indicated how changes in movement across all three phases after chemical exposure can be used to create "behavioral barcodes" representative of pharmacological target activity or environmental contaminant toxicity (Kokel et al., 2010; Reif et al., 2016).

Most of the screening with the PMR to date has been limited to one or a few concentrations of these compounds across a wide concentration range. Additionally, little to no data including the PMR are available for short duration exposures (1 h), which are important to help understand the health effects after acute exposures to acutely hazardous substances. General methods for rapidly screening compounds for acute toxicity are lacking, particularly with respect to potential exposure to military personnel in operational environments (National Academies of Sciences, Engineering, and Medicine, 2015). We investigated whether high throughput screening using the PMR could contribute to filling this gap.

In the present study, we analyzed several substances with established or unknown mechanisms of toxicity using a 1 h, or continuous, exposure paradigms to evaluate and compare PMR phase-specific effects across a concentration range that enabled us to observe phase-specific effects at different concentrations. These substances included inhibitors of aerobic respiration, acetyl cholinesterase inhibitors, and several chemical weapon precursor compounds with variable toxicity profiles and poorly understood mechanisms of toxicity.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents used

Chemicals selected for toxicity testing using the zebrafish model were acquired from Sigma Aldrich (Table 1). Chemical stock solutions were prepared by adding the proper amount of chemical to 1 mL of 99.9% dimethyl sulfoxide (DMSO) (Sigma Aldrich, CAS# 67–68-5) in a glass scintillation vial. Additionally, reagents required for the chemical process of embryo dechorionation included embryo media (Mandrell et al., 2012) and pronase (Sigma Aldrich, CAS# 9036-06-0, 25 PUK/mg).

2.2 | Zebrafish housing and breeding

Adult Tűbingen *Danio rerio* (zebrafish) from in-house breeding stocks were used. The stocks are outcrossed yearly with new stocks of Tűbingen zebrafish obtained from the Zebrafish International Resource Center (Eugene, OR, USA). These zebrafish were maintained at an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility at the United States Army Center for Environmental Health Research (USACEHR). The zebrafish colony was maintained on a 14-h light and 10-h dark photoperiod, with the light coming on at 5:00 AM and going off at 7:00 PM. Fish were housed in either custom semi-recirculating aquaculture racks or large, round, flow through, tanks. The water temperature was maintained at 26.4 ± 1.2 °C. Water quality was maintained under the following conditions: dissolved oxygen of 7.54 ± 0.06 mg/mL, pH of 7.54 ± 0.07, alkalinity of 105 ± 3 mg/L as CaCO, hardness of 218 ± 4 mg/L as CaCO, conductivity of 0.595 ± 0.025 μS/cm, and total ammonia of <0.1 mg/L as NH. Adult zebrafish were fed three-times daily during the week, twice with a commercial flake food (TetraMin Tropical Flakes, Blacksburg, VA, USA) and once with live brine shrimp nauplii (Brine Shrimp Direct, Ogden, UT, USA). Only two feedings were provided over the weekend (one flake and one live brine shrimp nauplii feeding).

Adult zebrafish aged 6–18 months were used to supply healthy embryos for chemical screening. The day prior to testing, ~60 zebrafish were selected used for breeding. Equal numbers of males and females were placed into two iSPAWN-S (Techniplast, West Chester, PA, USA) breeding chambers. A divider placed in each iSPAWN-S breeding chamber confined 15 females to the bottom of

TABLE 1 List of the testing chemicals that were used for the experiments described in the methods section

Chemical name	Chemical Abstracts Service Registry (CAS) Number	Lot Number	Purity
2,2′-Thiodiethanol	111–48-8	STBG4082V	≥99%
2-Chloroethanol	107–07-3	STBH1052	99%
2-(Diethylamino) ethanol	100–37-8	SHBH0708V	≥99.5%
2-(Diisopropylamino) ethanol	96–80-0	SHBH9458	≥99%
3-Quinuclidinol	1,618-34-7	MKBV3217V	99%
Arsenic (III) chloride	7,784-34-1	MKCC6076	99.99%
Carbonyl cyanide 3-chlorophenylhydrazone	555–60-2	MKCB6571	≥97%
Chlorpyrifos	2,921-88-2	BCBR6591V	NA
Cyanide	143–33-9	NA	97%
Dimethyl methylphosphonate	756–79-6	MKBP0689V	97%
Dimethyl phosphite	868–85-9	BCBQ7496V	98%
Methyl benzilate	76–89-1	NA	NA
Methylphosphonic dichloride	676–97-1	MKBV0016V	98%
N-Methyl-3-piperidinol	3,554-74-3	BCBN1449V	98%
Parathion	56–38-2	SZBF119XV	NA
Phosphorus (V) oxychloride	10,025–87-3	SHBG3231V	99%
Phosphorus trichloride	7,719-12-2	SHBG5466V	99%
Triethanolamine	102–71-6	BCBT6472	≥99.0%
Trimethyl phosphite	121–45-9	MKBR5761V	≥99%

the chamber, and 15 male zebrafish were placed into the top section. Lids prevented the zebrafish from jumping out of the chambers. I-SPAWN chambers used a recirculating water system, with temperature maintained at 28.5°± 1 °C. At about 6:30 AM or 1 h after the lights came on the next morning, the lid and divider were removed to allow breeding. The spawning platform of the breeding chamber was raised up to its upper most position to confine the zebrafish to shallow water and stimulate breeding. This method produced >1000 embryos per breeding event per breeding chamber. After 15 min, the spawning platform was lowered and the embryos were collected by placing a fine-mesh strainer under the valve located at the bottom of the breeding chamber and opening the valve only part of the way so that the embryos slowly come out without being damaged. Once the embryos were collected they were transferred to glass Petri dishes (~300–400 embryos/dish) containing fresh fish culture water and placed into an incubator shielded from light at 28.5°± 1 °C.

2.3 | Embryo screening and dechorionation

Collected zebrafish embryos were removed from the incubator and screened at the four cell stage (1 hpf) using a dissecting microscope. Embryos were discarded if they were not the right stage or if they were found to be necrotic or malformed; remaining embryos were returned to the incubator. The embryos were screened again at 3.5 hpf; abnormal or improperly staged embryos were again discarded. Remaining embryos were kept in the incubator until the 50% epiboly stage (5.25–5.5 hpf) (Kimmel et al., 1995). Once they reached this stage, ~500 embryos were chemically dechorionated in 25 mL freshly made embryo media in glass Petri dishes on a custom built dechorionator device (Mandrell et al., 2012) by the addition of 83 µL of 32 mg/mL Pronase. Dechorionation was used to remove the chorion to diminish any potential barrier effects the chorion might impose on the chemical uptake by the embryo (Kim & Tanguay, 2014; Mandrell et al., 2012; Pelka, Henn, Keck, Sapel, & Braunbeck, 2017). Excess media was removed from the Petri dishes, taking care to not damage or expose any of the freshly dechorionated embryos to the air. The dechorionated embryos were returned to the incubator for 30 min to allow them to rest before proceeding, and then screened again to remove any embryos that were still in their chorions or that were damaged by the Pronase; residual chorion debris was also removed at this time. The dechorionated embryos were again returned to the incubator for ~1 h.

2.4 | Chemical exposures

Initially, 96-well microtiter plates (Falcon U-Bottom Tissue Culture Plates, Sterile, Corning, Corning, NY) were filled with 100 µL MilliQ water using a Thermo Multidrop Comp Dispenser (Thermo Scientific, San Diego, CA, USA). At 7 hpf, one dechorionated zebrafish embryo was transferred to each well of the prefilled microtiter plates by hand using a flame polished glass Pasteur pipette. Once all the wells received an embryo, the plates were inspected under a dissecting microscope to ensure that none of the embryos had been damaged by the transfer process. Any damaged embryos were removed and

replaced with MilliQ water and a new embryo. After all the wells contained a healthy dechorionated embryo, the plates were placed back into the incubator until the embryos reached the 75%-epiboly stage (Kimmel et al., 1995) or ~8 hpf (1-h exposure plates were left in the incubator until they reached 24 hpf). At 8 hpf (24 hpf for 1-h exposure), the filled plates were removed from the incubator and dosed using a Tecan D300 digital dispenser (Tecan, Männedorf, Switzerland) following a custom dosing script with DMSO normalization of 0.1% (Maes et al., 2012). Each chemical that was tested had a minimum of two 96-well microtiter plates dosed for both the 1-h exposure and standard exposure assays. For each set of tests that were conducted, five chemical concentrations (n = 32 per concentration) and controls (n = 32) were evaluated. Controls embryos were located on columns 12 and 6 on the microtiter plates. Chemically exposed embryos were located in columns 5–1 and 11–7 in increasing concentrations (1, 5, 10, and 100 μM, except for cyanide where concentrations of 33.3, 67.3, 134.5, 269, and 583 μM were tested). All exposures were static exposures. After dosing, the plates were covered with a plate sealer (Universal Optical Microplate Sealing Tape, Corning, NY, USA) and aluminum foil, before being returned to the incubator. The aluminum foil shielded the embryos from light so that their PMR would not be impacted by pre-exposure to light. The plates were then kept in the incubator overnight for the 16-h exposure starting at 8 hpf (standard exposure) and to perform the 1-h exposures at 24 hpf. When the plates reached the appropriate developmental age (24 hpf) for the standard exposure or the exposure duration reached the 1 h mark for the 1-h exposure (25 hpf) the plates were removed from the incubator under dark room conditions. Then the plate sealers and aluminum foil were removed from the plates and the PMR tested using a custom built testing system (Reif et al., 2016). The plates were hand scored for their 24 hpf morphological endpoints under a dissecting microscope. At this point the 1-h exposure assay was ended and the embryos were euthanized with sodium hypochlorite. The standard exposure plates were covered with a plate sealer and returned to the incubator to develop until they reached 120 hpf, at which time the embryos were removed from the incubator to be hand scored for 120 hpf morphological endpoints before they were euthanized with sodium hypochlorite.

Chemical testing took place over the course of several weeks and additional sets of 1-h exposures were tested as either repeat exposures of the initial set of 1-h exposures at lower concentration due to high mortality or a higher concentration if no effects were noted at the original concentration. These exposures were tested for their PMR endpoints only and the time at which the PMR test was conducted was shifted from 25 to 28 hpf. This shift is reflective of results obtained (unpublished data) that indicated that a shift to a later time point provides a higher peak PMR from the embryos.

2.5 | PMR and morphological endpoints

To begin the PMR test, a single, filled microtiter plate was placed into the Photomotor Response Analysis Tool (PRAT) (Reif et al., 2016). The program that runs the PRAT was then started after the test had been given a unique identifier. Images were captured throughout the duration of the test with an infrared camera at a frame rate of 16 frames/s. The program executed a 50-s sequence containing three phases. The first phase of the test occurred from 0 to 30 s of the test (background phase), when the plates remained in the dark and spontaneous movement was captured. In the second, excitatory, phase, the dechorionated embryos were subjected to a 1 s flash of bright white light (18 000 lx) at 30 s followed by 9 s of dark. This flash of bright light triggers the PMR. The third phase extended from 40 to 50 s with a 1 s flash of bright white light (18 000 lx) at 40 s followed by 9 s of dark (Figure 1). This phase is termed the refractory phase, as under normal conditions the embryo fails to respond to the light flash. Following the conclusion of the PMR test the plates were hand scored under a dissecting microscope for a total of 23 morphological endpoints (1-h exposures were only scored for 24-hpf endpoints), four 24 hpf endpoints and 19, 120 hpf endpoints (Table 2). (Truong et al., 2013).

(Note: All test procedures described above were carried out by a single trained laboratory technician.)

2.6 | Statistical analysis

The images collected from the PMR test (~800 images/test) were modified using Image-J (National Institutes of Health [NIH], Bethesda, MD, USA) to create an Audio Video Interleave (AVI) file for each test. The AVI file was then converted into the mp4 format using a video converter. The mp4 file was uploaded into Ethovision software (Noldus, Leesburg, VA, USA) and embryo movement was tracked using the activity analysis function. The resulting output files scored movement as the percentage of pixel change in the embryo's position per frame per well. These files were then exported into an Excel

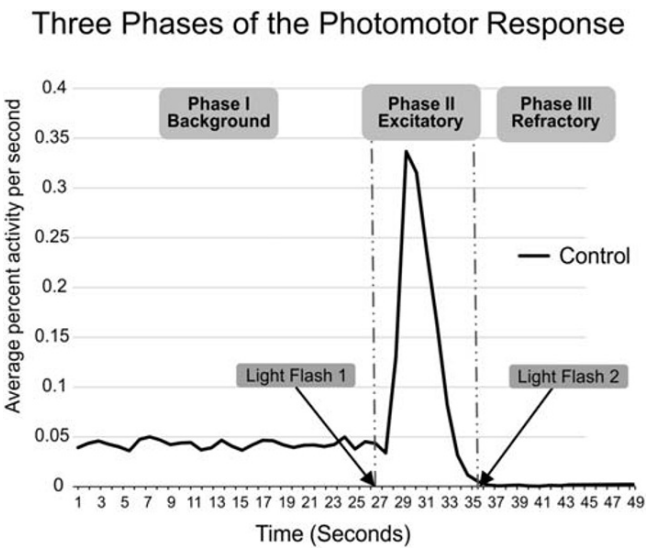

FIGURE 1 Photomotor response (PMR). This is a typical PMR response curve for control zebrafish embryos (n = 623) consisting of the three phases; the background, excitatory, and the refractory phase. The two light stimuli occur at 30 s and 40 s

TABLE 2 The endpoints that were recorded for each test at 24 h post-fertilization (hpf) and 120 hpf. Abbreviations for the endpoints are on the left side of the table with the corresponding descriptor on the right side

Endpoints Recorded at 24 hpf and 120 hpf (Key)	
MO24	Mortality at 24 hpf
DP24	Delayed Development at 24 hpf
NC24	Notochord Malformation at 24 hpf
MORT	Mortality at 120hpf
YSE	Yolk Sac Edema
AXIS	Axis Malformation
EYE	Eye Malformation
SNOU	Snout Malformation
JAW	Jaw Malformation
OTIC	Otic Vesicle(s) Malformation(s)
PE	Pericardial Edema
BRAI	Brain Malformation
SOMI	Somite Malformation
PFIN	Pectoral Fin Malformation
CFIN	Caudal Fin Malformation
PIG	Over Pigmentation or Lack of Pigmentation
CIRC	Lack of Circulation
TRUN	Trunk Malformation
SWIM	Swim Bladder Malformation
NC	Notochord Malformation
TR	Touch Response Deficiency
DNC	Do Not Count

template (two video files per exposure). Dechorionated embryos that were dead at 24 hpf (MO24) were excluded from the data collection. The offset function in Excel was used to average the percentage of pixel change per second for each plate. These averages were then used to calculate the area under the curve (AUC) for each concentration and each phase of the PMR. The AUC values were then reviewed to find and remove any non-responding embryos. The non-responders were defined as any embryos that had zero values across all three phases of the PMR. The remaining AUC values were analyzed by phase and chemical concentration for each test using a two-tailed *t*-test to determine statistical significance ($P < 0.05$). A positive response was indicative of a decrease (hypoactivity) or increase (hyperactivity) in movement compared to the control. Embryos were tested up to a maximum chemical concentration of 500 μM; if the exposed embryos showed no difference in their respective PMR profile when compared to the control embryos, then the PMR response was reported as negative. The morphological data were reported for malformation rates of >50% (Truong, Harper, & Tanguay, 2011). The concentration producing lethality in 50% of exposed animals (LC_{50}) for the duration of the test and the half maximal effective concentration (EC_{50}) for morphological endpoints were calculated using the Trimmed Spearmen–Karber method (Hamilton, Russo, & Thurston, 1977).

3 | RESULTS

Of the 20 chemicals tested, 10 tested positive for at least one phase of the PMR in either the standard (16 h) or 1-h exposure assay. In the standard assay, nine of the 20 test chemicals were positive for at least one phase of the PMR. For the 1-h assay, nine of the 20 chemicals also tested positive for PMR effects in at least one phase of the PMR (Table 3). There were two chemicals that produced positive PMR responses that differed between the two assays: methylphosphonic dichloride (only found positive in the 1-h exposure) and 2-(diethylamino) ethanol (only found positive in the standard exposure). The remaining eight chemicals had positive PMR responses in both the standard and 1-h exposure assay. The standard assay had positive PMR responses in the background phase and excitatory phase for seven of these chemicals, while the 1-h exposures only detected six of the chemicals in both phases. Both the standard and the 1-h exposure assay detected a positive PMR response in the refractory phase for chlorpyrifos. However, the standard exposure assay produced positive PMR responses across all three phases for the chlorpyrifos exposures, while the 1-h exposure produced positive PMR responses in the excitatory and refectory phases only. The 1-h assay for parathion was able to detect a positive PMR response in the refractory phase that was not detected in the standard exposure assay. Embryos exposed to 2-(diisopropylamino) ethanol produced positive PMR responses indicative of hypoactivity in both the background and the excitatory phase for the 1-h exposure, but the standard exposure assay only detected hypoactivity in the background phase. While embryos exposed to phosphorus (V) oxychloride produced PMR responses that were hypoactive during the background and the excitatory phase for the standard exposure, but were only found hypoactive in the excitatory phase for the 1-h exposure.

Two compounds with the same mechanism of action had similar phase-specific response profiles during the PMR test. The acetylcholinesterase-inhibiting organophosphate (OP) pesticides, chlorpyrifos and parathion, displayed hypoactivity in the background and excitatory phases, with hyperactivity during the refractory phase with increasing chemical concentrations (Figure 2). Hyperactivity in the refractory phase and background phase (at lower concentrations) was only seen in the 1-h exposure assay for parathion. Methylphosphonic dichloride also had a trend of increasing hyperactivity with increasing concentration in the refractory phase for the 1-h exposure. However, the statistical response threshold for this chemical was not met ($P = 0.0702$).

Rotenone, a mitochondrial complex I inhibitor (Li et al., 2003) induced hypoactive responses in both the background and the excitatory phase of the PMR test at 0.01 μM (Table 3); Reif et al. (2016) also found that rotenone induced hypoactivity at concentrations ≤0.064 μM. Cyanide and carbonyl cyanide *m*-chlorophenylhydrazine, also compounds that inhibit aerobic respiration, caused hypoactivity during the background and excitatory phases without affecting the refractory phase (Figure 2). Notably, these compounds reduced background activity at lower concentrations, while preserving the

TABLE 3 Chemical effects on zebrafish embryos. Photomotor response (PMR; 1-h and 16-h exposures) performed at 24 h post-fertilization (hpf) for standard (16-h) exposures and 25 hpf for 1-h exposures, and 120 hpf mortality and morphological endpoints (LC_{50} and EC_{50}) from the standard exposure treatments. Lowest effect concentration shown for PMR tests with calculated $P < 0.05$. Chemicals that were not reported in Table 3 for "EC_{50}-associated malformations (lowest effect concentration)" had malformations rates of ≤18.75%, with one exception of phosphorus (V) oxychloride, which had a malformation rate of 30.70% at the 100 μM concentration (which was above the LC_{50}). Malformations were observed in 0.95% of all the control embryos

Chemical name	PMR exposure duration, h	PMR (by phase)			XlogP3	Rodent LD_{50}, mg/kg	5-day LC_{50}, μM	5-day EC_{50}, μM	EC_{50}-associated malformations (lowest effect concentration)
		Background	Excitatory	Refractory					
		μM	μM	μM					
Carbonyl cyanide 3-chlorophenylhydrazone	16	1[a]	1[a]	None	3.4	8	<1	ND	None
	1	1	1	None					
Rotenone	16	0.01	0.01	None	4.1	2.8	0.03	ND	None
	1	0.01	0.05	None					
Chlorpyrifos	16	50	5	50*	5.3	60	52.34	9.48	YSE (10 μM); AXIS, TRUN, and NC (50 μM)
	1	None	50	100*					
Parathion	16	50	1	None	3.8	5	76.69	63.83	YSE (100 μM), AXIS (100 μM), EYE (100 μM), SNOU (100 μM), JAW (100 μM), PE (100 μM), NC (100 μM), TR (100 μM)
	1	1*	5	50*					
Cyanide	16	33.6	33.6	None	−1.69	4.7	90.35	40.08	PE (33.3 μM), YSE (67.3 μM), AXIS (67.3 μM), EYE (67.3 μM), SNOU (67.3 μM), JAW (67.3 μM), BRAI (67.3 μM), SOMI (67.3 μM), PFIN (67.3 μM), PIG (67.3 μM), SWIM (67.3 μM), NC (67.3 μM), TR (67.3 μM)
	1	33.6	134.5	None					
Phosphorus (V) oxychloride	16	100	50	None	1.7	327	92.06	ND	None
	1	None	100	None					
Methyl benzilate	16	50	50	None	2.6		>100	71.91	PE (100 μM), YSE (100 μM)
	1	100	100	None					
2-(Diethylamino) ethanol	16	100	None	None	0.3	1300	>100	80.35	TR (100 μM)
	1	None	None	None					
2-(Diisopropylamino) ethanol	16	100	None	None	1.2	770	>100	ND	None
	1	50	500	None					
Methylphosphonic dichloride	16	None	None	None	1	26	>100	ND	None
	1	None	10	None					
2,2′-Thiodiethanol	16	None	None	None	−0.6	6,610	>100	ND	None
	1	None	None	None					
2-Chloroethanol	16	None	None	None	−0.1	91	>100	ND	None
	1	None	None	None					
3-Quinuclidinol	16	None	None	None	0.2		>100	ND	None
	1	None	None	None					

(Continues)

TABLE 3 (Continued)

Chemical name	PMR exposure duration, h	PMR (by phase)			XlogP3	Rodent LD_{50}, mg/kg	5-day LC_{50}, μM	5-day EC_{50}, μM	EC_{50}-associated malformations (lowest effect concentration)
		Background	Excitatory	Refractory					
		μM	μM	μM					
Arsenic (III) chloride	16	None	None	None	1.61	48	>100	ND	To be added
	1	None	None	None					
Dimethyl methylphosphonate	16	None	None	None	−0.7	6,810	>100	ND	None
	1	None	None	None					
Dimethyl phosphite	16	None	None	None	0	1831	>100	ND	None
	1	None	None	None					
N-Methyl-3-piperidinol	16	None	None	None	0.1		>100	ND	None
	1	None	None	None					
Phosphorus trichloride	16	None	None	None	2.3	18	>100	ND	None
	1	None	None	None					
Triethanolamine	16	None	None	None	−1	5,846	>100	ND	None
	1	None	None	None					
Trimethyl phosphite	16	None	None	None	0.1	4,280	>100	ND	None
	1	None	None	None					

Notes: ND = 50% effect level could not be determined due to a low rate of malformations and/or the compound does not cause significant malformations. Further testing with some chemicals at higher or lower concentrations would be required to generate EC_{50}s and LC_{50}s.

[a]= Statistical significance could not be calculated due to high levels of mortality or zero movement caused by chemical toxicity.

[*]= These PMR responses were hyperactive; all other PMR responses were hypoactive.

excitatory PMR response during the 1-h exposures. Interestingly cyanide (33.3 μM) and carbonyl cyanide *m*-chlorophenylhydrazine (1 μM) caused delayed development during the standard exposures at low concentrations, which was similarly observed by Sips et al. (2018) for cyanide exposures.

Methyl benzilate induced hypoactive responses in both the background and the excitatory phase of the PMR at 100 and 50 μM respectively for the standard exposure (Figure 3). This response pattern resembles the PMR responses to diazepam, a known anxiolytic, reported by Kokel et al. (2010); thus, methyl benzilate may also be anxiolytic. This reduction in activity was further exacerbated when the zebrafish embryos were exposed to a 1-h exposure to concentrations of 500 μM. Methyl benzilate is a precursor for the incapacitant, BZ, which is a potent anti-cholinergic compound. Of the three BZ precursors tested in this study, only methyl benzilate showed bioactivity in the PMR assay.

The LC_{50}s could be calculated for five chemicals and embryo malformation EC_{50}s for five chemicals (Table 3). Rotenone had the lowest LC_{50} that could be generated with the concentrations that were tested. Carbonyl cyanide *m*-chlorophenylhydrazine also had high mortality, with an LC_{50} of <1 μM. Chlorpyrifos and cyanide were about three orders of magnitude less toxic than rotenone, while the remaining chemicals had LC_{50}s that were >500 μM.

Five chemicals had 120 hpf endpoints that caused malformations in ≥50% of the embryos (Table 3). The acetylcholinesterase-inhibiting OP pesticides, chlorpyrifos and parathion, induced similar morphological malformations to zebrafish embryos including yolk sac edema, axis malformations and notochord malformations. The main distinction between the two chemicals was that parathion induced malformations to the jaw, snout, and eye of the zebrafish embryos, while chlorpyrifos did not impact these areas but did effect the development of the trunk of the embryos. Both chemicals caused pericardial edema malformations in the developing zebrafish embryos. However, only parathion induced pericardial edema in ≥50% of the embryos. Methyl benzilate caused pericardial edema and yolk sac edema in ≥50% of the embryos at the 100 μM concentration.

We also performed a touch response assay. Embryos were gently touched with a probe to elicit their touch response (Truong et al., 2011); normally, embryos respond by swimming rapidly around their well for a short duration before coming to a rest. Cyanide, parathion and 2-(diethylamino) ethanol were the only chemicals that caused a touch response deficiency (TR). Embryos exposed to concentrations less than the LC_{50} of cyanide (LC_{50} = 90.35 μm, TR ≥ 50% = 67.3 μM) and 2-(diethylamino) ethanol (TR ≥50% = 100 μM) had severely reduced responses (slight caudal fin movement but no swimming motion) or no response at all to the touch stimuli. Embryos exposed to parathion also had a reduced touch response (caused at 100 μM) but at a concentration higher than the LC_{50} (76.69 μM). This suggests that the embryos, while they are still alive, could be moribund.

4 | DISCUSSION

The present study shows the potential of the zebrafish PMR to rapidly screen and differentiate compounds with uncharacterized

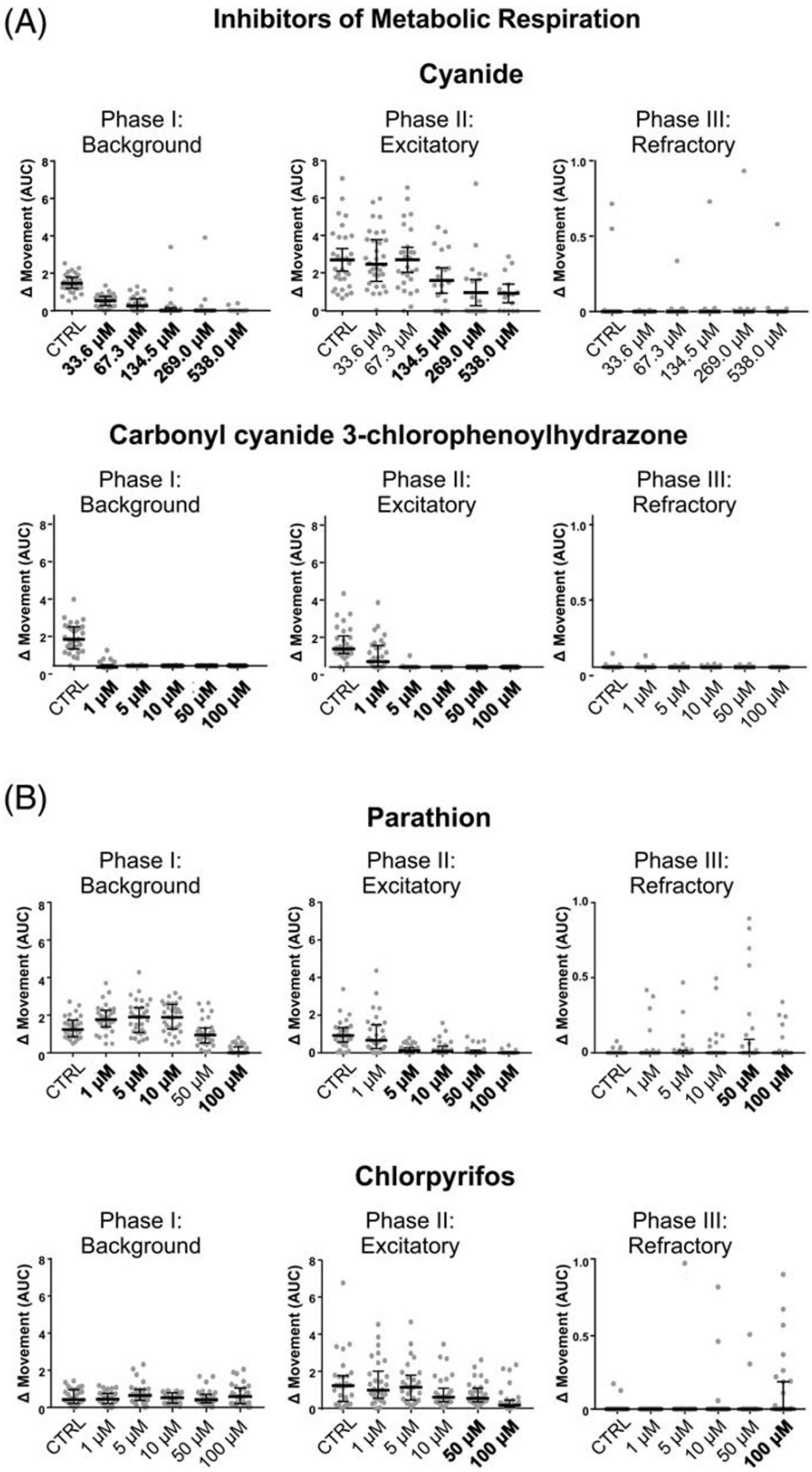

FIGURE 2 Phase-specific photomotor response (PMR). These are the PMRs for zebrafish embryos at 25 h post-fertilization (hpf) following a 1-h exposure initiated at 24 hpf. The raw data produced from the PMR was further condensed by phase and concentration using area under the curve as shown in the above scatter plot. The medians and interquartile ranges are shown above. Individual zebrafish embryo PMRs are shown on the charts as a red dot. Zebrafish embryos that died as a result of the exposure were excluded from the dataset above. Inhibitors of metabolic respiration (A.) caused decreased movement in both Phase I and II, but Phase III was unaffected. Acetyl cholinesterase inhibitors (B.) had elevated movement in Phase I, decreased movement in Phase II, and increased movement with increased concentrations during Phase III. Concentrations in bold show concentrations that were determined to be statistically significant after non-responders were removed ($\alpha = 0.05$)

toxicity, as well as provide information on chemicals that act via a specific mechanism of action. This model could be used in combination with other models in future work to quickly identify compounds with similar mechanisms of action by evaluating the phase-specific response pattern produced by the PMR after a short-term exposure. This behavioral response pattern could then be

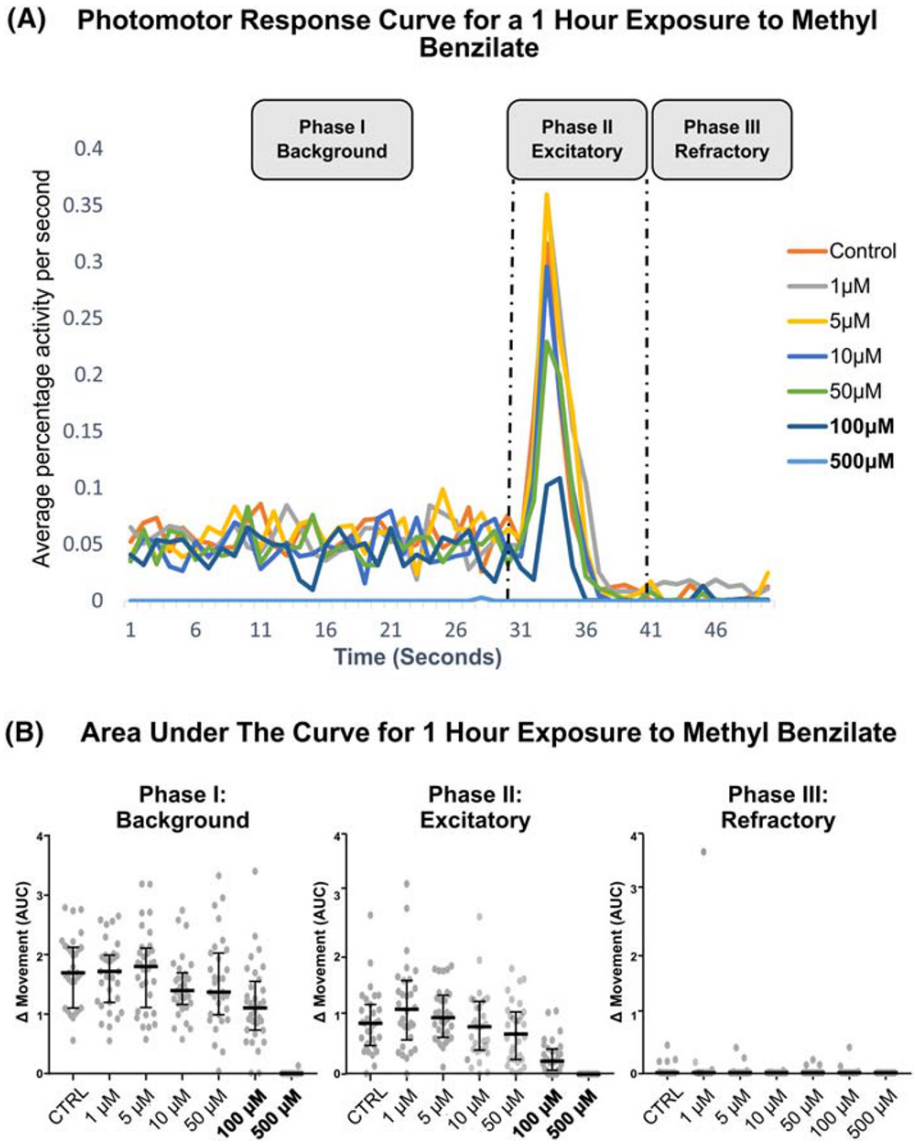

FIGURE 3 Photomotor response (PMR) for 1-h methyl benzilate exposure. A. Shows the PMR for the 1-h exposure to methyl benzilate beginning at 24 h post-fertilization (hpf) and ending at 25 hpf. Individual zebrafish embryo responses for each concentration were averaged together to generate an average PMR for each concentration (n = 32 per concentration, except for the 500 μM concentration n = 48). B. Is the raw data that was generated for the 1-h exposure to methyl benzilate put into the area under the curve statistic. The medians and interquartile ranges are shown above. Individual zebrafish embryo PMRs are shown on the charts as a red circle. Concentrations in bold indicates that the concentration was statically significant after non-responders were removed (α = 0.05)

compared to the behavioral response patterns of chemicals with known mechanisms of action to further characterize the toxicity of unknown compounds.

The two exposure paradigms (1-h vs 16-h exposures) presented in the present study complement each other, as each has strengths and limitations. The 1-h exposures to the 20 chemicals produced a total of 10 positive responses in the PMR assay, as did the standard exposures. However, two of the 10 chemicals that tested positive for the PMR response were detected by only one of the exposure paradigms, as previously mentioned. The remaining eight chemicals had positive PMR results in both assays. Furthermore, both the standard exposure assay and the 1-h exposure assay were able to detect the same amount of positive PMR responses overall across all phases of the PMR in the chemicals that were tested (17 positive PMR responses). Interestingly, there were slight differences in the behavioral patterns produced for the two OP compounds when comparing the standard exposure PMR barcode to the 1-h exposure PMR barcode. This observed difference could be caused by difference in the exposure duration of the compound to the embryo and/or the rate of absorption/metabolism of the chemical in the embryo. Upon further investigation we also found that parathion showed a unique pattern across all three passes of the PMR test for the 1-h exposure assay that was not observed by previous work by Reif et al. (2016). Reif et al. found that parathion caused hypoactivity only during the excitatory phase. However, the 1-h exposure is a much more rapid test (2 day run time from start to finish) compared to the standard exposure (5 day run time) and is more relevant where acute exposure scenarios are the primary concern, such as during military operations. The higher throughput 1-h exposure omits 120 hpf morphological data collection and possible early developmental malformations that could occur. For example, the standard exposures of cyanide and carbonyl cyanide 3-chlorophenlhydrazone caused delayed development in the zebrafish embryos but caused no effects in the 1-h exposures. For a

more comprehensive toxicity evaluation of unknown chemicals, it would be advantageous for both standard exposures and 1-h exposures to be conducted in parallel. By doing so, chemicals that have a high potential to cause acute lethality can be rapidly identified, but also chemicals causing developmental impairment can also be identified thereby creating a more robust and comprehensive understanding of the toxicity of unknown compounds.

However, a limitation of this assay is reflected in the low sensitivity to several of the chemical weapon precursor compounds with known toxicity in mammals (Table S1). This is likely an unavoidable limitation, because some the compounds are highly reactive [phosphorus trichloride, phosphorus (V) oxychloride, and methylphosphonic dichloride] in aqueous test systems and some were volatile (cyanide). In fact, several of these compounds were also tested in the ToxCast/Tox21 battery of assays (U.S. EPA, 2015) with very few hits (Table S2). Another reason for limited sensitivity can likely be attributed to logP (Zolotarev, Belyaeva, Mikhailov, & Mikhailova, 2017), which can skew the bioavailability of a test compounds relative to the nominal concentration in the media. For most of the chemicals that were positive in the assay, the LogP was >1 (Zolotarev et al., 2017).

PMR test results could be augmented by the addition of more complex behavioral assays to further discriminate the mechanisms of toxicity. For example, previous studies (Bruni et al., 2016; Kokel et al., 2010; McCarroll, Gendelev, Keiser, & Kokel, 2016; Rihel et al., 2010) have screened large sets of chemicals with varying known mechanisms of action to generate behavioral profiles utilizing the zebrafish model at different developmental stages. Bruni et al. (2016) developed a 7 days post-fertilization (dpf) zebrafish larval assay to screen a chemical library that consisted of >24 000 compounds, to identify compounds with unknown bioactivity by mapping known chemical behavioral barcodes to that of the unknowns. This type of behavioral assay differs from the PMR assay in that it includes a more complex set of stimuli presented to the zebrafish at a later developmental stage (7 dpf). The 7 dpf zebrafish assay includes the presentation of multiple wavelengths of light, as well as acoustic stimuli. However, the mechanisms that these stimuli target are not well known (Bruni et al., 2016). As a result of the larger number of stimuli presented, a greater variety of phenotypic response patterns for specific chemicals can be produced for each test. This additional information, while having lower throughput than the PMR, could provide additional specificity in the curation of behavioral barcodes to link chemicals with similar modes of action together.

Ultimately, applying a combination of PMR and other more complex behavioral assays can be used to identify and discriminate unknown compounds that impact neurological pathways and physiological processes from those that do not. Continued discovery of the mechanisms of action through which compounds impart toxicity will allow for a more refined, targeted approach to novel drug discovery and improve efficiencies through higher throughput screening. Current research efforts are mainly focused on finding countermeasures for chemical warfare agents and pesticides (OPs). While extensive research has been conducted on this set of chemicals, relatively few countermeasures have been produced to date. The current standard countermeasure for chemical warfare nerve agent and OP exposures is the administration of a combination of atropine and pralidoxime chloride (2-PAM). (FDA, 2010; Munro, Watson, Ambrose, & Griffin, 1990). Atropine counteracts the effects of the nerve agent on muscarinic receptors, while 2-PAM works to reactivate the cholinesterase activity following nerve agent exposure (Jokanovic & Protran, 2009). Pyridostigmine bromide has also been used as a prophylactic drug to protect against exposures to chemical warfare nerve agents through reversible competitive inhibition of acetylcholinesterase (Keeler, Hurst, & Dunn, 1991; Williams, 1984). However, pyridostigmine bromide exposure may have contributed to the etiology of 'Gulf War Illness' in soldiers that were deployed in the Persian Gulf War. (Joshi et al., 2019; Macht et al., 2019). The PMR model could help in the discovery of novel countermeasures and therapeutics due to the rapid throughput and low resource evaluations that can be achieved with the zebrafish model. The ability to quickly evaluate novel chemicals with a wider array of toxicity endpoints could potentially reduce the likelihood of fielding a therapeutic with harmful side-effects. However, before a targeted novel drug discovery method is developed, more data will need to be collected from a diverse set of compounds with well-known mechanisms of toxicity to further substantiate these results, which will be critical to furthering the utility of this model.

5 | DISCLAIMER

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CONFLICT OF INTEREST

The authors did not report any conflict of interest.

ORCID

Chance M. Carbaugh https://orcid.org/0000-0002-9949-691X

REFERENCES

Barbazuk, W. B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., … Johnson, S. L. (2000). The syntenic relationship of the zebrafish and

human genomes. *Genome Research*, *10*(9), 1351–1358. https://doi.org/10.1101/gr.144700

Bruni, G., Rennekamp, A. J., Velenich, A., McCarroll, M., Gendelev, L., Fertsch, E., … Kokel, D. (2016). Zebrafish behavioral profiling identifies multitarget antipsychotic-like compounds. *Nature Chemical Biology*, *12*(7), 559–566. https://doi.org/10.1038/nchembio.2097

Cornet, C., Calzolari, S., Miñana, P. R., Dyballa, S., van Doornmalen, E., Rutjes, H., … Terriente, J. (2017). ZeGlobalTox: An Innovative Approach to Address Organ Drug Toxicity Using Zebrafish. *International Journal of Molecular Sciences*, *18*(4), 864–882. https://doi.org/10.3390/ijms18040864

FDA. PROTOPAM Chloride (pralidoxime chloride) for injection. New Drug Application (NDA): U.S. Food and Drug Administration 2010 September 8, 2010 Contract No.: 014134/S-022.

Hagstrom, D., Truong, L., Zhang, S., Tanguay, R., & Collins, S. E. M. (2019). Comparative analysis of Zebrafish and planarian model Systems for Developmental Neurotoxicity Screens Using an 87- compound library. *Toxicological Sciences*, *167*(1), 15–25. https://doi.org/10.1093/toxsci/kfy180

Hamilton, A. H., Russo, C. R., & Thurston, V. R. (1977). Trimmed Spearman-Karber method for estimating median lethal concentration in toxicity bioassays. *Environmental Science & Technology*, *11*(7), 714–719. https://doi.org/10.1021/es60130a004

Horzmann, K. A., & Freeman, J. L. (2016). Zebrafish Get Connected: Investigating Neurotransmission Targets and Alterations in Chemical Toxicity. *Toxics*, *4*, 3–19. https://doi.org/10.3390/toxics4030019

Howe, K., Clark, M. D., Torroja, C. F., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. https://doi.org/10.1038/nature12111

Jokanovic, M., & Protran, M. (2009). Pyridinium oximes as cholinesterase reactivators. Structure-activity relationship and efficacy in the treatment of poisoning with organophosphorus compounds. *Current Medicinal Chemistry.*, *16*(17), 2177–2188. https://doi.org/10.2174/092986709788612729

Joshi, U., Pearson, A., Evans, J. E., Langlois, H., Saltiel, N., Ojo, J., … Abdullah, L. (2019). A permethrin metabolite is associated with adaptive immune responses in Gulf War Illness. *Brain, Behavior, and Immunity*, *81*, 545–559. https://doi.org/10.1016/j.bbi.2019.07.015

Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends in Pharmacological Sciences*, *35*(2), 63–75. https://doi.org/10.1016/j.tips.2013.12.002

Keeler, J. R., Hurst, C. G., & Dunn, M. A. (1991). Pyridostigmine Used as a Nerve Agent Pretreatment Under Wartime Conditions. *Journal of the American Medical Association.*, *266*(5), 693–695.

Kim, K. T., & Tanguay, R. L. (2014). The role of chorion on toxicity of silver nanoparticles in the embryonic zebrafish assay. *Environmental Health and Toxicology.*, *29*, 1–6, e2014021. https://doi.org/10.5620/eht.e2014021

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics.*, *203*, 253–310. https://doi.org/10.1002/aja.1002030302

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C. Y., Mateus, R., … Peterson, R. T. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nature Chemical Biology*, *6*(3), 231–237. https://doi.org/10.1038/nchembio.307

Kokel, D., Dunn, T. W., Ahrens, M. B., Alshut, R., Cheung, C. Y., Saint-Amant, L., & Peterson, R. T. (2013). Identification of nonvisual photomotor response cells in the vertebrate hindbrain. *The Journal of Neuroscience*, *33*(9), 3834–3843. https://doi.org/10.1523/JNEUROSCI.3689-12.2013

Kokel, D., & Peterson, R. T. (2008). Chemobehavioural phenomics and behaviour-based psychiatric drug discovery in the zebrafish. *Briefings in Functional Genomics & Proteomics*, *7*(6), 483–490. https://doi.org/10.1093/bfgp/eln040

Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., & Robinson, J. P. (2003). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *The Journal of Biological Chemistry*, *278*(10), 8516–8525. https://doi.org/10.1074/jbc.M210432200

Lieschke, J. G., & Currie, D. P. (2007). Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*, *8*, 353–367. https://doi.org/10.1038/nrg2091

Macht, V. A., Woodruff, J. L., Maissy, E. S., Grillo, C. A., Wilson, M. A., Fadel, J. R., & Reagan, L. P. (2019). Pyridostigmine bromide and stress interact to impact immune function, cholinergic neurochemistry and behavior in a rat model of Gulf War Illness. *Brain, Behavior, and Immunity*, *80*, 384–393. https://doi.org/10.1016/j.bbi.2019.04.015

MacRae, C. A., & Peterson, R. T. (2015). Zebrafish as tools for drug discovery. *Nature Reviews Drug Discovery*, *14*, 721–731. https://doi.org/10.1038/nrd4627

Maes, J., Verlooy, L., Buenafe, O. E., de Witte, P. A., Esguerra, C. V., & Crawford, A. D. (2012). Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. *PLoS ONE*, *7*(10), 1–9, e43850. https://doi.org/10.1371/journal.pone.0043850

Mandrell, D., Truong, L., Jephson, C., Sarker, M. R., Moore, A., Lang, C., … Tanguay, R. L. (2012). Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. *Journal of Laboratory Automation*, *17*(1), 66–74. https://doi.org/10.1177/2211068211432197

McCarroll, M. N., Gendelev, L., Keiser, M. J., & Kokel, D. (2016). Leveraging Large-scale Behavioral Profiling in Zebrafish to Explore Neuroactive Polypharmacology. *ACS Chemical Biology*, *11*(4), 842–849. https://doi.org/10.1021/acschembio.5b00800

Munro, N. B., Watson, A. P., Ambrose, K. R., & Griffin, G. D. (1990). Treating exposure to chemical warfare agents: implications for health care providers and community emergency planning. *Environmental Health Perspectives*, *89*, 205–215. https://doi.org/10.1289/ehp.9089205

National Academies of Sciences, Engineering, and Medicine. (2015). *Application of modern toxicology approaches for predicting acute toxicity for chemical defense*. Washington, DC: The National Academies Press. https://doi.org/10.17226/21775

Pelka, K. E., Henn, K., Keck, A., Sapel, B., & Braunbeck, T. (2017). Size does matter – Determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (Danio rerio) embryos. *Aquatic Toxicology*, *185*, 1–10. https://doi.org/10.1016/j.aquatox.2016.12.015

Reif, D. M., Truong, L., Mandrell, D., Marvel, S., Zhang, G., & Tanguay, R. L. (2016). High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. *Archives of Toxicology*, *90*(6), 1459–1470. https://doi.org/10.1007/s00204-015-1554-1

Rihel, J., Prober, D. A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., … Schier, A. F. (2010). Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science*, *327*, 348–351. https://doi.org/10.1126/science.1183090

Sips, P. Y., Shi, X., Musso, G., Nath, A. K., Zhao, Y., et al. (2018). Identification of specific metabolic pathways as druggable targets regulating the sensitivity to cyanide poisoning. *PLoS ONE*, *13*(6), 1–17, e0193889. https://doi.org/10.1371/journal.pone.0193889

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., & Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends in Neurosciences*, *37*(5), 264–278. https://doi.org/10.1016/j.tins.2014.02.011

Truong, L., Harper, S. L., & Tanguay, R. L. (2011). Evaluation of embryotoxicity using the zebrafish model. *Methods in Molecular Biology*, *691*, 271–279. https://doi.org/10.1007/978-1-60761-849-2_16

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D., & Tanguay, R. L. (2013). Multidimensional in vivo hazard assessment using zebrafish. *Toxicological Sciences*, *137*(1), 212–233. https://doi.org/10.1093/toxsci/kft235

U.S. EPA. (2015). ToxCast & Tox21 Summary Files from invitrodb_v3. Retrieved from https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data on October 28, 2018. Data released October 2018.

Wagner, C., Pan, Y., Sinha, V., & Zhao, P. (2016). Predicting the Effects of CYP3A Inducers on the Pharmacokinetics of Substrate Drugs Using Physiologically Based Pharmacokinetic (PBPK) Modeling: An Analysis of PBPK Submissions to the US FDA. *Clinical Pharmacokinetics*, *55*(4), 475–483. https://doi.org/10.1007/s40262-015-0330-y

Williams, J. I. (1984). *Human response to Pyridostigmine bromide*. Fairborn, OH: Macaulay-Brown, Inc. Available from the National Technical Information Service. NTIS/AD-A140960

Zolotarev, K. V., Belyaeva, N. F., Mikhailov, A. N., & Mikhailova, M. V. (2017). Dependence between LD_{50} for Rodents and LC_{50} for Adult Fish and Fish Embryos. *Bulletin of Experimental Biology and Medicine*, *162*(10), 439–444. https://doi.org/10.1007/s10517-017-3636-y

SUPPORTING INFORMATION

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