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Age matters: Developmental stage of *Danio rerio* larvae influences photomotor response thresholds to diazinon or diphenhydramine

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Abstract

Because basic toxicological data is unavailable for the majority of industrial compounds, High Throughput Screening (HTS) assays using the embryonic and larval zebrafish provide promising approaches to define bioactivity profiles and identify potential adverse outcome pathways for previously understudied chemicals. Unfortunately, standardized approaches, including HTS experimental designs, for examining fish behavioral responses to contaminants are rarely available. In the present study, we examined movement behavior of larval zebrafish over 7 days (4–10 days post fertilization or dpf) during typical daylight workday hours to determine whether intrinsic activity differed with age and time of day. We then employed an early life stage approach using the Fish Embryo Test (FET) at multiple developmental ages to evaluate whether photomotor response (PMR) behavior differed with zebrafish age following exposure to diazinon (DZN), a well-studied orthophosphate insecticide, and diphenhydramine (DPH), an antihistamine that also targets serotonin reuptake transporters and the acetylcholine receptor. 72 h studies were conducted at 1–4, 4–7 and 7–10 dpf, followed by behavioral observations using a ViewPoint system at 4, 7 and 10 dpf. Distance traveled and swimming speeds were quantified; nominal treatment levels were analytically verified by isotope-dilution LC-MSMS. Larval zebrafish locomotion displayed significantly different ($p < 0.05$) activity profiles over the course of typical daylight and workday hours, and these time of day PMR activity profiles were similar across ages examined (4–10 dpf). 10 dpf zebrafish larvae were consistently more sensitive to DPH than either the 4 or 7 dpf larvae with an environmentally realistic lowest observed effect concentration of 200 ng/L. Though ELS and FET studies with zebrafish typically focus on mortality or teratogenicity in 0–4 dpf organisms, behavioral responses of slightly older fish were several orders of magnitude more sensitive to DPH. Our observations highlight the importance of understanding the influence of time of day on intrinsic locomotor activity, and the age-specific hazards of aquatic contaminants to fish behavior.

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Keywords

Alternative toxicity test methods; Behavior; Comparative pharmacology and toxicology; Developmental toxicology; Photomotor response

1. Introduction

As global entities attempt to understand structural attributes associated with bioactivity and toxicity profiles of consumer products and industrial chemicals, issues concerning limited availability of relevant toxicological information continue to surface (Schaafsma et al, 2009). The roughly 68,000 chemicals lacking basic toxicological data present a significant hurdle for regulatory decision-making, but also require an exorbitant amount of resources to perform the traditional suite of toxicology studies (Rovida and Hartung, 2009; Weisbrod et al, 2007). In response to the burgeoning need to effectively evaluate the growing number of substances in commerce, the National Research Council (NRC) published Toxicity Testing in the 21st Century: A Vision and a Strategy, which effectively challenged the existing toxicology testing paradigm. This report identified the opportunities to move from historical chemical screening efforts with *in vivo* apical observations, to more cost and time effective *in vitro* observations aimed at describing the mechanisms responsible for adverse outcomes (NRC 2007). To meet these goals a two-pronged approach was recommended to facilitate the use of existing data, and to acquire new data on previously inadequately studied compounds using High Throughput Screening (HTS) methodologies typically utilized for drug discovery (Rusyn and Daston, 2010). Through the U.S. Environmental Protection Agency's (US EPA) Computational Toxicology research (CompTox) initiatives, data from programs such as Tox21, ToxCast™ and other reference databases are advancing HTS efforts to collect unprecedented information on biological activities of diverse contaminants (Kavlock et al., 2012).

In vitro extrapolations of HTS bioactivity information to *in vivo* toxicology data remains a challenging and critically important research need (Dobbins et al., 2008; Dreier et al, 2015; Huggett et al, 2003b). Parallel efforts to advance HTS efforts at the organismal level have also been undertaken and hold great promise for understanding contaminant at higher levels of biological complexity than *in vitro* systems (Padilla et al., 2012; Raftery et al., 2014; Truong et al., 2014). These *in vivo* HTS methodologies have largely examined early life stage studies with embryonic and larval zebrafish. For example, the Fish Embryo Acute Toxicity (FET; OECD 236) test, which is a standardized protocol for the evaluation of acute toxicity through apical endpoints such as mortality and teratogenicity (OECD, 2013), is increasingly used. Such alternative toxicity testing approaches are receiving much attention because if they can replace historical aquatic toxicology methods then animal welfare benefits and economic efficiencies are gained (Belanger et al., 2013). In addition to standardized endpoints, various histopathological, biochemical, molecular, and physiological sublethal responses are frequently included at the conclusion of early life stage studies.

There remains an increasing need to define adverse outcomes associated with behavioral alterations caused by aquatic contaminants (Brooks et al, 2003; Brooks, 2014; Ford and

Fong, 2015). Unlike standard toxicity endpoints, standardized protocols rarely provide requirements and recommendations for performing toxicology studies with nontraditional sublethal endpoints, including behavioral perturbations. A number of behavioral investigations have occurred with fish models, including adaptations of mammalian pharmacology tests on anxiety such as light/dark preference (Steenbergen et al., 2011), thigmotaxis (Schnorr et al., 2012), and open field and color preference (Ahmad and Richardson, 2013) tests. Additionally photomotor responses (PMR) are increasingly used to understand baseline, stimulatory and refractory zebrafish behaviors (Kokel et al., 2010; Raftery et al, 2014).

Whether various behavior modifications represent adverse outcomes relevant to hazard and risk assessment remains an active area of study. Existing experimental designs such as FET method focuses on early life stage responses to contaminants, but whether baseline behavior changes with age or during the course of typical daytime work hours is not well defined. In addition, the magnitude of toxicity differs across fish developmental stages for some molecules. For example, we recently identified a sensitivity gradient over the first 10 days of zebrafish development in which mortality responses to the acetylcholinesterase (AChE) inhibitor diazinon (DZN) and the antihistamine diphenhydramine (DPH) were more pronounced in slightly older organisms than are employed in early life stage studies (Kristofco et al., 2015). Whether such differential sensitivity throughout development extends to behavioral observations is not understood. Thus, the primary objectives of the current were (1) to define whether naïve PMR behaviors and locomotor activity of larval zebrafish differed during various times of day across ages commonly employed in early life stage studies (4–10 days post fertilization or dpf), and (2) to identify whether behavioral sensitivities to an insecticide and a pharmaceutical differed across early life stages of larval zebrafish.

2. Materials and methods

2.1. Zebrafish culture

Tropical 5D wild type zebrafish (*Danio rerio*) were maintained under standard culture conditions at Baylor University as previously described (Kristofco et al., 2015; Usenko et al., 2011). Briefly, zebrafish were raised 5–6 adult fish per 1.5 L tank in a filtered (Compact system, Lifeguard Aquatics, Cerritos, CA, USA) recirculating z-mod system (Marine Biotech Systems, Beverly, MA, USA) maintained at 28 °C on a 16 h light/8 h dark photoperiod. System culture water was formulated by addition of Instant Ocean® salts (Pentair AES, Apopka, FL, USA) to deionized water, which was buffered to pH 7 with sodium bicarbonate (50 mg/L). Zebrafish were fed twice daily with brine shrimp (*Artemia* sp. nauplii; Pentair AES, Apopka, FL, USA) and once per day with flake food (Pentair AES, Apopka, FL, USA). Culture and experimental conditions followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

2.2. Influence of age and time of day on larval zebrafish behavior

Standardized experimental designs for zebrafish toxicity such as the FET OECD no. 236, (OECD, 2013) method require experiments to be initiated and completed on various dpf, but

commonly do not require experimental observations to be performed during specific times during the day. We examined movement behavior of larval zebrafish over 7 days (4–10 dpf) during typical daylight workday hours to determine whether intrinsic activity differed with age and time of day. Adult zebrafish were spawned the evening prior to egg collection, and within two hours of the onset of daylight the embryos were collected. Organisms were then staged (Kimmel et al., 1995) and placed in petri dishes with clean culture water. Embryos were maintained at 28°C with a 14h light/10h dark photoperiod and received daily water changes. At 3 dpf spontaneously hatched larvae whose swim bladders were inflated were selected for baseline behavioral study.

Observations of untreated individuals were recorded for 40 min at the start of each hour commencing at 09:00 and then continuing until 18:00 h for each day from 4dpf until 10 dpf. One larvae was placed in a single well, each in twenty-four well plates with 2 mL culture water for observation. Each plate was assigned an hour, and behavioral recordings were performed at that time from 4 to 10 dpf using a Zebrabox (ViewPoint, Lyon, France). Starting at 4 dpf, after all behavioral recordings had concluded for the day, fifty percent water renewals were completed and larvae were fed 10 µL of 0.5 g/250 mL DI water Ziegler® Larval AP100 (Pentair AES, Apopka, FL).

2.3. Influence of diazinon or diphenhydramine on age-specific zebrafish behavior

In the present study, the experimental design of the OECD FET (OECD, 2013) method was used with minor modifications. In our previous research, three larval zebrafish ages over 10 dpf were identified to display differential mortality sensitivities to DPH and DZN (Kristofco et al., 2015). We thus extended these observations to examine whether behavioral responses were more sensitive than endpoints previously examined by the FET approach, and to determine whether behavioral toxicity thresholds differed among developmental ages. Three 72 h exposure windows were selected (1–4, 4–7, 7–10 dpf) for study; behavioral observations were performed at the conclusion of each 72 h experiment. Staging for the initiation of these studies followed descriptions provided by Kimmel et al. (1995).

DZN (CAS 333-41-5; >98% purity; Chem Service, Inc., West Chester, PA, USA) and DPH hydrochloride (CAS 147-24-0; 98% purity; Sigma-Aldrich Corp., St. Louis, MO, USA) were commercially obtained. For all experiments, 5 embryos or larvae were placed in each 100 mL experimental unit (glass beakers with 30 mL of solution) for treatment levels and negative controls; 8 replicate experimental units were included per treatment level. In addition to examining a range of sublethal concentrations, the highest treatment levels of DZN and DPH used in this study were age specific LC₅₀ values from our recent findings (Kristofco et al., 2015), and were selected to understand the magnitude of sensitivity differences between traditional mortality and alternative behavioral responses of different ages. These nominal treatment levels were 700, 250, and 50 mg/L for DPH, and 15, 12, and 5.5 mg/L for DZN for the 1–4, 4–7 and 7–10 dpf experiments, respectively.

For DPH, treatment levels were selected to include sublethal concentrations below a predicted therapeutic hazard value (THV). Building on our early work (Berninger et al, 2011; Valenti et al., 2012), the THV Eq. (1) of a pharmaceutical is a water concentration

predicted to result in fish accumulation to plasma levels equaling a human therapeutic dose ($C_{\max}$); (Brooks, 2014).

$$THV = \frac{\log P_{\text{Blood:Water}}}{C_{\max}} \quad (1)$$

It was motivated by a conceptual proposal by Huggett et al. (Huggett et al., 2003a); Eq. (2) to estimate pharmaceutical levels in fish plasma following aqueous exposure, which was derived

$$\text{Fish plasma concentration} = [\text{Aqueous}] \times \log P_{\text{Blood:Water}} \quad (2)$$

from initial plasma modeling by Fitzsimmons et al. (Fitzsimmons et al, 2001) to predict fish blood:water partitioning of hydrophobic chemicals. Because DPH is a weak base with a pK_a value of 9.1 (Nichols et al., 2015), the Fitzsimmons et al. model was modified to account for experimental pH (7) of the present study by using $\log D$ instead of $\log K_{ow}$ (Berninger et al., 2011); Eq. (3).

$$\log P_{\text{Blood:Water}} = 0.73 \times \log D - 0.88 \quad (3)$$

Larval zebrafish were fed 0.5g/250 mL Ziegler AP100 starting at 4 dpf until study initiation, or 150 μL per beaker. Experimental solutions of DZN or DPH were prepared in zebrafish culture water (Instant Ocean® in DI water buffered to pH 7 with sodium bicarbonate) at study initiation, and were stored at 4°C in the dark with aliquots warmed to experimental temperature prior to daily renewals. For DZN solutions, methanol was used as a solvent carrier, but not exceeding 0.01% v/v. Water samples of all nominal treatment levels were subsampled at study initiation for analytical verification of treatment levels by LC-MS/MS (see Subsection 2.6 below).

2.4. Behavioral observations

All behavioral observations were recorded in quantization mode using the ZebraBox and accompanying ZebraLab tracking software (ViewPoint, Lyon France). Treatment levels resulting in significant acute mortality or malformations were not included in behavioral studies. The calibration parameters for the plate and pixel detection thresholds were: plate width = 125 mm; pixel detection threshold = black, 13; movement thresholds: resting = <5mm/s; cruising = 5–20 mm/s; bursting = >20 mm/s; data bin = one minute. To reduce the amount of background noise from larval reflection on the walls of the well, tracking refreshed after each bin and only recorded movement when the organism traveled 2 pixels.

To determine potential influences of age and time of day on larval zebrafish, baseline observations were collected for 40 min, consisting of a 20 min dark period followed by a 20 min light period. As noted above, one plate was examined each hour from 09:00 to 18:00 h from 4 to 10 dpf. Hereafter, we refer to the 09:00 observation as the hour when light was

turned on in the morning. For all 09:00 h behavioral recordings a plate was removed from a dark incubator and transferred directly to a dark Zebrabox.

To determine influences of DZN or DPH on age-specific behavior of larval zebrafish, following each 72 h study period 4, 7 or 10 dpf zebrafish larvae were loaded one larva per well with 2 mL of corresponding treatment level solution in 24 well plates for observation. 3 larvae from each experimental replicate beaker were selected for behavioral observations, and all treatment levels were included on each plate to avoid potential bias due to time of day. Behavior of each plate then was recorded for 50 mins, and these recordings were initiated at ~ 10:30 to 11:00 h. The 50 min observation period consisted of a 10 min dark acclimation period followed by two consecutive cycles of a light stimulatory phase for 10 min followed by 10 min of a darkness refractory phase.

2.5. Analytical verification of diazinon and diphenhydramine treatment levels

Nominal treatment levels of DZN and DPH were verified for each experimental stock solution by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Agilent Infinity 1260 autosampler/quaternary pumping system, Agilent jet stream thermal gradient electrospray ionization source, and model 6420 triple quadrupole mass analyzer. A 500 μ L aliquot of undiluted or diluted stock solution was combined with 450 μ L of 0.1% formic acid (w/w) and spiked with 50 μ L of an internal standard (DPH-d₃ & DZN-d₁₀) in a standard 2mL analytical vial (Agilent Technologies, Santa Clara, CA, USA) before analyses. A gradient mobile phase condition that resulted in the elution of DPH at 4.1 min and DZN at 4.7 min was identified. Salts and other highly polar sample constituents were diverted to waste and away from the MS/MS during the first minute of each sample run.

Chromatography was performed using a 10 cm $\times$ 2.1 mm Poroshell 120 SB-AQ column (120 $\text{\AA}$, 2.7 μ m, Agilent Technologies, Santa Clara, CA, USA) preceded by a 5 mm $\times$ 2.1 mm Poroshell 120SB-C18 attachable guard column (120 $\text{\AA}$, 2.7 μ m, Agilent Technologies, Santa Clara, CA, USA). The ionization mode, monitored transitions, and instrumental parameters for DPH/DPH-d₃ and DZN/DZN-d₁₀ were as follows: ESI + DPH 256.2 > 167, fragmentor = 80, collision energy = 8; DPH-d₃ 259.2 > 167, fragmentor = 80, collision energy = 8; DZN 305.1 > 169.1, fragmentor = 125, collision energy = 24; DZN-d₁₀ 315.2 > 170.1, fragmentor = 130, collision energy = 24. Limit of detection (LOD) and limit of quantification (LOQ) were determined by running several method blanks and calculating the standard deviation. The LODs and LOQs for DPH and DZN were determined to be 0.017 μ g/L and 0.055 μ g/L, and 0.006 μ g/L and 0.020 μ g/L, respectively. The LOD and LOQ represented the lowest concentration that can be detected and quantified for the current study. Eight standards, ranging in concentration from 0.1 μ g/L to 500 μ g/L, were used to construct a linear calibration curve ($r^2 = 0.998$). Instrument calibration was monitored over time via analysis of continuing calibration verification (CCV) samples, which were run every five samples, with an acceptability criterion of $\pm 20\%$. Calibration standards and calibration verification samples were prepared in 0.1% formic acid (v/v). Nanopure water from a Thermo Barnstead Nanopure (Dubuque, IA, USA) Diamond UV water purification system with 18 M Ω and 0.1% formic acid were run to validate the purity of solutions and as method blanks. Analytically verified concentrations of DZN and DPH treatment levels ranged from 39 to 130% of nominal values; subsequently, all figures, tables and text

hereafter report analytically verified treatment levels, which are provided in the supplementary material (Table S1).

2.6. Statistical analyses

SigmaPlot version 11.0 (Systat Software Inc., San Jose, CA, USA) software was used for statistical analysis of biological observations. All data was examined for normality and equivalence of variance prior to additional analyses. The Wilcoxon Rank Sum Test was employed to examine whether significant mortality occurred ($\alpha = 0.05$). For locomotive studies, significant differences in distance traveled between groups were identified using analysis of variance (ANOVA); if the data did not meet normality and equal variance assumptions, ANOVA on ranks was conducted ($\alpha = 0.05$). PMR behavioral responses were analyzed by 2-factor ANOVAs with either treatment level (5 levels: DPH or DZN) or time of day (10 levels: 09:00 to 18:00 h) and lighting (2 levels: light, dark) as factors. Mean distance traveled was calculated in 1 min intervals and Dunnett's or Tukey's HSD post hoc tests, for treatment level and time of day, effects respectively, were performed.

3. Results

3.1. Influence of age and time of day on larval zebrafish behavior

Larval zebrafish behavior was significantly different ($p < 0.05$) over the course of typical daylight workday hours (Fig. 1), and these time of day PMR activity profiles were similar with increasing age from 4 to 10 dpf. Distance traveled by larval zebrafish at 09:00 h was significantly ($p < 0.05$) less than other times of day, and though this pattern remained significantly lower through 10 dpf it was most pronounced for organisms between 4 and 8 dpf (Fig. 1). Daily locomotor patterns consisted of relatively limited movements in the morning followed by sharp significant ($p < 0.05$) increases in activity with daily high distances traveled at ~12:00 h that then remained consistent between 14:00 and 18:00 h (Fig. 1).

Zebrafish larvae travel significantly different ($p < 0.05$) distances within the light and dark periods with some exceptions, including 09:00 h at 6, 7, 8, and 9 dpf, and for 6 other hours at 8 and 10 dpf (Fig. 1). Larval zebrafish activity levels were most variable between the hours of 09:00 and 12:00 across all age examined. For both the light and dark photoperiods, for all ages, locomotion was most stable after 14:00 hrs. The 13:00 and 14:00 h observation periods, although not significantly ($p > 0.05$) different from later afternoon times for larvae older than 7 dpf, displayed a decrease in activity relative to both morning and afternoon recordings between 4 and 6 dpf (Fig. 1).

3.2. Influence of diazinon or diphenhydramine on age-specific zebrafish behavior

3.2.1. Diazinon studies—Because significant decreases ($p < 0.05$) in survival were observed at the highest DZN treatment levels for each of the three age groups (Fig. 2 A, C, E), behavior of these treatment levels was not examined. No behavioral differences were observed between negative controls and solvent controls, and no malformations were evidenced at concentrations below the highest treatment levels, which corresponded to LC50 values (data not shown). DZN affected total distance traveled during both light and dark

photoperiods (Figure 2 A, C, E), but total distance traveled during the dark refractory phase was consistently significantly ($p<0.05$) greater than activity during the light stimulatory period (Fig. 2). Such behavior observations were consistent with our initial age-specific and time of day studies reported above. However, unlike our previous observations of increased DZN acute mortality with increasing zebrafish age (Kristofco et al., 2015), age-specific differences for DZN behavioral toxicity was not consistently observed in the present study (Fig. 2), though several treatment levels significantly decreased activity of 7 dpf and 10 dpf zebrafish compared to 4 dpf organisms. DZN significantly ($p<0.05$) decreased total distance traveled relative to controls at 1.7 $\mu\text{g/L}$ at 4 dpf (following a 1–4 dpf exposure period), at all concentrations tested at 7 dpf (following a 4–7 dpf exposure period), and at 0.3, 5.3 and 377.3 $\mu\text{g/L}$ at 10 dpf (following a 7–10 dpf exposure period; Fig. 2 A, C, E) during the dark photoperiod. However, the only significant decreases caused by DZN on zebrafish on locomotion during the light photoperiod were at 1.3 and 13.7 $\mu\text{g/L}$ at 7 dpf. Though the lowest treatment level resulting in significant decreases in distance traveled for all three ages examined was four orders of magnitude lower than their respective LC_{50} values, dose-dependent behavioral alterations were not consistently observed (Fig. 2 A, C, E). In addition to examining DZN effects on the total distance traveled by zebrafish, we further identified whether DZN differentially reduced or stimulated swimming speed. Though significant ($p<0.05$) stimulatory increases in locomotion were evident during the dark refractory phases at 1201.6 $\mu\text{g/L}$ and 886.6 $\mu\text{g/L}$ in the 4 and 7 dpf studies, respectively (Fig. 3, C and F), the greatest proportion of locomotion occurred at cruising speeds between 5 and 20 mm/s (Fig. 3).

3.2.2. Diphenhydramine studies—Similar to observations for DZN, no malformations were observed and significant decreases in survival were only observed for the highest DPH treatment level examined for each age group, which corresponded to and were consistent with age specific LC_{50} values from our recent research (Kristofco et al., 2015). Subsequently, behavior of surviving larvae from these highest treatment levels were not observed (Fig. 2; B, D, F). Also consistent with the DZN experiments and the initial age and time of day observations described above, locomotor activity for all other treatment levels was significantly ($p<0.05$) greater during the refractory dark photoperiod than in the stimulatory light period. Total distance traveled by larvae exposed to DPH was decreased relative to controls during only the dark photoperiod (Fig. 2). Unlike observations for DZN, DPH reductions in zebrafish locomotion were observed in a dose and age dependent fashion.

Contrary to observations for DZN but consistent with our previous observations of increased DPH acute mortality with increasing zebrafish age (Kristofco et al., 2015), DPH behavioral toxicity was more pronounced with increasing age in the present study (Fig. 2). Specifically, whereas only the highest two treatment levels for both the 1–4 and 4–7 dpf were significantly decreased, all treatment levels examined reduced distance traveled of 7–10 dpf larval zebrafish (Fig. 2; B, D, F). In fact, the lowest treatment levels adversely affecting locomotor behavior were 2, 3 and 5 orders of magnitude lower than the DPH LC_{50} for 4, 7 and 10 dpf larvae, respectively (Fig. 2; B, D, F). Similarly, acute to chronic ratio (ACR) values increased from 434 and 475 to 227,500 in the 1–4, 4–7 and 7–10 dpf experiments, respectively (Table 1). Additionally, only larvae exposed during 7–10 dpf exhibited reduced

total distance traveled by treatment levels below the predicted THV. In fact, significant behavioral responses were observed at just 200 ng/L (Fig. 2F), which was the lowest treatment level of DPH examined.

Similar to observations with DZN, the majority of locomotion occurred at swimming speeds between 5 and 20 mm/s and distance traveled was significantly greater during the dark photoperiod (Fig. 4). The only instances where distance traveled was not significantly different between the two photoperiods occurred at bursting speeds greater than >20 mm/s at 94.8 µg/L (following 1–4 dpf exposure period), 552.1 and 1638.3 µg/L (following 4–7 dpf exposure period) and 0.2 and 121.4 µg/L (following 7–10 dpf exposure period; Figure 4C, F, I). When examining whether DPH differentially reduced or stimulated swimming speed it was only noted that cruising speeds (5–20 mm/s) were significantly suppressed at 5.7 (µg/L following the 1–4 dpf exposure period (Fig. 4B), and significantly reduced during the stimulatory light photoperiod at 10 dpf (Fig. 4H).

4. Discussion

Limited toxicity data for most chemicals and legislation calling for the reduction of animal testing has stimulated a movement toward HTS screening approaches, including early life stage studies such as the FET method with larval zebrafish. Initial studies have reported relatively high agreement between FET results and data from historical acute toxicity testing (Belanger et al., 2013; Knöbel et al., 2012; Lammer et al., 2009; Nagel 2002). *In vivo* HTS efforts by the US EPA ToxCast program (Padilla et al., 2012) and other researchers (Noyes et al., 2015; Padilla et al., 2012; Raftery et al., 2014; Truong et al., 2014) are exponentially expanding the amount of toxicological data available for industrial chemicals. Such unprecedented datasets on biological activity of environmental contaminants are anticipated to support development of robust computational toxicology models, and the development of sustainable molecular design guidelines for less hazardous chemicals (Connors et al., 2014; Voutchkova et al., 2011; Voutchkova-Kostal et al., 2012). However, efforts are required to identify design guidelines that reduce hazards associated with important adverse outcomes, including behavioral perturbations.

In the present study we employed an early life stage approach initially to determine whether larval zebrafish behavior varied with age over typical daylight hours of a workday. Previous studies have examined the development of various larval fish behaviors through time (Kalueff et al., 2013; Saint-Amant and Drapeau, 1998) in efforts to parameterize influences of certain intrinsic and extrinsic variables on naïve larval fish locomotion. These characterizations included evaluations of well depth (Ingebretson and Masino, 2013), well diameter (Ingebretson and Masino, 2013; Padilla et al., 2011), light intensity (Burgess and Granato, 2007; Schnorr et al., 2012; Steenbergen et al., 2011), color preference (Ahmad and Richardson, 2013), solvent affect (Chen et al., 2011; MacPhail et al., 2009), and the number of descriptive behavioral endpoints (Ingebretson and Masino, 2013). Behavioral differences with larval age (Ingebretson and Masino, 2013; MacPhail et al., 2009) and time of day (MacPhail et al., 2009) have also been evaluated, but only for a select window during development. Initial motivation included determining an optimal time of day to conduct behavioral observations, while cataloguing locomotion during photoperiod cycling of

varying durations (MacPhail et al., 2009). In the present study, we expanded this work by examining the variability of locomotor activity between 09:00 and 18:00 h, and performing these observations for 7 consecutive days until zebrafish larvae reached an age of 10 dpf.

Similarly to observations by MacPhail et al. (2009) with 6 dpf zebrafish in dark conditions, baseline zebrafish behavior in the present study was significantly higher during late morning hours and less variable in the dark during afternoon hours (MacPhail et al., 2009). This trend remained consistent for all 7 days examined. Such observations suggest that future behavioral observations should carefully consider results from the first one to two hours following the initiation of the light photoperiod due to consistently significantly different locomotor behavior activities observed in the present study. In addition to reducing the intrinsic variability of the quantitative behavioral measurements, reducing variability of control observations will likely increase the sensitivity of the behavioral endpoints in toxicity studies. In fact, this recommendation is particularly relevant for startle response assays, because in addition to demonstrating significantly lower locomotion during the 09:00 h, zebrafish locomotory behaviors in early morning hours consistently demonstrated no significant difference in activity level between the light and dark photoperiods (Fig. 1).

After identifying time of day and age influences on locomotor behavior of untreated zebrafish, we examined whether age specific sensitivity of larval zebrafish behaviors differed following exposure to DZN or DPH in accordance with patterns previously observed for mortality responses (Kristofco et al., 2015). Our recent observations were consistent with a previous study with Japanese medaka for which difference in threshold sensitivities to an organophosphate were attributed to toxicokinetic (e.g., biotransformation to oxon metabolite) differences across developmental stages (Hamm and Hinton, 2000; Hamm et al., 2001). However, differences in behavioral responses to DZN exposure in the present study were not as pronounced across age groups as these age specific response thresholds for mortality of zebrafish (Kristofco et al., 2015) and medaka (Hamm and Hinton, 2000).

Though DZN could be expected to inhibit AChE enzymes, induce convulsions, and stimulate PMR behaviors, increased swim bursts were only evident at 10% of the nominal LC₅₀ values for 4 and 7 dpf organisms. For all other swimming speeds and total distance traveled measures across age groups, DZN elicited decreases in PMR behavior. Similar decreases in locomotion or PMR behaviors have been reported for larval zebrafish following exposure to DZN (Scheil et al., 2009; Yen et al., 2011) and other organophosphate insecticides across a variety of ages (Levin et al., 2004; Pérez et al., 2013; Richendrfer et al., 2012; i man, 2010; Tilton et al., 2011). However, this trend may not hold true for all cases, as previous work has indicated that specific types of behavioral responses may be compound specific among different organophosphates (Richendrfer and Creton, 2015).

The timing and duration of developmental exposure to cholinesterase inhibitors can influence the nature of behavioral response profiles. In the present study, we performed behavioral observations after a 72 h exposure period focusing on swimming speeds and distance traveled during light (stimulatory) and dark (refractory) conditions. But measurements following prolonged exposure may differ from shorter episodic responses if

the synapse is overwhelmed and its capacity to return to a resting state is exhausted due to a depletion of AChE. For example, Beauvais et al. (Beauvais et al., 2001) correlated decreased swimming speed of rainbow trout to increased AChE inhibition following 24 or 96 hour exposures, or 96 h exposure and a 48 h recovery period, to carbaryl or cadmium chloride. Other studies have also concluded that the severity of behavioral alterations by organophosphates stem from duration, compound and concentration specific AChE inhibition because decreases in fish locomotor activity levels were observed at higher treatment levels (Gaworecki et al., 2009). Because the type of behavioral responses may vary with timing and concentration, modulations in swimming speed were assessed (Ingebretson and Masino, 2013); we identified increased swimming bursts of larvae exposed to DZN at the highest doses but not at lower treatment levels. Such early development exposure to organophosphates may also have consequences later in life. For example, behavioral observations in adult zebrafish may differ from larval fish; startle response of adults that were developmentally exposed to organophosphates appear more sensitive (Eddins et al, 2010). Collectively, decreased swimming speed and locomotor activity provide physiological indicators of potential adverse outcomes in the field if individual organism encounters with potential prey are reduced, and if their predator avoidance, schooling behavior, mate attraction, or migration patterns are altered (Little and Finger, 1990; Little et al., 1993).

We further examined whether our previous age-specific mortality observations to DPH extended to similar changes in behavioral sensitivity. In fact, DPH influences on locomotory behavior were markedly greater in 7–10 dpf organisms than younger (1–4, 4–7 dpf) age groups (Fig. 2). An initial study of DPH with juvenile fathead minnows identified feeding behavior to be more sensitive than standardized apical endpoints (mortality, growth) with a LOEC of 5.8 µg/L (Berninger et al., 2011). In this previous work, an ACR value of 746 was determined for feeding behavior, which was an order of magnitude larger than another ACR value (85) calculated for the more commonly measured growth endpoint during standardized toxicity testing for regulatory purposes (Berninger et al., 2011). In the present study, PMR behavioral responses of 10 dpf larval zebrafish to DPH were markedly more sensitive than fathead feeding behavior with an environmentally relevant LOEC of 200 ng/L. Based on these PMR behavioral responses, DPH ACR values ranged from 434 to 227,500 in an age dependent manner (Table 1), and were larger than an ACR value of 2100 anticipated for DPH (Berninger and Brooks, 2010; Berninger et al., 2011). In addition, significant ($p < 0.05$) behavioral responses of 10 dpf organisms were lower than THV predicted concentrations, which were based on a fish plasma uptake model of DPH (see Methods 2.4). Thus, though fish plasma modeling appears to present promise to prioritize pharmaceuticals for advanced research and monitoring (Caldwell et al., 2014), additional comparative pharmacology and toxicology studies are needed in fish models (Brooks, 2014).

It is not clear why locomotor behavioral responses of 10 dpf organisms in the present study or mortality thresholds in our resent research were markedly more sensitive to DPH. As we previously discussed (Kristofco et al., 2015), a dispositional explanation for such observations due to comparative biotransformation does not appear reasonable, because DPH, unlike DZN, is not transformed in mammals to a more active metabolite and it does not appear to be appreciably transformed in aquatic species, based on in vitro S9 studies in

rainbow trout (Connors et al., 2013). It is also important to note that the relatively low thresholds for behavioral responses of larval zebrafish in the present study are likely to be more pronounced in surface waters with elevated pH (Valenti et al., 2011) because uptake of DPH to whole tissues and plasma is markedly increased when pH approaches its pKa value (Nichols et al., 2015). In the present study we maintained pH 7 across all experiments, but could not quantify DPH in plasma due to such limited volume in zebrafish larvae.

Unfortunately, whether uptake of DPH or other ionizable contaminants occurs differentially during fish development is not understood, but requires additional study. It is also possible that increased sensitivity of 10 dpf organisms could have resulted from toxicodynamic changes during development. Though DPH is commonly recognized as an antihistamine, it has at least three mechanisms of action in mammals, including anticholinergic and anti-serotonergic activities, that appear to be functionally conserved in aquatic vertebrates (Gunnarsson et al., 2008). Previous work in our lab identified fish antianxiety behaviors following exposure to the selective serotonin reuptake inhibitor sertraline during light conditions (Valenti et al., 2012). Though such observations were observed for adult male fathead minnows (Valenti et al., 2012), the stimulatory response profile and photoperiod were not consistent with locomotor activity observed in the present study during dark periods (Fig. 2). Alternatively, DPH is commonly used as a sleep agent (Sominex™) in humans because it elicits depression of the central nervous system. Previous studies have suggested that the histaminergic system, whose neurons appear around 85 hpf, may affect alertness in larval zebrafish (Eriksson et al., 1998; Rico et al., 2011) and thus could explain age dependent sensitivity observations of the current study. Further research is needed to define whether histaminergic activity alters specific behaviors of larval zebrafish and other aquatic vertebrates.

A movement toward HTS has exponentially increased the pace with which new toxicological and biological activity data is becoming available. Identifying the opportunities and challenges for utilizing this information to identify adverse outcome pathways during hazard and risk assessment represents a critically important research need, particularly for biologically active chemicals (Boxall et al., 2012; Rudd et al., 2014). Whether the experimental design assumptions of fish early life stage assays allow for applications to various classes of compounds and various toxicity pathways is not understood (Klüver et al., 2015). Though previous studies have demonstrated both greater sensitivity of several acute responses in fish embryos (Hamm and Hinton, 2000; Marty et al., 1990), and larvae (Fent and Meier, 1994; Gaikowski et al., 1996; Köprücü and Aydın, 2004), influence of age on various fish species responses to contaminants appears to be more complex than perhaps anticipated. For example, neurotoxic compounds (Klüver et al., 2015; Knöbel et al., 2012) and substances requiring metabolic activation (Knöbel et al., 2012) appear to more toxic to older organisms. It is also possible that internal plasma doses vary with age of zebrafish and other fish models. Most previous studies with zebrafish larvae focus on whole body concentrations in an effort to define internal concentrations; however, disposition of contaminants of emerging concern within various tissues and plasma of zebrafish larvae and most fish models is not known (Nichols et al., 2015). Such information is necessary to understand critical tissue levels associated with adverse outcomes (Brooks et al., 2009;

Boxall et al., 2012). Thus, future efforts are needed to advance an understanding of fish toxicokinetics with age.

With increased usage of larval fish behavior as an endpoint during hazard and risk assessment, increased scrutiny of the experimental design assumptions and reporting of these behavioral studies is warranted (Perkins et al., 2013). Observations in the present study support this perspective because age specific influences on DPH-induced behavioral effects were observed in slightly older larval zebrafish than typically included in early life stage studies. Species Sensitivity Distributions are commonly employed in environmental hazard and risk assessments to characterize differential sensitivities across species to a chemical. Developing a conceptually similar advanced understanding of the influences of age on species sensitivities to contaminants, particular those eliciting behavioral toxicity, appears warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2015.09.011>.

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Fig. 1. Mean ($\pm$ SE, $n = 24$) distance traveled per minute by naïve 4, 5, 6, 7, 8, 9, or 10 days post fertilization (dpf) zebrafish larvae between 09:00 and 18:00 h in light or dark conditions. Distance traveled was observed over 20 min of light (white bars) or dark (black bars). Different letters denote significant ($p < 0.05$) differences among hourly groups (2 way ANOVA with Tukey's HSD post hoc test).

Fig. 2.

Mean ($\pm$ SD, $N=8$) percent survival, and mean ($\pm$ SE, $N=8$) distance traveled (mm) per minute of 4, 7 or 10 days post fertilization (dpf) old *Danio rerio* larvae following exposure to diazinon (A, C, & E) or diphenhydramine (B, D, & F) for 72 h. Distance traveled was observed over two 10 min periods of light (white bars) or dark (black bars). Different letters indicate significant ($p < 0.05$) differences from controls. The vertical dashed line represents the Therapeutic Hazard Value (THV) for diphenhydramine; THV = 18.6 (μ L (at pH 7). At

concentrations with significant decreases in survival, no behavioral observations were recorded. N.M.: Not Measured.

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Fig. 3. Mean ($\pm$ SE, $N = 8$) distance traveled (mm) per minute for resting (<5 mm/s; A, D, G), cruising (5–20 mm/s; B, E, H) or bursting (>20 mm/s; C, F, I) swimming speeds of 4, 7 or 10 days post fertilization (dpf) old *Danio rerio* larvae following exposure to diazinon for 72 h. Distance traveled was observed over two 10 min periods of light (white bars) or dark (black bars). Different letters indicate significant ($p < 0.05$) differences from controls. At concentrations with significant decreases in survival, no behavioral observations were recorded. N.M.: Not Measured.

Fig. 4.

Mean ($\pm$ SE, $N = 8$) distance traveled (mm) per minute for resting (<5mm/s; A, D, G), cruising (5–20 mm/s; B, E, H) or bursting (>20 mm/s; C, F, I) swimming speeds of 4, 7 or 10 days post fertilization (dpf) old *Danio rerio* larvae following exposure to diphenhydramine for 72 h. Distance traveled was observed over two 10 min periods of light (white bars) or dark (black bars). Different letters indicate significant ($p < 0.05$) differences from controls. The vertical dashed line represents the Therapeutic Hazard Value (THV) for diphenhydramine; THV = 18.6 (μ g/L (at pH 7). At concentrations with significant decreases in survival, no behavioral observations were recorded. N.M.: Not Measured.

Table 1

No observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) for locomotor behavior responses and associated acute to chronic ratios (ACRs) of 4, 7 or 10 days post fertilization (dpf) old larval zebrafish following 72 h of exposure to diazinon or diphenhydramine.

Diazinon				
Age	LC50 (µg/L) ^a	LOEC (µg/L)	NOEC (µg/L)	ACR ^b
4dpf	14,700	1.66	<1.66	8855
7dpf	12,200	1.31	<1.31	9313
10 dpf	5410	0.32	<0.32	16,906
Diphenhydramine				
Age	LC50 (µg/L) ^a	LOEC (µg/L)	NOEC (µg/L)	ACR ^b
4dpf	692,000	1594.68	94.76	434
7dpf	262,000	552.1	33.3	475
10 dpf	45,500	0.2	<0.2	227,500

^aValues from Kristofco et al. (2015).

^bACR = LC50/LOEC.