ABSTRACT

Towards Less Hazardous Chemicals:
Identifying Chemical Bioactivity through Fish Behavioral Profiles

William Baylor Steele IV, Ph.D.

Mentor: Bryan W. Brooks, Ph.D.

Because most chemicals in commerce lack empirical toxicology information, innovative approaches are needed to identify substances presenting elevated hazards and risks to public health and the environment. When larval fish models are employed with automated tracking technologies, behavioral studies can be used to perform rapid, diagnostic toxicity screens for large volumes of chemicals. Beyond utility in chemical safety screening applications, behaviors are integrated processes that are critical for organism survival and reproduction. This dissertation developed novel approaches to diagnostically examine chemicals for toxicity using larval fish models, and further extended these efforts beyond lab raised models to examine effects of neuroactive substances on wild fish populations. In Chapter two, methods used in the biomedical sciences to study therapeutic attributes of novel molecules were adapted for environmental screening applications. Using automated tracking software, a protocol was developed to quantify locomotor and photomotor responses (PMRs) of two common larval fish models, the zebrafish and fathead minnow. These developed methods were

applied to study the behavioral effects of a common aquatic contaminant and neuro-stimulant, caffeine, which exerted photomotor and locomotor responses at environmentally relevant levels. In Chapter three, these methods were broadly applied to develop larval fish behavioral response profiles for a variety of different chemicals from diverse mode of action (MOA) categories. Both fish models demonstrated unique behavioral responses upon exposure to each chemical indicating that behavioral response may be informative of compound specific MOAs. Chapter four demonstrated that in the two most common larval fish models, refractory PMR and locomotor patterns appear informative of electrophilic properties associated with oxidative stress for S_N2 chemicals. Property-based quantum mechanical modeling of electrophile reaction energies were predictive of experimental in vivo acute and sublethal toxicity, which provide important implications for identifying and designing less hazardous industrial chemicals. Because lab fish models cannot be expected to be representative of wild fish populations, Chapter five examined behavioral effects of two commonly prescribed psychiatric medications, oxazepam and sertraline, on perch collected from natural waters. Results from this study identified time related effects on fish boldness and neuroactive pharmaceutical related effects on fish activity levels.

Towards Less Hazardous Chemicals:
Evaluating Compound Bioactivity through Fish Behavioral Toxicology

by

William Baylor Steele IV, B.B.A., M.S.

A Dissertation

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Approved by the Dissertation Committee

_____

Bryan Brooks, Ph.D., Chairperson

_____

Cole Matson, Ph.D.

_____

Spencer Williams, Ph.D.

_____

Ramon Lavado, Ph.D.

_____

Kevin Chambliss, Ph.D.

Accepted by the Graduate School

May 2019

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J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

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CHAPTER ONE

Introduction

Background and Significance

Humans and the environment are continuously exposed to complex mixtures of synthetic chemicals resulting in multiple adverse outcomes (Landrigan et al 2017; Malaj et al 2014; Schäfer et al 2016). Given the many risks associated with chemical exposure, there is a growing impetus to gather necessary toxicity information so that we may select chemicals that are useful but minimally hazardous. Such information may also be used to inform the design of safer industrial compounds (Coish et al 2017). Unfortunately, toxicology information for many of the tens and thousands of chemicals in commerce is lacking. Therefore, innovative testing methods are needed to rapidly screen chemicals for toxicity and gain insight into the chemical attributes associated with adverse effects.

Much of the current toxicity data available today is based on levels that cause mortality in animal models. While such information is useful for gauging the relative toxicity of a given chemical, it may not be relevant in certain scenarios. An ecological receptor, for example, may not die as a result of a chemical exposure, but sublethal effects on important survival processes (e.g. growth, reproduction) may ultimately impair individual fitness (Ankley et al 2007).

Behavioral effects are sublethal toxicity endpoints that warrant consideration in chemical hazard evaluation. Behavior is a sequence of measurable actions that operate through the central and peripheral nervous system to maintain an organism under

beneficial circumstances so that it can perform essential life functions (Little, 2002). Organism behavior can be viewed as the cumulative manifestation of biochemical, physiological, and ecological processes (Little, 2002; Tierney 2011). Therefore, toxicants that can impair organism behavior have clear implications at the individual, population, and community level. Behavioral assessments, however, are often overlooked in regulatory toxicity testing, but behaviors have long been used to support regulatory decisions and practices for the protection of ecological and human health as well as the conservation of wildlife.

The Endangered Species Act (ESA) of 1973 recognizes behavior as a critical function that must be protected in wildlife. "Take" prohibitions in ESA Sections 9 and 4(d), for example, make it unlawful to harm species listed as threatened or endangered. Under these sections, any act that will impair essential behavioral patterns including breeding, feeding, and shelter seeking are defined as harmful. These prohibitions are warranted as research is starting to recognize that behavioral theories, insights, and techniques are also important aspects in wildlife conservation practices (Shumway et al. 1999). Modifications to wildlife habitat, for example, have been demonstrated to have behavioral effects directly correlated with reproductive and survival success (Godfrey and Barreto 1995, Godfrey, 1996). Therefore, behavioral sciences can inform and improve conservation practices.

Behaviors effects are also considered in regulations concerned with the environmental safety of chemical substances and the discharge of hazardous materials into aquatic habitats. The Compensation and Liability Act of 1980 (CERCLA) provides for the recovery of damages to natural resources, including fish, as a result of the release

of hazardous substances into the environment. Natural Resource Damage Assessments (NRDA 1986) directed by CERCLA are site specific and focused on populations and communities unique to the site. These assessments include avoidance behaviors and clinical indications of toxicity as measures of injury (Little et al. 1992). In compliance with the Clean Water Act of 1972, industry must monitor waste water discharge to assure effluent is protective of aquatic life. The behavioral measure of ventilatory response which continuously monitors respiratory rate and gill purge response of exposed fish has been successfully used as an early warning system for failure in effluent treatment processes (Drummond and Carlson 1977, Diamond et al. 1990).

Behavioral studies extend beyond ecosystem and wild life protection. Human behavioral indicators have provided the motivation to adopt legislation to protect human health from harmful chemical effects. Lead exposure in young children, for example, is associated with several behavioral problems including hyperactivity, impulsivity, attention issues, and a number of learning disabilities. These impairments have been observed at blood levels ≤10 µg/deciliter (Liu et al. 2014). Findings on the neurobehavioral effects of lead in children has prompted several regulatory actions including amendments to the Lead-Safe Housing Rule (HUD, 2017), which requires prompt response to cases of children under 6 living in Housing and Urban Development (HUD) assisted housing who have elevated blood levels. In 2012, the Center for Disease Control (CDC) lowered the reference level for high blood levels from 10 to 5 µg/deciliter (Liu et al. 2014).

Beyond their importance in assessing human and ecological health, behaviors can act as biological read-outs of organism physiological processes. In pharmacology and

toxicology, behaviors of fish and mammalian models have been used to aid in the diagnosis of chemical-biological interactions. Behaviors in response to drug exposure, for example, can be informative of whether a pharmaceutical is interacting with an intended therapeutic target (Blanchard et al 2003; Da-Rocha 1993).

In aquatic ecotoxicology fish behavioral profiles or syndromes have been leveraged to classify chemicals according to mode of action (MOA) (Russom et al. 1997). With the development of new technologies, such as automated tracking platforms, there arises new opportunities to use behaviors as diagnostic indicators of toxicity. Fish are not only of high ecological importance (Weis et al. 2001), but they possess numerous enzyme/receptor systems similar to other vertebrates including humans (MacRae and Peterson, 2015). Therefore, fish demonstrate great potential to be used as behavioral models to explore chemicals bioactivity in relation to ecological and human health.

From this dissertation research I hope to understand how behavioral endpoints can be applied in aquatic toxicology to provide enhanced diagnostic screening and improved hazard characterization of aquatic contaminants. I intend that the data and knowledge I gain from these studies can be applied to better characterize bioactivity for compounds lacking important mechanistic data, and to provide improved understanding of chemical hazards through impacts on behaviors of fish species.

Leveraging New Approaches and Alternative Models in Behavioral Ecotoxicology

The growing popularity of zebrafish in neuropharmacology and psychiatric studies has led to observations of several behavioral phenotypes associated with various moods, psychological disorders, and social preferences that can be altered by exposure to various neuroactive compounds (Kalueff et al. 2016; Stewart et al. 2015a). This

understanding has the potential to be read across in ecotoxicology to test hypotheses and possibly predict effects of pharmaceuticals and other aquatic contaminants on natural fish populations. For example, it is well known in the biomedical sciences that particular larval and adult zebrafish behaviors such as wall hugging and light dark preferences are associated with anxiety levels (Maximino et al. 2014; Schnörr et al. 2012; Steenbergen et al. 2011a). These behaviors can be altered when fish are exposed to pharmaceuticals used to treat anxiety in humans. It is also demonstrated in ecotoxicology studies that anxiety influences predator avoidance behaviors in fish (Margiotta-Casaluci et al. 2014; Melvin and Wilson 2013; Valenti et al. 2012). Future research is needed to more quantitatively leverage data from psychiatric studies with zebrafish and other fish models to understand comparative effects of pharmaceuticals on ecologically relevant behaviors in natural fish populations, and in turn to support diagnostic evaluation of behavioral responses elicited by other industrial chemicals.

Because behaviors are rapid and sensitive indicators of toxicity (Gerhardt 2007; Melvin and Wilson 2013) behavioral endpoints can be used to quickly and effectively detect changes at the molecular and biochemical level to system changes in the individual organism (Kokel et al. 2010a). Advances in tracking technology and computational capabilities now allow for behavioral effects of pharmaceuticals to be determined in HTS using early life stage (ELS) fish models (Noyes et al. 2015a). Larval and embryonic fish models (FET, OECD) demonstrate the potential to be used to rapidly and sensitively screen compounds for behavioral responses using automated tracking software (Noyes et al. 2015a; Reif et al. 2015). Due to their small size and fast development, zebrafish embryos as young as 24 hours post fertilization (hpf) can be observed simultaneously in

well multi-channel well plates (Reif et al. 2015). Using ELS models in behavioral ecotoxicology with the aid of digital tracking software has the potential to greatly reduce time and resources and significantly enhance accuracy of behavioral observations (Sloman and McNeil 2012). Early life stage models, however, cannot be presumed to reflect behavioral responses representative of natural populations of fish. A key challenge for behavioral ecotoxicology is ensuring that laboratory models are representative of and can understand behavioral perturbations in the field (Little 1990; Sloman and McNeil 2012). Data and methods from pharmacological studies, however, can be used to elucidate how effects on known behavioral phenotypes in larval zebrafish may or may not translate to other fish species (Margiotta-Casaluci et al. 2014). Understanding the biochemical and physiological mechanisms underpinning these behavioral effects will provide the foundation to begin to translate behavioral effects in larval zebrafish to effects in other fish species/life stages (Parker 2016). Further research in comparative behavioral pharmacology and toxicology promises to also help fill the gaps among larval zebrafish behavior patterns and behavioral ecotoxicology of pharmaceuticals and other chemicals in various fish species. Understanding differences in natural behaviors among standard lab fish models as well as other fish is an important first step in this type of research (Kane et al. 2005).

Comparative Behavioral Toxicology: How do Two Common Larval Fish Models Differ?

One of the aims of 21st century toxicity testing put forth by the U.S. National Research Council is to developed and utilize in vitro tools that can be used to rapidly and effectively identify the underlying mechanisms and pathways by which chemicals adversely affect living systems (Andersen and Krewski 2009; Krewski et al. 2010).

Such testing methods would help prioritize chemicals for further assessment. To meet these goals, the U.S. EPA National Center for Computational Toxicology (NCCT) developed the ToxCast program in 2007 to screen thousands of chemicals using over 700 different types of high throughput in vitro assays that encompass a variety of cellular responses and approximately 300 signaling pathways (https://www.epa.gov/chemical-research/toxicity-forecasting).

While high throughput in vitro techniques utilizing cell based assays present many opportunities, and research efforts are continuing to utilize molecular and pathway based assays, translating in vitro data to predict whole animal toxicity is problematic. Cell based models lack biological complexity and therefore do not account for important whole system processes such as metabolism and cross talk between tissues (MacRae and Peterson 2015; Truong et al. 2014). Furthermore, cells represent an artificial biological environment for testing (Truong et al. 2014). Traditional testing methods such as those utilizing mammalian models, on the other hand, account for biological complexity, but these testing methods are time and resource intensive (Selderslaghs et al. 2010).

Larval fish models can be viewed as an ideal intermediate between cell based models and mammalian models, as fish are complete intact vertebrate systems, yet their smaller size, faster development, greater fecundity, and shorter generation time make larval fish models less time and resource intensive than mammalian models (Stewart et al. 2015c; Truong et al. 2014). Furthermore, fish are of high ecological relevance in aquatic ecosystems (Little et al. 1993), and they share considerable physiological and biochemical similarity with humans (Huggett et al. 2003; Irons et al. 2013; MacRae and Peterson 2015; Noyes et al. 2015b), so these models may be used as a means to gather

data for both ecological and human health risk assessment from a single study. In this way, fish models can be viewed as bridge to merge human health toxicology and ecotoxicology.

Standardized toxicology experimental designs, including the EPA WET test and the OECD FET, using larval fish have been developed and adopted by governmental and regulatory agencies around the world. In addition to embryonic and larval zebrafish, which has received increased attention as a behavioral model in toxicology (Kalueff et al. 2016; Kristofco et al. 2016), the larval fathead minnow (Pimephales promelas) has been primarily used as a model for ecotoxicology research and applications (Ankley and Villeneuve 2006). Both models have standardized experimental guidelines and are commonly used for regulatory purposes, but the fathead minnow model has seen comparatively little use in behavioral toxicology using automated tracking technologies when compared to zebrafish. There is, however, a growing interest in using the larval fathead minnow in automated behavioral platforms to assess toxicity of aquatic contaminants (Colón-Cruz et al. 2018). Given the relevance of the fathead minnow in ecotoxicology and the growing interest in both larval fish models in behavioral toxicology, further research is needed to understand differences in behavioral sensitivities and responses of these common models to aquatic contaminants. Unfortunately, comparative mechanistic (Corrales et al. 2016) and behavioral responses of fish models to diverse chemical MOAs are limited.

Exploring Relationships Between Chemical Modes of Action and Locomotor Responses

Beyond their use in pharmacology, fish behavioral endpoints have been leveraged as tools in the diagnostic screening of chemical hazards in toxicological studies.

Drummond et al. 1990, for example, observed behaviors of juvenile fathead minnows after acute exposure to several different industrial compounds with a variety of anticipated modes of action. From these observations the authors developed individual behavioral toxicity syndromes reflective of a general mode of action. These syndromes included Hypoactivity syndrome (depressed locomotor activity, little or no response to outside stimuli), Hyperactivity syndrome (accelerated locomotor activity, overreactive to outside stimuli,) Physical deformity syndrome (convulsions, spasms, tetany, scoliosis/lordosis and hemorrhage in the vertebral area). Russom et al. 1997 further expanded upon this work by leveraging each of these behavioral syndromes in conjunction with chemical descriptors, dose response assessments, and joint toxic action studies to classify over 600 industrial compounds according to mode of action. These syndromes were reflective of general modes of action and cannot be used to identify specific mechanisms or sites of action. Therefore, chemicals with differing modes of action may share the same syndrome. However minor variations in response within a general syndrome may provide important clues into toxic mechanisms and could be used to develop additional syndromes to better distinguish between classes of compounds.

With the development and advancement of digital tracking software, automated behavioral platforms can be used to analyze multiple fish locomotor endpoints simultaneously, and detect more subtle responses than would be observed by human visual observation alone (Legradi et al. 2015). Thus, these systems can be used to expand upon previous fish behavioral toxicity classification research by providing more detailed response profiles. Furthermore, because these systems are automated, they would allow

for behavioral assessment in a faster, more efficient manner than by visual observation (Kane et al. 2005).

In addition to providing insight into MoAs, behavioral endpoints can enhance the sensitivity of toxicity assays, and thus, improve detection of compound bioactivity (Velki et al. 2017; Weichert et al. 2017). Research by Kluver et al. 2015, for example, demonstrated fish embryo acute toxicity tests failed to identify toxicity in several compounds with a known neurotoxic mode of action. However, these compounds were identified by changes in embryonic locomotion. Other studies with organophosphate pesticides have also demonstrated that locomotor endpoints enhance method sensitivity of the fish embryo test (Selderslaghs et al. 2010; Velki et al. 2017; Weichert et al. 2017). While this concept is understood for compounds that are designed to target and modulate the nervous system, such as pesticides, less is known about the sensitivity of larval locomotor responses towards other commercial compounds with unintended biological activity.

Much of the unintended biological activity and toxicity of commercial chemicals is due to their covalent reactivity (Kostal et al. 2012). These reactions occur whereby electrophiles (electron deficient) form irreversible covalent bonds with biological macromolecules such as proteins, nucleic acids, and lipids (Enoch et al. 2011). In fact, these interactions are the most common to occur among commercial chemicals that are bioactive (Hermens 1990). In addition to demonstrating enhanced toxicity to aquatic life, reactive molecules can cause oxidative stress, an adverse outcome resulting from an imbalance between production of reactive oxygen species (ROS) and cellular antioxidant defenses (Corrales et al. 2016). Furthermore, electrophile adduct formation can disrupt

structure/function of macromolecules. Covalent interactions with nucleic acids are associated with genotoxicity whereas interactions with proteins has been associated with a number of organ specific toxicities including, neurotoxicity, cardiotoxicity, renal toxicity, and hepatotoxicity (Harder et al. 2003; LoPachin and DeCaprio 2005). Despite extensive research, the pathophysiological and molecular mechanisms by which these compounds elicit their effects are poorly understood (LoPachin and DeCaprio 2005).

Organic electrophiles can interact with biological targets through several different reaction chemistries including, nucleophilic substitution, Michael addition, Schiff base formation, and acylation (Enoch and Cronin 2010; Enoch et al. 2011). Additionally, electrophile softness/hardness influences specific target adduct formation (LoPachin et al. 2011). Styrene oxide, for example, is a hard electrophile that has a strong tendency to react with DNA whereas acrylamide, a soft electrophile, primarily interacts with amino acid targets on proteins (Harder et al. 2003b). Therefore, electrophiles can be classified according to a mechanistic basis like other specifically acting compounds, such as pesticides and pharmaceuticals.

Based on previous research with mammalian systems, several electrophiles are capable of eliciting organ specific toxicity (e.g. neurotoxicity). Protein adduct formation, for example, has been associated with the neurotoxicity of several electrophilic compounds, including pesticides (LoPachin and DeCaprio 2005). Further, these chemicals have demonstrated behavioral effects in mammals see (LoPachin 2004; LoPachin et al. 2002), yet electrophiles are understudied in fish behavioral toxicology compared to other biologically active compounds, such as pesticides and pharmaceuticals. If fish behavioral responses do occur at sublethal exposure to

electrophiles, larval fish behavioral endpoints may provide an effective means to detect whole system effects of chemical toxicity, and further investigate bioactivity of industrial compounds.

Possible Impacts of Pharmaceuticals to Fish Populations through Behavioral Alterations

Given that pharmaceuticals are designed to be biologically active but illicit minimal acute toxicity and are typically detected in aquatic habitats at concentrations far below those that cause lethality in aquatic species, the risk assessment of pharmaceuticals differs from traditional regulatory paradigms (Brooks 2014). Therefore, the study of subtle, non-lethal effects of pharmaceuticals are more appropriate endpoints than measurements such as acute mortality. However, little is known about the sub-lethal effects of pharmaceuticals to aquatic life (Ankley et al. 2007).

Behaviors are biological processes necessary for organism survival that are often more sensitive to aquatic contaminants than traditional regulatory endpoints (Melvin and Wilson 2013). Because pharmaceuticals such as psychiatric medications are designed to specifically modulate behavior in humans, and fish share considerable drug target homogoly with humans compared to other aquatic taxa, it can be anticipated that these compounds can modulate behaviors in fish species. In fact, extensive research in pharmacology with zebrafish has demonstrated that many psychiatric chemicals can modulate anxiety related behaviors in these models (López-Patiño et al. 2008; Maximino et al. 2014; Steenbergen et al. 2011a; Stewart et al. 2015a; Stewart et al. 2015c). However, it is unclear how behaviors of lab based models compares to natural populations of fish, as these populations, unlike zebrafish, are conditioned to respond to import threats from predatory fish.

Ecotoxicological research has recently begun to demonstrate impact of psychiatric medications at dilute, environmentally relevant concentrations on important predator avoidance behaviors associated with anxiety in fish from natural habitats. Oxazepam, for example, is a benzodiazepine commonly used to treat anxiety and depression in humans that has been detected in streams and waste water effluents in many parts of the world (Brodin et al. 2013; Calisto and Esteves 2009). This compound elicits its effects in mammals through agonism of GABA-A ion channels, which are targets also present in fish (Klaminder et al. 2016a). Lab based studies demonstrate that oxazepam exposure effects boldness, activity, and sociality of roach (Rutilus rutilus) and perch (Perca fluviatilis) collected from lakes in Sweden (Brodin et al. 2017a; Brodin et al. 2014b). These effects were confirmed with subsequent field studies in lake ecosystems populated by predatory fish. In these field assays, oxazepam exposed perch were also more bold and active, had a larger home range, and used pelagic habitats more than non-exposed perch (Klaminder et al. 2016a). Like oxazepam, sertraline is a psychiatric medication that has been frequently detected in the aquatic environment and is capable of impairing important predator avoidance behaviors in fish. Research with male fathead minnows demonstrated that binding of the drug to its target, the serotonin reuptake transporter (SERT), resulted in decreased shelter seeking in exposed fish (Valenti Jr et al. 2012). An important component lacking in many lab-based studies is the presence of predatory pressures, which are factors that influence fish behaviors.

Predatory olfactory signaling cues are an important stimulus fish use to assess predation risk (Mccormick and Manassa 2008). As such, fish naturally respond to these stimuli through avoidance behaviors. Because olfactory signaling cues represent a

predatory pressure stimuli present in natural systems, and anxiety is correlated with predator avoidance behaviors in fish, further research is needed to understand if pharmaceuticals intended to treat anxiety in humans can impair fish responses to predator signaling cues and if certain behaviors maybe overlooked in the absence of predatory pressure.

While little is known about pharmaceutical effects on aquatic life, less is known about the effects of concurrent drug exposure (Pomati et al. 2006). Given that drugs are discharged into aquatic habitats as mixtures of many biologically active ingredients, further research is warranted in understanding the interactions of drugs with differing MoAs.

CHAPTER TWO

Experimental Protocol for Examining Behavioral Response Profiles in Larval Fish: Application to the Neuro-Stimulant Caffeine

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Short Abstract

Here, we present a protocol to examine larval zebrafish and fathead minnow locomotor activities and photomotor responses (PMR) using an automated tracking software. When incorporated in common toxicology bioassays, analyses of these behaviors provide a diagnostic tool to examine chemical bioactivity. This protocol is described using caffeine, a model neurostimulant.

Long Abstract

Fish models and behaviors are increasingly used in the biomedical sciences; however, fish have long been the subject of ecological, physiological and toxicological studies. Using automated digital tracking platforms, recent efforts in neuropharmacology are leveraging larval fish locomotor behaviors to identify potential therapeutic targets for novel small molecules. Similar to these efforts, research in the environmental sciences and comparative pharmacology and toxicology is examining various behaviors of fish models as diagnostic tools in tiered evaluation of contaminants and real-time monitoring of surface waters for contaminant threats. Whereas the zebrafish is a popular larval fish model in the biomedical sciences, the fathead minnow is a common larval fish model in

ecotoxicology. Unfortunately, fathead minnow larvae have received considerably less attention in behavioral studies. Here, we develop and demonstrate a behavioral profile protocol using caffeine as a model neurostimulant. Though photomotor responses of fathead minnows were occasionally affected by caffeine, zebrafishes were markedly more sensitive for photomotor and locomotor endpoints, which responded at environmentally relevant levels. Future studies are needed to understand comparative behavioral sensitivity differences among fish with age and time of day, and to determine whether similar behavioral effects would occur in nature and be indicative of adverse outcomes at the individual or population levels of biological organization.

Introduction

Though fish models are increasingly used for biomedical studies, fish have been routinely employed for ecology and physiology studies, to examine contamination of surface waters, and to understand toxicological thresholds of chemicals. Such efforts are important because chemical contamination can impair aquatic ecosystems and jeopardize the quality of source water supplies[1,2]. Most of the chemicals in commerce, however, lack even basic toxicology information[3].

Animal model assays traditionally used in regulatory toxicity testing are resource intensive and cannot provide the high throughput, early tier screening needed for toxicity testing in the 21st century[4]. Subsequently, there is a growing impetus to adopt and utilize *in vitro* models that can more rapidly and efficiently screen compounds for biological activities[3,5]. Though cell based models present many opportunities, they often lack biological complexity, and thus do not account for many important whole organism processes, including metabolism[6].

The zebrafish is a common biomedical animal model that is gaining popularity as an alternative model in aquatic toxicology and ecotoxicology[7,8]. Given their small size, rapid development, and high fecundity, fish models can be used to rapidly and efficiently screen chemicals for bioactivity and toxicity at the whole organism scale[9]. With the aid of automated tracking software, larval zebrafish behaviors provide enhanced diagnostic utility in screening contaminants for toxicity[10,11]. Studies in the pharmaceutical sciences have demonstrated that locomotor endpoints are informative of chemical mechanisms of action, can be used to phenotype behaviors, and then may tentatively identify subcellular targets for novel molecules[12,13]. Whereas the zebrafish is a popular larval fish model in the biomedical sciences, the fathead minnow is a common, ecologically important fish model that is used for ecotoxicology studies and during prospective (*e.g.*, new chemical evaluations) and retrospective (*e.g.*, ambient surface water or wastewater effluent discharge monitoring) environmental assessments. Unfortunately, behavioral responses of larval fathead minnows have received markedly less attention than zebrafish. Our ongoing research with two common larval fish models, the zebrafish and fathead minnow, suggests that larval fish swimming patterns appear unique to anticipated modes or mechanisms of action for diverse chemicals. Thus, behavioral endpoints provide the potential to rapidly and sensitively examine chemicals for toxicity and to identify subcellular targets for industrial chemical and other contaminants, particularly during early tier assessments.

Here, we report a protocol for examining behavioral response profiles in larval fish. We demonstrate these methods using caffeine, a model neurostimulant and a common aquatic contaminant that is introduced to aquatic systems through discharge

from wastewater treatments plants following human consumption of foods, beverages, and pharmaceuticals formulated with caffeine[14]. We examine behavioral responses to caffeine in both larval zebrafish and fathead minnow, including to a sudden change in lighting condition, which is often referred to as a photomotor response (PMR) during pharmaceutical studies with embryonic and larval zebrafish[13,15]. We further identify effects of caffeine across several locomotor endpoints to develop chemical response profiles for each fish model. Caffeine treatment levels used in this study represent the upper centiles of exposure distributions based on measured environmental values of caffeine[16]. We also include treatments benchmarked to larval fish LC_{50} values, and the therapeutic hazard value (THV), a pharmaceutical concentration in water that is anticipated to result in plasma levels in fish consistent with a human therapeutic plasma dose.

Protocol

Studies in this protocol generally follow standardized experimental designs and recommended statistical analysis guidelines from the US Environmental Protection Agency (EPA no. 2000.0) for fathead minnows and the Organization for Economic Cooperation and Development (OECD no. 236) for zebrafish. These experimental designs (e.g., increasing replication) can be modified within the current protocol for future studies. Fish culture conditions follow previously published literature[17]. All experimental procedures and fish culture protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

1. Exposing Fish to Chemical Treatment

1. Prepare caffeine exposure solutions by dissolving caffeine in reconstituted hard water. Perform appropriate serial dilutions by diluting higher caffeine treatments with hard water to produce lower caffeine treatment levels. Table 1 summarizes each of the treatment levels used in this experiment.

Table 1: Experimental caffeine treatments for zebrafish and fathead minnow experiments. Nominal and measured values of caffeine for each treatment are given. *The caffeine treatments used in this study represent the upper centiles of exposure distributions based on measured environmental values of caffeine[16]. THV: Therapeutic Hazard Value. LOD: Limit of Detection

Zebrafish			Fathead Minnow		
Treatment	Nominal Caffeine Concentration (mg/L)	Measured Caffeine Concentration (mg/L)	Treatment	Nominal Caffeine Concentration (mg/L)	Measured Caffeine Concentration (mg/L)
Control	0	<LOD	Control	0	<LOD
75th Centile*	0.001	0.001	75th Centile*	0.001	0.001
95th Centile*	0.039	0.013	95th Centile*	0.039	0.009
99th Centile*	0.412	0.361	99th Centile*	0.412	0.310
THV	4.07	3.81	THV	4.07	4.12
10% LC50	48.46	46.66	10% LC50	14.1	14.7
40% LC50	193.82	186.67	40% LC50	56.38	53.91

2. Pour the prepared solution in individual exposure champers. Use 100 mL glass beakers filled with 20 mL of exposure solution for zebrafish exposure chambers and 500 mL beakers with 200 mL of exposure solution for fathead minnow exposure chambers.

3. Using a transfer pipette, place 10 zebrafish embryos aged 4-6 h post fertilization (hpf) in each of four replicate exposure chambers per treatment.

4. Place 10 fathead minnow larvae aged within 24 h of hatching in each of three replicate exposure chambers per treatment. To accommodate the larger size of fathead minnow larvae, cut the tip of the transfer pipette off prior to transfer.

5. Maintain zebrafish experiments at a 16:8 h light:dark photoperiod and a constant temperature of 28 ± 1 °C. Use the same photoperiod regime for fathead minnow studies, but at a temperature of 25 ± 1 °C.

6. After 96 h of chemical exposure, load individual fish in separate wells of 48 (for zebrafish) and 24 (for fathead minnow) well plates.

    1. To ensure that each well contains an equal volume of solution, transfer zebrafish larvae to 48 well plates using a 5,000 µL autopipette for a 1,000 µL volume per well. Use the autopipette to withdraw and transfer both the zebrafish larvae and exposure solution simultaneously.

    2. Due to their larger size, transfer fathead minnow larvae using a transfer pipette with the tip cut off. Prior to transferring fathead minnow larvae to individual

wells, fill each well to 2000 µL using an autopipette. When transferring individual fathead larvae to wells, place the tip of the transfer pipette in the well solution and allow the fish to swim from the pipette tip into the well.

2. Calibration of Video-Tracking Parameters

1. Prior to behavioral measures, set observation and calibration parameters in the video track software (see Table of Materials).

1. Place a well plate in the recording chamber with at least 1 larval fish in an individual well. Use the plate and associated fish as representations to set calibration parameters.

2. In the video track software, click "File | Generate Protocol", which will open a "protocol creation wizard" dialogue box. In the Location Count field, enter the number of individuals wells of the well plate and then click "OK".

3. At the top of the screen, click "View | Full Screen", which will prompt the system to display an overhead camera view of the well plate.

4. Click the "Draw Areas" icon, which appears as three multicolored shapes. To the right of the well plate viewing area, select the circle icon in the field labeled "Areas".

5. Use the cursor to delineate the circular video tracking area in the top left well of the well plate. Select "Top-Right Mark" and then outline the viewing area of the top right well. Then, select "Bottom Mark" to outline the bottom right well.

NOTE: After drawing the circular outline, its position will likely need to be adjusted. To adjust the position of the outline, click "select" and then use the cursor to move the outlined area. Also, outlines can be replicated by clicking "Copy**"** and then "Paste".

6. After the top left, top right, and bottom right well tracking areas have been defined, click "Build" to prompt the software to automatically delineate the viewing areas of the remaining wells.

7. In the area labeled "Calibration", click "Draw Scale". Use the cursor to draw a horizontal line across the plate. Once the line is drawn, a dialogue box labeled "Calibration measurement" will appear. Enter the well plate length and click "OK".

8. Exit the drawing manager by clicking the "Draw Areas" icon.

9. Click the "Tiles" icon. Using the cursor, highlight all the boxes that appear on the viewing screen so that each box is green.

NOTE: The Tiles icon appears as a group of six individual small squares.

10. “Click View| Full Screen”. To the right of the plate viewing area, click “Bkg” in the box labeled “Detection Threshold”. Use the threshold adjustment bar to set the pixel detection threshold. Once, the appropriate pixel detection threshold is selected, click “Apply to Group”.

Note: This protocol sets the detection threshold at 13 in black mode for zebrafish observations and at 110 in transparent mode for fathead minnow observations.

11. In the box labeled “Movement Threshold”, enter the desired movement speed tracking parameters. Once speed parameters are set, click “Apply to Group”.
NOTE: Note: This protocol sets small/large movements at 20 mm/s and inactive/small movements at 5 mm/s. These selections program the software to track larval fish movement at three different speed levels: inactive (freezing) = < 5 mm/s, small (cruising) = 5-20 mm/s, and large (bursting) = > 20 mm/s.

12. Click “Parameters | Protocol Parameters” from the drop-down menu. In the dialogue box, select the “Time” tab. Enter the observation time and the integration time. After parameters are entered click “Ok”.

13. To set the light/dark photoperiod times and light intensity for each photoperiod open the light driver settings dialogue box by selecting “Light Driving” from the “Parameters” drop down menu.
NOTE: See protocol video for setting multiple light-dark photoperiods.

14. After the video tracking parameters have been set, save the observation protocol.

NOTE: Note: This protocol observes fish behavior over a 50 min period that includes a 10 min acclimation phase followed by 4 altering light/dark phases consisting of two 10 min light periods and two 10 min dark periods. The integration time is set to measure behavior for each minute of the 50-min behavioral trial.

3. Observation of Larval Fish Locomotor and Photomotor Behavior

1. Place the well plate containing experimental fish in the behavioral recording chamber.

2. In the video tracking software, open the tracking protocol developed in step 2.

3. In the video tracking viewer, check to make sure that all larvae are visible on the computer screen, that only one individual larva is present in each well, and that individual wells are aligned within the observation areas that were defined in steps 2.1.5 and 2.1.6.

4. Click on “Experiment | Execute”.

NOTE: The system will prompt the user to provide a name and location to save the observation data.

5. Once the name and save location of the observation data have been specified, click on the “Several Live Images” icon to highlight all the pre-defined viewing areas

NOTE: This icon is located at the top of the computer screen and appears as a box divided into four smaller squares. Clicking on this icon will highlight all the pre-defined viewing areas.

6. Close the panel of the recording chamber and click “Background | Start” on the computer monitor.

4. Analyzing Behavioral Data

1. To retrieve larval fish activity data, open the spreadsheet, which is automatically compiled by the tracking software and is in the folder specified by the user before initiating behavioral trials (Step 3.4).

2. Refer to Figure 1A and 1B for representative measurements of naive locomotor activity of unexposed zebrafish and fathead minnow larvae, respectively. Refer to Figure 1C and 1D for PMR calculations, which effectively examine the magnitude of movement difference between light to dark or dark to light transitions.

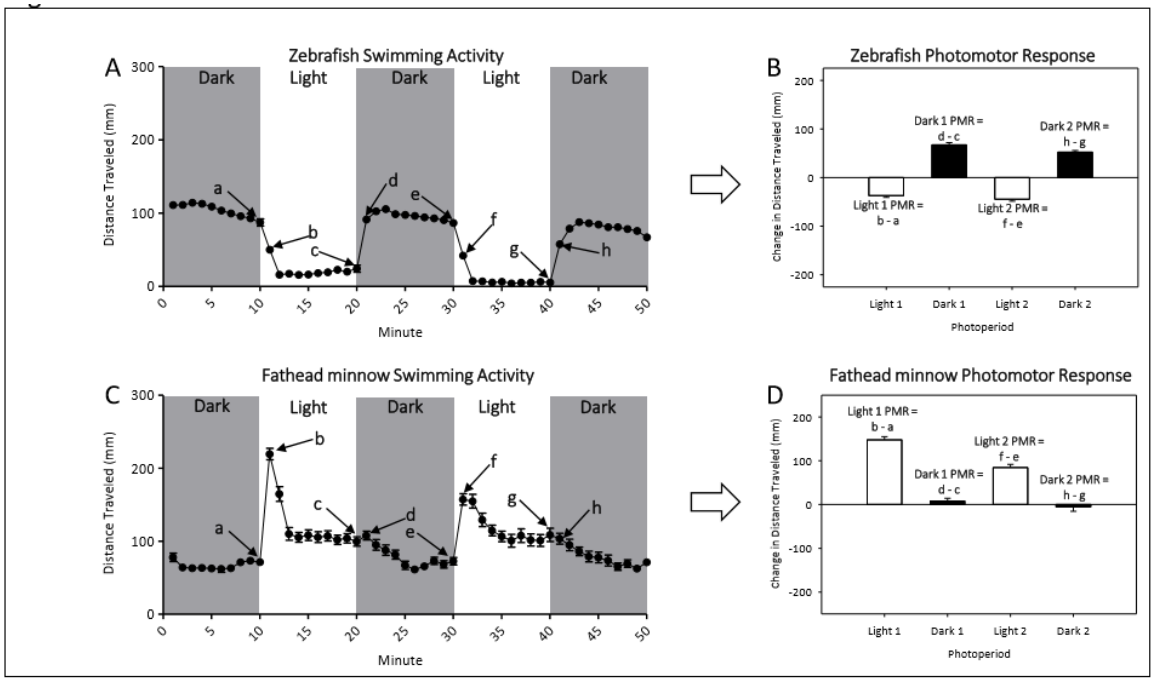


Figure 1: Example of baseline activity of unexposed zebrafish (A and B) and fathead minnow (C and D). The mean (± SEM) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing one-minute intervals of activity. Two dark and two light periods of photomotor responses are measured. The last (a, c, e, and g) and first (b, d, f, and h) minute of each photoperiod are used to calculate PMRs. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SEM) distance traveled between the last minute of an initial photoperiod and the first minute of the following period.

Representative Results:

Caffeine treatment levels did not appreciably vary during the 96 h experiments with zebrafish and fathead minnows. For example, Table 1 presents analytically verified concentrations of each treatment level. This protocol verified water samples for caffeine treatment levels by isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) generally following previously reported methods[28]. The formation of paraxanthine, the primary metabolite of caffeine, was also quantified. A description of these analytical procedures is provided in the supplemental analytical information. Because of the similarities between nominal and analytical verification of treatments, nominal treatment levels are presented throughout the remained of this manuscript.

Caffeine significantly altered zebrafish and fathead minnow behaviors. However, zebrafish locomotor responses were consistently more sensitive to caffeine than fathead minnows. The most sensitive behavioral endpoints for zebrafish and fathead minnow larvae were affected by caffeine at a concentration of 0.039 mg/L. Table 2 summarizes lowest observed effect concentrations (LOECs) and no observed effect concentrations (NOECs) for each behavioral endpoint in both fish models.

Table 2: Zebrafish and fathead minnow behavioral NOECs and LOECs for Caffeine. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) (mg/L) values for each of the light/dark swimming activity endpoints and photomotor responses for zebrafish and fathead minnows exposed to caffeine. Dashes indicate that no effects were observed at a particular endpoint across all treatment levels.

Zebrafish			Fathead minnow		
Endpoint	LOEC (mg/L)	NOEC (mg/L)	Endpoint	LOEC (mg/L)	NOEC (mg/L)
Total Distance Dark	0.412	0.039	Total Distance Dark	–	56.38
Total Distance Light	48.46	4.07	Total Distance Light	–	56.38
Total Counts Dark	0.412	0.039	Total Counts Dark	–	56.38
Total Counts Light	48.46	4.07	Total Counts Light	–	56.38
Bursting Distance Dark	–	193.82	Bursting Distance Dark	–	56.38
Bursting Distance Light	193.82	48.46	Bursting Distance Light	–	56.38
Bursting Counts Dark	193.82	48.46	Bursting Counts Dark	–	56.38
Bursting Counts Light	193.82	48.46	Bursting Counts Light	–	56.38
Bursting Duration Dark	193.82	48.46	Bursting Duration Dark	–	56.38
Bursting Duration Light	–	193.82	Bursting Duration Light	–	56.38
Cruising Distance Dark	0.412	0.039	Cruising Distance Dark	–	56.38
Cruising Distance Light	48.46	4.07	Cruising Distance Light	–	56.38
Cruising Counts Dark	0.412	0.039	Cruising Counts Dark	–	56.38
Cruising Counts Light	48.46	4.07	Cruising Counts Light	–	56.38
Cruising Duration Dark	0.412	0.039	Cruising Duration Dark	–	56.38
Cruising Duration Light	48.46	4.07	Cruising Duration Light	–	56.38
Freezing Distance Dark	0.412	0.039	Freezing Distance Dark	0.039	0.001
Freezing Distance Light	0.039	0.001	Freezing Distance Light	–	56.38
Freezing Counts Dark	0.412	0.039	Freezing Counts Dark	–	56.38
Freezing Counts Light	48.46	4.07	Freezing Counts Light	–	56.38
Freezing Duration Dark	–	193.82	Freezing Duration Dark	56.38	14.10
Freezing Duration Light	48.46	4.07	Freezing Duration Light	–	56.38
Dark 1 PMR	48.46	4.07	Dark 1 PMR	0.039	0.001
Light 1 PMR	48.46	4.07	Light 1 PMR	–	56.38
Dark 2 PMR	48.46	4.07	Dark 2 PMR	–	56.38
Light 2 PMR	48.46	4.07	Light 2 PMR	–	56.38
Bursting Dark 1 PMR	–	193.82	Bursting Dark 1 PMR	–	56.38
Bursting Light 1 PMR	–	193.82	Bursting Light 1 PMR	–	56.38
Bursting Dark 2 PMR	193.82	48.46	Bursting Dark 2 PMR	–	56.38
Bursting Light 2 PMR	–	193.82	Bursting Light 2 PMR	–	56.38
Cruising Dark 1 PMR	48.46	4.07	Cruising Dark 1 PMR	–	56.38
Cruising Light 1 PMR	48.46	4.07	Cruising Light 1 PMR	–	56.38
Cruising Dark 2 PMR	48.46	4.07	Cruising Dark 2 PMR	–	56.38
Cruising Light 2 PMR	193.82	48.46	Cruising Light 2 PMR	56.38	14.10
Freezing Dark 1 PMR	48.46	4.07	Freezing Dark 1 PMR	–	56.38
Freezing Light 1 PMR	193.82	48.46	Freezing Light 1 PMR	–	56.38
Freezing Dark 2 PMR	48.46	4.07	Freezing Dark 2 PMR	–	56.38
Freezing Light 2 PMR	193.82	48.46	Freezing Light 2 PMR	–	56.38

Figure 2 presents total locomotor activity and PMRs of zebrafish and fathead minnow following 96 h exposure to caffeine. Fathead minnow larvae PMRs were altered by caffeine at lower treatment levels (0.038 mg/L) than zebrafish, but a markedly larger number of photomotor endpoints were affected in zebrafish. The highest treatment level of caffeine (193.82 mg/L) altered PMR in zebrafish, in which these responses were

exactly opposite from controls. At this elevated treatment level, however, PMRs decreased in dark and increased in light conditions.

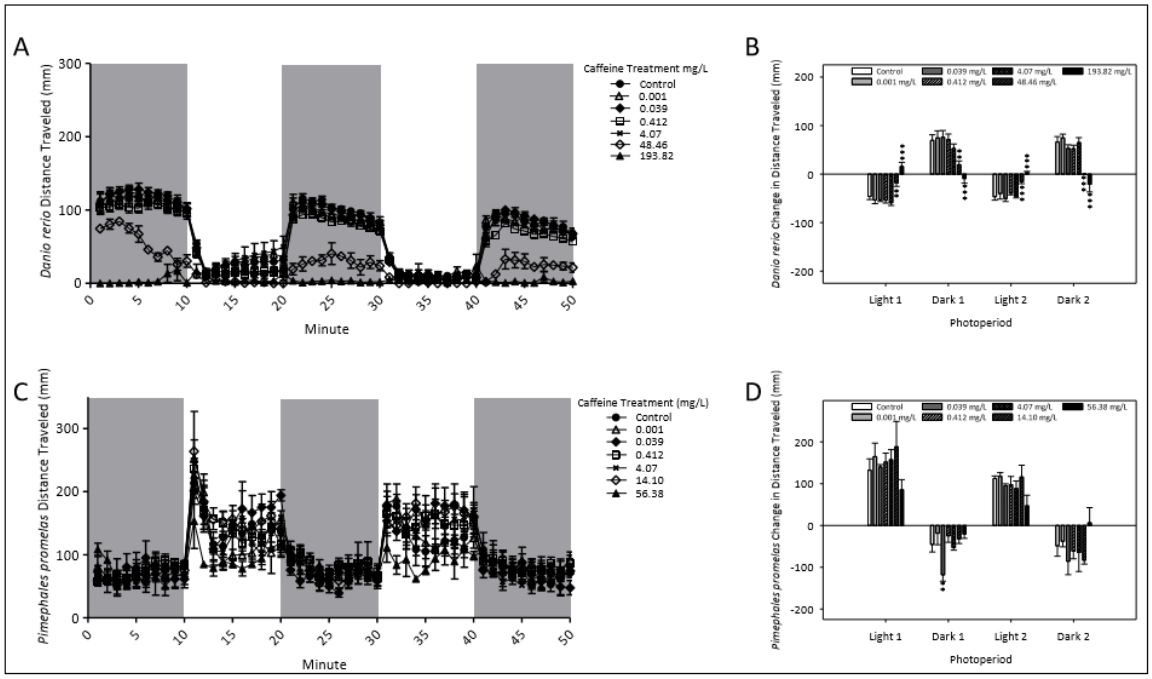
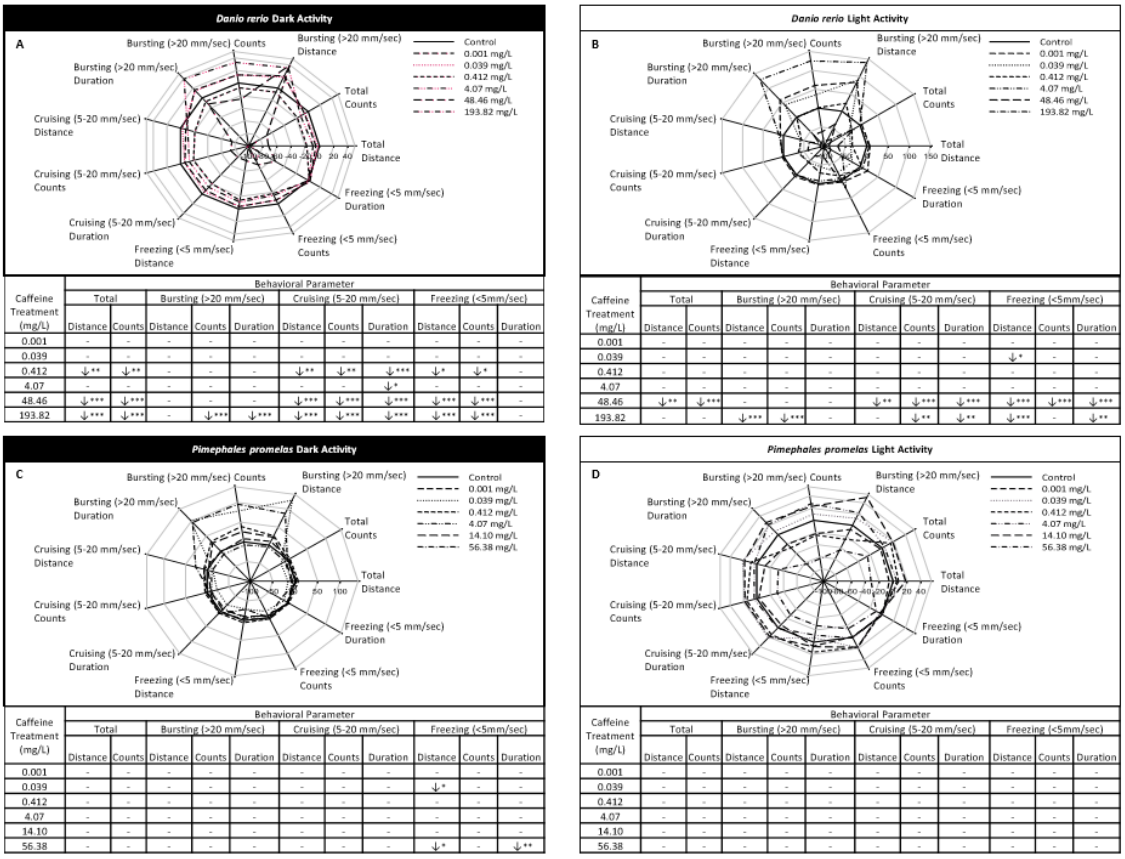


Figure 2: Swimming activity and photomotor responses of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to caffeine. The mean (± SEM) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1-min intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (± SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnows were used for behavioral observation. *p <0.10; ** p <0.05; *** p <0.01.

In addition to measuring larval PMRs, light and dark locomotor activity was analyzed across three speed thresholds for distance moved, number of movements, and duration of movements. This data is used to develop behavioral response profiles for caffeine (Figure 3, Supplemental Figure 19). In both of the fish models, caffeine inhibited activity at all significantly affected locomotor endpoints. Both fish models demonstrated increased activity at the bursting speed thresholds following exposure to caffeine, though not significantly. Similar to the results of the PMR observations, caffeine effected a

greater number of zebrafish locomotor endpoints. In fact, caffeine significantly altered several locomotor responses under dark conditions at environmentally realistic levels below the THV. However, fathead minnow locomotor activity was not significantly affected under light conditions by any treatment level.



Caffeine Treatment (mg/L)	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.001	-	-	-	-	-	-	-	-	-	-	-
0.039	-	-	-	-	-	-	-	-	-	-	-
0.412	↓**	↓**	-	-	-	↓**	↓**	↓***	↓*	↓*	-
4.07	-	-	-	-	-	-	-	↓*	-	-	-
48.46	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	-
193.82	↓***	↓***	-	↓***	↓***	↓***	↓***	↓***	↓***	↓***	-

Caffeine Treatment (mg/L)	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.001	-	-	-	-	-	-	-	-	-	-	-
0.039	-	-	-	-	-	-	-	-	↓*	-	-
0.412	-	-	-	-	-	-	-	-	-	-	-
4.07	-	-	-	-	-	-	-	-	-	-	-
48.46	↓**	↓***	-	-	-	↓**	↓***	↓***	↓***	↓***	↓***
193.82	-	-	↓***	↓***	-	-	↓**	↓**	↓***	-	↓**

Caffeine Treatment (mg/L)	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.001	-	-	-	-	-	-	-	-	-	-	-
0.039	-	-	-	-	-	-	-	-	↓*	-	-
0.412	-	-	-	-	-	-	-	-	-	-	-
4.07	-	-	-	-	-	-	-	-	-	-	-
14.10	-	-	-	-	-	-	-	-	-	-	-
56.38	-	-	-	-	-	-	-	-	↓*	-	↓**

Caffeine Treatment (mg/L)	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.001	-	-	-	-	-	-	-	-	-	-	-
0.039	-	-	-	-	-	-	-	-	-	-	-
0.412	-	-	-	-	-	-	-	-	-	-	-
4.07	-	-	-	-	-	-	-	-	-	-	-
14.10	-	-	-	-	-	-	-	-	-	-	-
56.38	-	-	-	-	-	-	-	-	-	-	-

Figure 3: Response profiles of larval zebrafish and fathead minnows after 96 h exposure to caffeine. Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to caffeine. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam, and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p <0.10; ** p <0.05; *** p <0.01.

Discussion

When selecting chemical treatment levels for behavioral toxicology studies, several factors must be considered. Caffeine treatment levels in the present study were selected based on upper centile values for predicted environmental exposure scenarios from wastewater effluent[16]. When possible, we routinely select treatment levels for aquatic toxicology studies using probabilistic exposure assessments of environmental observations[19,20]. A THV, which is calculable for medicines, was also included as a treatment level in the present study. THV values (Eq. 1)[22,23] are defined as predicted water concentrations leading to human therapeutic doses (Cmax) of pharmaceuticals in fish[23], are inspired from initial plasma modeling efforts[24], and are calculated based on blood:water chemical partitioning coefficients (Eq. 2)[25].

$$THV = C_{max} / \log P_{BW} \qquad \text{Eq. 1}$$

$$\log P_{BW} = \log [(10^{0.73 \,.\, \log Kow} \cdot 0.16)+0.84] \qquad \text{Eq. 2}$$

Here, we also select sublethal treatment levels relative to zebrafish and fathead minnow LC50 values. We consider this approach a useful benchmarking procedure for behavioral responses, particularly when comparing thresholds of specific behaviors with a fish model across multiple chemicals. It further facilitates calculations of acute to chronic ratios, which can be diagnostically useful in aquatic toxicology for mechanistic studies and assessments. LC50 values were obtained from preliminary toxicity bioassays following the standardized guidelines given in step 2.1.

In this protocol, we employ common experimental designs and statistical techniques recommended by the US EPA and OECD standardized methods for toxicology studies with fish models. Though we report *p* values (*e.g.*, < 0.01, <0.05,

<0.10), significant differences (α=0.10) in activity levels are identified among treatments using analysis of variance (ANOVA) if normality and equivalence of variance assumptions are met. Dunnett's or Tukey's HSD post hoc tests are performed to identify treatment level differences. We select this alpha (α=0.10) value to reduce type II errors, particularly for early tier assays and when an understanding of biologically important effect size is limited for understudied behavioral endpoints and model organisms[26], instead employing procedures more common in the biomedical sciences for multiple comparisons (*e.g.*, Bonferroni correction for RNA-Seq data)[27]. Future studies are needed to understand variability of these behavioral responses and potentially modify experimental designs (e.g., increase replication) accordingly.

A number of factors can influence behavior of larval fish in addition to chemical exposure. For example, time of day, age, well size, temperature, lighting condition, and volume of exposure solution in each well represent important considerations[11,30]. For these reasons, precautions should be taken to minimize the effects of external factors that could influence locomotor behavior of the larval fish during experimentation. Behavioral observations should be performed in narrow time windows (3 to 4 h) and across time periods when time of day effects are expected to have minimal influence on larval locomotor behavior[11]. Additionally, larval fish should be maintained at a consistent temperature (28 ± 1 °C for zebrafish and 24 ± 1 °C for FHM) and on a defined light/dark cycle in temperature-controlled incubators throughout the exposure period. In addition, the temperature of the laboratory where behaviors are recorded should be maintained to conditions approximating experimental conditions to avoid temperature influences on

behaviors. Further, wells used during behavioral observations should be maintained at a consistent volume for each individual fish.

Larval and embryonic zebrafish PMRs have been previously used in the biomedical sciences to identify potential therapeutic targets for novel compounds[12,13]. This protocol expands on previous behavioral research with zebrafish by utilizing 38 endpoints to investigate chemical bioactivity of environmental contaminants. Although caffeine is a common aquatic contaminant with an understood mechanism of action (MoA), many compounds in commerce lack important mechanistic data. Therefore, this protocol can be employed to gain insight of MoAs for compounds lacking toxicity data, including commercial chemicals[39]. Furthermore, the protocol provides methods for two of the most commonly used fish models. As noted above, whereas the zebrafish is a common biomedical fish model that is becoming increasingly popular in ecotoxicology, the fathead minnow is commonly used as an ecological model for environmental assessment applications but has received comparatively less attention in behavioral studies with automated systems compared to the zebrafish. Though there remains no standardized regulatory methods for fish behavioral toxicology studies, this protocol provides an approach to support future efforts.

Caffeine elicited behavioral responses in each of the fish models at levels that have been detected in the aquatic environment[16]. Rodriguez-Gil *et al.* 2018 developed global environmental exposure distributions in aquatic systems based on measured values of caffeine[16]. Specifically, 95% of predicted wastewater effluent concentrations would fall below the LOECs for the most sensitive behavioral endpoints of zebrafish and fathead minnow in the present study (Table 2). Though several behavioral effects of

caffeine were observed in zebrafish (particularly in dark conditions) at environmentally relevant levels, it is unclear whether these behavioral modifications might occur in natural fish populations or result in ecologically important adverse outcomes. Though useful for sensitive, diagnostic screening purposes, larval fish behavioral thresholds may not be representative of other life history stages or of fish in natural populations. Further research is warranted to determine whether similar behavioral response thresholds would occur in nature and be indicative of adverse outcomes at the individual or population levels of biological organization.

Disclosures

None

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References

1 Malaj, E., et al. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. Proceedings of the National Academy of Sciences. 111 (26), 9549-9554 (2014).

2 Schäfer, R. B., Kühn, B., Malaj, E., König, A. & Gergs, R. Contribution of organic toxicants to multiple stress in river ecosystems. Freshwater Biology. 61 (12), 2116-2128 (2016).

3 Andersen, M. E. & Krewski, D. Toxicity testing in the 21st century: bringing the vision to life. Toxicological Sciences. 107 (2), 324-330 (2008).

4 Rovida, C. & Hartung, T. Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals—a report by the transatlantic think tank for toxicology (t (4)). Altex. 26 (3), 187-208 (2009).

5 Council, N. R. Toxicity testing in the 21st century: a vision and a strategy. (National Academies Press, 2007).

6 Mehta, G., Hsiao, A. Y., Ingram, M., Luker, G. D. & Takayama, S. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. Journal of Controlled Release. 164 (2), 192-204 (2012).

7 Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T. & Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environmental Science and Pollution Research. 15 (5), 394-404 (2008).

8 Fraysse, B., Mons, R. & Garric, J. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotoxicology and Environmental Safety. 63 (2), 253-267 (2006).

9 Noyes, P. D., Haggard, D. E., Gonnerman, G. D. & Tanguay, R. L. Advanced morphological—behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicological Sciences. 145 (1), 177-195 (2015).

10 Colón-Cruz, L., et al. Alterations of larval photo-dependent swimming responses (PDR): New endpoints for rapid and diagnostic screening of aquatic contamination. Ecotoxicology and Environmental Safety. 147, 670-680 (2018).

11 Kristofco, L.A., et al. Age matters: developmental stage of Danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic Toxicology. 170 344-354 (2016).

12 Rihel, J., et al. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science. 327 (5963), 348-351 (2010).

13 Kokel, D., et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nature Chemical Biology. 6 (3), 231-237 (2010).

14 Bruton, T., Alboloushi, A., De La Garza, B., Kim, B.-O. & Halden, R. U. in Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations 257-273 (ACS Publications, 2010).

15 van Woudenberg, A.B., et al. Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. Reproductive Toxicology. 49, 101-116 (2014).

16 Rodríguez-Gil, J., Cáceres, N., Dafouz, R. & Valcárcel, Y. Caffeine and paraxanthine in aquatic systems: Global exposure distributions and probabilistic risk assessment. Science of the Total Environment. 612, 1058-1071 (2018).

17 Corrales, J., et al. Toward the Design of Less Hazardous Chemicals: Exploring Comparative Oxidative Stress in Two Common Animal Models. Chemical Research in Toxicology. 30 (4), 893-904 (2017).

18 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. Developmental Dynamics. 203 (3), 253-310 (1995).

19 Kristofco, L. A. & Brooks, B. W. Global scanning of antihistamines in the environment: Analysis of occurrence and hazards in aquatic systems. Science of the Total Environment. 592 477-487 (2017).

20 Saari, G. N., Scott, W. C. & Brooks, B. W. Global assessment of calcium channel blockers in the environment: Review and analysis of occurrence, ecotoxicology and hazards in aquatic systems. Chemosphere. (2017).

21 Corrales, J., et al. Toward the Design of Less Hazardous Chemicals: Exploring Comparative Oxidative Stress in Two Common Animal Models. Chemical Research in Toxicology. 30 (4), 893-904 (2017).

22 Berninger, J.P., et al. Effects of the antihistamine diphenhydramine on selected aquatic organisms Environmental Toxicology and Chemistry. 30 (9), 2065-2072 (2011).

23 Brooks, B. W. Fish on Prozac (and Zoloft): ten years later. Aquatic Toxicology. 151, 61-67 (2014).

24 Huggett, D., Cook, J., Ericson, J. & Williams, R. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Human and Ecological Risk Assessment. 9 (7), 1789-1799 (2003).

25 Fitzsimmons, P. N., Fernandez, J. D., Hoffman, A. D., Butterworth, B. C. & Nichols, J. W. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology. 55 (1-2), 23-34 (2001).

26 Scheiner, S. M. & Gurevitch, J. Design and Analysis of Ecological Experiments. (Oxford University Press, 2001).

27 Nakagawa, S. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behavioral Ecology. 15 (6), 1044-1045 (2004).

28 Bean, T.G., et al. Pharmaceuticals in water, fish and osprey nestlings in Delaware River and Bay. Environmental Pollution. 232 533-545 (2018).

29 Richendrfer, H., Pelkowski, S., Colwill, R. & Creton, R. On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behavioural Brain Research. 228 (1), 99-106 (2012).

30 Padilla, S., Hunter, D., Padnos, B., Frady, S. & MacPhail, R. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicology and Teratology. 33 (6), 624-630 (2011).

31 Sukardi, H., Chng, H. T., Chan, E. C. Y., Gong, Z. & Lam, S. H. Zebrafish for drug toxicity screening: bridging the in vitro cell-based models and in vivo mammalian models. Expert Opinion on Drug Metabolism & Toxicology. 7 (5), 579-589 (2011).

32 Ankley, G. T. & Villeneuve, D. L. The fathead minnow in aquatic toxicology: past, present and future. Aquatic Toxicology. 78 (1), 91-102 (2006).

33 Hutson, L. D. & Liang, J. O. Making an impact: zebrafish in education. Zebrafish. 6, 119+ (2009).

34 Hutson, L. D., Liang, J. O., Pickart, M. A., Pierret, C. & Tomasciewicz, H. G. Making a difference: education at the 10th international conference on zebrafish development and genetics. Zebrafish. 9 (4), 151-154 (2012).

35 Kane, A., Salierno, J. & Brewer, S. Fish models in behavioral toxicology: automated techniques, updates and perspectives. Methods in Aquatic Toxicology. 2, 559-590 (2005).

36 Rodriguez, A., et al. ToxTrac: a fast and robust software for tracking organisms. Methods in Ecology and Evolution. 9 (3), 460-464 (2018).

37 Hamm, J., Wilson, B. & Hinton, D. Increasing uptake and bioactivation with development positively modulate diazinon toxicity in early life stage medaka (Oryzias latipes). Toxicological Sciences. 61 (2), 304-313 (2001).

38 Kristofco, L. A., Haddad, S. P., Chambliss, C. K. & Brooks, B. W. Differential uptake of and sensitivity to diphenhydramine in embryonic and larval zebrafish. Environmental Toxicology and Chemistry. 37, 1175–1181 (2018).

39 Steele, W.B., Kristofco, L.A., Corrales, J., Saari, G.N., Haddad, S.P,. Gallagher, E.P., Kavanagh, T.J., Kostal, J., Zimmerman, J.B., Voutchkova-Kostal, A., Anastas, P.T. & Brooks, B.W. Comparative behavioral toxicology of two common larval fish models: exploring relationships between modes of action and locomotor responses. Science of the Total Environment 460-461, 1587-1600 (2018).

CHAPTER THREE

Comparative Behavioral Toxicology with Two Common Larval Fish Models: Exploring Relationships Among Modes of Action and Locomotor Responses

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Abstract

Behavioral responses inform toxicology studies by rapidly and sensitively detecting molecular initiation events that propagate to physiological changes in individuals. These behavioral responses can be unique to chemical specific mechanisms and modes of action (MOA) and thus present diagnostic utility. In an initial effort to explore the use of larval fish behavioral response patterns in screening environmental contaminants for toxicity and to identify behavioral responses associated with common chemical specific MOAs, we employed the two most common fish models, the zebrafish and the fathead minnow, to define toxicant induced swimming activity alterations during interchanging photoperiods. Though the fathead minnow (*Pimephales promelas*) is a common model for aquatic toxicology research and regulatory toxicology practice, this model has received little attention in behavioral studies compared to the zebrafish, a common biomedical model. We specifically compared behavioral responses among 7 different chemicals (1-heptanol, phenol, R-(-)-carvone, citalopram, diazinon, pentylenetetrazole (PTZ), and xylazine) that were selected and classified based on anticipated MOA (nonpolar narcosis, polar narcosis, electrophile, specific mechanism)

according to traditional approaches to examine whether these comparative responses differ among chemicals with various structure-based predicted toxicity. Following standardized experimental guidelines, zebrafish embryos and fathead minnow larvae were exposed for 96 h to each compound then were observed using digital behavioral analysis. Behavioral observations included photomotor responses, distance traveled, and stimulatory, refractory and cruising locomotor activity. Though fathead minnow larvae displayed greater behavioral sensitivity to 1-heptanol, phenol and citalopram, zebrafish were more sensitive to diazinon and R-(-)-carvone. Both fish models were equally sensitive to xylazine and PTZ. Further, the pharmaceuticals citalopram and xylazine significantly affected behavior at therapeutic hazard values, and each of the seven chemicals elicited unique behavioral response profiles. Larval fish behaviors appear useful as early tier diagnostics to identify mechanisms and pathways associated with diverse biological activities for chemicals lacking mechanistic data.

1. Introduction

There remains a global need to protect human health and the environment from chemical exposures, yet the majority of the tens of thousands of chemicals in commerce lack sufficient toxicology data to conduct hazard and risk assessments (Becker et al., 2015; Perkins et al., 2015). Due to the costly nature of traditional toxicity testing methods and policy decisions, regulatory agencies are often constrained by resource scarcity. These challenges have resulted in a growing impetus for more efficient, high throughput toxicity screening methods (Rovida and Hartung, 2009). When mechanistically coupled with sustainable and green chemistry and engineering advances, such efforts promise to identify attributes of chemicals initiating toxicity pathways, to support chemical

alternatives assessments and substitutions, and to advance sustainable molecular design of less hazardous compounds (Voutchkova et al., 2010; Coish et al., 2016).

Whereas high throughput in vitro techniques present exciting and unprecedented opportunities (Krewski et al., 2010), translating in vitro data to predict whole animal toxicity remains challenging. Cell based models have lower biological complexity than in vivo models and therefore do not account for important whole system processes such as metabolism and cross talk between tissues, including neurotransmission (MacRae and Peterson, 2015; Truong et al., 2014; Emmert et al., 2016). Traditional in vivo testing methods account for biological complexity, but these approaches are more time and resource intensive (Rovida and Hartung, 2009). Herein, larval fish models represent intermediate alternative test systems between cell based models and mammalian models that are less time and resource intensive, including during experimentation (Stewart et al., 2015; Truong et al., 2014). Furthermore, fish behavioral perturbations are of high ecological importance (Little and Finger, 1990; Little et al., 1993; Weis et al., 2001; Melvin and Wilson, 2013; Wong and Candolin, 2015). Because fish possess numerous enzyme/receptor systems similar to other vertebrates, including humans (Gunnarsson et al., 2008; Verbruggen et al., 2017; Huggett et al., 2003; MacRae and Peterson, 2015), these models are being used for coupled ecological and human health assessments (Perkins et al., 2013). Herein, basic and translational studies with specifically acting compounds (e.g., pharmaceuticals) are advancing comparative physiology, pharmacology and toxicology efforts across species (Brooks, 2018).

Larval fish behavior provides promising utility in identifying compound specific mechanisms and modes of action (MOAs) in pharmacology and toxicology (Brooks and

Steele, 2018). Behavioral responses are often more sensitive than other whole organism endpoints (mortality, growth, etc.), and behavioral thresholds and patterns can be informative of chemical bioactivity domains (Brooks, 2018). With the aid of automated tracking software and larval zebrafish, large-scale screening approaches in drug discovery have been advanced to identify molecular targets for novel therapeutics (Kokel et al., 2010; Rihel et al., 2010). For example, Kokel et al. (2010) specifically examined 14,000 different molecules through which 982 unique chemicals that transformed specific aspects of the photomotor response (PMR) were identified in 24 h post fertilization (hpf) zebrafish embryos. After identifying a proposed behavioral phenotype elicited by each compound, hierarchal clustering organized molecules by these profiles was then used to identify molecular targets for novel compounds using a "guilt by association" method (Kokel et al., 2010). Rihel et al. (2010) employed a similar approach using hatched zebrafish larvae that were 4 days post fertilization in which rest wake activity during altering photoperiods was observed. More recently Reif et al. (2015) employed PMRs with 24 hpf dechorionated zebrafish to screen thousands of ToxCast compounds. Such observations were mapped against previously published in vitro results (Sipes et al., 2013) to potentially identify specific molecular targets for chemicals eliciting behavioral responses in these PMR assays. Thus, such behavioral responses appear to represent useful early tiered assay approaches.

In comparison to embryonic and larval zebrafish, which have received increased attention as a behavioral model in the biomedical sciences such as developmental, drug discovery and toxicology disciplines (Kalueff et al., 2016; Kristofco et al., 2016), the larval fathead minnow (Pimephales promelas) has been primarily used as a model for

ecotoxicology research and environmental protection applications (Ankley and Villeneuve, 2006). Both models have standardized experimental guidelines and are commonly used for regulatory purposes, but the fathead minnow model has seen comparatively little use in behavioral toxicology using automated tracking technologies when compared to zebrafish. There is, however, a growing interest in using other species, including the ecologically important fathead minnow model, in automated behavioral platforms to understand organism responses to aquatic contaminants (Kristofco et al., 2016; Colón-Cruz et al., 2018; Steele et al., 2018). In fact, decades of experience in aquatic toxicology and behavior ecology promise to reciprocally inform and advance behavioral studies with fish models within biomedical efforts.

Here we present an initial effort to examine whether methods previously used for pharmaceuticals in the biomedical sciences can be translated within an aquatic behavioral toxicology framework for diverse environmental contaminants. We specifically selected seven chemicals based on a common aquatic toxicity classification scheme (Verhaar et al., 1992) for anticipated MOAs (nonpolar narcosis, polar narcosis, electrophiles, specific mechanisms) using standardized experimental designs with the two most common fish models to examine whether larval fish behavioral responses differed among chemicals. We hypothesized that behavioral locomotor and photomotor response profiles would differ among MOAs. Further, if fish behavioral endpoints are to be used diagnostically for screening purposes, it is critical to understand how experimental results may be interpreted based on choice of common larval fish models and associated regulatory experimental designs employed by academic, business and regulatory institutions.

Unfortunately, comparative mechanistic (Corrales et al., 2017) and behavioral responses among fish models to diverse chemical MOAs are limited.

2. Methods

2.1. Study Chemicals and Chemical Classification

Each study compound was selected using an anticipated MOA approach. This common classification scheme followed an approach proposed by Verhaar et al. (1992b), whereby each chemical was assigned to one of four categories traditionally used for aquatic contaminants. Chemical classifications included: 1-heptanol (class I: non-polar narcosis; 98.0% pure), phenol (class II: polar narcosis; ≥99.0 % pure), R-(-)-Carvone (class III: electrophile/proelectrophile activity; 98.0% pure), citalopram hydrobromide (class IV: specifically acting - selective serotonin reuptake inhibitor; 99.8% pure), diazinon (class IV: specifically acting - acetylcholinesterase inhibitor; 98.9% pure), pentylenetetrazole (PTZ) (class IV: specifically acting - GABA receptor antagonist; 100% pure), and xylazine hydrochloride (class IV: specifically acting - α-2 adrenergic agonist; 100% pure). Specifically acting chemicals were selected to target different receptors and enzymes associated with neurotransmission. Each chemical was obtained at the highest available purity (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions were prepared through serial dilution with reconstituted hard water (APHA et al 1998) prior to the onset of each study.

2.2. Fish Culture

Fathead minnow (P. promelas) cultures were maintained at Baylor University using a flow through system that introduced aged dechlorinated tap water to individual aquaria. Water

temperature was held at a constant temperature of 25 ± 1 °C. Fish were cultured under a 16:8 h light:dark photoperiod and fed artemia (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily. Embryos used for exposures were collected from sexually mature adults aged to at least 120 days (Valenti et al., 2009, Valenti et al., 2012; Berninger et al., 2011; Corrales et al., 2017). Collected embryos were exposed within 24 h after hatching. Tropical 5D wild type zebrafish (D. rerio) were also cultured at Baylor University using a z-mod recirculating system (Marine Biotech Systems, Beverly, MA, USA). Fish were maintained at a density of <4 fish per liter in 260 ppm instant ocean with a pH of 7.0. Similar to fathead minnows, zebrafish were cultured under a 16:8 h light:dark photoperiod and fed artemia (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily (Kristofco et al., 2016, Kristofco et al., 2018; Corrales et al., 2017). All experimental procedures and fish culture protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

2.3. Experimental Design

2.3.1. Acute studies. To maximize comparability of our work with other efforts we employed common standardized regulatory guidelines for toxicity studies with zebrafish (OECD FET; OECD, 2013) and fathead minnow (US EPA WET; US EPA, 2002) models. Prior to performing behavioral experiments, preliminary acute 96 h toxicity experiments were conducted to define mortality thresholds of both fish models. Briefly, zebrafish embryo studies were initiated with 4–6 hpf organisms, while experiments with fathead minnow larvae were initiated within 24 h post hatch according

to US EPA methods. Hatching for zebrafish occurs between 48 and 72 hpf. Fathead minnows hatching typically occurs at 96 h post fertilization. Because water pH can affect chemical ionization state, bioavailability and toxicity (Valenti et al., 2009), experimental conditions were maintained at pH 7.5 for all toxicity experiments with both fish models. Before the start of each experiment, solutions used for exposure were titrated to a pH of 7.5 following standard methods (US EPA, 1991). Water quality parameters (dissolved oxygen, pH, hardness, alkalinity, temperature) of reconstituted hard water (APHA et al., 1998) were routinely monitored following standard methods (APHA et al., 1998). During experiments, temperature was maintained in climate controlled incubators at 25 ± 1 °C for fathead minnows at 28 ± 1 °C for zebrafish. For each treatment level, a total of 20 (2 replicates of each treatment level with 10 individuals in each experimental unit) zebrafish and fathead minnows were used. Throughout the duration of each 96 h experiment, organisms were maintained in individual replicate beakers consisting of solution volumes of 200 mL and 20 mL for fathead minnows and zebrafish, respectively. These volumes were selected to ensure that the loading density did not exceed acceptable levels for standardized EPA and OECD guidelines (Steele et al., 2018).

2.3.2. Sublethal studies. Similar to the acute toxicity studies, sublethal behavioral experiments followed standardized toxicology experimental designs from the US EPA (US EPA, 2002) for fathead minnows and the OECD (FET OECD no. 236; OECD, 2013) for zebrafish but included behavioral responses (Steele et al., 2018). Sublethal experiments utilized 40 (4 replicates of each treatment level with 10 individuals in each experimental unit) zebrafish and 30 fathead minnows (3 replicates of each treatment level with 10 individuals in each experimental unit).

From each of the acute toxicity studies, nominal LC_{50} values were used as benchmarks for sublethal treatment levels used in behavioral experiments (Steele et al., 2018). Specifically, treatment levels were 40%, 20%, 10%, and 5% of the LC_{50} value for 1-heptanol, phenol, and R-(-)-carvone. Because specifically acting (class IV) chemicals are designed to target enzymes or receptors, these compounds are expected to affect biological systems at lower concentrations than less-specific chemicals. Subsequently, we employed treatment levels for class IV compounds that included 40%, 10%, 5%, 1%, 0.1% of each compound's respective LC_{50}. Diazinon included a 0.01% DMSO solvent control treatment. Additionally, therapeutic hazard values (THV) were calculated from mammalian pharmacology and physicochemical data for xylazine and citalopram (Brooks, 2014), but not PTZ because it is an experimental compound lacking human therapeutic dose information. THV values (Eq. (1); (Berninger et al., 2011; Brooks, 2014)) are defined as predicted water concentrations leading to human therapeutic doses (Cmax) of pharmaceuticals in fish, advance initial plasma modeling efforts (Huggett et al., 2003), and are calculated based on blood:water chemical partitioning coefficients (Eq. (2); (Fitzsimmons et al., 2001)).

(1) THV=Cmax/logPBW

(2) logPBW=log100.73logKow⋯0.16+0.84

A plasma hazard value (PHV) was calculated for diazinon in a similar fashion as citalopram and xylazine except that a PHV for diazinon was based on plasma levels predicted to cause sublethal toxicological effects in mammals (Santonastaso et al., 2014; Schulz et al., 2012; Viglino et al., 2008).

At the end of 96 h of exposure, larvae were placed in well plates for behavioral observation. Animals were maintained in solution at pH 7.5 from respective experimental units during behavioral observations. Each fathead minnow larvae were loaded in individual wells on 24-well plates, while zebrafish were loaded in 48-well plates due to species specific size differences. Solution volumes in 24 and 48 well plates were 2 mL and 1 mL, respectively. For each treatment level in these sublethal experiments, a total of 24 (4 replicates of each treatment level with 6 individuals in each experimental unit) zebrafish larvae and 12 fathead minnow larvae (3 replicates of each treatment level with 4 individuals in each experimental unit) were used for behavioral observations. After organisms were loaded on a given plate in solution from their respective experimental unit, it was placed in an incubator until the previous plate was finished with behavioral testing. Plate loading took approximately 15–25 min, so wait time (~30 min) was intentionally minimized (Steele et al., 2018).

2.3.3. Behavioral observations. Following methods previously described by our laboratory (Kristofco et al., 2016; Steele et al., 2018), larval swimming patterns were observed and recorded using automated tracking software (ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France). For each sublethal experiment, behavioral observations were initiated in the afternoon between 14:00 and 15:00 h because recent observations from our laboratory identified significant control behavioral differences between morning hours and other times time of day (Kristofco et al., 2016). If a treatment level resulted in significant mortality then these treatment levels were not examined for behavioral perturbations. The ViewPoint system was set in tracking mode and behavioral recordings occurred over 50 min. This time period included a 10 min dark

acclimation period followed by a 40 min observation period consisting of two altering 10 min light/dark cycles. Observations were recorded for total distance swam and total number of movements. Additionally, larval distance swam, number of movements, and duration of movements were recorded for activity across three different speed thresholds. These speeds include bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s) to characterize stimulatory and refractory behaviors. To measure larvae swimming responses to a sudden change in light condition, a PMR was observed following methods previously used (van Woudenberg et al., 2013) with slight modifications. PMR for each photoperiod transition (2 light and 2 dark responses) was calculated as the change in mean distance traveled (in mm) between the last minute of an initial photoperiod and the first minute of the following period. PMRs were observed across each speed threshold (bursting, cruising, and freezing) in addition to total distance.

2.3.4. Analytical measures. Water samples were collected at 0 h and 96 h from behavioral experiments with the specifically acting chemicals (citalopram, diazinon, PTZ, xylazine). Each treatment level was analytically confirmed using isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) with an Agilent Infinity 1260 autosampler/quaternary pumping system, Agilent jet stream thermal gradient electrospray ionization source (ESI), and model 6420 triple quadrupole mass analyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were prepared by taking a 500 µL aliquot of appropriately diluted stock solution, 450 µL of 0.1% aqueous formic acid (v v−1), and 50 µL of the appropriate internal standard solution combined in a 2 mL analytical vial (Agilent Technologies, Santa Clara, CA, USA). Four separate binary gradient mobile phase conditioned methods, consisting of 0.1% aqueous formic acid (v v−1) as solvent A

and MeOH as solvent B, resulted in the elution of citalopram at 4.5 mins, diazinon at 5.2 mins, pentylenetetrazole at 4.2 mins, and xylazine at 4.4 mins. 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. Separation was performed using a 10 cm × 2.1 mm Poroshell 120 SB-AQ column (120 Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) preceded by a 5 mm × 2.1 mm Poroshell 120 SB-C18 attachable guard column (120 Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA). The flow rate was held constant at 0.5 mL/min with a column temperature maintained at 60 °C. The injection volume was 10 µL. Cycle time was adjusted to 100 ms for acquisition of data. Multiple reaction monitoring (MRM) transitions for target analytes and associated instrument parameters were automatically determined using MassHunter Optimizer Software (Agilent Technologies, Santa Clara, CA, USA) by flow injection analysis. The ionization mode, monitored transitions, and instrumental parameters for citalopram/citalopram-d6, diazinon/diazinon-d10, pentylenetetrazole/pentylenetetrazole-d6, and xylazine/xylazine-d6 were as follows: ESI+ citalopram 352.2 > 109, fragmentor = 103, collision energy = 33, citalopram-d6 331.2 > 109, fragmentor = 103, collision energy = 33, diazinon 305.1 > 169.1, fragmentor = 125, collision energy = 24, diazinon-d10 315.2 > 170.1, fragmentor = 130, collision energy = 24, pentylenetetrazole 139.1 > 96.1, fragmentor = 103, collision energy = 17, pentylenetetrazole-d6 145.1 > 102.1, fragmentor = 103, collision energy = 17, xylazine 321.1 > 90.1, fragmentor = 103, collision energy = 21, and xylazine-d6 227.1 > 90.1, fragmentor = 103, collision energy = 25.

Quantitation was performed using an isotope dilution calibration method. Calibration standards, containing internal standards and variable concentrations of target compound, were prepared in 95:5 0.1% (v v−1) aqueous formic acid–methanol. The linear range for each analyte (0.1–500 ng mL−1) was confirmed from plots of sensitivity (i.e., response factor; RF) versus analyte concentration. Our criterion for linearity required that the relative standard deviation of RFs for standards spanning the noted range was ≤15%. Internal standard calibration curves were constructed for each analyte using eight standards that were within the corresponding linear range. Calibration data were fit to a linear regression, and correlation coefficients (r2) for all analytes were ≥0.995. Quality assurance and quality control measures included running a continued calibration verification (CCV) sample every five samples to check calibration validity during the run. A criteria of ±20% of CCV concentration was held to be acceptable for all analytes. One blank (i.e., reference water with internal standards only) and one solvent blank were included in each analytical sample batch. Nominal and measured values for each compound are reported in Supplemental Tables 1 and 2. Measured values for each of the specifically acting compounds are represented in parenthesis.

2.4. Statistical Analyses

LC50 values from initial acute studies with each of the compounds were calculated using the Toxicity Relationship Analysis Program version 1.30a (EPA). Sigma Plot 13.0 (Systat Software Inc., San Jose, CA, USA) software was used for statistical analysis of behavioral data, which generally followed previous work with the ViewPoint system and zebrafish. Consistent with our previously reported methods (Steele et al., 2018), we employed common experimental designs and statistical techniques

recommended by US EPA and OECD standardized methods for toxicology studies with fish models and extended these designs to include behavioral responses. Prior to analysis, data was examined for normality and equivalence of variance. Behavioral observations included a nested design, in which statistical analyses were performed for each treatment level with 6 individuals from each experimental replicate (N = 4) of zebrafish larvae and 4 individuals from each experimental replicate (N = 3) of fathead minnow larvae. Significant differences ($\alpha = 0.10$) in movement patterns were identified among treatments using analysis of variance (ANOVA) if normality and equivalence of variance assumptions were met. Because fish behavioral screening assays are likely employed in tiered approaches, we selected $\alpha = 0.10$ to decrease type II errors, which is particularly important when an understanding of biologically important effect sizes are limited for understudied behavioral endpoints and model organisms (Scheiner and Gurevitch, 2001). Dunnett's post hoc tests were performed to identify treatment level differences; mean distance traveled, number of movements (counts), and duration of movements were calculated in 1 min intervals. For data not meeting ANOVA assumptions, data was log transformed or ANOVA on ranks was performed as recommended by standard methods.

3. Results

3.1. Naïve Larval Fish Photomotor Behavior

Control 96 hpf zebrafish displayed increased activity in response to dark periods and diminished activity during light photoperiods. Fathead minnow larvae demonstrated a different response from zebrafish, whereby larval fish activity dramatically increased in response the light but showed little change during dark photoperiods. Figure 4

summarizes light/dark locomotor activity (Fig. 4 A and C) and PMRs (Fig. 4 B and D) of naïve zebrafish and fathead minnow larvae.

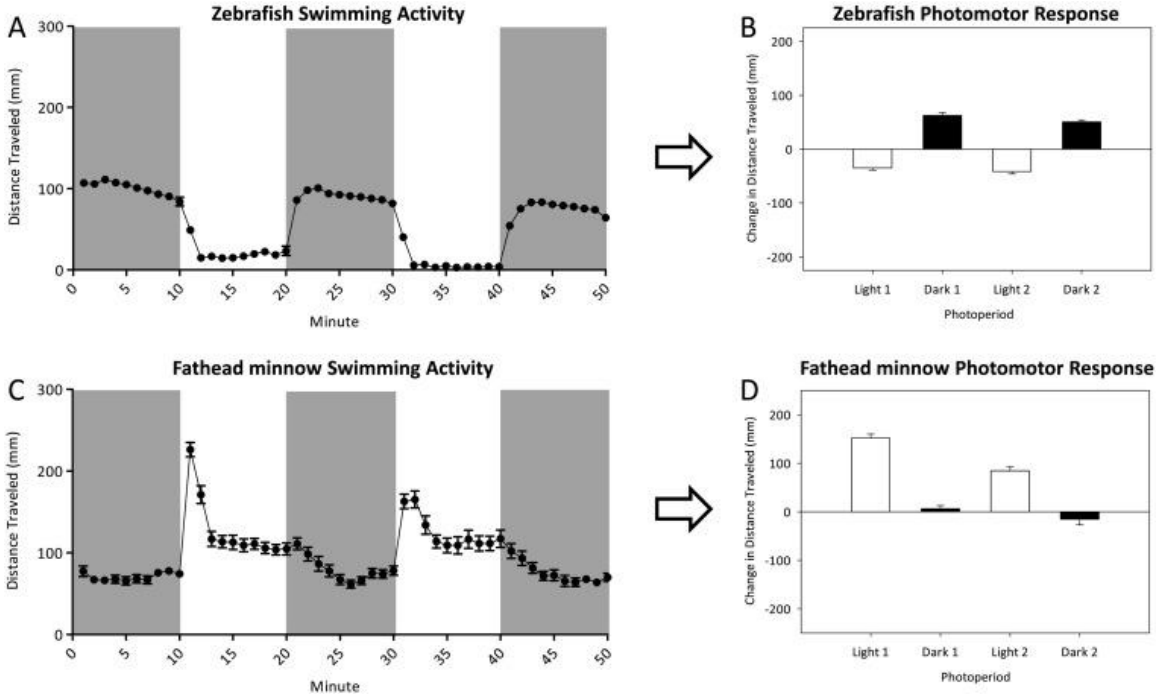


Figure. 4. Baseline activity of unexposed zebrafish (A and B) and fathead minnow (C and D). The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 min intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. A total of 168 zebrafish (28 replicates each with 6 larvae) and 84 (21 replicates each with fish) fathead minnow were used for each baseline behavioral observation.

3.2. Acute Toxicity and Behavioral Response Thresholds

Nominal and measured values for treatment levels are reported in Supplemental Tables 1 and 2. Acute toxicity differed among study chemicals and between fish models. LC50 values (Table 3) for each of the study compounds varied by over two orders of magnitude with diazinon being the most acutely toxic, and PTZ, also a class IV compound, demonstrating the lowest toxicity to both fish models. Acute toxicity of diazinon, PTZ, xylazine, and R(-)carvone was fairly similar between the fish models,

whereas LC50 values for 1-heptanol and citalopram differed between models with fathead minnow values 2 to 4 times lower than zebrafish (Table 3).

Table 3. LC50 values for zebrafish and fathead minnow following 96 h exposure to seven compounds.

Chemical	Zebrafish LC_{50} (mg/L)	Fathead minnow LC_{50} (mg/L)
1-heptanol	110.3	40.3
Phenol	116.5	121.3
R-(-)-carvone	58.2*	58.6*
Citalopram	38.4	9.3
Diazinon	11.4	11.5
Pentylenetetrazole	2644.9	2785.6
Xylazine	37.1	48.1

Behavioral response thresholds similarly varied between model organisms and among the study chemicals. For example, lowest observed effect concentrations (LOECs) and no observed effect concentrations (NOECs) are summarized in Table 4 for the most sensitive behavioral endpoint among four PMR transitions (2 light and 2 dark) across three speed thresholds and 11 locomotor behaviors (total distance moved; total number of movements; distance, number and duration of movements for each speed threshold) in light and dark conditions. Fathead minnow larvae displayed greater behavioral sensitivity to 1-heptanol, phenol and citalopram while zebrafish were more sensitivity to diazinon, and R-(-)-carvone (Table 2). Similar sensitivities of both fish models were observed for PTZ and xylazine (Table 4).

Table 4. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC)(mg/L) values for themost sensitive behavioral endpoints across light/dark swimming activityendpoints and photomotor responses for zebrafish and fathead minnows exposed to seven compounds. Behavioral endpoint for each LOEC is given in parenthesis next to each value. Cnts: Counts;Dist: Distance; Dur: Duration; PMR: Photomotor Response.

	Zebrafish		Fathead minnow	
Chemical	LOEC (mgl/L)	NOEC (mg/L)	LOEC (mg/L)	NOEC (mg/L)
1-Heptanol	5.5 (Bursting Cnts)	<5.5	2.0 (Freezing PMR)	<2.0
Phenol	23.3 (Freezing Dist)	11.7	6.1 (Freezing Dist)	<6.1
R-(-)-Carvone	2.91 (Total Counts)	<2.91	11.72 (Cruising PMR)	5.86
Citalopram	0.02 (Cruising Dist)	<0.02	0.01 (Freezing PMR)	<0.01
Diazinon	0.003 (Freezing PMR)	0.0005	0.17 (Cruising PMR)	0.04
Pentylenetetrazole	130.00 (Cruising PMR)	26.00	120.00 (Bursting Dist)	26.00
Xylazine	0.04 (Cruising PMR)	<0.04	0.04 (Bursting Dist)	<0.04

3.3. Photomotor Responses Among Chemical Mode of Action Categories

Each of the study chemicals employed for the current study elicited unique PMR responses in both fish models. For example, dark photomotor responses of larval zebrafish to 1-heptanol demonstrated a progressive decrease in magnitude as 1-hepanol treatments increased while the light PMR was only affected at freezing speed thresholds (Supplemental Fig. 25 B, E, F, and G). Zebrafish exposed to phenol, conversely, demonstrated increased activity in response to light at the 40% LC50 (46.6 mg/L; $p < 0.05$) treatment and a diminished response to dark at the 20% (23.29 mg/L; $p < 0.05$) and 40% (46.58 mg/L; $p < 0.001$) LC50 treatments (Supplemental Fig. B). However, fathead minnow PMRs were only significantly ($p < 0.05$) affected by 1-heptanol at the freezing speed threshold in the dark, but remained unaffected by phenol across all treatments and speed thresholds (Supplemental Fig. 25 D, H, I, and J; Supplemental Fig. 26 D, H, I, and J). R-(-)-carvone effected PMRs of both fish models. Specifically, fathead minnow PMRs were effected at the 20% (11.7 mg/L; $p < 0.01$), and 40% LC50 (23.4 mg/L; $p < 0.01$) treatment levels while zebrafish were effected al lower (5% LC50, 2.9 mg/L; $p < 0.01$) treatment levels (Supplemental Fig. 27). Furthermore, R-(-)-carvone

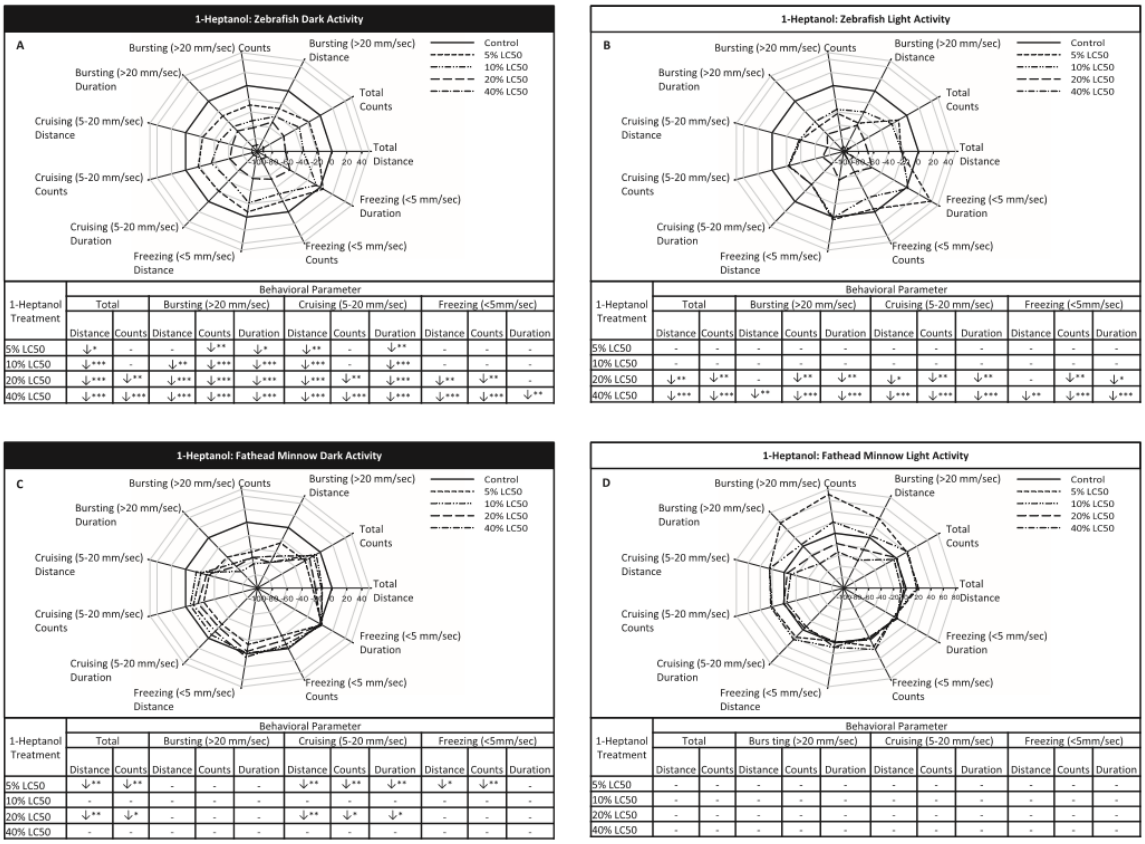
elicited an inverted PMR in zebrafish during light 1 and dark 1 photoperiods whereby both PMR responses were opposite of the controls and the 40% LC50 (23.3 mg/L) treatment level (Supplemental Fig. 27 B). A similar inverted response occurred in exposed fathead minnows at cruising speed thresholds (Supplemental Fig. 27 I).

With the exception of PTZ, larval fish PMRs were generally more sensitive to specifically acting compounds than other categories (classes I, II, III) of chemicals examined, and light PMRs were more sensitive to these compounds than dark PMRs (Supplemental Figs. 28–31). Zebrafish PMRs were more sensitive than fathead minnows for all of the class IV compounds except citalopram. Citalopram specifically affected zebrafish light PMRs at the THV (0.02 mg/L; $p < 0.05$)and fathead minnow light PMRs below the THV (0.1% LC50; 0.01 mg/L; $p < 0.05$) at freezing speed thresholds (Supplemental Fig. 28 H). Zebrafish light PMRs were also significantly ($p < 0.1$) affected at the THV (0.04 mg/L; $p < 0.1$) by xylazine (Supplemental Fig. 31 B and F), while diazinon affected zebrafish light PMRs at the 0.1% LC50 (0.003 mg/L; $p < 0.05$) treatment level (Supplemental Fig. 29 B and E). At the highest (40% LC50; 1.1 mg/L; $p < 0.05$) treatment level examined, diazinon exposed zebrafish demonstrated increased dark PMR activity at bursting speed thresholds (Supplemental Figure 29 G). Fathead minnow larvae PMRs were affected by the 5% LC50 value for diazinon (0.17 mg/L; $p < 0.05$) and xylazine (2.4 mg/L; $p < 0.05$; Supplemental Figs. 29 D, H, I, J; 31 D, H, I, J). Similar to observations R-(-)-carvone, the 1ight PMR of fathead minnows exposed to xylazine at the 40% LC50 (21 mg/L) treatment level was directly opposite from control fish responses at cruising speed thresholds (Supplemental Fig. 31 I).

In the current study, PTZ was the least potent class IV chemical studied; however, PTZ produced abnormal PMRs in both fish models. In zebrafish, PTZ elicited PMRs that were diametrically opposite from control fish at the 40% LC50 (1000 mg/L) treatment (Supplemental Fig. 30 B). Similar to exposed zebrafish, PTZ elicited an unusual PMR response in fathead minnow larvae exposed to the 10% LC50 (230 mg/L; $p < 0.05$) treatment level in which significant increases in activity occurred during the dark PMR, a response that was again opposite from control fish (Supplemental Fig. 30 D).

3.4. Locomotor Behavioral Profiles Among Chemical Mode of Action Categories

Behavioral response variables (Fig. 5, Fig. 6, and Supplemental Figs. 20-24) were structured to include number (counts) and duration of stimulatory (bursting), cruising, and refractory (freezing) behaviors in different regions of each plot. Through each behavioral endpoint measured, clear differences in larval behavioral responses among each of the chemicals were apparent. In the case of 1-heptanol (Fig. 5) and PTZ (Fig. 6) exposures, for example, markedly different response profiles were observed.



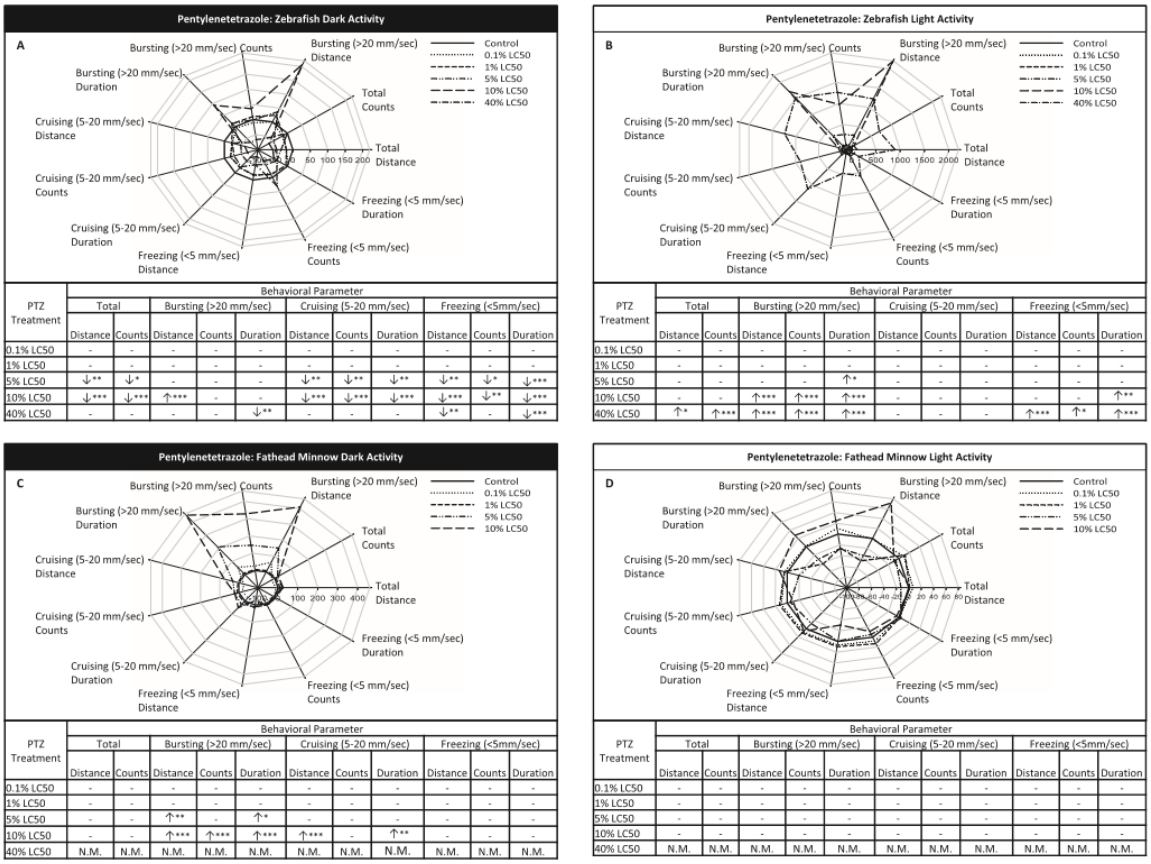
1-Heptanol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	↓*	-	-	↓**	↓*	↓**	-	↓**	-	-	-
10% LC50	↓***	-	↓**	↓***	↓***	↓***	-	↓***	-	-	-
20% LC50	↓***	↓**	↓***	↓***	↓***	↓***	↓**	↓***	↓**	↓**	-
40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**

1-Heptanol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	↓**	↓**	-	↓**	↓**	↓*	↓**	↓**	-	↓**	↓*
40% LC50	↓***	↓***	↓**	↓***	↓***	↓***	↓***	↓***	↓**	↓***	↓***

1-Heptanol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	↓**	↓**	-	-	-	↓**	↓**	↓**	↓*	↓**	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	↓**	↓*	-	-	-	↓**	↓*	↓*	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

1-Heptanol Treatment	Behavioral Parameter										
	Total		Burs ting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

Figure 5. Behavioral response profile plots with mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to 1-heptanol, a model category 1 (nonpolar narcosis) chemical. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *$p < 0.10$;**$p < 0.05$; ***$p < 0.01$.



PTZ Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	↓**	↓*	-	-	-	↓**	↓**	↓**	↓**	↓*	↓***	
10% LC50	↓***	↓***	↑***	-	-	↓***	↓***	↓***	↓***	↓**	↓***	
40% LC50	-	-	-	-	↓**	-	-	-	↓**	-	↓***	

PTZ Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	-	↑*	-	-	-	-	-	-	
10% LC50	-	-	↑***	↑***	↑***	-	-	-	-	-	↑**	
40% LC50	↑*	↑***	↑***	↑***	↑***	-	-	-	↑***	↑*	↑***	

PTZ Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	↑**	-	↑*	-	-	-	-	-	-	
10% LC50	-	-	↑***	↑***	↑***	↑***	-	↑**	-	-	-	
40% LC50	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	

PTZ Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	-	-	-	-	-	-	-	-	
10% LC50	-	-	-	-	-	-	-	-	-	-	-	
40% LC50	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	

Figure 6. Behavioral response profiles with mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to PTZ, a model category 4 (specifically acting) chemical. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *$p < 0.10$;**$p < 0.05$; ***$p < 0.01$. N.M.: Not Measured.

In larval zebrafish, 1-heptanol caused a significant decrease in overall swimming activity at each of the locomotor endpoints, and larval swimming activity declined as 1-heptanol concentrations increased (Fig. 5 A and B). Fathead minnow larvae displayed a similar response profiles to zebrafish during dark photoperiods when exposed to 1-heptanol, but were not affected across any of the locomotor endpoints during light

photoperiods (Fig. 5 C and D). When fathead minnow and zebrafish larvae were exposed to phenol (class II), a markedly different response profile than 1-heptanol emerged (Table 5; Supplemental Fig. 20A and B). Larval fish exposed to phenol demonstrated a large increase in activity for several endpoints, but most notably for the bursting speed threshold, while fish exposed to 1-heptanol demonstrated a decrease in activity at all affected endpoints. Phenol elicited differential light and dark responses in zebrafish larvae compared to 1-heptanol. For example, cruising activity of phenol exposed fish in the dark decreased ($p < 0.05$) in comparison to control fish, but during light conditions cruising activity drastically increased ($p < 0.01$) in comparison to control larvae. Phenol suppressed ($p < 0.01$) the duration of movements of the fathead minnow model at the freezing speed threshold, but elicited hyperactivity of all other effected endpoints during both photoperiods (Table 5; Supplemental Fig. 20 C and D). Fathead minnow activity was not effected by R-(-)-carvone exposure in either light or dark conditions (Table 6). However, zebrafish swimming activity was effected by R(−)-carvone during light and dark photoperiods across all behavioral parameters (Table 6). A larger number of zebrafish endpoints were effected by at the lowest treatment level in the light, indicating that larval zebrafish maybe more sensitive to R-(-)-carvone during light photoperiods.

Table 5. Mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to phenol. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p < 0.10;**p < 0.05; ***p < 0.01.

Species	Photoperiod	Phenol Treatment	Behavioral Parameter										
			Total		Bursting (>20 mm/s)			Cruising (5–20 mm/s)			Freezing (<5 mm/s)		
			Distance	Counts	Distance	Counts	¨Duration	Distance	Counts	Duration	Distance	Counts	Duration
Zebrafish	Dark	5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		20% LC50	–	–	↑*	–	–	–	–	–	↓***	–	↓***
		40% LC50	–	↓*	↑**	–	–	–	↓**	↓**	↓***	↓**	–
Zebrafish	Light	5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		20% LC50	–	–	–	–	–	–	–	–	↓**	–	–
		40% LC50	↑***	↑***	↑**	↑**	↑**	↑***	↑***	↑**	–	↑**	↑***
Fathead minnow	Dark	5% LC50	–	–	–	–	–	–	–	–	↑**	–	–
		10% LC50	–	–	–	–	–	–	–	–	↑**	–	–
		20% LC50	–	–	–	–	–	–	–	–	↑*	–	–
		40% LC50	–	–	↑***	↑**	↑**	–	–	–	–	–	↓*
Fathead minnow	Light	5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	↑*	–
		20% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	–	–	↑***	↑***	↑***	–	–	–	–	↑*	↓***

Table 6. Mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure R-(-)-carvone. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p < 0.10;**p < 0.05; ***p < 0.01.

Species	Photoperiod	R-carvone Treatment	Behavioral Parameter										
			Total		Bursting (>20 mm/s)			Cruising (5–20 mm/s)			Freezing (<5 mm/s)		
			Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
Zebrafish	Dark	5% LC50	–	–	–	–	–	–	–	–	–	–	↓**
		10% LC50	–	↓*	–	–	–	–	–	–	–	↓**	↓***
		20% LC50	↓*	↓***	–	–	–	–	↓***	↓***	↓**	↓***	↓***
		40% LC50	↓***	↓***	↓**	↓**	↓**	↓***	↓***	↓***	↓***	↓***	↓***
Zebrafish	Light	5% LC50	–	↓***	–	–	–	↓**	↓***	–	–	↓***	↓***
		10% LC50	↓*	↓***	–	–	–	↓**	↓***	–	–	↓***	↓***
		20% LC50	↓***	↓***	–	–	–	↓***	↓***	↓***	↓***	↓***	↓***
		40% LC50	↓***	↓***	↓**	↓**	↓**	↓***	↓***	↓***	↓***	↓***	↓***
Fathead minnow	Dark	5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		20% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	–	–	–	–	–	–	–	–	–	–	–
Fathead minnow	Light	5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		20% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	–	–	–	–	–	–	–	–	–	–	–

Diazinon increased stimulatory activity for the bursting speed thresholds at the 5% (0.17 mg/L; $p < 0.1$) and 10% LC50 (0.28 mg/L; $p < 0.05$) treatment levels but decreased activity at the other speed thresholds in zebrafish (Supplemental Fig. 23; Table 8). Furthermore, diazinon effects on zebrafish locomotor activities were only significant in the dark. Fathead minnow larvae, in contrast, significantly responded to diazinon at the 5% LC50 (0.17 mg/L; $p < 0.05$) treatment at several endpoints during light photoperiods, but in the dark, diazinon only affected ($p < 0.05$) distance moved at the freezing speed threshold (Supplemental Fig. 23; Table 8).

PTZ exposure at the 40% LC50 (1000 mg/L) value elicited an aberrant swimming pattern in larval zebrafish as exposure caused swimming activity to increase in the light, but activity to decrease in the dark (Supplemental Fig. 30 A). This locomotor activity was essentially opposite from control fish, and was similar to PMR observations. At the 10%

LC50 (260 mg/L; $p < 0.001$) treatment level, PTZ elicited increased activity in the dark at bursting speed thresholds but caused a decrease in activity at all other endpoints where significant effects were observed. In the light, however, larval zebrafish demonstrated hyperactivity across all significantly affected endpoints (Fig. 6 A and B). PTZ significantly increased fathead minnow activity but only at the bursting and cruising thresholds in the dark. PTZ did not significantly affect fathead minnow activity in the light (Fig. 6 C and D).

In both fish models, xylazine demonstrated a similar response pattern to 1-heptanol (Supplemental Fig. 31) in the dark. During light conditions, however, this compound differed from 1-heptanol by increasing activity of several endpoints in zebrafish larvae at the 1% LC50 (0.40 mg/L) treatment level and increasing bursting distance in fathead minnow larvae at the THV (0.04 mg/L; $p < 0.05$) (Table 9; Supplemental Fig. 31). Xylazine also produced unique responses whereby larval zebrafish displayed increased activity for several endpoints at the 1% LC50 (0.39 mg/L), but activity decreased at the 40% LC50 (16 mg/L). A similar effect of xylazine occurred in fathead minnow larvae for the stimulatory bursting distance endpoint at the THV (0.04 mg/L; $p < 0.05$).

Zebrafish larvae exposed to citalopram were significantly affected by all treatment levels including the THV (0.02 mg/L). Citalopram (Supplemental Fig. 22 A and B) elicited a locomotor profile that appeared to be very similar to 1-heptanol (Fig. 5 A and B). However, citalopram only significantly decreased stimulatory swimming in the light whereas 1-heptanol affected all locomotor endpoints in light conditions. For fathead minnow larvae, citalopram significantly decreased activity in the light and the dark, but

larval swimming behaviors were more sensitive to the antidepressant in the light in contrast to the zebrafish that displayed greater sensitivity during dark photoperiods (Table 7).

Table 7. Mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to citalopram. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p < 0.10;**p < 0.05; ***p < 0.01.

Species	Photoperiod	Citalopram Treatment	Behavioral Parameter										
			Total		Bursting (>20 mm/s)			Cruising (5–20 mm/s)			Freezing (<5 mm/s)		
			Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
Zebrafish	Dark	THV	↓**	↓*	–	–	–	↓***	↓*	↓**	–	↓*	–
		0.1% LC50	↓***	↓**	↓*	↓**	↓*	↓***	↓**	↓***	–	↓**	–
		1% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	–	↓***	–
		5% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	–
		10% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**
		40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**
Zebrafish	Light	THV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	↓*	–	↓*	–	–	–	–	–	–
		40% LC50	–	–	–	–	↓*	–	–	–	–	–	–
Fathead minnow	Dark	0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	–	–	↓**	–	↓***
		10% LC50	–	–	–	–	–	–	–	–	–	–	↓**
		40% LC50	↓**	–	–	–	–	–	↓***	↓**	↓***	–	↓***
Fathead minnow	Light	0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	↓**	–	↓*	–	–	–	–	–	–
		5% LC50	–	–	↓**	–	↓*	–	–	–	–	–	↓**
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	↓***	↓***	↓***	–	↓**	–	↓***	↓***	↓***	↓***	↓***

Table 8. Mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to diazinon. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (N = 4 replicates each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p < 0.10;**p < 0.05; ***p < 0.01.

Species	Photoperiod	Diazinon Treatment	Behavioral Parameter										
			Total		Bursting (>20 mm/s)			Cruising (5–20 mm/s)			Freezing (<5 mm/s)		
			Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
Zebrafish	Dark	TxHV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	↓*	–	–	–	–	–	–	–	↓*	–
		5% LC50	–	↓*	↑*	–	–	–	↓*	–	↓**	↓**	–
		10% LC50	–	–	↑**	–	–	–		–	↓*	–	–
		40% LC50	↓**	↓***	–	–	–	–	↓***	↓**	↓***	↓***	↓*
Zebrafish	Light	TxHV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	–	–	–	–	–	–	–	–	–	–	–
Fathead minnow	Dark	TxHV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	–	–	–	–	–	–	–	–	↓**	–	–
Fathead minnow	Light	TxHV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	↓**	↓**	–	–	–	↓**	↓**	↓**	–	↓**	–
		10% LC50	↓**	↓**	–	–	–	–	↓**	↓**	–	↓**	–
		40% LC50	↓***	↓**	–	↓***	–	↓*	↓***	↓**	↓**	↓**	–

Table 9. Mean zebrafish dark (A) and light (B) swimming activities compared to mean fathead minnow dark (C) and light (D) activities after 96 h exposure to xylazine. Plotted data represents activity over two 10 min dark photoperiods and two 10 min light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/s), cruising (5–20 mm/s), and freezing (<5 mm/s). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity and ↓ indicates a significant decrease in activity in comparison to control, respectively. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p < 0.10;**p < 0.05; ***p < 0.01.

Species	Photoperiod	Xylazine Treatment	Behavioral Parameter										
			Total		Bursting (>20 mm/s)			Cruising (5–20 mm/s)			Freezing (<5 mm/s)		
			Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
Zebrafish	Dark	0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	–	–	–	–	–
		10% LC50	↓***	↓***	–	–	–	↓***	↓***	↓***	↓**	↓**	–
		40% LC50	↓***	↓***	↓***	↓***	↓**	↓***	↓***	↓***	↓***	↓***	–
Zebrafish	Light	0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	↑*	–	–	–	–	–	↑*	–	↑***
		5% LC50	–	–	↑*	–	–	–	–	–	–	–	–
		10% LC50	–	–	–	–	–	–	–	–	–	–	–
		40% LC50	↓***	↓***	–	–	–	↓***	↓***	↓***	↓***	↓***	↓***
Fathead minnow	Dark	THV	–	–	–	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	–	–	–	–	–	–	–	–	–	–
		5% LC50	–	–	–	–	–	–	↓*	–	–	–	–
		10% LC50	↓***	↓***	–	–	–	↓**	↓***	↓***	↓***	↓***	–
		40% LC50	↓***	↓***	–	↓**	–	↓***	↓***	↓***	↓***	↓**	–
Fathead minnow	Light	THV	–	–	↑**	–	–	–	–	–	–	–	–
		0.1% LC50	–	–	–	–	–	–	–	–	–	–	–
		1% LC50	–	↓**	–	–	–	↓*	↓**	–	↓**	↓**	–
		5% LC50	–	↓***	–	↓*	–	↓**	↓***	–	↓***	↓**	–
		10% LC50	↓*	↓***	–	↓*	–	↓**	↓***	–	↓***	↓***	↓***
		40% LC50	↓**	↓***	↓*	↓***	↓**	↓***	↓***	↓**	↓***	↓***	↓***

4. Discussion

Though the present study represents an initial effort to understand PMRs in larval fathead minnows, previous research has revealed sudden increases in movement in larval fathead minnows and zebrafish after tactile stimulation (Russom et al., 1997; Weber, 2006; Painter et al., 2009). These coordinated bursts of swimming, also called fast-starts, are responses to predator stimulation that appear to be conserved across teleost lineages (Painter et al., 2009). A commonly measured tactile fast-start response, the c-start, has been demonstrated to be affected by a number of different compounds, including neurologically active chemicals (e.g., antidepressants) (McGee et al., 2009; Painter et al., 2009; Weber, 2006). Typical naïve PMR behaviors observed in zebrafish are likely evolutionary-linked adaptive responses to predator threats or prey capture (Burgess and Granato, 2007; Colwill and Creton, 2011). Such sudden increases in activity responses to changing light conditions exhibited in the present study may parallel tactile startle responses as a visually invoked predator avoidance behavior (Colwill and Creton, 2011) or a foraging strategy (Burgess and Granato, 2007), and thus, warrants future comparative investigation.

In pharmacological and toxicological studies, larval and embryonic zebrafish PMRs have been employed as a rapid and effective means to investigate bioactivity of a diverse range of chemicals (Copmans et al., 2016; Kokel et al., 2010; Noyes et al., 2015; Reif et al., 2015; Rihel et al., 2010; van Woudenberg et al., 2014; van Woudenberg et al., 2013). Embryonic zebrafish exhibit increased motion in response to sudden pulses of light (Kokel et al., 2010; Noyes et al., 2015). Similar to zebrafish embryos, hatched zebrafish larvae demonstrated consistent responses to light/dark stimulus. Previous

research demonstrated that larval zebrafish remain fairly inactive during light photoperiods, but once lights are turned off, larvae activity dramatically increases (de Esch et al., 2012; Irons et al., 2010; Kristofco et al., 2016; Zhao et al., 2014). Several studies have used PMRs in larval zebrafish to screen compounds for bioactivity. Noyes et al. (2015), for example, observed PMR profiles in 120 hpf larval zebrafish by recording activity during an initial 10 min period (light 1), followed by two 5 min dark phases in which zebrafish activity dramatically increased (dark stimulation), and then gradually declined (dark acclimation). These dark phases were then followed by observation of a final 5 min light period (light 2). Kokel et al. (2010) observed PMRs in 30 to 42 hpf embryos whereby frequency of body flexions and tail oscillations were measured in responses to a sudden pulses of light.

Other researchers have quantified activity of 96 hpf zebrafish across alternating 15 min light/dark periods (van Woudenberg et al., 2014) Specifically, larval fish habituation responses to dark were measured in addition to a photoperiod "startle response," which identifies differences in activity between the end of initial photoperiod and the beginning of the following photoperiod (van Woudenberg et al., 2014). In the present study, we similarly examined such PMRs of both larval fish models during interchanging light/dark periods. We also quantified each PMR across three speed thresholds. Consistent with the previous literature (Kristofco et al., 2016; Zhao et al., 2014), we observed control 96 hpf zebrafish to consistently display increased activity in response to dark periods and diminished activity during light periods (Fig. 4 A and B). However, control fathead minnows revealed a dramatically different PMR than larval zebrafish. Activity of fathead minnows only changed slightly in response to dark periods,

but demonstrated a large increase in activity in response to light conditions (Fig. 4 C and D). As mentioned previously, PMRs exhibited by larval zebrafish could be a evolutionarily adapted predator avoidance behavior (Colwill and Creton, 2011). Research by Burgess and Granato (2007) conversely indicate that these larval zebrafish behaviors are more closely associated with a food foraging strategy. Whether such PMR responses in zebrafish larvae are associated with predation, foraging, or both predation and foraging, requires further study.

There has apparently been no research on the evolutionary and ecological origins of PMRs in fathead minnow larvae. Therefore, the underpinnings behind the behavioral differences between each fish model are not currently understood, but could be associated with predatory avoidance or foraging. However, photomotor behaviors could be age dependent in these and other fish models. In studies examining light/dark preference of zebrafish, it has been demonstrated that adults prefer dark areas whereas larvae preferred light areas (Maximino et al., 2014). This differential behavior is likely associated with the development of melanophores. Larval zebrafish lack any pigmentation, and thus prefer lighter areas (Fuiman and Magurran, 1994). A recent study by Vignet and Parrott (2017) with fathead minnow larvae demonstrated similar results to zebrafish, where adult and juvenile fathead minnows demonstrated a preference for dark areas, but larval fathead minnows spent more time in light areas.

In addition to examining PMR thresholds of two common fish models for representative chemicals from the traditional MOA categories (Verhaar et al., 1992), we further examined total distance traveled, refractory (freezing), cruising, and stimulatory (bursting) behaviors under light and dark conditions. Because neuroactive compounds

can have differential effects on movement patterns in light and dark conditions, locomotor studies have quantified zebrafish activity during altering photoperiods (Irons et al., 2010). Distances traveled or mean speed of movements are among the most commonly measured locomotor endpoints (Spulber et al., 2014; Steenbergen et al., 2011; Wang et al., 2011; Zhang et al., 2012; Zhao et al., 2014). Other previously measured endpoints include duration of movements, number of movements, and turning activity (number of turns and turn angles) (Baraban et al., 2005; Budick and O'Malley, 2000; Chen et al., 2011; de Esch et al., 2012; Spulber et al., 2014). Some studies have used speed thresholds in conjunction with distances to more effectively distinguish between refractory and stimulatory responses (Steele et al., 2018). For example, Winter et al. (2008) observed larval zebrafish distances traveled across three speed thresholds (bursting: >20 5 mm/s, cruising: 5–20 mm/s, and freezing: <5 mm/s) to examine effects of PTZ on locomotor behaviors. These speed thresholds were also employed in recent studies that identified developmental age specific effects of DZN and diphenhydramine on locomotor behaviors of 96 hpf, 168 hpf, and 240 hpf zebrafish (Kristofco et al., 2016). The present study observed distance, number (counts), and duration of stimulatory (bursting), cruising, and refractory (freezing) behaviors in larval zebrafish and fathead minnows.

To compare PMRs and other locomotor behavioral profiles among compounds with unique bioactivities, we selected seven compounds from four MOA categories traditionally employed in aquatic toxicology (Verhaar et al., 1992). These representative compounds were selected because their MoAs have previously been established. 1-heptanol, a class I compound, is an industrial chemical known to elicit toxicity through

non-polar narcosis, a non-specific MOA whereby toxicity is reversible and occurs through arrested activity of protoplasmic structures (Veith et al., 1983). Class I chemicals have been referred to as “inert” compounds with minimum or “baseline” toxicity because they are believed to lack specific biological activity and toxicity is related to lipophilicity (e.g., increasing acute toxicity with increasing log Kow). In the current study, 1-heptanol reduced activity at each significantly affected endpoints for both fathead minnows and zebrafish. Russom et al. (1997) reported similar behavioral responses in juvenile fathead minnows exposed to 1-heptanol in which fish exhibited suppressed locomotion and reduced reaction to outside stimuli (e.g., prodding).

Class II compounds, such as phenol, elicit toxicity through polar narcosis (Bradbury et al., 1989), a process related to the polar attribute of a molecule and is thought to be mediated through hydrogen bonding. These compounds are more toxic than would be predicted by their log Kow alone and often have lower lethal body burdens in fish than class I compounds (Vaes et al., 1998; Verhaar et al., 1992). Larval fish exposed to phenol demonstrated a clearly different response from those exposed to 1-heptanol. Phenol exposed fish exhibited a large increase in activity for several endpoints, but most notably for the bursting speed threshold. Similar to the observations from the present study, Drummond and Russom (1990) previously observed significant bouts of hyperactivity in juvenile fathead minnows exposed for 96 h to polar narcosis type chemicals (e.g., phenol).

Conversely, R-carvone effected fathead minnow light PMRs but did not affect locomotor activity in either light or dark conditions. However, zebrafish swimming activity was effected by R(-)-carvone during light and dark photoperiods across all

behavioral parameters. R-(-)-carvone is categorized in class III as having electophile/proelctrophile activity because its toxicity profile is expected to be associated with its reactivity. In particular, R-(-)-carvone is understood to be a Michael acceptor (Corrales et al., 2017), so it exerts toxicity following reaction with nucleophilic sites in biological macromolecules, such as proteins and nucleic acids (Hermens, 1990). To our knowledge, this is currently the only study to have observed behavioral endpoints of fish exposed to R-(-)-carvone, and highlights the importance of further understanding fish behavioral responses to class III chemicals

Class IV compounds illicit toxicity through specific actions on subcellular targets. Each of the class IV compounds (citalopram (selective serotonin re-uptake inhibitor, SSRI), diazinon (acetylcholinesterase inhibitor), PTZ (GABAA receptor antagonist), and xylazine (α2-adrenergic receptor agonist)) examined in this study were chosen because their MOAs are well studied and these compounds are expected to have fairly high specificity for their targets based on evolutionary conservation across vertebrates (Gunnarsson et al., 2008). Diazinon toxicity is primarily mediated by its active metabolite, diazoxon, and has previous been examined in behavioral studies with fish (Scholz et al., 2000). Following inhibition of acetylcholinesterase (AChE) by diazoxon, acetylcholine (ACh) accumulates in the synaptic cleft causing persistent stimulation of cholinergic receptors on the postsynaptic cells of the nervous system, and changes in postsynaptic cell function resulting in disrupted loss of nervous system function (Pope et al., 2005). Bursting swimming patterns and light/dark response profiles similar to the results in this study were also consistent with previous research that observed behaviors of 96 hpf larval zebrafish exposed to diazinon (Kristofco et al., 2016). Previous studies

with larval rainbow trout (Oncorhynchus mykiss) exposed to diazinon showed correlations with impaired swimming velocity and brain AChE levels (Beauvais et al., 2000). Similar correlations have been demonstrated with swimming velocity and brain AChE levels in European seabass (Dicentrarchus labrax) exposed to the organophosphate pesticide fenitrothion (Almeida et al., 2010). Others have reported reduced movement durations and distances in larval zebrafish exposed to diazinon (Scheil et al., 2009; Yen et al., 2011).

Both zebrafish and fathead minnow exposed to PTZ demonstrated erratic, hyperactive behaviors and abnormal PMR response patterns. Such erratic behavior observed in fish exposed to PTZ results from binding to and blocking the GABAA receptor, a ligand gated ion channel that mediates inhibitory functions of gamma-aminobutyric acid (GABA) (Farahmandfar et al., 2017). Binding of GABAA targets results in anxiogenic behavioral responses and uncontrolled convulsions in fish and mammalian models. PTZ chemically induces seizures, which is why it is often used in epilepsy research studies with vertebrate models (Baraban et al., 2005; Giorgi et al., 1996). Consistent with results from the present study, previous research with larval zebrafish have also observed PTZ to cause hyperactivity and to alter responses following changes in lighting conditions (Baraban et al., 2005; Berghmans et al., 2007; Peng et al., 2016; Torres-Hernández et al., 2016; Winter et al., 2008).

Xylazine is a sedative, anesthetic, and muscle relaxant commonly used in veterinary medicine (England et al., 1992; Hsu, 1981). As an α2-adrenergic agonist, xylazine exerts its effects through stimulation of α2-receptors in the central nervous system (Ruiz-Colón et al., 2014). Several classes of α2 adrenergic receptor agonists have

been shown to suppress locomotor activity in larval and adult zebrafish (Renier et al., 2007; Rihel et al., 2010; Ruuskanen et al., 2005). Similar to these previous findings, higher treatments of xylazine in the present study decreased activity in each of the larval fish models. Though the mechanism resulting in increased activity following exposure to lower treatments levels of xylazine is not understood, ethanol, another central nervous system depressant, has been demonstrated to elicit hyperactivity at low doses and hypoactivity at high doses in larval zebrafish (Irons et al., 2010).

Citalopram, a SSRI that antagonizes serotonin reuptake transporters (SERT) in fish and mammals, is a common pharmaceutical used to treat anxiety and depression in humans (Gould et al., 2007). This pharmaceutical primarily had inhibitory effects on each of the larval zebrafish and fathead minnow behavioral endpoints. Several studies with SSRIs, including efforts with zebrafish and fathead minnow, have demonstrated behavioral responses (Stanley et al., 2007; Chiffre et al., 2016; Kellner et al., 2016; Kokel et al., 2010; Sackerman et al., 2010; Valenti et al., 2009, Valenti et al., 2012; Connors et al., 2014; Weinberger and Klaper, 2014) and SERT binding in fish brain tissues (Gould et al., 2007; Sackerman et al., 2010; Valenti et al., 2012). Furthermore, many of these effects may be associated with reduced anxiety, resulting in unique behavioral response profiles in larval fish models.

Behavioral response thresholds and acute toxicity varied between organisms for the study chemicals. Because we followed standardized EPA and OECD guidelines used by regulatory and academic institutions around the world, the results from this study were intended to be more broadly comparable, and more directly expanded by future work. Following these guidelines, fathead minnows were exposed within 24 h post hatch and

zebrafish were exposed 4 to 6 h post fertilization, respectively. It is thus important to note, as recently reported by our research team (Corrales et al., 2017; Kristofco et al., 2018), that differences in metabolic activation, bioaccumulation and toxicodynamics can occur with fish age, resulting in differential sensitivity to contaminants. It has been previously demonstrated that age can specifically influence behavioral responses in larval fish models (Kristofco et al., 2016), and therefore, this study was not designed to directly examine such age and species specific differences between the fish models. Whether behavioral responses to contaminants and differences among common fish models are age and/or species related warrants future study.

Regardless of differences in species and age, each of the pharmaceutical compounds affected both fish models at or below the THV, a treatment level predicted to result in an internal plasma dose equaling a human therapeutic plasma concentration. Previous studies have examined fish plasma modeling and the THV concept through predictions and observations of fish plasma levels of pharmaceuticals associated with therapeutic internal doses in mammals. For example, Valenti et al. (2012) observed significant changes in serotonin receptor binding and antianxiety behaviors of adult fathead minnows when internal fish plasma levels exceeded human therapeutic concentrations of sertraline. Margiotta-Casaluci et al. (2014) made similar behavioral observations with the SSRI fluoxetine. In the present study, xylazine, an α2 adrenergic receptor agonist, significantly altered fish behavior at a treatment level corresponding to the THV (0.04 mg/L) in both fathead minnow and zebrafish models. Citalopram altered zebrafish behavior at the THV (0.02 mg/L) and fathead minnow behaviors at the lowest treatment level (0.01 mg/L), which is lower than the THV for this antidepressant.

Interestingly, fathead minnow larvae demonstrated a more sensitive PMR to citalopram than any of the other compounds. These observations suggest that there may be an association among inhibition of the SERT transporter, anxiety levels, and larval fish responses to sudden changes in light condition. Here again, whether such responses translate to ecologically important behavioral modifications was outside the scope of the present study but deserve further attention.

5. Conclusions

Understanding chemical MOAs is a critical step in pharmacology, toxicology, and hazard and risk assessment. It is also important during the design of inherently less hazardous and more sustainable chemicals, an important goal of green chemistry (Anastas and Warner, 1998). To evaluate the use of larval fish behavioral endpoints as sensitive, high throughput means of gaining insight to chemical MOAs at the whole organism level, we specifically expanded on previous research with zebrafish by examining diverse endpoints to determine locomotor profiles during light and dark periods and also quantified startle responses to changing light conditions. We compared such response profiles and PMRs to larval fathead minnow, another common fish model. Through this approach, we observed divergent behavioral effects and identified specific differences in response profiles between fish models and among study chemicals. Our results suggest that larval fish behaviors may be useful diagnostics to identify mechanisms and pathways associated with biological activities, and potentially adverse outcomes for chemicals lacking mechanistic data. Such efforts are particularly needed for class III and class IV compounds. Similar to class IV compounds, electrophiles (class III) can interact with biological targets through various mechanisms, including Michael

addition, nucleophilic substitution and Schiff's base formation, each of which may influence electrophile interactions with susceptible biological nucleophiles (Harder et al., 2003). Clearly, future efforts are needed to examine other chemicals with diverse MOAs. Exploring use of behavioral pattern analysis to discriminate unique MOAs across stages of organismal development will further an understanding of age and species specific behaviors in fish models. Lastly, the current research utilized larval fish behaviors to evaluate toxicity and bioactivity of commercial chemicals on an individual basis. This approach, however, may also provide diagnostic utility in monitoring ambient surface waters, effluent discharge and chemical mixtures. Fish behavioral research with mixtures of compounds is needed because surface waters routinely contain multiple chemical contaminants.

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References

Ali, S., Champagne, D.L., Richardson, M.K., 2012. Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. Behavioural Brain Research 228, 272-283.

Almeida, J.R., Oliveira, C., Gravato, C., Guilhermino, L., 2010. Linking behavioural alterations with biomarkers responses in the European seabass Dicentrarchus labrax L. exposed to the organophosphate pesticide fenitrothion. Ecotoxicology 19, 1369-1381.

Andersen, M.E., Krewski, D., 2009. Toxicity testing in the 21st century: bringing the vision to life. Toxicological Sciences 107, 324-330.

Ankley, G.T., Villeneuve, D.L., 2006. The fathead minnow in aquatic toxicology: past, present and future. Aquatic Toxicology 78, 91-102.

Baraban, S., Taylor, M., Castro, P., Baier, H., 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 131, 759-768.

Beauvais, S.L., Jones, S.B., Brewer, S.K., Little, E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (Oncorhynchus mykiss) and their correlation with behavioral measures. Environmental Toxicology and Chemistry 19, 1875-1880.

Becker, R.A., Ankley, G.T., Edwards, S.W., Kennedy, S.W., Linkov, I., Meek, B., Sachana, M., Segner, H., Van Der Burg, B., Villeneuve, D.L., Watanabe, H., Barton-Maclaren, T.S., 2015. Increasing Scientific Confidence in Adverse Outcome Pathways: Application of Tailored Bradford-Hill Considerations for Evaluating Weight of Evidence. Regulatory Toxicology and Pharmacology 72, 514-537.

Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacology Biochemistry and Behavior 94, 75-80.

Berghmans, S., Hunt, J., Roach, A., Goldsmith, P., 2007. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy research 75, 18-28.

Berninger, J.P., Du, B., Connors, K.A., Eytcheson, S.A., Kolkmeier, M.A., Prosser, K.N., Valenti, T.W., Chambliss, C.K., Brooks, B.W., 2011. Effects of the antihistamine diphenhydramine on selected aquatic organisms. Environmental Toxicology and Chemistry 30, 2065-2072.

Bradbury, S.P., Carlson, R.W., Henry, T.R., 1989. Polar narcosis in aquatic organisms. Aquatic Toxicology and Hazard Assessment: 12th Volume. ASTM International.

Brooks, B.W., 2014. Fish on Prozac (and Zoloft): ten years later. Aquatic Toxicology 151, 61-67.

Brooks, B.W., 2018. Urbanization, environment and pharmaceuticals: Advancing comparative physiology,pharmacology and toxicology. Conservation Physiology 6(1): cox079 10.1093/conphys/cox079

Brooks, B.W., Steele, W.B., 2018. Ecotoxicological Perspectives on Healthcare and the Environment. Chapter 4 in: Boxall, A.B.A., Kookana, R. (Eds). Healthcare and Environmental Contaminants, Elsevier, London, UK. pp. 41-66.

Budick, S.A., O'Malley, D.M., 2000. Locomotor repertoire of the larval zebrafish: swimming, turning and prey capture. Journal of Experimental Biology 203, 2565-2579.

Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., Kyzar, E., Wu, N., Kalueff, A.V., 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. PloS One 6, e17597.

Chen, T.-H., Wang, Y.-H., Wu, Y.-H., 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquatic Toxicology 102, 162-166.

Chiffre, A., Clérandeau, C., Dwoinikoff, C., Le Bihanic, F., Budzinski, H., Geret, F., Cachot, J., 2016. Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (Oryzias latipes) larvae above environmental concentrations. Environmental Science and Pollution Research 23, 4964-4977.

Coish, P., Brooks, B.W., Gallagher, E.P, Kavanagh, T.J., Voutchkova-Kostal, A., Zimmerman, J.B., Anastas, P.T., 2016. Current status and future challenges in the molecular design for reduced hazard. ACS Sustainable Chemistry and Engineering 4, 5900−5906.

Colón-Cruz, L., Kristofco, L., Crooke-Rosado, J., Acevedo, A., Torrado, A., Brooks, B.W., Sosa, M.A., Behra, M., 2018. Alterations of larval photo-dependent swimming responses (PDR): New endpoints for rapid and diagnostic screening of aquatic contamination. Ecotoxicology and Environmental Safety 147, 670-680.

Colwill, R.M., Creton, R., 2011. Imaging escape and avoidance behavior in zebrafish larvae. Reviews in the Neurosciences 22, 63-73.

Connors, K.A., Valenti, T.W., Lawless, K., Sackerman, J., Onaivi, E.S., Brooks, B.W., Gould, G.G., 2014. Similar anxiolytic effects of agonists targeting serotonin 5-HT 1A or cannabinoid CB receptors on zebrafish behavior in novel environments. Aquatic Toxicology 151, 105-113.

Copmans, D., Meinl, T., Dietz, C., van Leeuwen, M., Ortmann, J., Berthold, M.R., de Witte, P.A., 2016. A KNIME-Based Analysis of the Zebrafish Photomotor Response Clusters the Phenotypes of 14 Classes of Neuroactive Molecules. Journal of Biomolecular Screening 21, 427-436.

Corrales, J., Kristofco, L.A., Steele, W.B., Saari, G.N., Kostal, J., Williams, E.S., Mills, M., Gallagher, E.P., Kavanagh, T.J., Simcox, N., 2017. Toward the design of less hazardous chemicals: exploring comparative oxidative stress in two common animal models. Chemical Research in Toxicology 30, 893-904.

de Esch, C., van der Linde, H., Slieker, R., Willemsen, R., Wolterbeek, A., Woutersen, R., De Groot, D., 2012. Locomotor activity assay in zebrafish larvae: Influence of age, strain and ethanol. Neurotoxicology and Teratology 34, 425-433.

Drummond, R.A., Russom, C.L., 1990. Behavioral toxicity syndromes: A promising tool for assessing toxicity mechanisms in juvenile fathead minnows. Environmental Toxicology and Chemistry 9, 37-46.

Emmert, M., Witzel, P., Heinrich, D., 2016. Challenges in tissue engineering–towards cell control inside artificial scaffolds. Soft Matter 12, 4287-4294.

England, G., Clarke, K., Goossens, L., 1992. A comparison of the sedative effects of three α2-adrenoceptor agonists (romifidine, detomidine and xylazine) in the horse. Journal of Veterinary Pharmacology and Therapeutics 15, 194-201.

Farahmandfar, M., Akbarabadi, A., Bakhtazad, A., Zarrindast, M.-R., 2017. Recovery from ketamine-induced amnesia by blockade of GABA-A receptor in the medial prefrontal cortex of mice. Neuroscience 344, 48-55.

Fitzsimmons, P.N., Fernandez, J.D., Hoffman, A.D., Butterworth, B.C., Nichols, J.W., 2001. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology 55, 23-34.

Giorgi, O., Carboni, G., Frau, V., Orlandi, M., Valentini, V., Feldman, A., Corda, M., 1996. Anticonvulsant effect of felbamate in the pentylenetetrazole kindling model of epilepsy in the rat. Naunyn-Schmiedeberg's Archives of Pharmacology 354, 173-178.

Gould, G.G., Brooks, B.W., Frazer, A., 2007. [3H] citalopram binding to serotonin transporter sites in minnow brains. Basic & Clinical Pharmacology & Toxicology 101, 203-210.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessment. Environmental Science and Technology 42, 5807–5813.

Harder, A., Escher, B.I., Landini, P., Tobler, N.B., Schwarzenbach, R.P., 2003. Evaluation of bioanalytical assays for toxicity assessment and mode of toxic action classification of reactive chemicals. Environmental Science & Technology 37, 4962-4970.

Hermens, J., 1990. Electrophiles and acute toxicity to fish. Environmental Health Perspectives 87, 219.

Hsu, W.H., 1981. Xylazine-induced depression and its antagonism by alpha adrenergic blocking agents. Journal of Pharmacology and Experimental Therapeutics 218, 188-192.

Huang, R.-Q., Bell-Horner, C.L., Dibas, M.I., Covey, D.F., Drewe, J.A., Dillon, G.H., 2001. Pentylenetetrazole-induced inhibition of recombinant γ-aminobutyric acid type A (GABAA) receptors: mechanism and site of action. Journal of Pharmacology and Experimental Therapeutics 298, 986-995.

Huang, S.S.Y., Benskin, J.P., Chandramouli, B., Butler, H., Helbing, C.C., Cosgrove, J.R., 2016. Xenobiotics Produce Distinct Metabolomic Responses in Zebrafish Larvae (Danio rerio). Environmental Science & Technology.

Huggett, D., Cook, J., Ericson, J., Williams, R., 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Human and Ecological Risk Assessment 9, 1789-1799.

Hyttel, J., 1982. Citalopram—pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. Progress in Neuro-Psychopharmacology and Biological Psychiatry 6, 277-295.

Irons, T., MacPhail, R., Hunter, D., Padilla, S., 2010. Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. Neurotoxicology and Teratology 32, 84-90.

Irons, T.D., Kelly, P.E., Hunter, D.L., MacPhail, R.C., Padilla, S., 2013. Acute administration of dopaminergic drugs has differential effects on locomotion in larval zebrafish. Pharmacology Biochemistry and Behavior 103, 792-813.

Kalueff, A.V., Echevarria, D.J., Homechaudhuri, S., Stewart, A.M., Collier, A.D., Kaluyeva, A.A., Li, S., Liu, Y., Chen, P., Wang, J., 2016. Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. Aquatic Toxicology 170, 297-309.

Kellner, M., Porseryd, T., Hallgren, S., Porsch-Hällström, I., Hansen, S.H., Olsén, K.H., 2016. Waterborne citalopram has anxiolytic effects and increases locomotor activity in the three-spine stickleback (Gasterosteus aculeatus). Aquatic Toxicology 173, 19-28.

Klüver, N., König, M., Ortmann, J., Massei, R., Paschke, A., Kühne, R., Scholz, S., 2015. Fish embryo toxicity test: Identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. Environmental Science & Technology 49, 7002-7011.

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C.Y.J., Mateus, R., Healey, D., Kim, S., Werdich, A.A., Haggarty, S.J., MacRae, C.A., Shoichet, B., Peterson, R.T., 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat Chem Biol 6, 231-237.

Krewski, D., Acosta Jr, D., Andersen, M., Anderson, H., Bailar III, J.C., Boekelheide, K., Brent, R., Charnley, G., Cheung, V.G., Green Jr, S., 2010. Toxicity testing in the 21st century: a vision and a strategy. Journal of Toxicology and Environmental Health, Part B 13, 51-138.

Kristofco, L.A., Cruz, L.C., Haddad, S.P., Behra, M.L., Chambliss, C.K., Brooks, B.W., 2016. Age matters: Developmental stage of Danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic Toxicology 170, 344-354.

Kristofco, L.A., Haddad, S.P., Chambliss, C.K., Brooks, B.W., 2018. Differential uptake of and sensitivity to diphenhydramine in embryonic and larval zebrafish. Environmental Toxicology and Chemistry 37, 1175-1181.

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiology & Behavior 90, 54-58.

Little, E.E., Fairchild, J.F., DeLonay, A.J., 1993. Behavioral methods for assessing impacts of contaminants on early life stage fishes. American Fisheries Society Symposium[AM FISH SOC SYMP] 1993.

Little, E.E., Finger, S.E., 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology and Chemistry 9, 13-19.

MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nature Reviews Drug Discovery 14, 721-731.

Maximino, C., da Silva, A.W.B., Araújo, J., Lima, M.G., Miranda, V., Puty, B., Benzecry, R., Picanço-Diniz, D.L.W., Gouveia Jr, A., Oliveira, K.R.M., 2014. Fingerprinting of psychoactive drugs in zebrafish anxiety-like behaviors. PloS One 9, e103943.

McGee, M.R., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Schoenfuss, H.L., 2009. Predator avoidance performance of larval fathead minnows (Pimephales promelas) following short-term exposure to estrogen mixtures. Aquatic Toxicology 91, 355-361.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. Chemosphere 93, 2217-2223.

Noyes, P.D., Haggard, D.E., Gonnerman, G.D., Tanguay, R.L., 2015. Advanced morphological—behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicological Sciences 145, 177-195.

OECD. 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Guidelines for Testing of Chemicals Section 2: Effects on Biotic Systems, OECD Publishing, Paris, France.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (Pimephales promelas). Environmental Toxicology and Chemistry 28, 2677-2684.

Peng, X., Lin, J., Zhu, Y., Liu, X., Zhang, Y., Ji, Y., Yang, X., Zhang, Y., Guo, N., Li, Q., 2016. Anxiety-related behavioral responses of pentylenetetrazole-treated zebrafish larvae to light-dark transitions. Pharmacology Biochemistry and Behavior 145, 55-65.

Perkins, E.J., Ankley, G.T., Crofton, K.M., Garcia-Reyero, N., LaLone, C.A., Johnson, M.S.,

Tietge, J.E., Villeneuve, D.L., 2013. Current perspectives on the use of alternative species in human health and ecological hazard assessments. Environmental Health Perspectives 121, 1002–1010.

Perkins, E., Garcia-Reyero, N., Edwards, S., Wittwehr, C., Villeneuve, D., Lyons, D., Ankley, G., 2015. The Adverse Outcome Pathway: A Conceptual Framework to Support Toxicity Testing in the Twenty-First Century. Computational Systems Toxicology, 1-26.

Pope, C., Karanth, S., Liu, J., 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environmental Toxicology and Pharmacology 19, 433-446.

Reif, D.M., Truong, L., Mandrell, D., Marvel, S., Zhang, G., Tanguay, R.L., 2015. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Archives of Toxicology, 1-12.

Renier, C., Faraco, J.H., Bourgin, P., Motley, T., Bonaventure, P., Rosa, F., Mignot, E., 2007. Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmacogenetics and Genomics 17, 237-253.

Rihel, J., Prober, D.A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S.J., Kokel, D., Rubin, L.L., Peterson, R.T., 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327, 348-351.

Rihel, J., Schier, A.F., 2012. Behavioral screening for neuroactive drugs in zebrafish. Developmental Neurobiology 72, 373-385.

Rovida, C., Hartung, T., 2009. Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals—a report by the transatlantic think tank for toxicology (t (4)). ALTEX 26, 187-208.

Ruiz-Colón, K., Chavez-Arias, C., Díaz-Alcalá, J.E., Martínez, M.A., 2014. Xylazine intoxication in humans and its importance as an emerging adulterant in abused drugs: a comprehensive review of the literature. Forensic Science International 240, 1-8.

Russom, C.L., Bradbury, S.P., Broderius, S.J., Hammermeister, D.E., Drummond, R.A., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 16, 948-967.

Ruuskanen, J.O., Peitsaro, N., Kaslin, J.V., Panula, P., Scheinin, M., 2005. Expression and function of α2-adrenoceptors in zebrafish: drug effects, mRNA and receptor distributions. Journal of Neurochemistry 94, 1559-1569.

Sackerman, J., Donegan, J.J., Cunningham, C.S., Nguyen, N.N., Lawless, K., Long, A., Benno, R.H., Gould, G.G., 2010. Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of Danio rerio line. International Journal of Comparative Psychology/ISCP; sponsored by the International Society for Comparative Psychology and the University of Calabria 23, 43.

Santonastaso, A., Hardy, J., Cohen, N., Fajt, V., 2014. Pharmacokinetics and pharmacodynamics of xylazine administered by the intravenous or intra-osseous route in adult horses. Journal of Veterinary Pharmacology and Therapeutics 37, 565-570.

Scheil, V., Kienle, C., Osterauer, R., Gerhardt, A., Köhler, H.-R., 2009. Effects of 3, 4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (Danio rerio) embryos and larvae. Ecotoxicology 18, 355-363.

Scholz, N.L., Truelove, N.K., French, B.L., Berejikian, B.A., Quinn, T.P., Casillas, E., Collier, T.K., 2000. Diazinon disrupts antipredator and homing behaviors in chinook salmon (Oncorhynchus tshawytscha). Canadian Journal of Fisheries and Aquatic Sciences 57, 1911-1918.

Schulz, M., Iwersen-Bergmann, S., Andresen, H., Schmoldt, A., 2012. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Critical Care 16, 1-4.

Sipes, N.S., Martin, M.T., Kothiya, P., Reif, D.M., Judson, R.S., Richard, A.M., Houck, K.A., Dix, D.J., Kavlock, R.J., Knudsen, T.B., 2013. Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays. Chemical Research in Toxicology 26, 878-895.

Sloman, K., McNeil, P., 2012. Using physiology and behaviour to understand the responses of fish early life stages to toxicants. Journal of Fish Biology 81, 2175-2198.

Spulber, S., Kilian, P., Ibrahim, W.N.W., Onishchenko, N., Ulhaq, M., Norrgren, L., Negri, S., Di Tuccio, M., Ceccatelli, S., 2014. PFOS Induces Behavioral Alterations, Including Spontaneous Hyperactivity That Is Corrected by Dexamfetamine in Zebrafish Larvae. PloS One 9, e94227.

Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 69, 9-16.

Steele, W.B., Mole, R., Brooks, B.W., 2018, In press. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neurostimulant caffeine. Journal of Visualized Experiments

Steenbergen, P.J., Richardson, M.K., Champagne, D.L., 2011. The use of the zebrafish model in stress research. Progress in Neuro-Psychopharmacology and Biological Psychiatry 35, 1432-1451.

Stewart, A.M., Ullmann, J.F., Norton, W.H., Parker, M., Brennan, C., Gerlai, R., Kalueff, A.V., 2015. Molecular psychiatry of zebrafish. Molecular Psychiatry 20, 2-17.

Torres-Hernández, B.A., Colón, L.R., Rosa-Falero, C., Torrado, A., Miscalichi, N., Ortíz, J.G., González-Sepúlveda, L., Pérez-Ríos, N., Suárez-Pérez, E., Bradsher, J.N., 2016. Reversal of pentylenetetrazole-altered swimming and neural activity-regulated gene expression in zebrafish larvae by valproic acid and valerian extract. Psychopharmacology, 1-15.

Truong, L., Reif, D.M., St Mary, L., Geier, M.C., Truong, H.D., Tanguay, R.L., 2014. Multidimensional in vivo hazard assessment using zebrafish. Toxicological Sciences 137, 212-233.

US EPA. 1991. Methods for Aquatic Toxicity Identification Evaluation Phase I: Toxicity Confirmation Procedures for Samples Exhibiting Acute and Chronic Toxicity Office of Research and Development, Washington, DC.

US EPA. 2002. Methods for Measuring Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Office of Research and Development, Washington, DC.

Vaes, W.H., Ramos, E.U., Verhaar, H.J., Hermens, J.L., 1998. Acute toxicity of nonpolar versus polar narcosis: Is there a difference? Environmental Toxicology and Chemistry 17, 1380-1384.

Valenti, T.W., Gould, G.G., Berninger, J.P., Connors, K.A., Keele, N.B., Prosser, K.N., Brooks, B.W., 2012. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRI) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. Environmental Science & Technology 46, 2427-2435.

Valenti, T.W., Perez-Hurtado, P., Chambliss, C.K., Brooks, B.W., 2009. Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. Environmental Toxicology and Chemistry 28, 2685-2694.

van Woudenberg, A.B., Snel, C., Rijkmans, E., de Groot, D., Bouma, M., Hermsen, S., Piersma, A., Menke, A., Wolterbeek, A., 2014. Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. Reproductive Toxicology.

van Woudenberg, A.B., Wolterbeek, A., te Brake, L., Snel, C., Menke, A., Rubingh, C., de Groot, D., Kroese, D., 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reproductive Toxicology 41, 35-44.

Veith, G.D., Call, D.J., Brooke, L., 1983. Structure-toxicity relationships for the fathead minnow, Pimephales promelas: Narcotic industrial chemicals. Canadian Journal of Fisheries and Aquatic Sciences 40, 743-748.

Verbruggen, B., Gunnarsson, L., Kristiansson, E., Österlund, T., Owen, S.F., Snape, J.R., Tyler, C.R., 2017. Nucleic Acids Research gkx1024.

Verhaar, H.J., Van Leeuwen, C.J., Hermens, J.L., 1992a. Classifying environmental pollutants. Chemosphere 25, 471-491.

Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J.L.M., 1992b. Classifying environmental pollutants. Chemosphere 25, 471-491.

Viglino, L., Aboulfadl, K., Prévost, M., Sauvé, S., 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. Talanta 76, 1088-1096.

Wang, M., Chen, J., Lin, K., Chen, Y., Hu, W., Tanguay, R.L., Huang, C., Dong, Q., 2011. Chronic zebrafish PFOS exposure alters sex ratio and maternal related effects in F1 offspring. Environmental Toxicology and Chemistry 30, 2073-2080.

Weber, D., 2006. Dose-dependent effects of developmental mercury exposure on C-start escape responses of larval zebrafish Danio rerio. Journal of Fish Biology 69, 75-94.

CHAPTER FOUR

Towards Less Hazardous Industrial Compounds: Coupling Quantum Mechanical Computations and Behavioral Profiles Identify Chemical Bioactivity of S_N2 Electrophiles in Fathead Minnow and Zebrafish Models

This chapter will be submitted for publication in Environmental Health Perspectives

Abstract

Background: Sustainable molecular design of less hazardous chemicals promises to reduce risks to public health and the environment. Computational chemistry modeling, when coupled with alternative toxicology models (larval fish), present unique high-throughput opportunities to understand structural characteristics eliciting adverse outcomes.

Objectives: We examined a common chemical mechanism (electrophile bimolecular substitution (S_N2)) associated with oxidative stress using property based computational modeling coupled with acute (mortality) and sublethal (glutathione, photomotor behavior) responses in the zebrafish (Danio rerio) and the fathead minnow (Pimephales promelas) models.

Methods: Following standardized methods, embryonic zebrafish and larval fathead minnows were exposed separately to eight different S_N2 compounds for 96 hrs. Acute and sublethal responses were compared to computationally derived in silico chemical descriptors.

Results: Quantum chemical computations (ω) were significantly related to acute LC_{50} values and photomotor response (PMR) No Observed Effect Concentrations (NOECs) in both fathead minnow and zebrafish. Behavioral PMR profiles routinely

demonstrated refractory responses to these SN2 chemicals. This reactivity index, LC50 and PMR NOECs were also significantly related to whole body glutathione (GSH) levels, indicating that acute and chronic toxicity may be driven by protein adduct formation for SN2 electrophiles.

Conclusions: Our study demonstrates that in the two most common larval fish models, refractory PMR and locomotor patterns appear informative of electrophilic properties associated with oxidative stress for S_N2 chemicals. Property-based quantum mechanical modeling of electrophile reaction energies were predictive of experimental in vivo acute and sublethal toxicity. These observations provide important implications for identifying and designing less hazardous industrial chemicals.

Introduction

Humans and the environment are continuously exposed to diverse synthetic chemicals resulting in diverse adverse outcomes (Landrigan et al. 2017; Malaj et al. 2014; Schäfer et al. 2016). Unfortunately, toxicology information for many of these compounds is lacking. Whereas the fourth principle of Green Chemistry aims to select or design sustainable chemicals with lower hazards to public health and the environment, it remains to be the least developed discipline of the twelve principles that define the field (Erythropel et al. 2018), with a few proposed solutions to date (Clymer et al. 2019; Kostal et al. 2015). Thus, there is a growing impetus to identify safer chemical alternatives and develop guidelines for de novo synthesis of compounds with preserved efficacy but minimal toxicity (Coish et al. 2018).

Computational methods capable of predicting chemical toxicity and identifying attributes of less hazardous substances provide tremendous potential in chemical hazard

assessment and sustainable molecular design (Voutchkova et al. 2010). Developing reliable in silico models requires a mechanistic understanding of chemical-biological interactions at the subcellular level and associated adverse outcomes at higher levels of biological organization (Coish et al. 2018). Since mechanistic toxicity data only exist for a small percentage of the tens of thousands of chemicals in commerce (Perkins et al. 2015), rapid screening techniques with diagnostic capabilities to identify chemical hazards and gain insights to chemical bioactivity are needed (Andersen and Krewski 2008).

Unintended biological activity and toxicity of industrial compounds often results from covalent reactivity (Kostal et al. 2012). These reactions occur when electrophilic xenobiotics form irreversible covalent bonds with biological macromolecules such as proteins, nucleic acids, and lipids (Enoch and Cronin 2010; Enoch et al. 2011). Beyond eliciting acute toxicity, reactive molecules can cause oxidative stress, resulting in adverse outcomes associated with a number of chronic diseases including cancers, diabetes, atherosclerosis and neurodegenerative disorders (Aruoma 1998; Corrales et al. 2017). These reactive compounds form adducts with macromolecules through several types of chemistries, which are determined by molecular attributes of a chemical (Enoch et al. 2011). Chemical mechanisms of electrophile-biological nucleophile interaction, also known as reactive domains, include nucleophilic substitution, Schiff base formation, Michael addition, acylation, nucleophilic aromatic substitution, among others (Enoch et al. 2011). Recently, we have investigated molecular initiation events involving electrophiles and subcellular targets, such as protein thiols, by examining the physicochemical properties of these compounds, rather than through traditional structure-

based approaches (Kostal and Voutchkova-Kostal 2015; Corrales et al. 2017; Melnikov et al. 2019). However, whether fish behavioral responses can be correlated to chemical electrophilicity remains poorly studied and understood (Russom et al 1997; Steele et al. 2018a).

Larval and embryonic zebrafish are useful in high throughput screening designs with automated behavioral tracking systems to identify therapeutic targets for thousands of novel molecules (Kokel et al. 2010; Rihel et al. 2010). Potential targets identified using behavioral profiling or "fingerprinting", whereby locomotor and photomotor response (PMR) patterns of fish exposed to drugs with unknown bioactivities, are matched to profiles of those exposed to compounds with understood subcellular targets. We recently extended this research to two common larval fish models, the zebrafish and the fathead minnow model, to demonstrate that industrial and specialty chemicals with diverse underlying modes and mechanisms of action (MOAs) elicited unique behavioral response profiles in both fish models, indicating that behavioral responses may be informative of MOAs (Steele et al. 2018a; Steele et al. 2018b). Though this approach appears promising, research is necessary to identify relationships among biological responses at the organismal level and molecular attributes of environmental contaminants.

In the present study, we examined PMR patterns of larval zebrafish and fathead minnow following exposure to eight different electrophiles sharing S_N2 reactive chemistry. We determined whether electrophilic reactivity indices calculated for each study compound were related to a common biochemical biomarker of oxidative stress (GSH) in addition to acute (mortality) and sublethal (behavior) toxicity endpoints.

Because our previous research demonstrated that chemicals with different MOAs produced divergent fish behavioral response patterns, we tested whether these S_N2 compounds would produce similar response profiles. We also hypothesized that acute and sublethal toxicity endpoints would be related to estimates of chemical reactivity.

Methods

Study Chemicals and Chemical Classification

Study compounds were selected from class III compounds (electrophile/proelectrophiles) according to a traditional classification scheme (Verhaar et al. 1992). More specifically, we selected electrophiles with reactive chemistries through bimolecular nucleophilic substitution (S_N2). These compounds include 3-bromo-1-propanol, 3-chloro-1,2-propanediol, dibromoacetonitrile, glycidol, sodium decyl sulfate, styrene oxide, tris(2,3-dibromopropyl) phosphate (TBPP), and tris(1,3-dichloro-2-propyl) phosphate (TDCPP). Each chemical was obtained at the highest available purity (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions were prepared through serial dilution with reconstituted hard water (APHA, 1998) prior to initiating each study.

Fish Culture

A flow through system that introduced aged dechlorinated tap water to individual aquaria was used to maintain fathead minnow (P. promelas) cultures at Baylor University. Water temperature was kept at a constant temperature of 25±1° C and fish were cultured under a 16:8 hour light:dark photoperiod. Fathead minnows were fed artemia (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily. Embryos used for exposures were collected from

sexually mature adults aged to at least 120 days (Berninger et al. 2011; Corrales et al. 2017; Valenti et al. 2009) and exposed within 24 hours after hatching. Tropical 5D wild type zebrafish (D. rerio) were also cultured at Baylor University using a z-mod recirculating system (Marine Biotech Systems, Beverly, MA, USA). Fish were maintained at a density of <4 fish per liter in 260 ppm instant ocean with a pH of 7.0. Similar to fathead minnows, zebrafish were cultured under a 16:8 hour light:dark photoperiod and fed artemia (Artemia sp. nauplii; Pentair AES, Apopka, FL, USA) with flake food (Pentair AES, Apopka, FL, USA) twice daily (Corrales et al. 2017; Kristofco et al. 2016). All experimental procedures and fish culture protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

Experimental Design

Acute studies. We employed common standardized regulatory guidelines for toxicity experiments with zebrafish (OECD FET; OECD 2013) and fathead minnow (US EPA WET; US EPA 2002) models to maximize comparability of our work with other efforts. Prior to performing behavioral experiments, preliminary acute 96-hr toxicity studies were conducted to define mortality thresholds of both fish models. Briefly, zebrafish embryo studies were initiated with 4-6 hpf organisms, while experiments with fathead minnow larvae were initiated within 24 hours post hatch according to US EPA methods. Hatching for zebrafish occurs between 48 and 72 hpf. Fathead minnows hatching typically occurs at 96 hours post fertilization. Because water pH can effect chemical ionization state, bioavailability and toxicity (Valenti et al. 2009), experimental conditions were maintained at pH 7.5 for all toxicity experiments with both fish models.

Before initiating each experiment, solutions used for exposure were titrated to a pH of 7.5 following standard methods (US EPA 1991). Water quality parameters (dissolved oxygen, pH, hardness, alkalinity, temperature) of reconstituted hard water (APHA 1998) were routinely monitored following standard methods (APHA 1998). During experiments, temperature was maintained in climate controlled incubators at 25±1° C for fathead minnows and at 28±1° C for zebrafish. Following US EPA methods for toxicity identification evaluation (US EPA 1992), 2 replicates of each treatment level with 10 individuals in each experimental unit were used to identify acute zebrafish and fathead minnow thresholds. Throughout the duration of each 96-hr experiment, organisms were maintained in individual replicate beakers consisting of solution volumes of 200 mL and 20 mL for fathead minnows and zebrafish, respectively. These volumes were selected to ensure that fish density did not exceed acceptable levels for standardized EPA and OECD guidelines.

Sublethal studies. Consistent with the acute toxicity studies, sublethal behavioral experiments followed standardized toxicology experimental designs from the US EPA (US EPA 2002) for fathead minnows and the OECD (FET OECD no. 236; OECD 2013) for zebrafish but included behavioral responses (Steele et al. 2018a; Steele et al. 2018b). Each sublethal experiments with zebrafish employed 4 replicates of each treatment level with 15 individuals in each experimental unit, and fathead minnow experiments included 3 replicates of each treatment level with 10 individuals in each experimental unit. From each of the acute toxicity studies, nominal LC_{50} values were used as benchmarks to select treatment levels for sublethal experiments (Steele et al. 2018a; Steele et al. 2018b). Specifically, treatment levels were 40%, 20%, 10%, 5% and 1% of the LC_{50} value for 3-

chloro-1,2-propanediol, dibromoacetonitrile, glycidol, sodium decyl sulfate, styrene oxide, tris(2,3-dibromopropyl) phosphate (TBPP), and tris(1,3-dichloro-2-propyl) phosphate (TDCPP). For 3-bromo-1-propanol treatment levels were 40%, 10%, 1%, 0.1%, and 0.01% of the LC50 value. In addition to those used for behavioral measurements at 96 hr exposure, larvae were collected in cryoviles and frozen at -80 °C for subsequent glutathione tissue measurements. Larvae were collected in three replicates and each replicate included a pool of five 96-hr-posthatch (hph) fathead minnow or ten 96-hr-postfertilization (hpf) zebrafish.

Glutathione tissue measurements. GSH measurements in larval fish were carried out following previously described methods (Corrales et al. 2017). After fish were homogenized with a hand-held homogenizer, GSH levels were determined using a modified Tietze assay using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA). Samples were first deproteinated with 1.25 M metaphosphoric acid and 0.2 M triethanolamine. DTNB (5,5-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) was then added. The sulfhydryl group of GSH present in the fish homogenates reacted with DTNB to produce 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production was measured, which is directly proportional to the concentration of GSH in a sample.

Behavioral observations. At the end of 96 hours of exposure, larvae were placed in well plates for behavioral observation. Animals were maintained in solution at pH 7.5 from respective experimental units during behavioral observations. Each fathead minnow larvae were loaded in individual wells on 24-well plates, while zebrafish were loaded in

48-well plates due to species-specific size differences. Solution volumes in 24 and 48 well plates were 2 mL and 1 mL, respectively. For each treatment level in these sublethal experiments, a total of 24 (4 replicates of each treatment level with 6 individuals in each experimental unit) zebrafish larvae and 12 fathead minnow larvae (3 replicates of each treatment level with 4 individuals in each experimental unit) were used for behavioral observations. After organisms were loaded on a given plate in solution from their respective experimental unit, it was placed in an incubator until the previous plate was finished with behavioral testing. Plate loading took approximately 15-25 minutes, so wait time (~30 minute) was intentionally minimized (Steele et al. 2018a; Steele et al. 2018b).

Following methods previously described by our laboratory (Steele et al. 2018a; Steele et al. 2018b), larval swimming patterns were observed and recorded using automated tracking software (ViewPoint, Lyon, France) and associated platform (Zebrabox, ViewPoint, Lyon France). For each sublethal experiment, behavioral observations were initiated in the afternoon between 14:00 and 15:00 hrs because we recently observed significant control behavioral differences between morning hours and other times time of day (Kristofco et al. 2016). If a treatment level resulted in significant

mortality, then these treatment levels were not examined for behavioral perturbations. The ViewPoint system was set in tracking mode and behavioral recordings occurred over 50 minutes. This time period included a 10 minute dark acclimation period, followed by a 40 minute observation period consisting of two altering 10 minute light/dark cycles. Observations were recorded for total distance swam and total number of movements. Additionally, larval distance swam, number of movements, and duration of movements were recorded for activity across three different speed thresholds. These

speeds include bursting (>20 mm/s), cruising (5-20 mm/s), and freezing (<5 mm/s) to characterize stimulatory, more typical and refractory, respectively, behaviors. To measure larvae swimming responses to a sudden change in light condition, we also recorded PMR. PMR for each photoperiod transition (2 light and 2 dark responses) was calculated as the change in mean distance traveled (in mm) between the last minute of an initial photoperiod and the first minute of the following period. PMRs were observed across each speed threshold (bursting, cruising, and freezing) in addition to total distance.

Quantum mechanical reaction models with glutathione. The frontier molecular orbital (FMO) theory, pioneered by Kenichi Fukui, demonstrates the importance of frontier orbitals on chemical reactivity (Kato 2000). Quantum mechanical models can be used to calculate FMO energies for a wide variety of chemicals by applying suitable ab initio or density functional level of theory to accurately determine the electronic structure. Energy separation between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) has long served as a simple yet powerful measure of kinetic stability (Corrales et al. 2017). However, HOMO and LUMO energies can be used to determine a host of other chemical properties, such as chemical softness (S), which measures the ease of electron redistribution upon bonding, and the electrophilicity index (ω), which considers both chemical potential and softness. The latter is considered a more comprehensive measure of electrophilic reactivity (LoPachin et al. 2011).

$\omega = \mu^2 / 2\eta$ Eq. 1

where

$\mu = (ELUMO + EHOMO)/2$

η = ELUMO – EHOMO

GSH is a tripeptide (γ-glutamylcysteinylglycine) widely distributed in organisms; it serves as a co-substrate to glutathione transferases in the detoxification of xenobiotics and is often used as a model nucleophile when developing computational reactivity matrices (Corrales et al. 2017; Schultz et al. 2006). In the current study, electrophilicity was calculated by considering the HOMO of GSH and HOMO(N+1) of the respective xenobiotic, which estimates vertical electron affinity according to the extension of Koopman's theorem. Orbital energies were computed using density functional theory, mPW1PW91/MIDIX+, in the gas phase. We found computed energies of HOMO(N+1), i.e. the HOMO of a system with an electron added, to be more reliable than LUMO, which is a virtual orbital without a well-founded physical interpretation, and its energy is sensitive to the choice of basis set used in the calculation. Further, our past studies showed that using HOMO of the nucleophile in reactive indices calculations yields significantly more predictive models than HOMO of the electrophile (Kostal et al. 2012).

Statistical Analysis

Toxicity Relationship Analysis Program version 1.30a (EPA) was used to calculate LC_{50} values from initial acute studies with each of the compounds. Behavioral data analysis generally followed previous work using Sigma Plot 13.0 (Systat Software Inc., San Jose, CA, USA) software (Steele et al. 2018a; Steele et al. 2018b). We employed common experimental designs and statistical techniques recommended by the US EPA and OECD standardized methods for toxicology studies with fish models and extended these designs to include behavioral responses. Prior to analysis, data was examined for normality and equivalence of variance. Behavioral observations included a

nested design, in which statistical analyses were performed for each treatment level with 6 individuals from each experimental replicate (N=4) of zebrafish larvae and 4 individuals from each experimental replicate (N=3) of fathead minnow larvae.

Significant differences (α=0.10) in movement patterns were identified among treatments using analysis of variance (ANOVA) if normality and equivalence of variance assumptions were met. Because fish behavioral screening assays are employed in tiered approaches, we selected $\alpha = 0.10$ to decrease type II errors, which is particularly important when an understanding of biologically important effect sizes are limited for understudied behavioral endpoints and model organisms (Scheiner and Gurevitch 2001). Dunnett's post hoc tests were performed to identify treatment level differences; mean distance traveled, number of movements (counts), and duration of movements were calculated in 1-minute intervals. For data not meeting ANOVA assumptions, data was log transformed prior to analysis or ANOVA on ranks was performed. Regression analysis between reactivity estimates and different toxicity endpoints was also performed using Sigma Plot 13.0.

Results

Acute Toxicity

LC_{50} values for each of the study compounds are summarized in table 10. In both fish models, dibromoacetonirile was most acutely toxic and 3-chloro-1,2-propanediol was least acutely toxic. Acute toxicity of dibromoacetonitrile, glycidol, and styrene oxide was similar between each of the fish models, whereas 3-chloro-1,2-propanediol, SDS,

and TDCPP were more acutely toxic to zebrafish and 3-bromo-1-propanol and TBPP were more acutely toxic to fathead minnows.

Table 10. LC_{50} values for zebrafish and fathead minnow following 96 h exposure to seven compounds.

Chemical	Zebrafish LC50 (mmol/L)	Fathead minnow LC50 (mmol/L)
3-Bromo-1-Propanol	1.1397	0.4410
3-chloro-1,2-propanediol	50.2843	83.5448
Dibromoacetonitrile	0.0021	0.0015
Glycidol	0.8828	0.8828
Sodium decyl sulfate	0.8906	1.9278
Styrene oxide	0.1238	0.1047
TBPP	0.0044	0.0005
TDCPP	0.0063	0.0118

Behavioral Effect Thresholds

Behavioral effects occurred at sublethal concentrations for all study compounds except glycidol, which affected zebrafish but not fathead minnow activity (Table 11). These effects occurred at concentrations ranging from 0.0001 mmol/L dibromoacetonitrile to 2.51 mmol/L 3-chloro-1,2-propanediol in zebrafish and from 0.000015 mmol/L dibromoacetonitrile to 8.35 mmol/L3-chloro-1,2-propanediol in fathead minnow. Seven of the compounds used in zebrafish studies, and five of the chemicals used in fathead minnow experiments, had acute toxicity thresholds that were one order of magnitude or greater than behavioral effect thresholds. Nominal treatment concentrations for each of the study compounds during zebrafish and fathead minnow sublethal experiments are summarized in supplemental Tables S1 and S2. For most of the compounds, larval zebrafish and fathead minnow PMRs were more sensitive to chemical effects than fish light/dark locomotor activity. Though behaviors of both fish models were affected at sublethal levels, zebrafish typically demonstrated greater sensitivity to these electrophiles and a higher number of zebrafish behavioral endpoints were affected for each study compound. While 3-bromo-1-propanol was more acute toxic to fathead

minnow than to zebrafish, larval zebrafish were much more sensitive to behavioral effects of this compound.

Table 11. No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) (mmol/L) values for the most sensitive behavioral endpoints across light/dark swimming activity endpoints and photomotor responses for zebrafish and fathead minnows exposed to eight compounds. Behavioral endpoint for each LOEC is given in parenthesis next to each value. Cnts: Counts; Dist: Distance; Dur: Duration; PMR: Photomotor Response.

	Zebrafish		Fathead minnow	
Chemical	LOEC (mmol/L)	NOEC (mmol/L)	LOEC (mmol/L)	NOEC (mmol/L)
3-Bromo-1-Propanol	0.0001 (Cruising PMR)	<0.0001	0.61 (Cruising PMR)	0.06
3-chloro-1,2-propanediol	2.5142 (Total Cnts)	0.5028	923.5 (Freezing Dist)	461.75
Dibromoacetonitrile	0.0001 (Cruising PMR)	0.0002	0.003 (Total PMR)	<0.003
Glycidol	0.1766 (Cruising PMR)	0.0882	13.08 (Freeze PMR)	6.54
Sodium decyl sulfate	0.0089 (Bursting Dist)	<0.0089	50.19 (Burst PMR)	25.09
Styrene oxide	0.0062 (Cruising PMR)	0.0012	0.63 (Bursting Cnts)	0.13
TBPP	0.00004(Freezing Cnts)	<0.00004	0.004 (Total PMR)	<0.004
TDCPP	0.03 (Total PMR)	<0.03	2.040 (Freezing Dist)	1.02

Locomotor Profile Evaluation of Eight S_N2 Electrophiles

For each study chemical, locomotor profiles are represented in Figures 7 and 8 for zebrafish and fathead minnow, respectively. Plotted data includes distance moved, number of movements, and movement duration across three speed thresholds (Bursting: >20 mm/s, Cruising: 5-20 mm/s, and freezing <5 mm/s) in light and dark. Total distance moved and total number of movements are also included. Supplemental figures 32-39 include plotted data across all treatment levels and significant increases (↑) or decreases (↓) in larval fish locomotor for each of the study compounds.

With the exception of styrene oxide, each of the study chemicals suppressed zebrafish locomotor activity in the dark at each of the significantly altered endpoints (Fig. 7). Styrene oxide elicited significant increases in zebrafish activity at the bursting speed thresholds in the dark as indicated by a locomotor profile (Fig. 7F) where activity skewed towards stimulatory speeds at the 10% LC50 (0.012 mmol/L) treatment. All study chemicals suppressed zebrafish activity in the light for all significantly affected endpoints

(Fig. 7A-H). Glycidol (Fig. 8D) and sodium decyl sulfate (Fig. 8E) did not affect larval fathead minnow swimming activity in the light or the dark across treatment levels examined. The remaining study compounds significantly affected fathead minnow activity, but for fewer endpoints compared to zebrafish (Figs. 7 and 8). While 3B1P elicited a similar response profile in the fathead minnow model (Fig. 2A) compared to zebrafish (Fig. 1A), other compounds produced differential response patterns between the species. Fathead minnow locomotor profiles for 3B1P (Fig. 8A) and styrene oxide (Fig. 8F) had similar inhibitory effects whereas other compounds produced profiles that appear more stimulatory. For dibromoacetonitrile (Fig. 8C), however, activity profiles shifted toward both stimulatory and refractory speeds in the dark, but more refractory in the light.

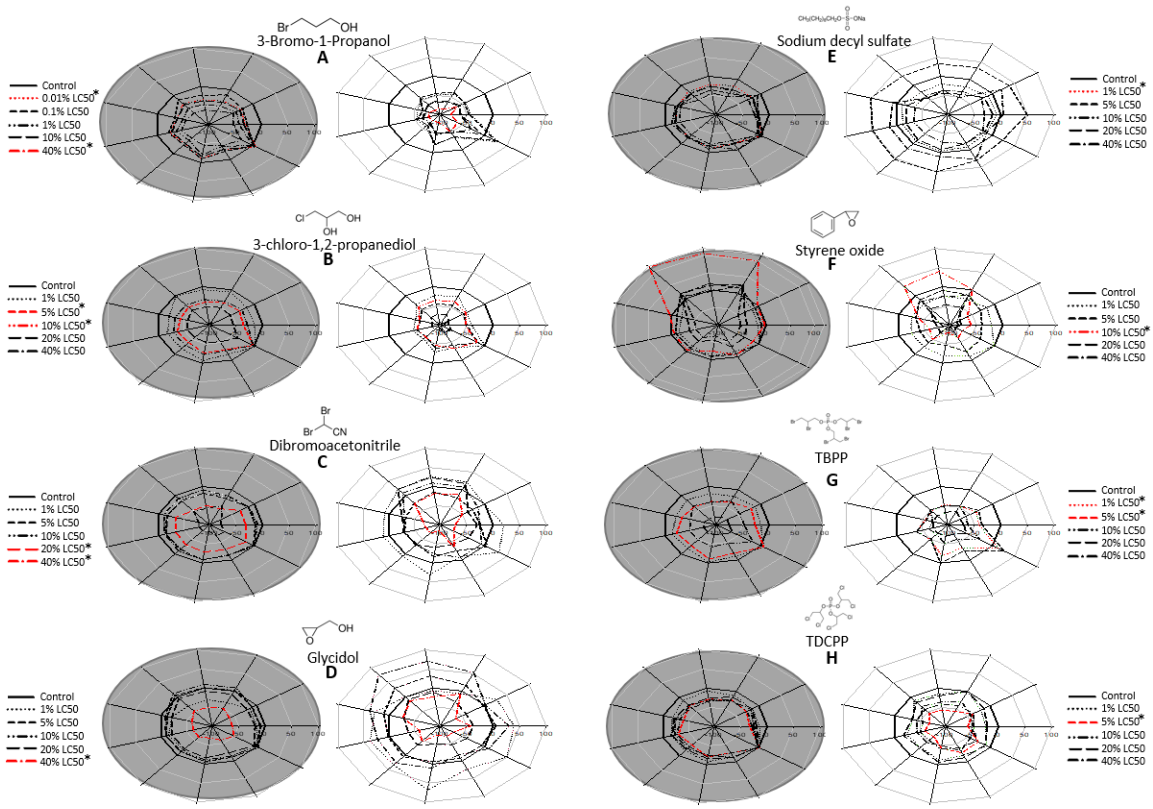

Figure 7. Mean (n=24; N=4) zebrafish swimming activity during dark and light photoperiods after 96 h exposure to A. 3-Bromo-1-propanol, B. 3-chloro-1,2-propanediol, C. dibromoacetonitrile, D. glycidol, E. sodium decyl sulfate, F. styrene oxide, G. Tris(2,3-dibromopropyl) phosphate (TBPP), and H. Tris(1,3-dichloro-2-propyl) phosphate (TDCPP). Plotted data of larval swimming activity in dark (grey plots) and light (white plots) for each chemical exposure are normalized to control, which is represented at the 0 axis in each figure. Asterisks on each legend signify the lowest observed effect concentration (LOEC) for each chemical in dark and light photoperiods. LOECs are also highlighted in red on each plot. Starting at the right side of each plot were axis number labels appear and moving counterclockwise measured endpoints on each plot are total distance moved, total number of movements, distance moved at bursting speeds (>20 mm/sec), number of movement at bursting speeds, duration of movements at bursting speeds, distance moved at cruising speeds (5-20 mm/sec), number of movements at cruising speeds, duration of movement at cruising seeds, distance moved at freezing speeds (<5 mm/sec), number of movements at freezing speeds, and duration of movements at freezing speeds.

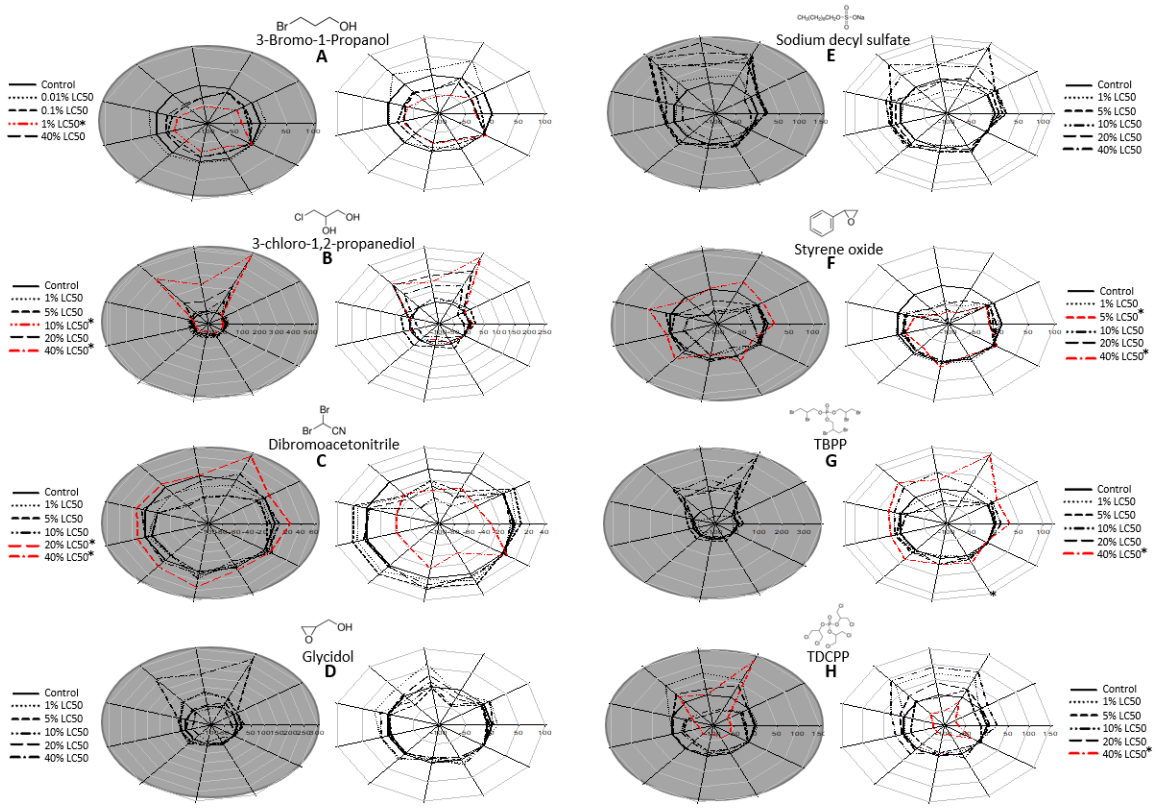

Figure 8. Mean (n=12; N=3) fathead minnow swimming activity during dark and light photoperiods after 96 h exposure to A. 3-Bromo-1-propanol, B. 3-chloro-1,2-propanediol, C. dibromoacetonitrile, D. glycidol, E. sodium decyl sulfate, F. styrene oxide, G. Tris(2,3-dibromopropyl) phosphate (TBPP), and H. Tris(1,3-dichloro-2-propyl) phosphate (TDCPP). Plotted data of larval swimming activity in dark (grey plots) and light (white plots) for each chemical exposure are normalized to control, which is represented at the 0 axis in each figure. Asterisks on each legend signify the lowest observed effect concentration (LOEC) for each chemical in dark and light photoperiods. LOECs are also highlighted in red on each plot. Starting at the right side of each plot were axis number labels appear and moving counterclockwise measured endpoints on each plot are total distance moved, total number of movements, distance moved at bursting speeds (>20 mm/sec), number of movement at bursting speeds, duration of movements at bursting speeds, distance moved at cruising speeds (5-20 mm/sec), number of movements at cruising speeds, duration of movement at cruising seeds, distance moved at freezing speeds (<5 mm/sec), number of movements at freezing speeds, and duration of movements at freezing speeds.

PMR Evaluation of Eight S_N2 Electrophiles

All of the study compounds except for sodium decyl sulfate caused significant changes in zebrafish dark PMRs (Fig. 9). Each of these chemicals elicited refractory responses to dark conditions, in which activity changes in response to dark were reduced compared to controls. Only half of the chemicals elicited significant changes on light PMRs. Similar to dark PMRs, dibromoacetonitrile (Fig. 9C) and glycidol (Fig. 9D) produced refractory larval fish responses to light. Styrene oxide (Fig. 9F), on the other hand, elicited a stimulatory light response at the 20% LC50 (0.025 mmol/L) treatment level, whereby sudden differences in activity levels between dark and light conditions were increased. TBPP (Fig. 9G) reduced zebrafish activity in responses to light at the the 20% LC50 (0.0009 mmol/L) treatment level, but caused an opposite light response from unexposed fish at the 40% LC50 (0.0018 mmol/L) treatment level. Although sodium decyl sulfate (Fig. 9E) did not effect zebrafish total distance PMRs , this chemical affected cruising (5-20 mm/s) speed dark PMRs (Supplemental Fig. 44F) at lower treatment levels (1% LC50; 0.0089 mmol/L). Similarly, the 40% LC50 (0.46 mmol/L) treatment was the lowest concentration of 3-bromo-1-propanol (Fig. 3A) to affect zebrafish total PMRs, but at cruising speeds (Supplemental Fig. 40F) 3-bromo-1-propanol significantly altered zebrafish light PMR at the 0.01% LC50 (0.0001 mmol/L) concentration.

3-Bromo-1-propanol, dibromoacetonitrile, TBPP, and TDCPP, each reduced fathead minnow activity levels in response to a sudden change to light photoperiods (Figs 10A,C,G,H). The remaining compounds (3-chloro-1,2-propanediol , glycidol, sodium decyl sulfate, and styrene oxide) did not significantly alter fathead minnow total PMRs.

Glycidol (Supplemental Fig. 43H) and sodium decyl sulfate (Supplemental Fig. 44I), however, reduced fathead minnow light PMRs at freezing speeds and cruising speeds. PMR measurements across three speed thresholds (Bursting: >20 mm/s, Cruising: 5-20 mm/s, and freezing <5 mm/s) for each compound and both fish models are given in supplemental figures 40-47 E-J.

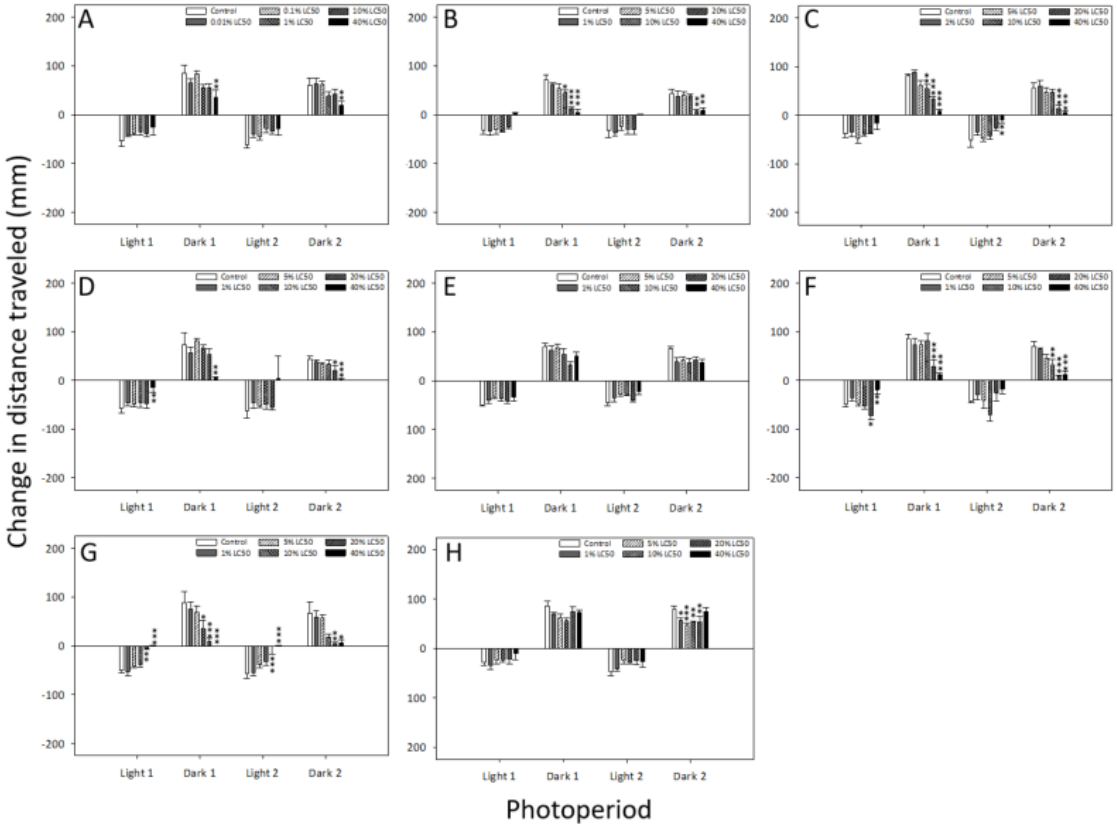

Figure 9. Photomotor responses (PMRs) of zebrafish after 96 h exposure to A. 3-Bromo-1-propanol, B. 3-chloro-1,2-propanediol, C. dibromoacetonitrile, D. glycidol, E. sodium decyl sulfate, F. styrene oxide, G. Tris(2,3-dibromopropyl) phosphate (TBPP), and H. Tris(1,3-dichloro-2-propyl) phosphate (TDCPP). Each PMR is measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.

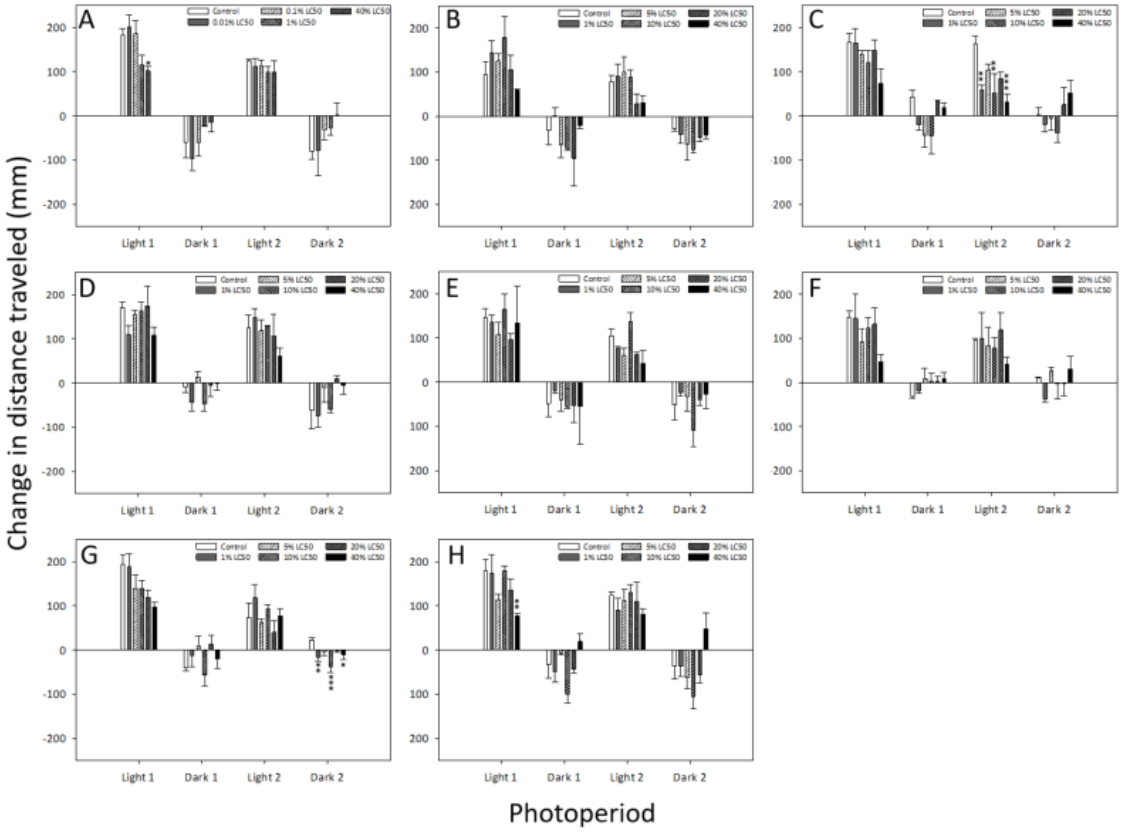

Figure 10. Photomotor responses (PMRs) of fathead minnow after 96 h exposure to A. 3-Bromo-1-propanol, B. 3-chloro-1,2-propanediol, C. dibromoacetonitrile, D. glycidol, E. sodium decyl sulfate, F. styrene oxide, G. Tris(2,3-dibromopropyl) phosphate (TBPP), and H. Tris(1,3-dichloro-2-propyl) phosphate (TDCPP). Each PMR is measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *$p<0.10$;**$p<0.05$; ***$p<0.01$.

In silico Reactivity Predictions of GSH Levels

No observed effects concentrations on larval GSH levels ranged from 0.0004 mmol/L TBPP to 20.11 mmol/L 3-chloro-1,2-propanediol in zebrafish (Table 12) and from 0.000005 mmol/L TBPP to 16.71 mmol/L 3-chloro-1,2-propanediol in fathead minnows (Table 13). We observed significant ($p<0.05$) relationships between electrophilic index (ω) values of eight S_N2 electrophiles and GSH of the zebrafish and fathead minnow models (Figure 11A and B). Further, GSH threshold (NOECs) effects in

both zebrafish and fathead minnow models were significantly ($p < 0.001$) related to mortality (LC_{50}) and behavioral PMR (NOECs) responses (Figure 11C and D). Lastly, ω values for these S_N2 electrophiles were significantly ($p < 0.05$) related to both mortality (LC_{50}) and behavioral PMR (NOECs) responses for both larval fish models (Figure 11E and F).

Table 12. Chemical electrophilicity index (ω) as well as No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) (mmol/L) for chemical effects on larval zebrafish dark 1 PMR and GSH tissue levels.

		PMR		GSH	
Chemical	ω	LOEC (mmol/L)	NOEC (mmol/L)	LOEC (mmol/L)	NOEC (mmol/L)
3-Bromo-1-Propanol	0.5540	0.4559	0.1140	>0.4559	0.4559
3-chloro-1,2-propanediol	0.4622	5.0284	2.5142	>20.1137	20.1137
Dibromoacetonitrile	1.0198	0.0002	0.0001	0.0004	0.0002
Glycidol	0.4426	0.3531	0.1766	>0.3531	0.3531
Sodium decyl sulfate	0.2015	>0.3563	0.3563	0.1781	0.0891
Styrene oxide	0.4828	0.0248	0.0124	0.0248	0.0124
TBPP	1.1834	0.0004	0.0002	0.0009	0.0004
TDCPP	0.8410	>0.0025	0.0025	1.0900	0.0013

Table 13. Chemical electrophilicity index (ω) as well as No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) (mmol/L) for chemical effects on larval fathead minnow light 1 PMR and GSH tissue levels.

		PMR		GSH	
Chemical	ω	LOEC (mmol/L)	NOEC (mmol/L)	LOEC (mmol/L)	NOEC (mmol/L)
3-Bromo-1-Propanol	0.5540	0.1764	0.0044	0.1764	0.00
3-chloro-1,2-propanediol	0.4622	>33.4179	33.4179	33.4179	16.71
Dibromoacetonitrile	1.0198	>0.0006	0.0006	0.0001	0.000
Glycidol	0.4426	>0.3531	0.3531	>0.35	0.35
Sodium decyl sulfate	0.2015	>0.7711	0.7711	>0.77	0.77
Styrene oxide	0.4828	>0.0419	0.0419	0.0052	0.00
TBPP	1.1834	>0.0002	0.0002	0.0000	0.000
TDCPP	0.8410	0.0047	0.0024	0.0001	>0.0001

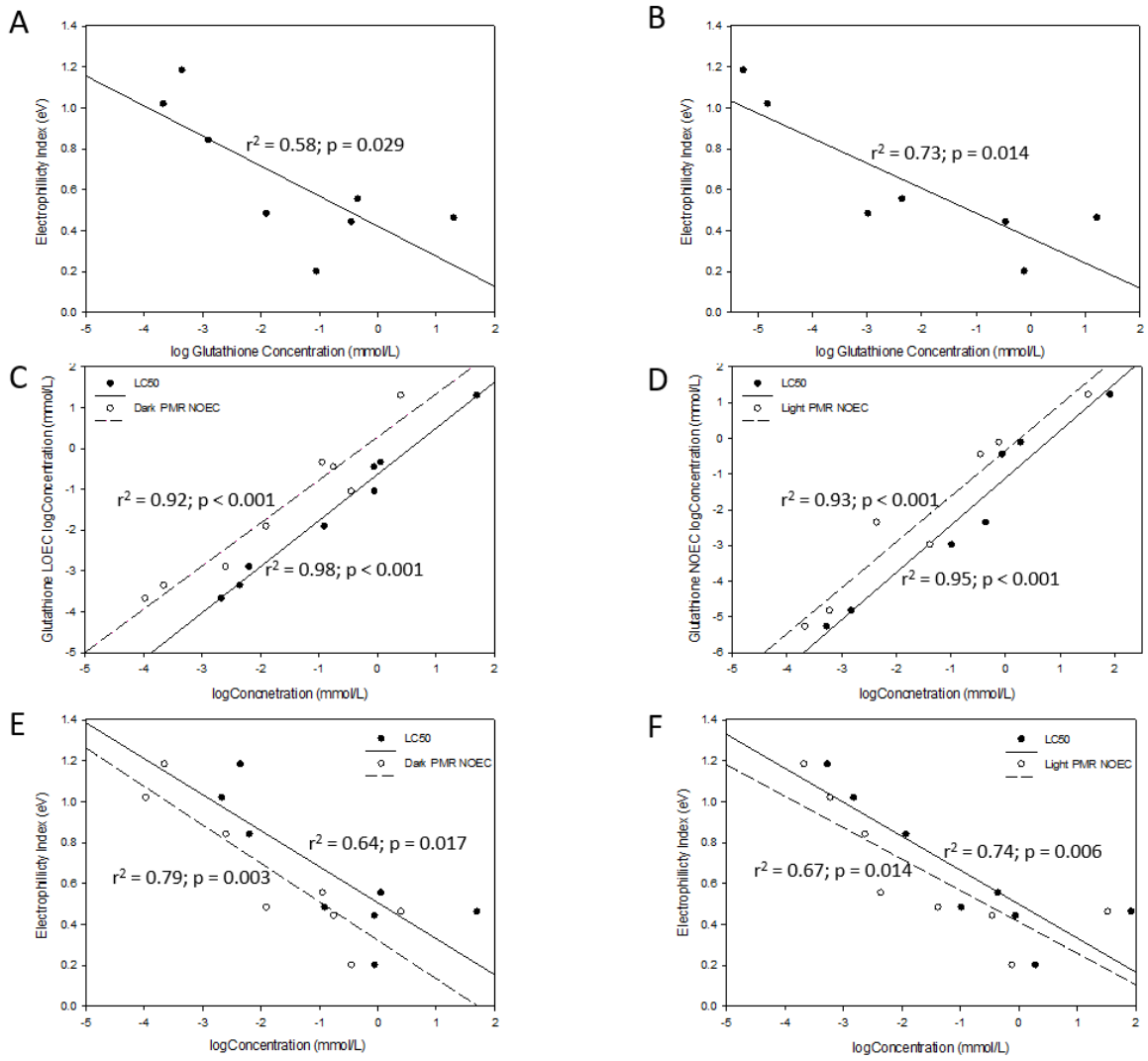

Figure 11. Relationship among whole organism GSH levels, toxicity endpoints, and *in silico* reactivity matrices for eight industrial chemicals. Toxicity values include each compounds LC_{50} (represented by black circles) and No Observed Effect Concentrations (NOECs) (mg/L) for zebrafish dark and fathead minnow light photomotor responses (PMRs) (represented by unfilled circles). Zebrafish (A, C, and E) and Fathead minnow (B, D, and F) GSH levels are compared to reactivity matrices in figures A and B. Toxicity endpoints are also compared to GSH levels (C and D) and reactivity computations (E and F).

Discussion

Predicting attributes of commercial chemicals with desired functions and limited hazards has recently been identified as priority research questions to protect public health and the environment (Furley et al. 2018; Van den Brink et al. 2018). Previous research by our team reported several computational parameters that appear useful to identify

attributes of contaminants associated with lower and higher acute and chronic toxicity from standardized experimental designs ((Voutchkova et al. 2011, Voutchkova et al. 2012; Kostal et al. 2015). In fact, Connors et al (2014) observed that if two chemical design guidelines, log Ko/w and ΔE, were followed, then the proportion of chemicals eliciting high toxicity to these standardized acute and chronic toxicity responses would be markedly diminished. Though we recently observed relationships between computational predictions for oxidative stress and GSH in vitro, similar efforts coupling computational, biochemical and behavioral activity patterns in vivo have not been engaged. In the present study, we advanced our earlier work to examine a specific chemical domain (S_N2) for compounds with electrophilic reactivity using computational modeling of an electrophilic index (ω), and identified significant relationships between ω, GSH and subtle sublethal behavioral PMR and refractory responses in two common alternative vertebrate models.

Glutathione has long been considered as a model nucleophile for protein binding (Schultz et al. 2006). This thiol group provides a rapid, cost-effective nucleophile for in chemico reactivity assays and is an important biological antioxidant (Böhme et al. 2009; Clarke et al. 1998; McCarthy et al. 1994). Chemicals that react with GSH can deplete it and form adducts with proteins, including in the alternative vertebrate models employed here. Substantial GSH depletion or protein binding can lead to cell death and oxidative stress through a number of mechanisms (Aruoma 1998). Therefore, GSH has been successfully used as a model nucleophile in a variety of in *silico, in vitro, and in vivo* experiments to study electrophile toxicity (Freidig et al. 1999; Freidig and Hermens 2000). In the present study, GSH was used as a model nucleophile to develop in silico

chemical descriptors for eight electrophiles that share the same reactive mechanistic domain. Electrophilicity index (ω) was the chemical descriptor that emerged to have the highest correlation with experimental metrics examined in this study, and was significantly ($p < 0.05$) related to S_N2 electrophile influences on antioxidant (GSH) levels in both larval fish models. The relevance and predictive power of FMO-derived reactivity indices demonstrated herein is a natural extension of our previous efforts in the context of acute and chronic aquatic toxicity (Kostal et al. 2015; Melnikov et al. 2016; Voutchkova-Kostal et al. 2012; Voutchkova et al. 2011), skin sensitization (Kostal and Voutchkova, 2016), and oxidative stress (Melnikov, 2018). While often overlooked for more sophisticated computational and statistical modeling approaches, calculations of global and local reactivity indices, are not only invaluable in rapid screening of large datasets of chemicals, or in assessing general reactivity across multiple MOAs, but due to significant advancements in hybrid density functional methods, can also provide highly accurate and reliable, endpoint-specific models (CADRE-SS, CADRE-AT).

After leveraging reactivity computations at the biochemical level, we examined potential relationships between ω and whole organism toxicity and bioactivity responses. For the eight S_N2 electrophiles, significant ($p < 0.05$) relationships between acute (LC50) toxicity and ω were observed in both zebrafish and fathead minnow bioassays. Such observations indicate that ω provides a useful in silico predictor of electrophilic influences on acute fish toxicity, potentially influenced by protein adduct formation, consistent with our previous findings (Kostal et al, 2015). Though acute mortality represents a commonly measured endpoint with fish models (Freidig and Hermens 2000; Hermens 1990; Lipnick et al. 1987; Veith and Mekenyan 1993), little is known about

potential relationships between sublethal fish behavioral toxicity for electrophiles (Russom et al. 1997). While such relationships have not been robustly explored, larval fish PMRs have been previously used to identify therapeutic targets for novel chemicals in pharmaceutical studies and to investigate industrial chemical bioactivity in toxicity studies (Kokel et al. 2010; Noyes et al. 2015; Rihel et al. 2010; Steele et al. 2018a; Steele et al. 2018b). These measurements are rapid, sensitive and provide nonlethal toxicity endpoints with diagnostic utility, and therefore, present unique opportunities to examine computational predictions of electrophilic bioreactivity.

Zebrafish dark PMRs and fathead minnow light PMRs were selected for analysis with reactivity indices due to consistent behaviors observed in zebrafish, which increase activity during light to dark transitions, and in fathead minnows, which exhibit a similar PMR when dark to light conditions change (Colón-Cruz et al. 2018; Steele et al. 2018a; Steele et al. 2018b). In the present study with S_N2 electrophiles, both zebrafish dark PMR and fathead minnow light PMR NOECs were significantly ($p < 0.05$) predicted by the electrophilicity index, which was similar to computational predictions of mortality (LC_{50}) endpoints described above for these common fish models. Significant ($p < 0.001$) relationships between computational predictions and GSH levels, LC_{50} values and PMR thresholds in both fish models support involvement of electrophilic mechanisms in the toxicity responses to these S_N2 chemicals. Though most previous work with larval zebrafish PMRs has investigated drug candidates and apparent interactions with therapeutic targets (Kokel et al. 2010; Rihel et al. 2010), our observations indicate that PMRs may be useful in examining bioactivity of reactive electrophiles, and may provide

a nonlethal alternative approach to acute mortality when investigating electrophilic toxicity.

In the current study, we further investigated behavioral response profiles, which can be used to define chemical bioactivity at the whole organism level (Truong et al. 2013) and provide unique signatures associated with various MOAs (Steele et al. 2018a). Prior to development of robust automated tracking capacity, which we employed here for the first time for S_N2 electrophiles, fish behavioral profiles were reported for electrophilic and other types of industrial compounds through observations of various toxicity syndromes in juvenile fathead minnows (Drummond et al. 1986; McKim et al. 1987; Russom et al. 1997). Russom et al. (1997), for example, used three distinct behavioral syndromes in conjunction with other toxicity and physiochemical descriptors to classify over 600 industrial compounds across eight MOA categories. Chemicals eliciting toxicity through a non-polar narcosis MOA primarily elicited inhibitory behavioral syndromes, whereby the fathead minnow model became lethargic and less responsive to outside stimuli (Russom et al. 1997). Conversely, substances classified with a polar narcosis MOA primarily resulted in stimulatory responses, in which fish were hyperactive and over responsive to external stimuli (Russom et al. 1997). Electrophilic chemicals, however, produced a variety of stimulatory and refractory responses (Russom et al. 1997).

In the current study, S_N2 electrophiles commonly elicited refractory larval zebrafish behavioral response profiles, with the exception of styrene oxide, which caused significant increases in activity at bursting speed thresholds in the dark. Specifically, S_N2 chemicals significantly reduced locomotor activity in the dark for multiple endpoints.

Refractory effects were also observed under light conditions, though fewer significant behavioral effects were observed in the present study. These primarily refractory responses are in stark contrast to our previous studies (Steele et al. 2018a) with chemicals eliciting aquatic toxicity through different MOAs. For example, the acetylcholinesterase inhibitor diazinon significantly increased zebrafish activity at bursting speed thresholds under dark, but not light, conditions; however, xylazine, an α-2 adrenergic agonist, significantly inhibited activity in dark and light but significantly increased bursting distance, freezing distance and freezing duration in the light. Such observations are differentiated from 1-heptanol, which elicits acute toxicity through non-polar narcosis, and significantly reduced activity across all locomotor endpoints in both light and dark conditions. Thus, these relatively consistent refractory behavioral response profiles in zebrafish appear indicative of S_N2 electrophilic activity, though further research is warranted to elucidate specific mechanisms by which such refractory behaviors are propagated by electrophiles.

Whereas zebrafish and fathead minnow behavioral responses demonstrated similar refractory behavioral responses to 3B1P exposure, the remaining compounds elicited differential responses between these two common fish models. As noted above, zebrafish locomotor responses were predominantly inhibitory, but fathead minnows demonstrated greater diversity of stimulatory and refractory responses in both light and dark conditions. Such observations are generally consistent with previous studies with juvenile fathead minnows by Russom et al. (1997), who identified reactive chemicals to elicit different behavioral syndromes, though these measures were recorded in response to exposure at acutely lethal (LC_{50}) concentrations. Fathead minnow locomotor responses

to S_N2 electrophiles were further less consistent than zebrafish, which is in contrast to our previous research with several specifically acting compounds (e.g., pharmaceuticals, pesticides) that elicited consistent behavioral effects in the fathead minnow and zebrafish models (Steele et al. 2018a).

It is important to note that we followed standardized OECD and EPA guidelines in which zebrafish exposure was initiated 4-6 h post fertilization and fathead minnow larvae exposure began within 24 h post hatch, respectively, to maximize broader comparability of results presented here with other studies. In addition to influencing behavioral responses (Kristofco et al. 2016), development age of fish can influence biotransformation, bioaccumulation and toxicodynamics of various chemical contaminants (Corrales et al. 2017; Kristofco et al. 2016). Therefore, this study was not designed to directly examine whether differential behavioral response thresholds exhibited between larval zebrafish and fathead minnows are related to differences in age and/or species. However, observations in the present study indicate that larval zebrafish behaviors may provide greater diagnostic utility than larval fathead minnows for S_N2 and other electrophilic bioactivity. Future research is needed to examine propagation of molecular initiation events to fish photomotor and locomotor activities. The latter represents an important step in leveraging larval fish behaviors to identify subcellular targets of industrial chemicals. Though underlying mechanisms associated with electrophile effects on larval photomotor and locomotor responses are not understood, covalent binding with a nucleophilic target represents an important molecular initiation event for reactive toxicity (Enoch and Cronin 2010; Enoch et al. 2011; Schultz et al. 2006) linked to measurable adverse outcomes (Ellison et al. 2016; Freidig and Hermens

2000; Schultz et al. 2006). Observations in the present study identify utility of quantum mechanical computations as an initial step in identifying mechanisms of electrophilic contaminants.

Acknowledgments

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References

Andersen ME, Krewski D. 2008. Toxicity testing in the 21st century: Bringing the vision to life. Toxicological sciences 107:324-330.

Aruoma OI. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American oil chemists' society 75:199-212.

Association AAPH. 1999. Standard methods for the examination of water and wastewater 20th ed. Prepared and published jointly by: APHA, AWWA and WPCF.

Berninger JP, Du B, Connors KA, Eytcheson SA, Kolkmeier MA, Prosser KN, et al. 2011. Effects of the antihistamine diphenhydramine on selected aquatic organisms. Environmental Toxicology and Chemistry 30:2065-2072.

Böhme A, Thaens D, Paschke A, Schüürmann G. 2009. Kinetic glutathione chemoassay to quantify thiol reactivity of organic electrophiles · application to α, β-unsaturated ketones, acrylates, and propiolates. Chemical research in toxicology 22:742-750.

Clarke ED, Greenhow DT, Adams D. 1998. Metabolism-related assays and their application to agrochemical research: Reactivity of pesticides with glutathione and glutathione transferases. Pesticide science 54:385-393.

Clymer T, Vargas V, Corcoran E, Kleinberg R, Kostal J. 2019. Redesigning hazardous chemicals by learning from structure-based drug discovery. Green Chemistry.

Coish P, Brooks BW, Gallagher EP, Mills M, Kavanagh TJ, Simcox N, et al. 2018. The molecular design research network. Toxicological Sciences 161:241-248.

Colón-Cruz L, Kristofco L, Crooke-Rosado J, Acevedo A, Torrado A, Brooks BW, et al. 2018. Alterations of larval photo-dependent swimming responses (pdr): New endpoints for rapid and diagnostic screening of aquatic contamination. Ecotoxicology and environmental safety 147:670-680.

Connors KA, Voutchkova-Kostal AM, Kostal J, Anastas P, Zimmerman JB, Brooks BW. 2014. Reducing aquatic hazards of industrial chemicals: Probabilistic assessment of sustainable molecular design guidelines. Environmental toxicology and chemistry 33:1894-1902.

Corrales J, Kristofco L, Steele W, Saari G, Kostal J, Williams E, et al. 2017. Toward the design of less hazardous chemicals: Exploring comparative oxidative stress in two common animal models. Chemical research in toxicology 30:893-904.

Drummond RA, Russom CL, Geiger DL, Defoe DL. 1986. Behavioral and morphological changes in fathead minnow (pimephales promelas) as diagnostic endpoints for screening chemicals according to mode of action. In: Aquatic toxicology and environmental fate: Ninth volume:ASTM International.

Ellison CM, Piechota P, Madden JC, Enoch SJ, Cronin MT. 2016. Adverse outcome pathway (aop) informed modeling of aquatic toxicology: Qsars, read-across, and interspecies verification of modes of action. Environmental science & technology 50:3995-4007.

Enoch S, Cronin M. 2010. A review of the electrophilic reaction chemistry involved in covalent DNA binding. Critical Reviews in Toxicology 40:728-748.

Enoch S, Ellison C, Schultz T, Cronin M. 2011. A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. Critical reviews in toxicology 41:783-802.

EPA U. Environmental protection agency (2002) methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms.US Environmental Protection Agency Office of Water (4303T).

Erythropel HC, Zimmerman JB, de Winter TM, Petitjean L, Melnikov F, Lam CH, et al. 2018. The green chemistree: 20 years after taking root with the 12 principles. Green Chemistry 20:1929-1961.

Freidig AP, Verhaar HJ, Hermens JL. 1999. Quantitative structure-property relationships for the chemical reactivity of acrylates and methacrylates. Environmental toxicology and chemistry 18:1133-1139.

Freidig AP, Hermens JL. 2000. Narcosis and chemical reactivity qsars for acute fish toxicity. Quantitative Structure-Activity Relationships: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis 19:547-553.

Furley TH, Brodeur J, Silva de Assis HC, Carriquiriborde P, Chagas KR, Corrales J, et al. 2018. Toward sustainable environmental quality: Identifying priority research questions for latin america. Integrated environmental assessment and management 14:344-357.

Hermens J. 1990. Electrophiles and acute toxicity to fish. Environmental health perspectives 87:219.

Kato S. 2000. Perspective on “a molecular orbital theory of reactivity in aromatic hydrocarbons”. In: Theoretical chemistry accounts:Springer, 219-220.

Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, et al. 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nature chemical biology 6:231-237.

Kostal J, Voutchkova-Kostal A, Weeks B, Zimmerman JB, Anastas PT. 2012. A free energy approach to the prediction of olefin and epoxide mutagenicity and carcinogenicity. Chemical research in toxicology 25:2780-2787.

Kostal J, Voutchkova-Kostal A, Anastas PT, Zimmerman JB. 2015. Identifying and designing chemicals with minimal acute aquatic toxicity. Proceedings of the National Academy of Sciences 112:6289-6294.

Kostal J. 2016. Computational chemistry in predictive toxicology: Status quo et quo vadis? In: Advances in molecular toxicology, Vol. 10:Elsevier, 139-186.

Kostal J, Voutchkova-Kostal A. 2016. Cadre-ss, an in silico tool for predicting skin sensitization potential based on modeling of molecular interactions. Chemical Research in Toxicology 29:58-64.

Kristofco LA, Cruz LC, Haddad SP, Behra ML, Chambliss CK, Brooks BW. 2016. Age matters: Developmental stage of danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic Toxicology 170:344-354.

Landrigan PJ, Fuller R, Acosta NJ, Adeyi O, Arnold R, Baldé AB, et al. 2017. The lancet commission on pollution and health. The Lancet.

Lipnick R, Watson K, Strausz A. 1987. A qsar study of the acute toxicity of some industrial organic chemicals to goldfish. Narcosis, electrophile and proelectrophile mechanisms. Xenobiotica 17:1011-1025.

LoPachin RM, Gavin T, DeCaprio A, Barber DS. 2011. Application of the hard and soft, acids and bases (hsab) theory to toxicant–target interactions. Chemical research in toxicology 25:239-251.

Malaj E, Peter C, Grote M, Kühne R, Mondy CP, Usseglio-Polatera P, et al. 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. Proceedings of the National Academy of Sciences 111:9549-9554.

McCarthy TJ, Hayes EP, Schwartz CS, Witz G. 1994. The reactivity of selected acrylate esters toward glutathione and deoxyribonucleosides in vitro: Structure-activity relationships. Fundamental and applied toxicology 22:543-548.

McKim JM, Bradbury SP, Niemi GJ. 1987. Fish acute toxicity syndromes and their use in the qsar approach to hazard assessment. Environmental Health Perspectives 71:171.

Melnikov F, Kostal J, Voutchkova-Kostal A, Zimmerman JB, Anastas PT. 2016. Assessment of predictive models for estimating the acute aquatic toxicity of organic chemicals. Green Chemistry 18:4432-4445.

Melnikov F, Botta D, White CC, Schmuck SC, Winfough M, Schaupp CM, et al. 2018. Kinetics of glutathione depletion and antioxidant gene expression as indicators of chemical modes of action assessed in vitro in mouse hepatocytes with enhanced glutathione synthesis. Chemical research in toxicology.

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. 2015. Advanced morphological—behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicological Sciences 145:177-195.

OECD. 2013. Test no. 236: Fish embryo acute toxicity (fet) test.

Perkins E, Garcia-Reyero N, Edwards S, Wittwehr C, Villeneuve D, Lyons D, et al. 2015. The adverse outcome pathway: A conceptual framework to support toxicity testing in the twenty-first century. In: Computational systems toxicology:Springer, 1-26.

Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327:348-351.

Russom CL, Bradbury SP, Broderius SJ, Hammermeister DE, Drummond RA. 1997. Predicting modes of toxic action from chemical structure: Acute toxicity in the fathead minnow (pimephales promelas). Environmental toxicology and chemistry 16:948-967.

Schäfer RB, Kühn B, Malaj E, König A, Gergs R. 2016. Contribution of organic toxicants to multiple stress in river ecosystems. Freshwater Biology 61:2116-2128.

Scheiner SM, Gurevitch J. 2001. Design and analysis of ecological experiments:Oxford University Press.

Schultz T, Carlson R, Cronin M, Hermens J, Johnson R, O'Brien P, et al. 2006. A conceptual framework for predicting the toxicity of reactive chemicals: Modeling soft electrophilicity. SAR and QSAR in Environmental Research 17:413-428.

Steele WB, Kristofco LA, Corrales J, Saari GN, Haddad SP, Gallagher EP, et al. 2018a. Comparative behavioral toxicology with two common larval fish models: Exploring relationships among modes of action and locomotor responses. Science of The Total Environment 640:1587-1600.

Steele WB, Mole R, Brooks BW. 2018b. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neurostimulant caffeine. Journal of visualized experiments: JoVE.

Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. 2013. Multidimensional in vivo hazard assessment using zebrafish. Toxicological Sciences 137:212-233.

US-EPA. 1993a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, epa/600/4–90/027f.

US-EPA. 1993b. Methods for aquatic toxicity identification evaluations: Phase i toxicity identification procedures for samples exhibiting acute and chronic toxicity:US Environmental Protection Agency, Office of Research and Development.

Valenti TW, Perez-Hurtado P, Chambliss CK, Brooks BW. 2009. Aquatic toxicity of sertraline to pimephales promelas at environmentally relevant surface water ph. Environmental Toxicology and Chemistry 28:2685-2694.

Van den Brink PJ, Boxall AB, Maltby L, Brooks BW, Rudd MA, Backhaus T, et al. 2018. Toward sustainable environmental quality: Priority research questions for europe. Environmental toxicology and chemistry 37:2281-2295.

Veith GD, Mekenyan OG. 1993. A qsar approach for estimating the aquatic toxicity of soft electrophiles [qsar for soft electrophiles]. Quantitative Structure-Activity Relationships 12:349-356.

Verhaar HJ, Van Leeuwen CJ, Hermens JL. 1992. Classifying environmental pollutants. Chemosphere 25:471-491.

Voutchkova-Kostal AM, Kostal J, Connors KA, Brooks BW, Anastas PT, Zimmerman JB. 2012. Towards rational molecular design for reduced chronic aquatic toxicity. Green Chemistry 14:1001-1008.

Voutchkova AM, Osimitz TG, Anastas PT. 2010. Toward a comprehensive molecular design framework for reduced hazard. Chemical reviews 110:5845-5882.

Voutchkova AM, Kostal J, Steinfeld JB, Emerson JW, Brooks BW, Anastas P, et al. 2011. Towards rational molecular design: Derivation of property guidelines for reduced acute aquatic toxicity. Green Chemistry 13:2373-2379.

CHAPTER FIVE

Individual and Interactive Effects of Oxazepam and Sertraline on Perch (Perca fluviatilis) Behavior With or Without a Predator Cue

Abstract

Two commonly used psychiatric medications, oxazepam (a benzodiazepine) and sertraline (a selective serotonin reuptake inhibitor, SSRI), affect anxiety related behaviors at dilute concentrations in lab reared and wild fish populations. These drugs are often present in the environment together, yet there is little understanding of whether co-exposure to these compounds may affect fish behavior. Previous research with mammalian models has demonstrated that benzodiazepines can counteract the anti-depressant behavioral activity of SSRIs. This study aimed to understand the effects oxazepam and sertraline on perch boldness and predatory avoidance behaviors when fish are exposed to each drug individually and in combination. Because predatory stimuli are important factors governing fish behavior in the wild, this study also aimed to understand how predatory olfactory signaling cues may influence perch behaviors in response to each pharmaceutical treatment. Juvenile European perch (*Perca fluviatilis*) were caught from Bjensjon (Bear Lake, a remote uncontaminated lake) 20 km Southwest of Umea, Sweden. Behavioral observations of boldness (scototaxis), activity, and sociality before and after 7 days of exposure to each drug treatment consisting of 10 µg/L oxazepam, 10 µg/L sertraline, a combination of both pharmaceuticals, and controls (n = 30) in ~3 l experimental units for 7 days. These treatments, which were analytically verified by LC-MSMS, and controls were also subjected in parallel to a predatory olfactory cue (n = 30),

for a total of 240 experimental units. Sertraline but not oxazepam significantly ($p < 0.05$) increased activity levels of perch. Fish exposed to a combination of sertraline and oxazepam, however, demonstrated no significant increases in activity compared to controls. Furthermore, sertraline did not significantly ($p > 0.05$) increase activity compared to control fish in the presence of a predatory signaling cue. There were also no significant ($p > 0.05$) differences in boldness among treatments, but control and sertraline exposed fish demonstrated increases in boldness between pre and post-experimental behavioral observations. These pre/post exposure effects were not apparent in the presence of a predator signaling cue, but such differences were apparent in fish exposed to a combination of sertraline and oxazepam in the presence of a predatory olfactory signaling cue. No significant ($p > 0.05$) differences in sociality were observed among the drug treatments either with and without predatory olfactory cues. Our observations demonstrate that neuroactive pharmaceutical effects in lab studies may differ from those in the field due to drug-drug interactions and predatory pressures present in urban aquatic ecosystems.

Introduction

Pharmaceuticals are emerging aquatic contaminants that are ubiquitous in urbanized water sheds throughout the world, yet there is little understanding about the adverse impacts that these compounds may have on aquatic life (Boxall et al. 2012; Brooks et al. 2012; Brooks et al. 2003; Kümmerer 2010; Metcalfe et al. 2004). After discharge from wastewater effluent, pharmaceuticals typically enter surface waters at dilute concentrations, but given their inherent biological activity, they demonstrate the potential to effect non-target aquatic species at low exposure levels (Brooks 2014).

However, current risk paradigms may not capture MoA relevant effects of pharmaceuticals as these compounds are designed to be therapeutic with minimal toxicity. Therefore, common toxicity endpoints, such as mortality, may not be relevant in evaluating potential environmental risks of pharmaceutical compounds (Arnold et al. 2014; Brooks 2014; Connors et al. 2014).

Many psychiatric compounds, for example, are present in the environment at levels far below those that cause mortality in aquatic organisms, but several of these drugs have been demonstrated to effect behaviors in fish species at dilute, environmentally relevant levels (Brodin et al. 2013; Brodin et al. 2017; Klaminder et al. 2014; Kristofco et al. 2016; Martin et al. 2017; Painter et al. 2009; Steele et al. 2018c). Given that fish share considerable drug target homology with humans and psychiatric medicines are designed to modify human behavior, it is apparent why these medicines are capable of effecting behavior in fish (Huggett et al. 2003; LaLone et al. 2014). Such evidence underlies the importance of behavioral endpoints when evaluating the effects of psychiatric medicines to aquatic life (Brodin et al. 2014; Klaminder et al. 2014).

Indeed, there is a growing body of evidence indicating that behavioral alterations can influence individual performance, ecosystem function, and even species evolution. Further, it is recognized that such alterations can occur through chemical contaminants (Saaristo et al. 2018). Although behavioral effects of contaminants have been studied for several decades in the field of ecotoxicology, it is not until recently that researchers have begun to focus on more ecologically relevant behaviors, such as predator avoidance and prey capture, that are known to directly influence individual performance (Little 2002).

Given the importance of fear in avoiding predation, antianxiety medications demonstrate potential to alter predatory threat perception in fish, and ultimately render prey fish more susceptible to predation (Brodin et al. 2014). Two commonly studied groups of psychiatric medication, benzodiazepines and selective serotonin reuptake inhibitors (SSRIs), have been demonstrated to effect anxiety related behaviors in lab reared and wild fish populations. The benzodiazepine, oxazepam, has been demonstrated to increase boldness, and subsequently reduce predator avoidance behaviors in wild caught perch (*Perca fluviatilis*). Fish exposed to low, environmentally relevant levels (1.8 µg/L) of oxazepam exhibited reduced sociality and increased activity levels (Brodin et al. 2013). Similar effects have been observed in wild caught roach (*Rutilus rutilus*) exposed to dilute levels (0.84 µg/L) of oxazepam (Brodin et al. 2017). The SSRI, sertraline has been demonstrated to effect shelter seeking, an important predator avoidance behavior, in adult male fathead minnows (*Pimephales promelas*) (Valenti et al. 2012).

The observed effects of antianxiety medications also extend into the field. After performing laboratory based behavioral assays, Klaminder et al. 2016 conducted field studies with oxazepam exposed perch, whereby fish behaviors were tracked via acoustic telemetry in lake ecosystem populated with a predatory fish species, pike (*Esox lucius*). Similar to results from the lab-based assays, field observations indicated that exposed perch exhibited greater boldness and were more active. Furthermore, exposed perch had a larger home range, and used pelagic habitats more than the non-exposed perch. Although observations were similar between field and laboratory studies, effects were

more obvious in the field study. Therefore, short-term behavioral assays may fail to detect all the effects expressed in natural environments.

One important factor present in the natural environment that is often missing in lab based behavioral studies is the presence of predatory threats. Predatory olfactory signaling cues are an important stimulus fish use to assess predation risk (Van Donk et al. 2016). As such, prey fish naturally respond to these stimuli through defensive behaviors (Martin et al. 2010; Ylönen et al. 2007). Because olfactory signaling cues represent a predatory pressure stimuli present in natural systems, and anxiety is correlated with predator avoidance behaviors in fish, further research is needed to understand if pharmaceuticals intended to treat anxiety in humans can impair fish responses to predator signaling cues and if certain behaviors maybe overlooked in the absence of predatory pressure. Another important, overlooked factor is that aquatic organisms undergo concurrent exposure to pharmaceuticals because drugs are discharged into the environment as complex mixtures.

Oxazepam and sertraline are present in the environment together, yet there is little understanding of how co-exposure to these compounds may affect fish behavior. While both compounds are used to treat anxiety, they each have differing mechanisms of therapeutic action. Oxazepam exerts its therapeutic effects through acting as an agonist of $GABA_A$ receptors, whereas sertraline blocks serotonin re-uptake transporters and inhibits the re-uptake of serotonin in the post synaptic cleft of neuronal cells (Frazer 2001; Vinkers and Olivier 2012). Previous research with mammalian models has demonstrated that benzodiazepines can counteract the anti-depressant behavioral activity of SSRIs (Da-Rocha et al. 1997). Although the mechanisms of this counteraction is not

clear, benzodiazepines reduce the activity of noradrenaline (NA), dopamine (DA), and serotonin (5-HT) neurons. Thus, the reduction in neurotransmission of these monoamine transporters by benzodiazepines may ultimately inhibit the therapeutic effects of SSRIs (Haefely 1983).

Given that benzodiazepines and SSRIs are likely to exist in urbanized aquatic environments together and studies with mammals indicate benzodiazepines can alter the behavioral effect of SSRIs, this study aims to understand the effects of two commonly prescribed anxiety medications, oxazepam ($GABA_A$ agonist) and Sertraline (SSRI), on perch behaviors when fish are exposed to each drug individually and in combination. Lastly, we aim to understand how predatory olfactory signaling cues may influence perch behaviors in response to each drug treatment as predatory stimuli are important factors governing fish behavior in the wild.

Materials and Methods

Study Animals

Juvenile perch (N=240) used in this study were collected from Bjensjön, a remote, uncontaminated lake 16 km southeast of Umeå, Sweden. Collected fish were transported to a flow through holding tank (2000 L) in the Department of Ecology and Environmental Science, Umeå University using 200L aerated transportation tubes. Before the first set of behavioral assays commenced, fish were given a 10 d period to acclimate to lab conditions in the holding tank. During this time, perch were fed frozen *chironomid* larvae once per day.

Chemical Exposure

Twenty-four hours before pre-exposure behavioral observations, each fish was transferred to individual aerated aquaria (N = 240, 14 cm high x 14 cm wide x 22 cm long) made of transparent plastic. Aquaria were filled with 3 L of aged dechlorinated tap water. Following pre-exposure behavioral observations on day 0 of the study, each perch was randomly assigned to one of the eight treatments consisting of controls, 10 µg/L oxazepam, 10 µg/L sertraline, or a combination of both drugs each at 10 µg/L concentrations. These treatments were also replicated, but instead, also contained additions of predator olfactory signaling cues resulting in a total of eight treatments. During drug exposure each fish was contained separately in individual aerated aquaria (N = 240, 14 cm high x 14 cm wide x 22 cm long) identical to the aquaria fish were contained in before pre-exposure (Day 0) behavioral observations. Each aquaria was filled with 3 L of exposure solutions prepared with dechlorinated tap water. Predator olfactory cues consisted of water taken from tanks (500 L) that housed 4 pike (*Esox lucius*). These cues were transferred to each predator cue exposure replicate tank each day at 8 ml volumes. Following seven days of exposure, behavioral trials were conducted for each fish. Fish were fed 24 hrs before pre-exposure behavioral observation (Day 0) and on day 6 of the study, which was 24 hrs before behavioral observations on day 7. Food consisted of frozen *chironomid* larvae.

Behavioral Analyses

Two behavioral experiments were conducted to measure three ecologically important behavioral traits (boldness, activity, and sociality). Boldness, which describes the degree of risk taking of an individual, was quantified through scototaxis trials following previously developed protocols (Brodin et al. 2017; Maximino et al. 2010). This test

measures the time an individual fish spends in the white compartment of a tank that is dived between two zones where the bottom half of one zone is black (perceived as safer) and the bottom half of the other zone is white (perceived as more dangerous). The longer an individual fish spends in the white compartment, the more bold that fish is considered to be. Scototaxis tanks consisted of glass tanks (42 cm x 50 cm x 80 cm) with water column kept at 10 cm. Tanks were dived equally into one-half black (42 cm x 50cm x 40 cm) and one half white (42 cm x 50cm x 40 cm) compartments. Each perch was introduced into the center of each scototaxis tank during which behavior was recorded for 12 min with a SONY Handycam HDR-PJ50VE. This 12 minute period consisted of a 2 min phase whereby recording was excluded as it was considered an acclimation period. The ten minute period of time following the acclimation period was used to observe perch behavior and analyze spatial use of the tank.

Following a previously developed protocol (Brodin et al. 2013), sociality and activity levels were observed in glass aquaria (30 cm x 50 cm x 50 cm; 10 cm water column) divided into three compartments with the observation fish contained in the middle compartment, a group of conspecific fish (social fish) contained in one of the adjacent compartments, and an empty compartment on the side opposite of the social fish compartment. The total time spent at distances from the shoal was multiplied with a zone-specific sociality factor (8, 6, 4, 2, 1, 0, -1, -2, -4, -6, -8). Activity was measured, simultaneously with sociality, as the number of fish to swim during the observation period. Similar to scototaxis trials, sociality trials also consisted of a 2 min acclimation period followed by a 10 min observation period.

Chemical Analyses

Stock solution of oxazepam and sertraline were prepared by dissolving the drugs in water, and then the respective amounts of stock solution added to the different exposure containers. The nominal target concentration in each of the treatments was 10 µg/L for each drug. This was also the target nominal concentration for each drug in the sertraline and oxazepam mixture treatments. Water samples were collected on day 0 and day 7 of the study. After collection, all samples were immediately stored at −20°C for subsequent analyses. Oxazepam and sertraline concentrations in water samples (exposed and controls) were determined by chemical analysis. A triple stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used as analytical system. For a detailed description of the analysis and pretreatment see Brodin et al. (2013, 2014).

Statistical Analysis

Prior to analysis, data was examined for normality and equivalence of variance. Kruskal-Wallis tests were used to analyze among treatment differences in behavioral traits of boldness and sociality as the distributions were non-normal. Student t-tests were used to analyze before and after exposure differences for each treatment. Chi-square tests were used to determine differences in activity among the treatments and between each observation period. All statistical analysis was performed using Sigma Plot 13.0 (Systat Software Inc., San Jose, CA, USA) and SPSS 24 (IBM, Armonk, NY, USA) statistical software.

Results

Behavioral Effects

Boldness of control and sertraline exposed fish significantly increased from pre-exposure behavioral observations (Day 0) to post exposure observations (Day 7) (Figure 12a). When boldness measurements were only analyzed for shy fish, or fish that remain in black for entire the entire observation period during pre-exposure behavioral trials, significant before/after differences were apparent across all treatments including those co-exposed with a predatory olfactory signaling cue (Figure 13). However, no significant differences were observed among any of the treatments after exposures (Figures 12 and 13).

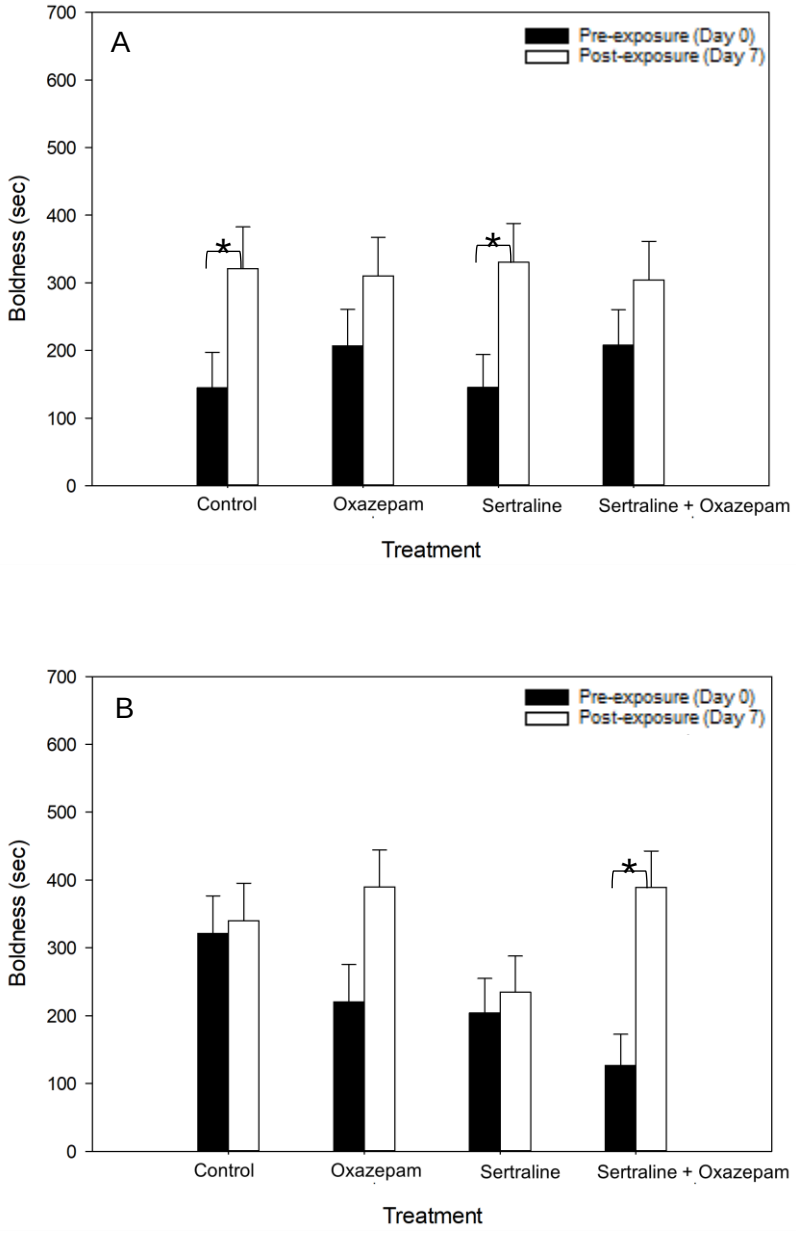

Figure 12: Boldness of perch, measured as time spent in the white half of the scototaxis tank during 10 min. A. Behavior of fish before (dark bars, day 0) and after (white bars, day 7) exposure to dissolved oxazepam, sertraline, or oxazepam and sertraline. B. Before and after effects of drugs co-administered with a predator signaling cue. *$p < 0.05$. n = 24-30.

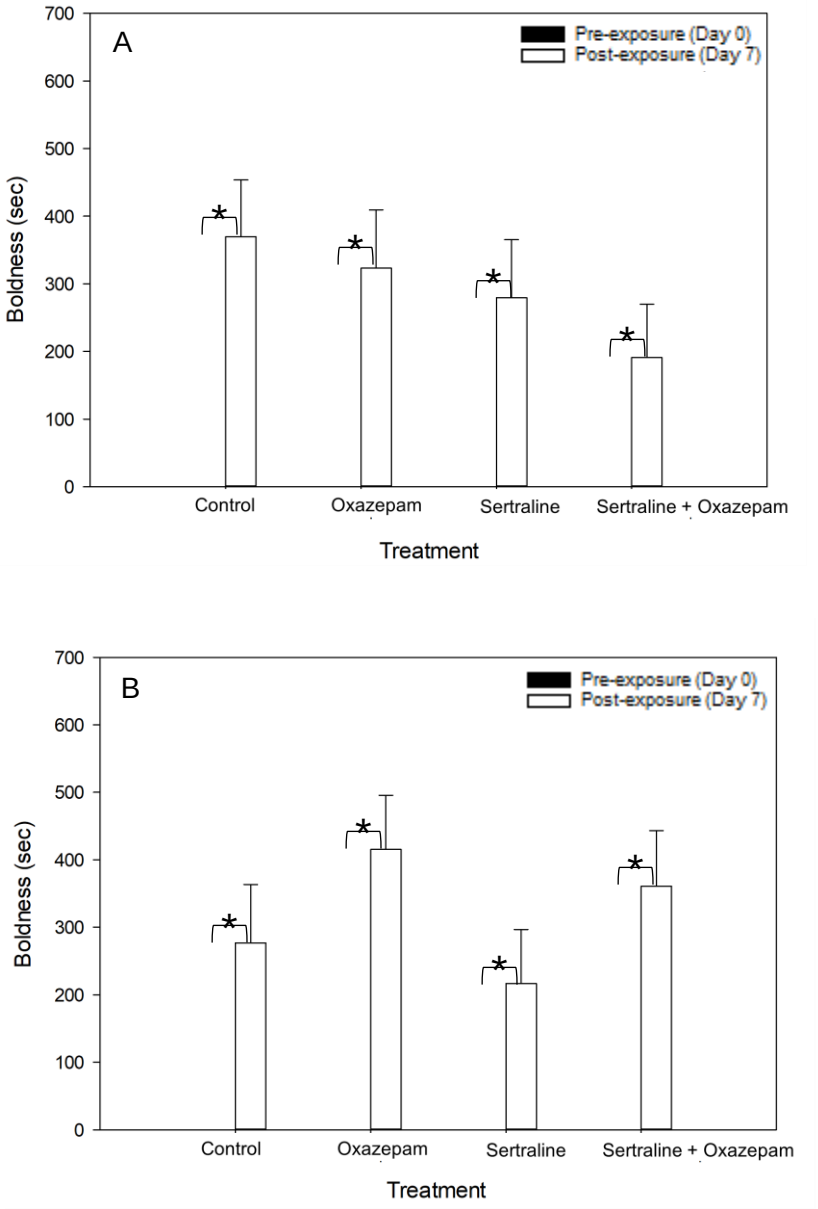

Figure 13: Boldness of 13 randomly selected shy perch, measured as time spent in the white half of the scototaxis tank during 10 min. A. Behavior of fish before (dark bars, day 0) and after (white bars, day 7) exposure to dissolved oxazepam, sertraline, or oxazepam and sertraline. B. Before and after effects of drugs co-administered with a predator signaling cue. *$p < 0.05$, $n = 24$-30.

Only a fraction of fish in each exposure group swam during pre-exposure trials (Figure 14). No observable differences in activity were apparent during pre-exposure trials, but after exposures a significantly greater number of sertraline fish swam compared to control fish. This increased activity was not apparent in sertraline exposed fish that were co-exposed to oxazepam or a predatory olfactory signaling cue.

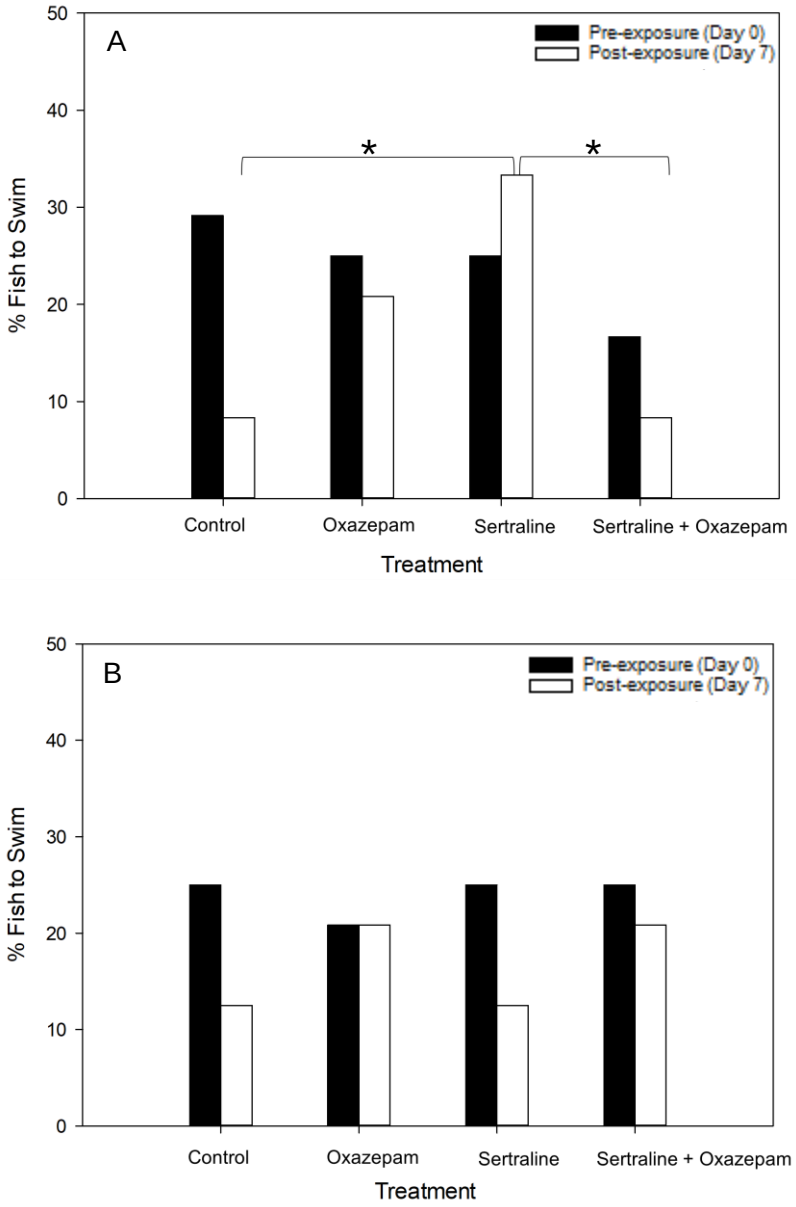

Figure 14: Activity of perch, measured as proportion of fish to swim during 10 min observation period. A. Behavior of fish before (dark bars, day 0) and after (white bars, day 7) exposure to dissolved oxazepam, sertraline, or oxazepam and sertraline. B. Before and after effects of drugs co-administered with a predator signaling cue. *$p < 0.05$, n=24.

Figure 15 summarizes sociality measurements of each treatment before and after the 7-day exposure treatment period. None of the drug treatments significantly effected perch sociality behavior. Similarly, predator cue treatments did not affect fish sociality behaviors.

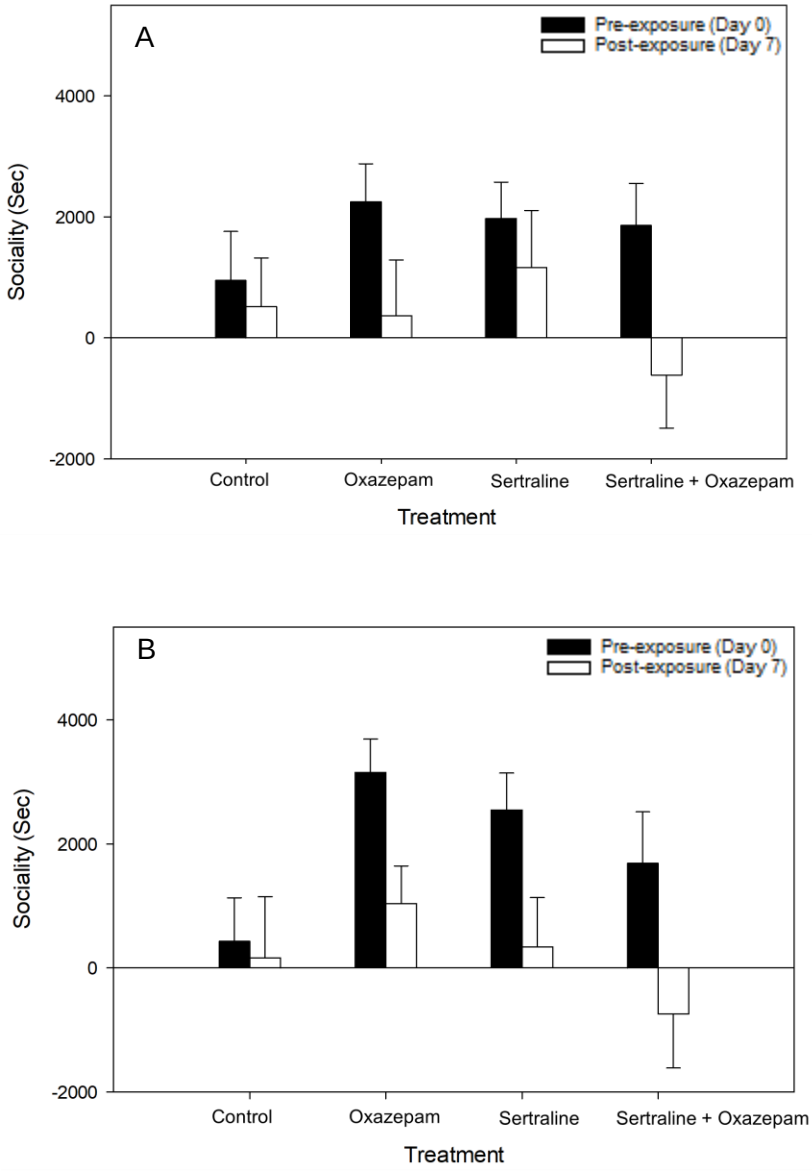

Figure 15: Sociality of perch, measured as cumulative time spent close to a group of conspecifics. A. Behavior of fish before (dark bars, day 0) and after (white bars, day 7) exposure to dissolved oxazepam, sertraline, or oxazepam and sertraline. B. Before and after effects of drugs co-administered with a predator signaling cue. *$p < 0.05$, $n = 24$.

Fish size, which is typically correlated with fish age, can potentially influence fish behavior Hellstrom et al., (2017, 2016) as well as behavioral sensitivity (Kristofco et al., 2016) to drug exposure. In this study, there were no significant differences in fish weight (control: 6.16 ± 1.28 g; control with predator cue: 6.40 ± 1.55 g; oxazepam: 6.34 ± 1.40 g; Oxazepam with predator cue: 6.17 ± 1.27 g; Sertraline: 6.02 ± 1.20 g; sertraline with predator cue: 6.24 ± 1.47 g; sertraline and oxazepam: 6.57 ± 1.58 g; sertraline and oxazepam with predator cue; 6.28 ± 1.51 g, mean weight ± SD) or length (control: 8.82 ± 0.59 cm; control with predator cue: 8.75 ± 0.69 cm; oxazepam: 8.93 ± 0.75 cm; oxazepam with predator cue: 8,75 ± 0.69 cm; sertraline: 8.78 ± 0.75 cm; sertraline with predator cue: 8.82 ± 0.73 cm; sertraline and oxazepam: 9.07 ± 0.83 cm; sertraline and oxazepam with predator cue: 8.79 ± 0.86 cm, mean length ± SD) among each of the treatments. Therefore, fish size is not expected to influence the results of this study.

Analytical results

Water samples were collected from each treatment to verify nominal exposure levels via LC/MS/MS. Sertraline was detected far below nominal (10 µg/L) values at 0.04 µg/L. Oxazepam, on the other hand was detected at 5.5 µg/L, an average value that is closer to nominal (10 µg/L) levels.

Discussion

Oxazepam and Sertraline are ubiquitous neuro active compounds that exhibit the ability to effect fish behaviors at dilute water concentrations (Brodin et al. 2013; Brodin et al. 2017; Valenti et al. 2012). These drugs have been detected in the environment concurrently, yet there is little understanding of their interactive effects on fish behavior.

Herein, the goal of this study was to examine the interactive effects of these two compounds on important fish behaviors in the presence of predatory olfactory signaling cues.

When conducting behavioral assays with fish models, it is important to consider differences between lab reared and wild fish populations. Although useful for answering important questions about the effects of psychoactive compounds on fish behavior, lab reared fish models cannot be anticipated to exhibit similar behaviors and behavioral responses to drug exposure as those of wild fish populations (Alvarez and Nicieza 2003; Johnsson and Abrahams 1991; Wright et al. 2006). Therefore, this study observed behaviors of perch taken from the wild. When observing boldness of these fish, it is apparent that time of observation had a greater influence over perch behavior than drug treatment. Boldness of control fish was significantly greater during observations after the exposure period compared to observations before the exposure period. When observations of only shy fish from each treatment group were analyzed, before and after exposure increase in boldness occurred across all treatments.

The cause behind these time related effects warrant further research and consideration as these effects could easily influence experimental results and interpretation. For example, shifts in behavior over time may over shadow drug effects thereby inhibiting one's ability to identify important drug related differences among groups. Previous research observing boldness of wild caught perch populations demonstrated no effects of time on perch boldness. However, the boldness measurements performed in these experiments were assessed using a different behavioral protocol. Rather than using the sototaxis trial, the authors measured boldness as latency to enter an open environment (Brodin et al. 2013). Scototaxis measurements have also been observed in wild caught

roach, but no before/after differences were observed in this study either (Brodin et al. 2017).

Oxazepam and other benzodiazepines are positive allosteric modulators of the γ-aminobutyric acid (GABA)-A receptor, a ligand-gated chloride-selective ion channel. Binding of the GABA-A receptor induces a conformational change in the receptor's chloride ion channel that hyperpolarizes the cell and ultimately reduces the excitability of neurons throughout the central nervous system. This inhibitory action accounts for a calming effect in the brain, which is why benzodiazepines are commonly prescribed to treat anxiety, insomnia, and epilepsy (Griffin et al. 2013). GABA receptors have been identified in fish brains, and a number of studies have demonstrated that oxazepam can influence important fish behaviors (Brodin et al. 2014; Hellström et al. 2016; Wilkinson et al. 1983). In particular, oxazepam has influenced sociality, boldness, and activity of wild caught perch and other fish species at levels similar to the ones used in this study (Brodin et al. 2013; Brodin et al. 2017; Klaminder et al. 2014). In the current study, however, oxazepam had no significant effects on any of the perch behaviors. One likely contributor to this discrepancy is differences in fish ages between the perch used in the current study and those used in previous studies. This study utilized fish with average ± SD weight and length of 6.16 ± 1.28 g and 8.82 ± 0.59 cm, respectively. Experiments by Brodin et al. (2013), on the other hand, used smaller perch with respective average ± SE weight and length of 2.9 ± 0.1 g and 6.9 ± 0.1 cm. The older larger fish in this study were less active and social than those used in previous studies by Brodin et al. (2013). Therefore, the older fish models in this study may be less susceptible to predation and thus do not demonstrate predator avoidance behaviors to the same degree as younger perch. Studies performed by

Hellström et al. (2016) and Helstrom et al. (2107) demonstrated that juvenile salmon (high risk of predation) displayed significantly altered behaviors when exposed to oxazepam, but behaviors of adults (low risk of predation) remained un-altered when exposed to similar concentrations of oxazepam. Another age-related factor that could be influencing the discrepancies between this study and previous research with oxazepam is age related drug sensitivity. Previous work by Kristofco et al. 2016 demonstrated that younger zebrafish larvae were less sensitive to behavioral effects caused by diphenhydramine and diazinon compared to older fish larvae. Thus, physiological factors, such as differences in metabolism, could also play an important role in the observed age-related differences in response to oxazepam exposure.

SSRIs elicit therapeutic effects through serotonin transporter (SERTs) binding and inhibition in presynaptic neurons. SERT inhibition in synapses results in increased serotonin (5-HT) binding in post synaptic nerve cells causing a reduction in anxiety (Frazer 2001). SSRIs have been demonstrated to interact with drug targets and have a number of whole organism effects in a wide variety of aquatic species, but among the most sensitive measured effects are behaviors (Brooks et al. 2003; Gould et al. 2007; McDonald 2017; Painter et al. 2009; Valenti et al. 2012). Many of these measured behaviors have important ecological implications as they are critical in fish predator avoidance, nest building, and reproduction (Dzieweczynski and Hebert 2012; Martin et al. 2017; Painter et al. 2009; Pelli and Connaughton 2015; Saaristo et al. 2017; Sebire et al. 2015; Valenti et al. 2012). In their study with sertraline, Valenti et al. (2012) observed binding at the therapeutic target (SERT) in brain tissues and subsequent reduced shelter seeking of exposed male fathead minnows. In the current study, sertraline had no significant effects on perch sociality or

boldness, but the SSRI did elicit significant increases in activity of exposed perch. A greater proportion of sertraline exposed perch swam compared to control fish. Fish activity is an important behavior in relation to foraging and predator avoidance. Fish that are more active, will have more opportunity to find food, but these fish will also be more conspicuous, and thus more susceptible to predation.

The sertraline and oxazepam co-exposure treatment, on the other hand, had a significantly lower proportion of fish to move compared to the sertraline treatment and the proportion of co-exposed fish to move was equal to that of the control treatment. It appears that oxazepam diminishes the activity of effects of sertraline. Research with mice using the forced swimming test, a procedure widely accepted for its predictive value of anti-depressant activity in humans, demonstrated that benzodiazepines can inhibit the behavioral activity of SSRIs (Da-Rocha et al.1997). One of the possible mechanisms behind this inhibition in mice is the reinforcement of negative control exerted by GABAergic neurons on other neurotransmitter systems. That is, benzodiazepines reduce the activity of noradrenaline, dopamine, and 5-HT neurons, and therefore, these drugs can reverse the antimobility effects caused by SSRIs (Haefely et al., 1983). Weather such drug interactions between benzodiazepines and SSRIs is occurring in fish species warrants further research.

Detecting the presence of predators and responding to predator stimuli are critical for prey fish survival in the wild. In many aquatic systems, prey fish use visual, olfactory, tactile, and auditory cues to detect and appropriately respond to predator threats (Smith 1992). While both vision and olfaction are recognized as the most important sensory cues used by prey in shallow-water environments, chemosensory inputs might be the most

important source of information about predator presence in darkness and for hiding animals (Mikheev et al. 2006; Ylönen et al. 2007). Thus, predator chemosensory inputs are critical aspects that influence prey fish behavior in the wild, but these stimuli are typically not accounted for in lab based behavioral studies. For this reason, this study aimed to understand how predator olfactory cues in the form of water taken from pike aquaria may influence fish responses to exposure to each of the drug treatments. Although no significant differences were observed between control fish and fish exposed to predator stimuli, sertraline exposed fish treated with a predator stimulus did not demonstrate the significant increases in activity that sertraline exposed fish did. These observations suggest that predator olfactory stimuli may be counteracting the effects of sertraline on perch activity levels. The mechanism behind this possible interaction is not understood and warrants further consideration.

Conclusions

The results in the current study collectively demonstrate the importance of accounting for time related factors that can affect fish boldness behavioral measurements. These results further demonstrate the importance of accounting for factors present in urbanized aquatic ecosystems that can influence experimental results of lab studies. Future research should aim to identify the causes effecting the time related behavioral influences observed.

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References

Alvarez D, Nicieza A. 2003. Predator avoidance behaviour in wild and hatchery-reared brown trout: The role of experience and domestication. Journal of Fish Biology. 63(6):1565-1577.

Arnold KE, Brown AR, Ankley GT, Sumpter JP. 2014. Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. The Royal Society.

Boxall AB, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T. 2012. Pharmaceuticals and personal care products in the environment: What are the big questions? Environmental health perspectives. 120(9):1221-1229.

Brodin T, Fick J, Jonsson M, Klaminder J. 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science. 339(6121):814-815.

Brodin T, Nordling J, Lagesson A, Klaminder J, Hellström G, Christensen B, Fick J. 2017. Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish,(rutilus rutilus). Journal of Toxicology and Environmental Health, Part A. 80(16-18):963-970.

Brodin T, Piovano S, Fick J, Klaminder J, Heynen M, Jonsson M. 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Phil Trans R Soc B. 369(1656):20130580.

Brooks BW. 2014. Fish on prozac (and zoloft): Ten years later. Aquatic Toxicology. 151:61-67.

Brooks BW, Berninger JP, Kristofco LA, Ramirez AJ, Stanley JK, Valenti TW. 2012. Pharmaceuticals in the environment: Lessons learned for reducing uncertainties in environmental risk assessment. Progress in molecular biology and translational science. Elsevier. p. 231-258.

Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, La Point TW. 2003. Aquatic ecotoxicology of fluoxetine. Toxicology letters. 142(3):169-183.

Connors KA, Valenti TW, Lawless K, Sackerman J, Onaivi ES, Brooks BW, Gould GG. 2014. Similar anxiolytic effects of agonists targeting serotonin 5-ht1a or cannabinoid cb receptors on zebrafish behavior in novel environments. Aquatic toxicology. 151:105-113.

Da-Rocha MA, Puech AJ, Thiébot M-H. 1997. Influence of anxiolytic drugs on the effects of specific serotonin reuptake inhibitors in the forced swimming test in mice. Journal of Psychopharmacology. 11(3):211-218.

Dzieweczynski TL, Hebert OL. 2012. Fluoxetine alters behavioral consistency of aggression and courtship in male siamese fighting fish, betta splendens. Physiology & behavior. 107(1):92-97.

Frazer A. 2001. Serotonergic and noradrenergic reuptake inhibitors: Prediction of clinical effects from in vitro potencies. The Journal of clinical psychiatry. 62:16-23.

Gould GG, Brooks BW, Frazer A. 2007. [3h] citalopram binding to serotonin transporter sites in minnow brains. Basic & clinical pharmacology & toxicology. 101(3):203-210.

Haefely W. 1983. Neuropharmacology of benzo-diazepines: Synaptic mechanisms and neural basis of action. The benzodiazepines: from molecular biology to clinical practice.21-66.

Hellström G, Klaminder J, Finn F, Persson L, Alanärä A, Jonsson M, Fick J, Brodin T. 2016. Gabaergic anxiolytic drug in water increases migration behaviour in salmon. Nature Communications. 7:13460.

Huggett D, Cook J, Ericson J, Williams R. 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Human and Ecological Risk Assessment. 9(7):1789-1799.

Johnsson JI, Abrahams MV. 1991. Interbreeding with domestic strain increases foraging under threat of predation in juvenile steelhead trout (oncorhynchus mykiss): An experimental study. Canadian Journal of Fisheries and Aquatic Sciences. 48(2):243-247.

Klaminder J, Hellström G, Fahlman J, Jonsson M, Fick J, Lagesson A, Bergman E, Brodin T. 2016. Drug-induced behavioral changes: Using laboratory observations to predict field observations. Frontiers in Environmental Science. 4:81.

Klaminder J, Jonsson M, Fick J, Sundelin A, Brodin T. 2014. The conceptual imperfection of aquatic risk assessment tests: Highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants. Environmental Research Letters. 9(8):084003.

Kristofco LA, Cruz LC, Haddad SP, Behra ML, Chambliss CK, Brooks BW. 2016. Age matters: Developmental stage of danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic Toxicology. 170:344-354.

Kümmerer K. 2010. Pharmaceuticals in the environment. Annual review of environment and resources. 35:57-75.

LaLone CA, Berninger JP, Villeneuve DL, Ankley GT. 2014. Leveraging existing data for prioritization of the ecological risks of human and veterinary pharmaceuticals to aquatic organisms. Phil Trans R Soc B. 369(1656):20140022.

Little EE. 2002. Behavioral measures of environmental stressors in fish.

Martin CW, Fodrie FJ, Heck KL, Mattila J. 2010. Differential habitat use and antipredator response of juvenile roach (rutilus rutilus) to olfactory and visual cues from multiple predators. Oecologia. 162(4):893-902.

Martin JM, Saaristo M, Bertram MG, Lewis PJ, Coggan TL, Clarke BO, Wong BB. 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environmental pollution. 222:592-599.

Maximino C, De Brito TM, de Mattos Dias CAG, Gouveia Jr A, Morato S. 2010. Scototaxis as anxiety-like behavior in fish. Nature protocols. 5(2):209.

McDonald MD. 2017. An aop analysis of selective serotonin reuptake inhibitors (ssris) for fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 197:19-31.

Metcalfe C, Miao X-S, Hua W, Letcher R, Servos M. 2004. Pharmaceuticals in the canadian environment. Pharmaceuticals in the environment. Springer. p. 67-90.

Mikheev V, Wanzenböck J, Pasternak A. 2006. Effects of predator-induced visual and olfactory cues on 0+ perch (perca fluviatilis l.) foraging behaviour. Ecology of Freshwater Fish. 15(2):111-117.

Painter MM, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, Furlong ET, Schultz MM, Schoenfuss HL. 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (pimephales promelas). Environmental Toxicology and Chemistry. 28(12):2677-2684.

Pelli M, Connaughton VP. 2015. Chronic exposure to environmentally-relevant concentrations of fluoxetine (prozac) decreases survival, increases abnormal behaviors, and delays predator escape responses in guppies. Chemosphere. 139:202-209.

Saaristo M, Brodin T, Balshine S, Bertram MG, Brooks BW, Ehlman SM, McCallum ES, Sih A, Sundin J, Wong BB. 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proc R Soc B. 285(1885):20181297.

Saaristo M, McLennan A, Johnstone CP, Clarke BO, Wong BB. 2017. Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (poecilia reticulata). Aquatic toxicology. 183:38-45.

Sebire M, Davis JE, Hatfield R, Winberg S, Katsiadaki I. 2015. Prozac affects stickleback nest quality without altering androgen, spiggin or aggression levels during a 21-day breeding test. Aquatic Toxicology. 168:78-89.

Smith RJF. 1992. Alarm signals in fishes. Reviews in Fish Biology and Fisheries. 2(1):33-63.

Steele WB, Mole RA, Brooks BW. 2018. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neuro-stimulant caffeine. Journal of visualized experiments: JoVE. (137).

CHAPTER SIX

Conclusions

With a large and ever-growing number of chemicals in commerce lacking appropriate toxicity data for safety evaluation, there remains a need to adopt methods to efficiently and effectively screen compounds for toxicity to protect human health and the environment (Andersen and Krewski 2009). However, current testing paradigms with traditional models are time and resource intensive. Further, traditional endpoints, such as mortality, may not capture MoA relevant effects of chemicals (Connors et al. 2014). Pharmaceuticals, for example, are ubiquitous aquatic contaminants in urban water sheds that are designed to be biologically active, but minimally toxic (Arnold et al. 2014). In fish, several antidepressant compounds typically cause mortality at much higher concentrations than would otherwise be detected in surface waters but behavioral effects have been measured at low, environmentally relevant concentrations for several of these chemicals (Brodin et al. 2013; Brodin et al. 2017; Painter et al. 2009). Indeed, a large variety of different types of compounds have been demonstrated to effect fish behaviors at much lower levels than those that effect growth or cause mortality (Gerhardt 2007; Little and Finger 1990; Robinson 2009). Given the importance of behaviors for organism survival and that behaviors are sensitive measurements that can be informative of chemical MoA, behavioral endpoints demonstrate relevance in aquatic toxicology. However, due to the time involvement and difficulty in producing accurate quantifiable data from human behavioral observation, conducting behavioral studies has been

challenging (Gerhardt 2007). Advances in technology offer solutions to alleviate many of these challenges.

Approaches in the biomedical sciences have leveraged embryonic and larval fish behaviors in conjunction with automated tracking technologies to identify therapeutic targets for thousands of novel compounds (Kokel et al. 2010; Rihel et al. 2010). Such an approach demonstrates potential in the environmental sciences to sensitively, rapidly, and diagnostically screen chemicals for toxicity. Standardized guidelines toxicity guidelines with larval fish models may be adapted for behavioral studies with automated platforms. While the larval zebrafish is a popular model in the biomedical sciences that is growing in popularity as models in ecotoxicology, the larval fathead minnow is a common ecological model, but has seen considerably less attention than the zebrafish in behavioral studies. There is, however, a growing interest in using other species, including the ecologically important fathead minnow model, in automated behavioral platforms to understand organism responses to aquatic contaminants (Kristofco et al, 2016; Colón-Cruz et al, 2018; Steele et al, 2018). In fact, decades of experience in aquatic toxicology and behavior ecology promise to reciprocally inform and advance behavioral studies with fish models within biomedical efforts.

In this dissertation methods, methods were developed to incorporate behavioral measurements with an automated tracking platform into toxicity assays following standardized guidelines (OECD FET and EPA WET) with larval zebrafish and fathead minnows. Chapter two demonstrated that each fish model displayed consistent responses to changing photoperiods. These responses, however, were markedly different between each of the fish models. The developed methods were then tested with caffeine, a

common aquatic contaminant and model neurostimulant. Caffeine affected larval fish photomotor and locomotor activities at dilute levels far below those causing mortality, indicating that the developed methods can be used to effectively examine behavioral effects of various chemicals.

Because understanding chemical MOAs is critical step in pharmacology, toxicology, and hazard and risk assessment, methods developed in Chapter two were used to investigate the use of behavioral profiles as a means of gaining insight into chemical bioactivity. Both larval fish models were exposed to seven chemicals (-heptanol, phenol, R-(-)-carvone, citalopram, diazinon, pentylenetetrazol (PTZ), and xylazine) each having distinctly different anticipated MOAs. Each of the compounds was classified according to Verharr et al. 1992 (Class I, Class II, Class III, and Class IV) and behavioral response following exposure were profiled to compare response patterns among each study compound. For zebrafish and fathead minnow larvae, distinctly different response patterns emerged for each of the chemicals indicating that behaviors maybe informative of chemical MOAs. Our results suggest that larval fish behaviors may be useful diagnostics to identify mechanisms and pathways associated with biological activities, and potentially adverse outcomes for chemicals lacking mechanistic data. Such efforts are particularly needed for class III and class IV compounds. Similar to class IV compounds, electrophiles (class III) can interact with biological targets through various mechanisms, including Michael addition, nucleophilic substitution and Schiff's base formation, each of which may influence electrophile interactions with susceptible biological nucleophiles (Harder et al., 2003).

To directly expand upon the findings in Chapter three and further examine if behavioral responses are associated with anticipated MOAs, I profiled larval fish behavioral responses after exposure to eight class III industrial compounds all sharing the same anticipated reactivity domain, nucleophilic substitution. Behavioral responses of zebrafish to each of the compounds, with the exception of styrene oxide, demonstrated refractory behavioral patterns in both dark and light conditions. These responses appeared much more similar to one another than those of the Chapter three study compounds having a variety of behavioral responses. Therefore results from Chapter four provide further support of chapter three conclusions, that larval fish behaviors can be informative of chemical MOA. Further research in this field can be used to develop tools to use larval fish photomotor and locomotor responses as a means to effectively identify hazardous attributes of molecules at the whole organism level.

Because coupled computational modeling and whole animal toxicity data can be used to develop guidelines for the selection and production of less hazardous substances, *in silico* estimates of electrophile reactivity were compared to lethal and sublethal (PMR) chemical effects of each S_N2 electrophile. Quantum mechanical models using GSH, a common surrogate for protein binding, indicate that acute and sublethal toxicity of each compound is likely driven by adduct formation and that computer-based modeling can effectively be used to computed reaction energies to experimental *in vivo* toxicity data. Thus, coupled quantum mechanical modeling and larval fish toxicity studies demonstrate potential to effectively identify attributes of less hazardous substances. While effects on larval fish PMRs demonstrate correlation with reactivity matrices, the precise protein

targets and mechanism by which reactive compounds elicit behavioral effects in larval fish models is not understood, and therefore, further research is warranted.

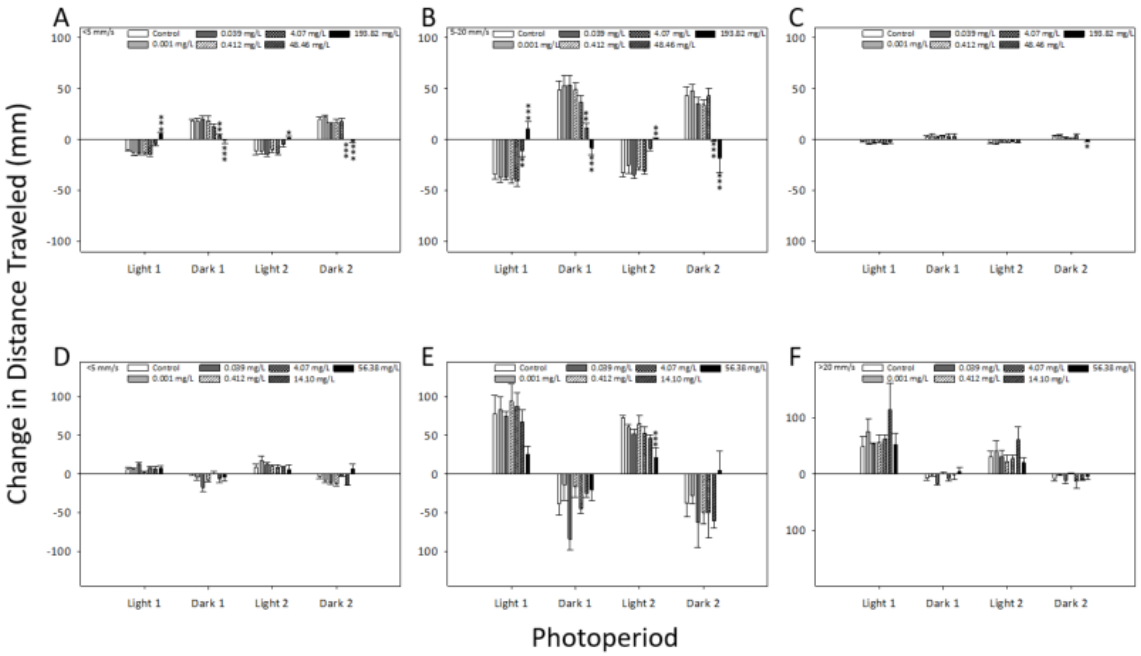
While lab raised fish models are useful for testing hypothesis and answering specific questions, these models cannot be anticipated to be representative of fish from natural habitats. Therefore, Chapter five extended efforts beyond studies on larval fish locomotor and photmotor behaviors to examine effects of neuroactive chemicals on important predator avoidance behaviors of perch taken from the wild. This study observed the individual and interactive effects of two common phsychiatric medications, sertraline and oxazepam, on perch sociality, boldness, and activity in response to predator olfactory signaling cues. In this study, time (pre-exposure observations vs post exposure observations) but not drug or predator cue treatments effected fish boldness. Further, sertraline significantly increased perch activity levels, but this increase was not observed when fish were co-exposed to oxazepam or a predator cue.

The results from Chapter five collectively demonstrate the importance of accounting for time related factors that can affect fish boldness behavioral measurements. Such effects have important implications in behavioral ecotoxicology because time related effects can potentially overshadow effects of study chemicals. These results further demonstrate the importance of accounting for factors present in urbanized aquatic ecosystems (drug mixture interactions and predatory pressures) that can influence experimental results of lab studies. Future research should aim to identify the causes effecting the time related behavioral influences on perch boldness.

APPENDICES

APPENDIX A

Supplemental Information for Chapter Two



Supplementary: Figure 19: Photomotor responses of zebrafish (A and B) and fathead minnow (C and D) across three speed thresholds. Zebrafish (A, B, and C) and fathead minnow larvae (D, E, and F) photomotor responses across three speed thresholds (Freezing: 20 mm/s) after 96hr exposure to caffeine. Photomotor responses of zebrafish and fathead minnow are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.

APPENDIX B

Supplemental Information for Chapter Three

Supplementary: Table 12: Nominal and measured concentrations for each of the study compounds during fathead minnow exposures. Measured concentrations are indicated in parenthesis.

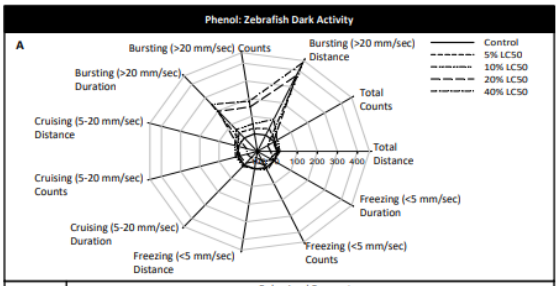
	Treatment (mg/L)													
	THV/TxHV (measured)		0.1% LC_{50} (measured)		1% LC_{50} (measured)		5% LC_{50} (measured)		10% LC_{50} (measured)		20% LC_{50} (measured)		40% LC_{50} (measured)	
Chemical	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr
1-Heptanol	-	-	-	-	-	-	2.015	2.015	4.029	4.029	8.058	8.058	16.116	16.116
Phenol	-	-	-	-	-	-	6.064	6.064	12.127	12.127	24.254	24.254	48.508	48.508
R-(-)-Carvone	-	-	-	-	-	-	2.930	2.930	5.860	5.860	11.720	11.720	23.440	23.440
Citalopram	0.02* (NA)	0.0203* (NA)	0.0093 (0.01)	0.0093 (0.01)	0.0933 (0.10)	0.0933 (0.11)	0.4665 (0.53)	0.4665 (0.54)	0.9330 (0.92)	0.9330 (0.93)	-	-	3.7318 (3.7)	3.7318 (3.7)
Diazinon	0.0005 (0.0003)	0.0005 (0.0003)	0.0115 (0.003)	0.0115 (0.003)	0.1146 (0.04)	0.1146 (0.03)	0.5730 (0.18)	0..5730 (0.16)	1.146 (0.3)	1.146 (0.26)	-	-	4.584 (1.2)	4.584 (1.0)
PTZ	-	-	2.79 (2.7)	2.79 (2.7)	27.86 (25)	27.86 (26)	139.28 (120)	139.28 (120)	278.56 (230)	278.56 (230)	-	-	1114.24 (1100)	1114.24 (1000)
Xylazine	0.0375 (0.04)	0.0375 (0.04)	0.0481 (0.05)	0.0481 (0.05)	0.4812 (0.53)	0.4812 (0.53)	2.4062 (2.4)	2.4062 (2.3)	4.8123 (4.7)	4.8123 (4.7)	-	-	19.2492 (21)	19.2492 (21)

*THV values is between 0.1% LC50 value and 1% LC50 value, so THV was not used as a treatment. THV: Therapeutic hazard value. TxHV: Toxic hazard value.

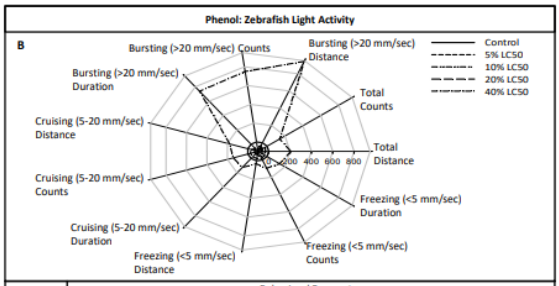
Supplementary: Table 13: Nominal and measured concentrations for each of the study compounds during zebrafish exposures. Measured concentrations are indicated in parenthesis.

	Treatment (mg/L)													
	THV/TxHV (measured)		0.1% LC_{50} (measured)		1% LC_{50} (measured)		5% LC_{50} (measured)		10% LC_{50} (measured)		20% LC50 (measured)		40% LC_{50} (measured)	
Chemical	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr	0 hr	96 hr
1-Heptanol	-	-	-	-	-	-	5.517	5.517	11.013	11.013	22.068	22.068	44.136	44.136
Phenol	-	-	-	-	-	-	5.823	5.823	11.646	11.646	23.292	23.292	46.584	46.584
R-(-)-Carvone	-	-	-	-	-	-	2.910	2.910	5.820	5.820	11.640	11.640	23.280	23.280
Citalopram	0.0203 (0.02)	0.0203 (0.03)	0.0384 (0.04)	0.0384 (0.05)	0.3841 (0.46)	0.3841 (0.43)	1.9205 (2.0)	1.9205 (2.0)	3.8410 (3.9)	3.8410 (4.2)	-	-	15.3640 (18)	15.3640 (19)
Diazinon	0.0005 (0.0005)	0.0005 (0.0004)	0.0114 (0.003)	0.0114 (0.003)	0.1141 (0.03)	0.1141 (0.03)	0.5706 (0.16)	0.5706 (0.17)	1.1411 (0.3)	1.1411 (0.26)	-	-	4.5644 (1.1)	4.5644 (1.0)
PTZ	NA	NA	2.65 (2.4)	2.65 (2.4)	26.45 (26)	26.45 (26)	132.25 (130)	132.25 (130)	264.49 (260)	264.49 (260)	-	-	1057.96 (1000)	1057.96 (1000)
Xylazine	0.0375* (NA)	0.0375* (NA)	0.0371 (0.04)	0.0371 (0.04)	0.3707 (0.39)	0.3707 (0.41)	1.8535 (1.8)	1.8535 (1.9)	3.7070 (3.8)	3.7070 (3.9)	-	-	14.8280 (16)	14.8280 (16)

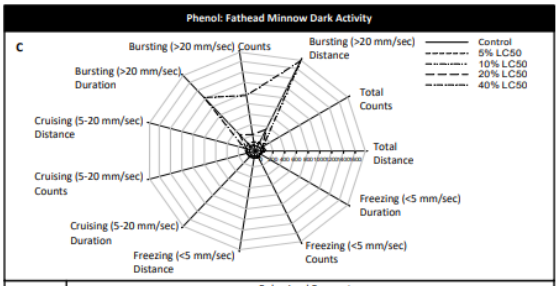
*THV values is between 0.1% LC50 value and 1% LC50 value, so THV was not used as a treatment. THV: Therapeutic hazard value. TxHV: Toxic hazard value.



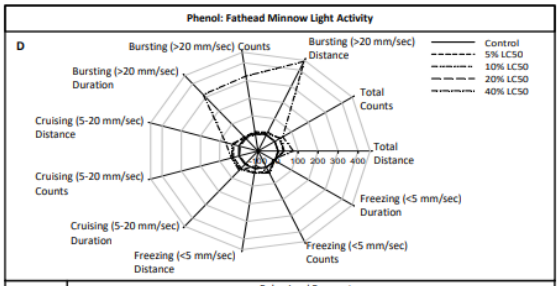
Phenol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	↑*	-	-	-	-	-	↓***	-	↓***
40% LC50	-	↓*	↑**	-	-	-	↓**	↓**	↓***	↓**	-



Phenol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	↓**	-	-
40% LC50	↑***	↑***	↑**	↑**	↑**	↑***	↑***	↑**	-	↑**	↑***

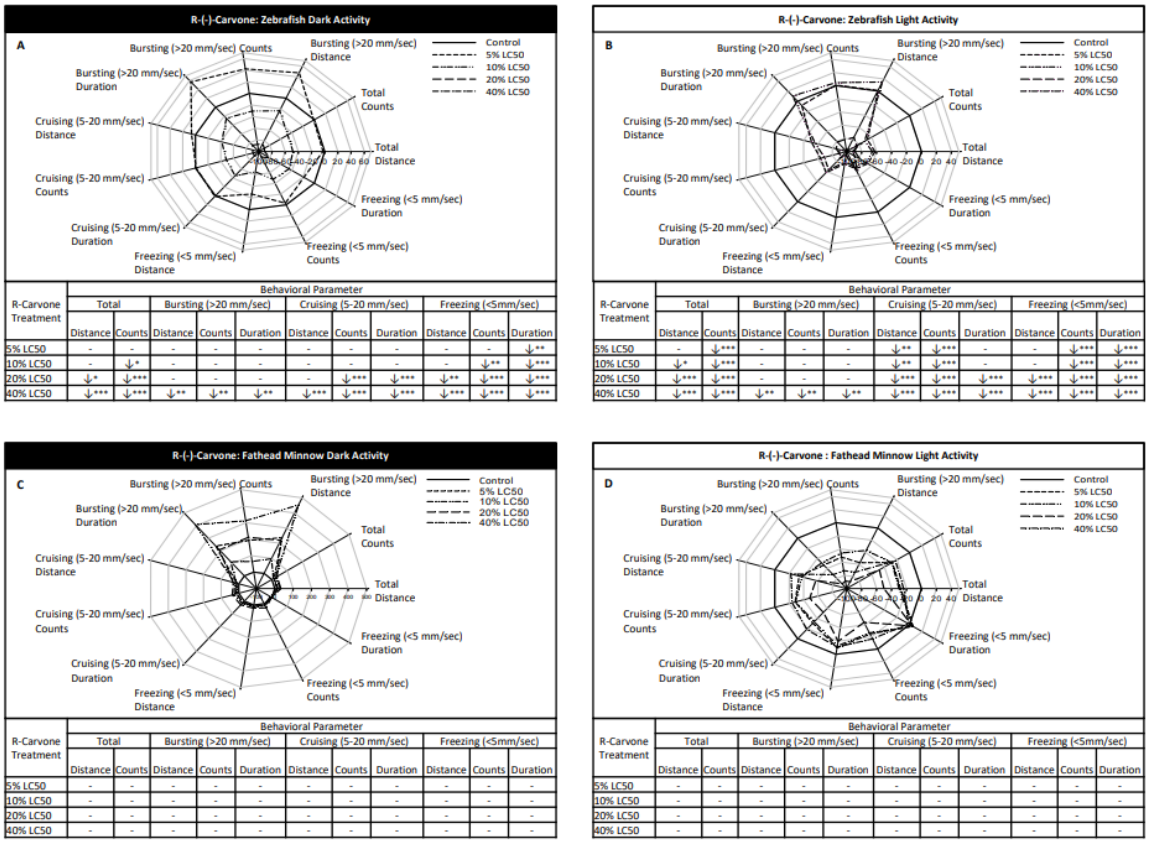


Phenol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	↑**	-	-
10% LC50	-	-	-	-	-	-	-	-	↑**	-	-
20% LC50	-	-	-	-	-	-	-	-	↑*	-	-
40% LC50	-	-	↑***	↑**	↑**	-	-	-	-	-	↓*



Phenol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	↑*	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	↑***	↑***	↑***	-	-	-	-	↑*	↓***

Supplementary: Figure 20: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to phenol. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



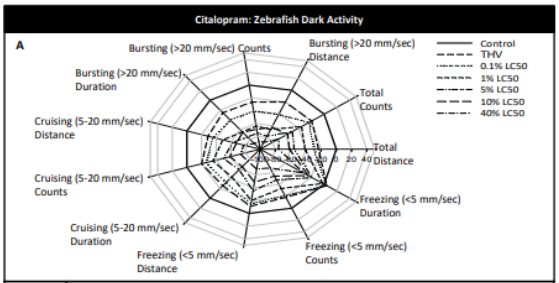
R-(-)-Carvone: Zebrafish Dark Activity											
R-Carvone Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	↓**
10% LC50	-	↓*	-	-	-	-	-	-	-	↓**	↓***
20% LC50	↓*	↓***	-	-	-	-	↓***	↓***	↓**	↓***	↓***
40% LC50	↓***	↓***	↓**	↓**	↓**	↓***	↓***	↓***	↓***	↓***	↓***

R-(-)-Carvone: Zebrafish Light Activity											
R-Carvone Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	↓***	-	-	-	↓**	↓***	-	-	↓***	↓***
10% LC50	↓*	↓***	-	-	-	↓**	↓***	-	-	↓***	↓***
20% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	↓***
40% LC50	↓***	↓***	↓**	↓**	↓**	↓***	↓***	↓***	↓***	↓***	↓***

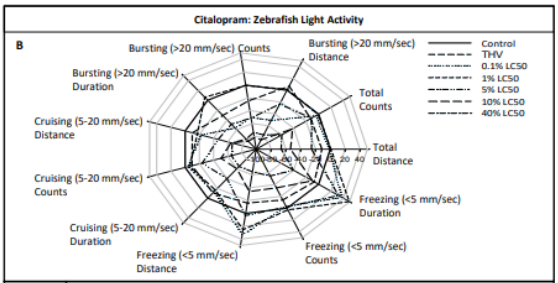
R-(-)-Carvone: Fathead Minnow Dark Activity											
R-Carvone Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

R-(-)-Carvone : Fathead Minnow Light Activity											
R-Carvone Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

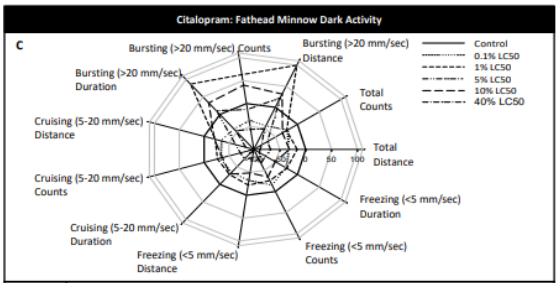
Supplementary: Figure 21: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to R-(-)-carvone. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



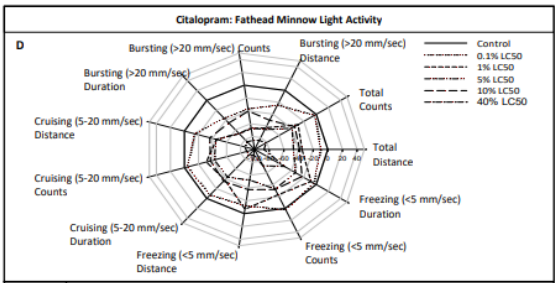
Citalopram Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
THV	↓**	↓*	-	-	-	↓***	↓*	↓**	-	↓*	-
0.1% LC50	↓***	↓**	↓*	↓**	↓*	↓***	↓**	↓***	-	↓**	-
1% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	-	↓***	-
5% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	-
10% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**
40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**



Citalopram Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
THV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	↓*	-	↓*	-	-	-	-	-	-
40% LC50	-	-	-	-	↓*	-	-	-	-	-	-

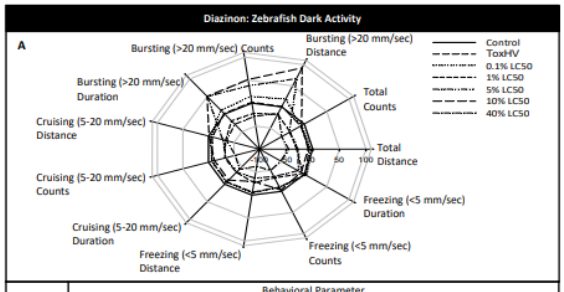


Citalopram Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	↓**	-	↓***
10% LC50	-	-	-	-	-	-	-	-	-	-	↓**
40% LC50	↓**	-	-	-	-	-	↓***	↓**	↓***	-	↓***

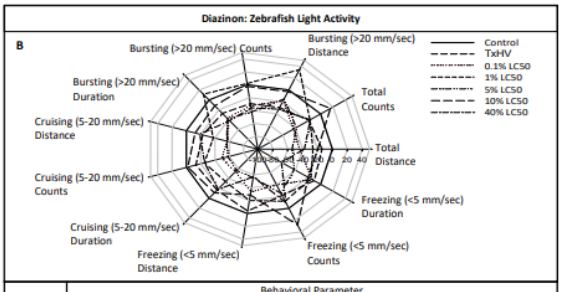


Citalopram Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	↓**	-	↓*	-	-	-	-	-	-
5% LC50	-	-	↓**	-	↓*	-	-	-	-	-	↓**
10% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓***	↓***	↓***	-	↓**	-	↓***	↓***	↓***	↓***	↓***

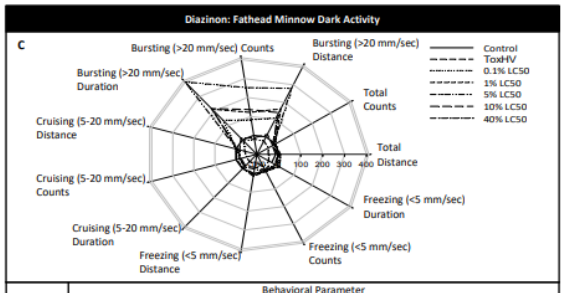
Supplementary: Figure 22: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to citalopram. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01. THV: Therapeutic Hazard Value.



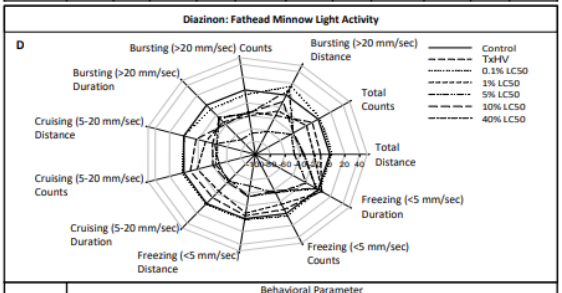
Diazinon Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
PHV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	↓*	-	-	-	-	-	-	-	↓*	-
5% LC50	-	↓*	↑**	-	-	-	↓*	-	↓**	↓**	-
10% LC50	-	-	↑***	-	-	-	-	-	↓*	-	-
40% LC50	↓**	↓***	-	-	-	-	↓***	↓**	↓***	↓***	↓*



Diazinon Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
PHV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

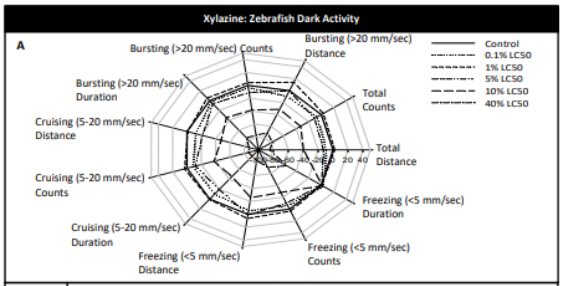


Diazinon Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
PHV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	↓**	-	-

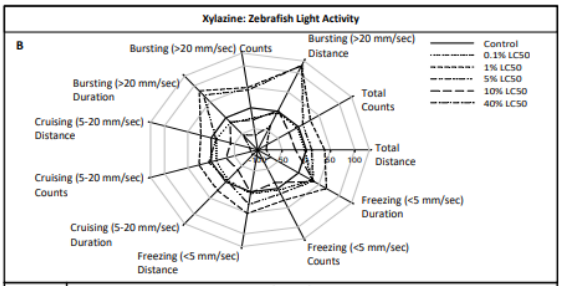


Diazinon Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
PHV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	↓**	↓**	-	-	-	↓**	↓**	↓**	-	↓**	-
10% LC50	↓**	↓**	-	-	-	-	↓**	↓**	-	↓**	-
40% LC50	↓***	↓**	-	↓***	-	↓*	↓***	↓**	↓**	↓**	-

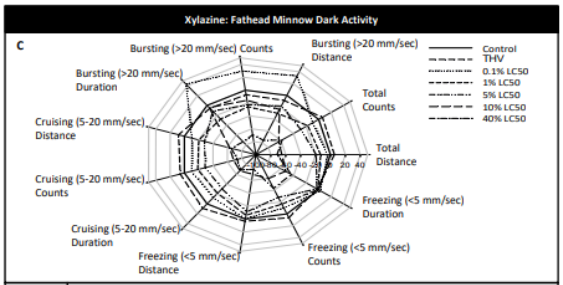
Supplementary: Figure 23: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to diazinon. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



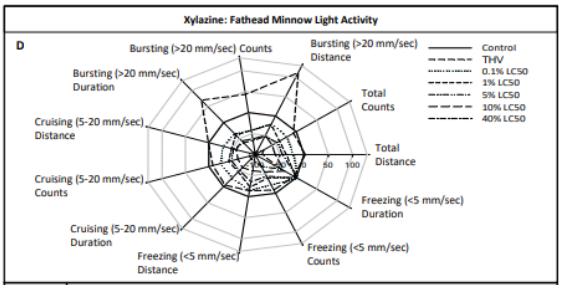
Xylazine Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓**	↓**	-
40% LC50	↓***	↓***	↓***	↓***	↓**	↓***	↓***	↓***	↓***	↓***	-



Xylazine Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	↑*	-	-	-	-	-	↑*	-	↑***
5% LC50	-	-	↑*	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	↓***

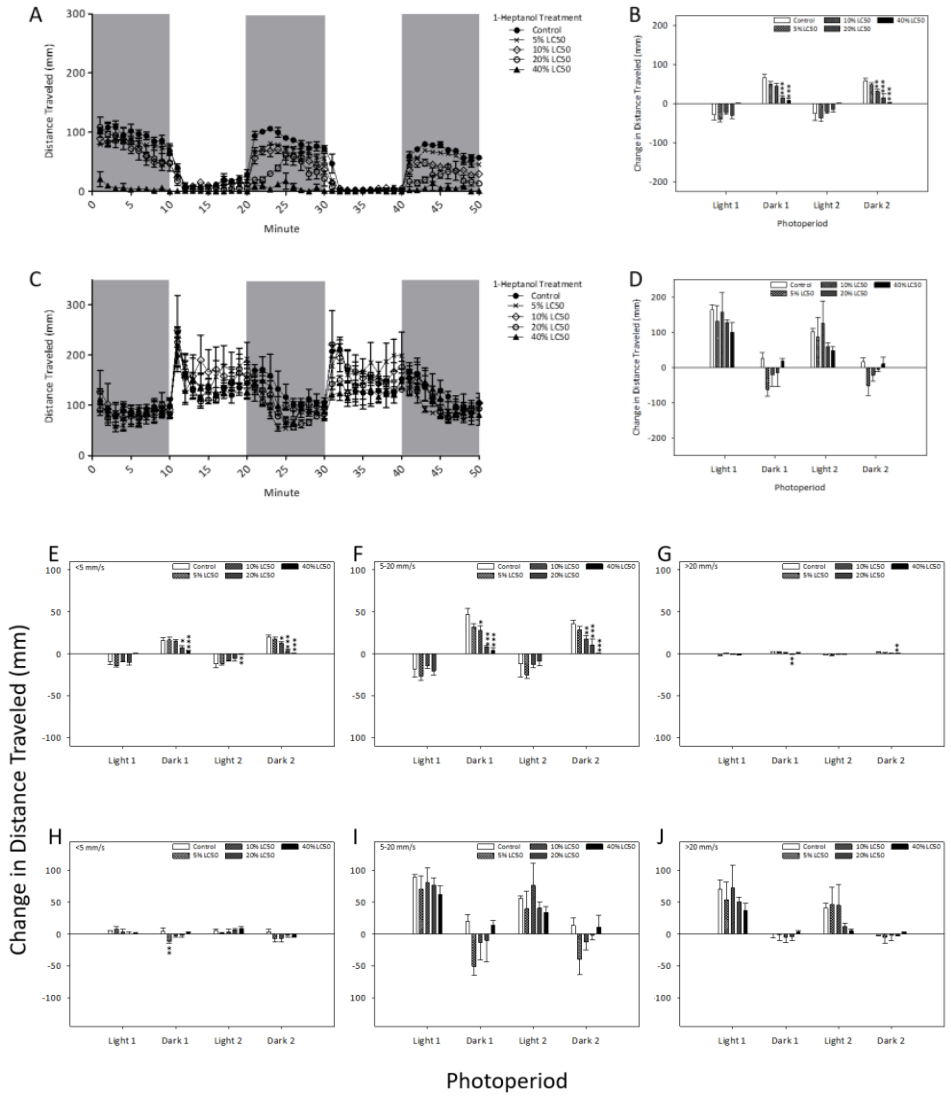


Xylazine Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
THV	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	↓*	-	-	-	-
10% LC50	↓***	↓***	-	-	-	↓**	↓***	↓***	↓***	↓***	-
40% LC50	↓***	↓***	-	↓**	-	↓***	↓***	↓***	↓***	↓**	-

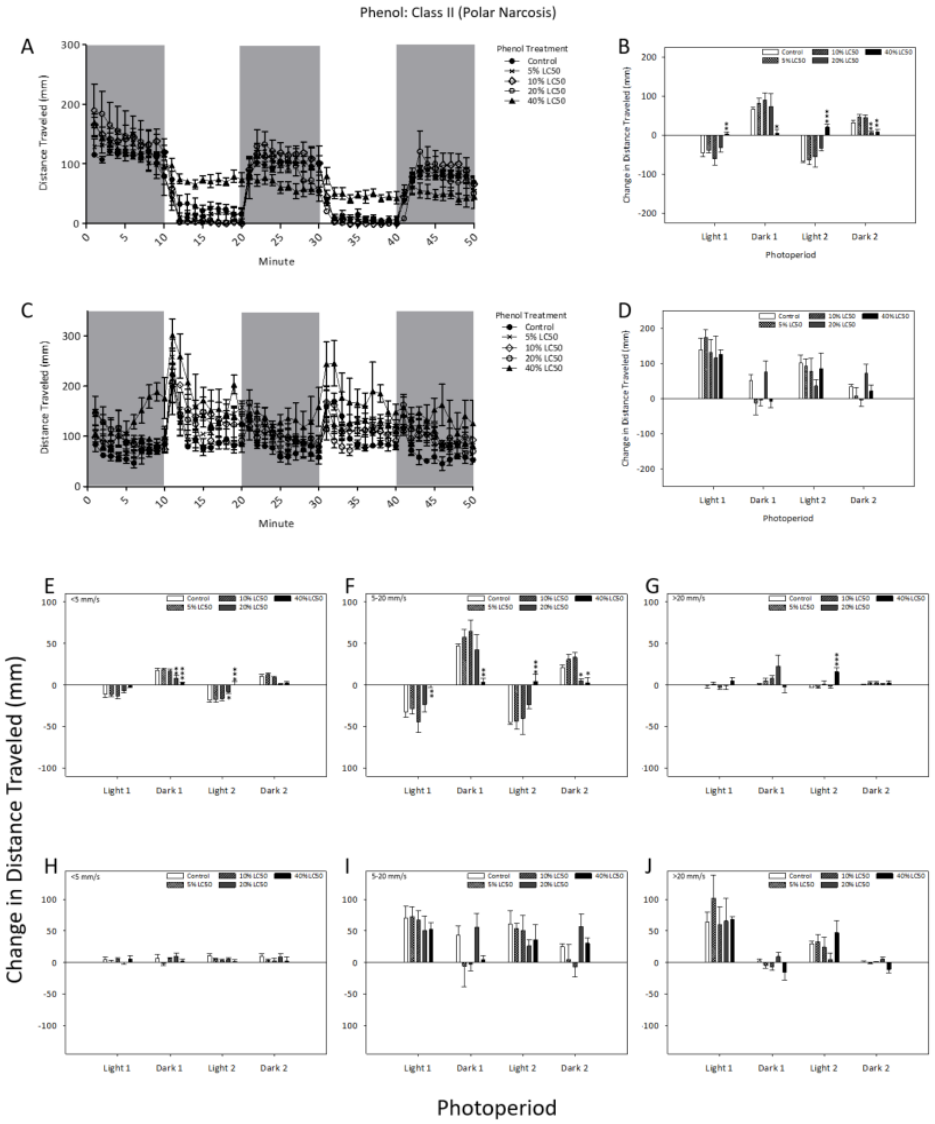


Xylazine Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
THV	-	-	↑**	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	↓**	-	-	-	↓*	↓**	-	↓**	↓**	-
5% LC50	-	↓***	-	↓*	-	↓**	↓***	-	↓***	↓**	-
10% LC50	↓*	↓***	-	↓*	-	↓**	↓***	-	↓***	↓***	↓***
40% LC50	↓**	↓***	↓*	↓***	↓**	↓***	↓***	↓**	↓***	↓***	↓***

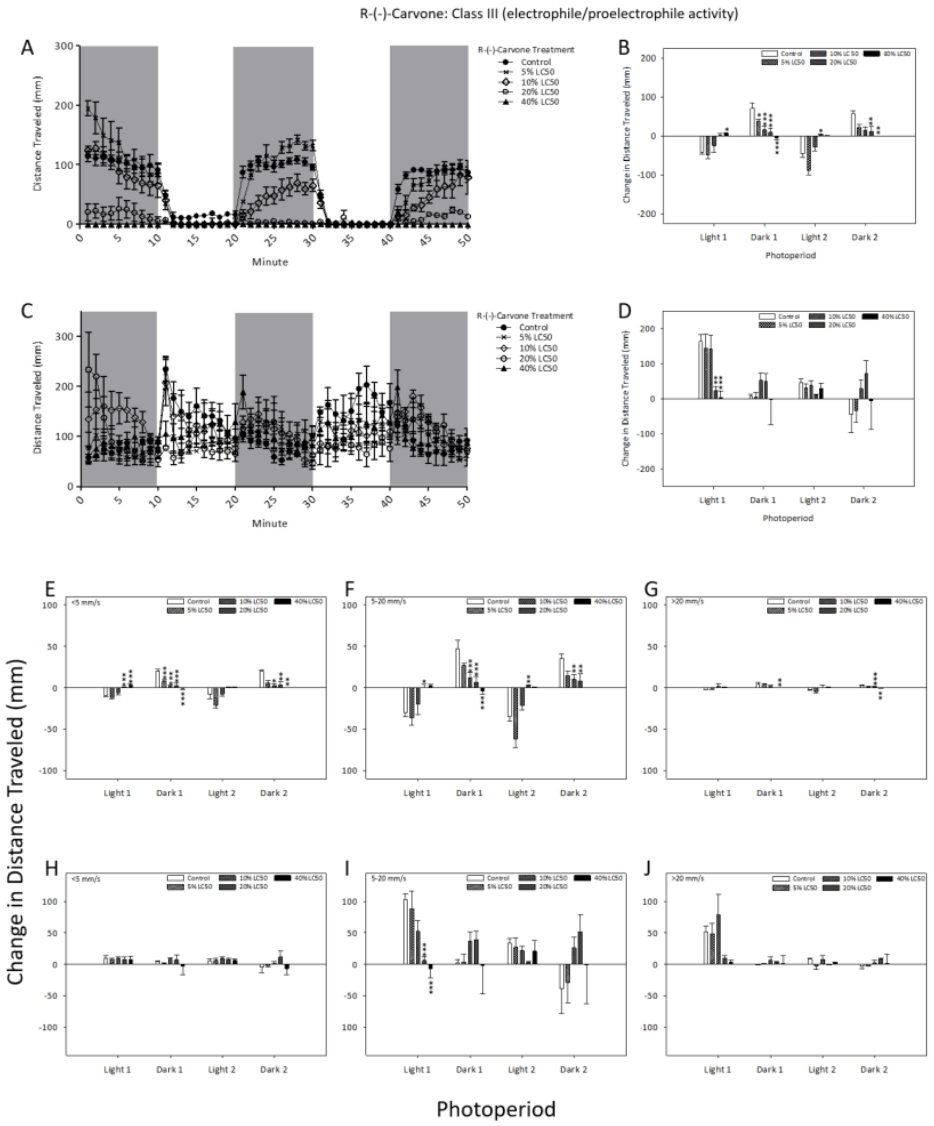
Supplementary: Figure 24: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to xylazine. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates each of 4 larvae) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01. THV: Therapeutic Hazard Value.



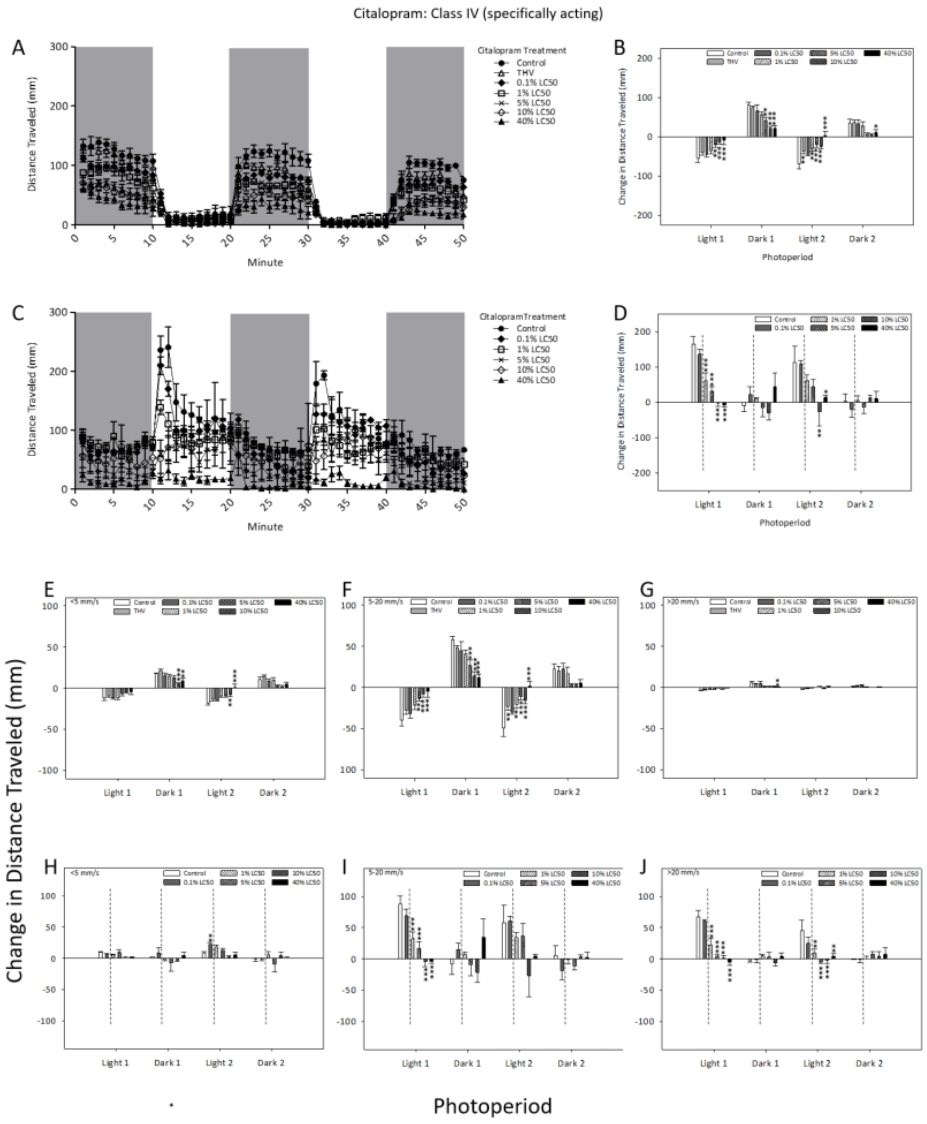
Supplementary: Figure 25: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to 1-heptanol. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



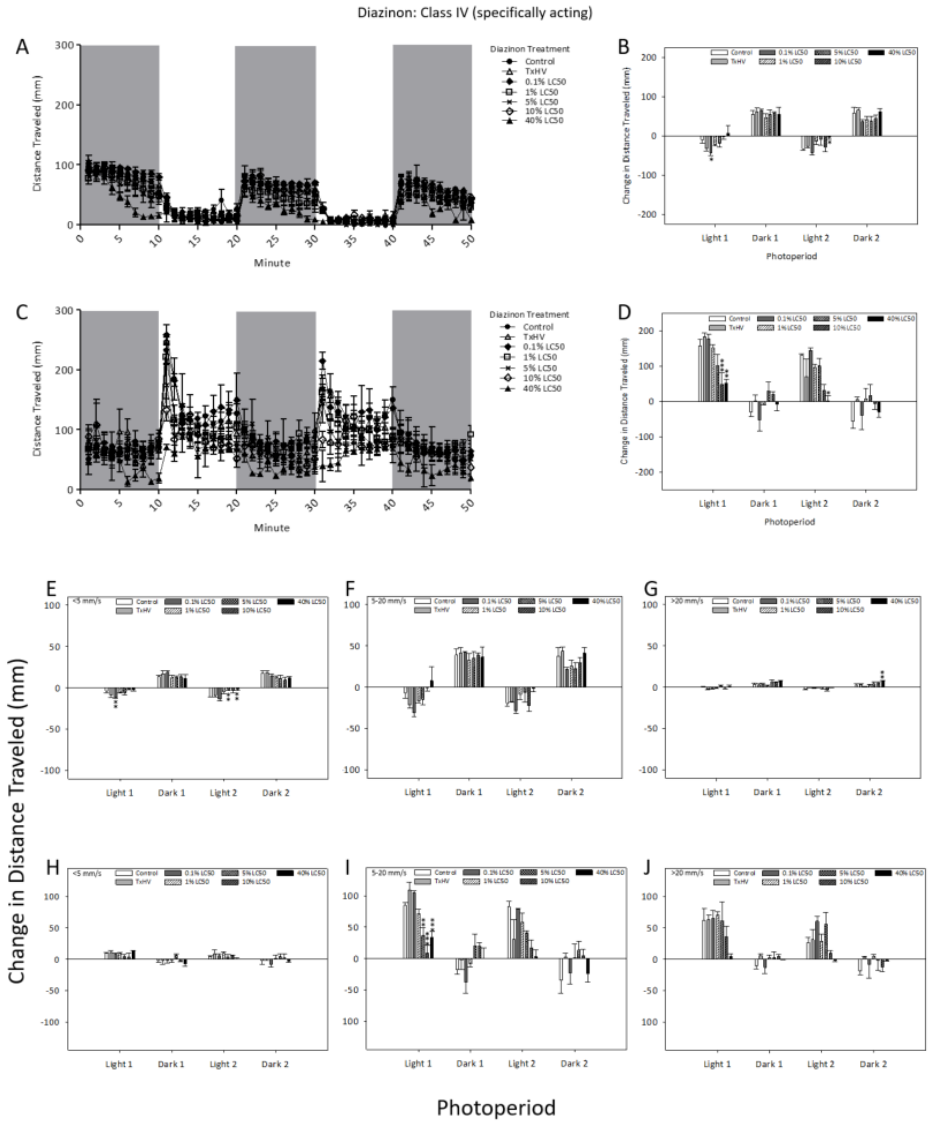
Supplementary: Figure 26: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to phenol. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01



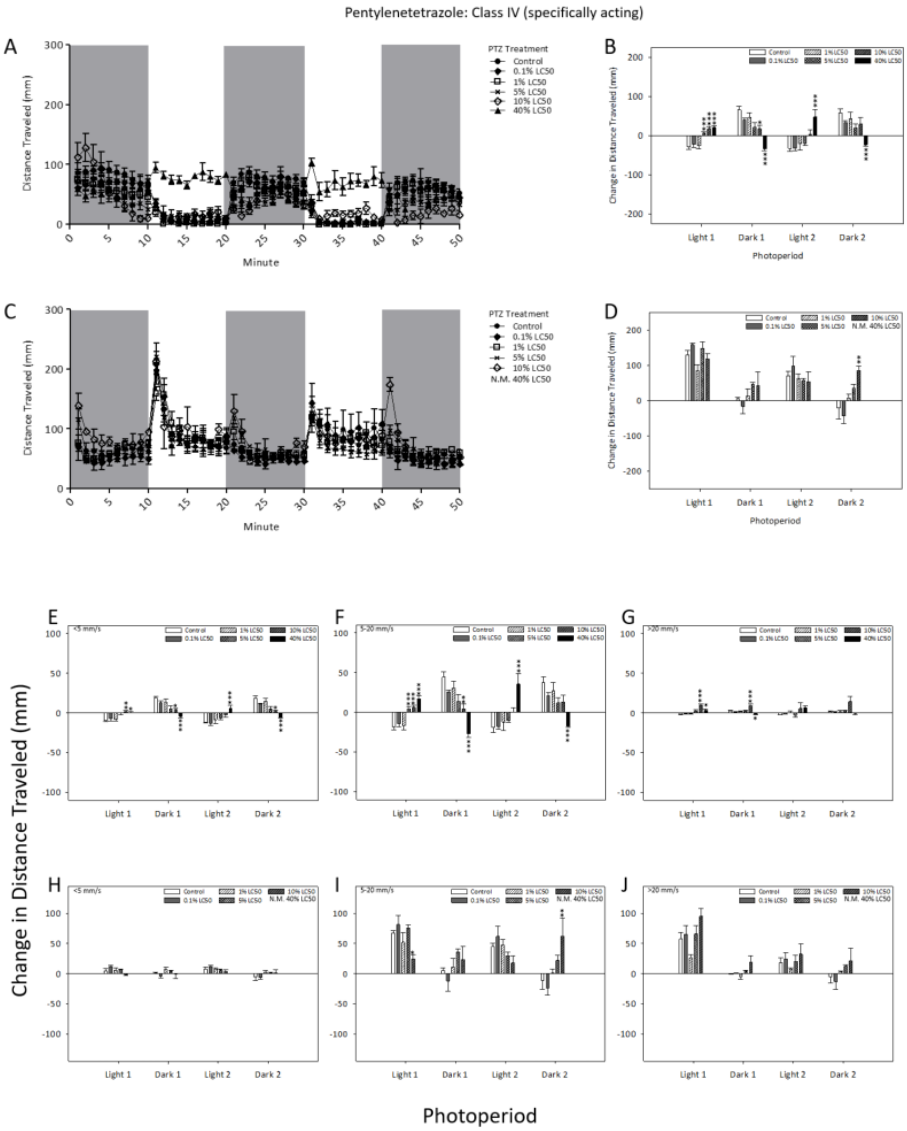
Supplementary: Figure 27: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to R-(-)-Carvone. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



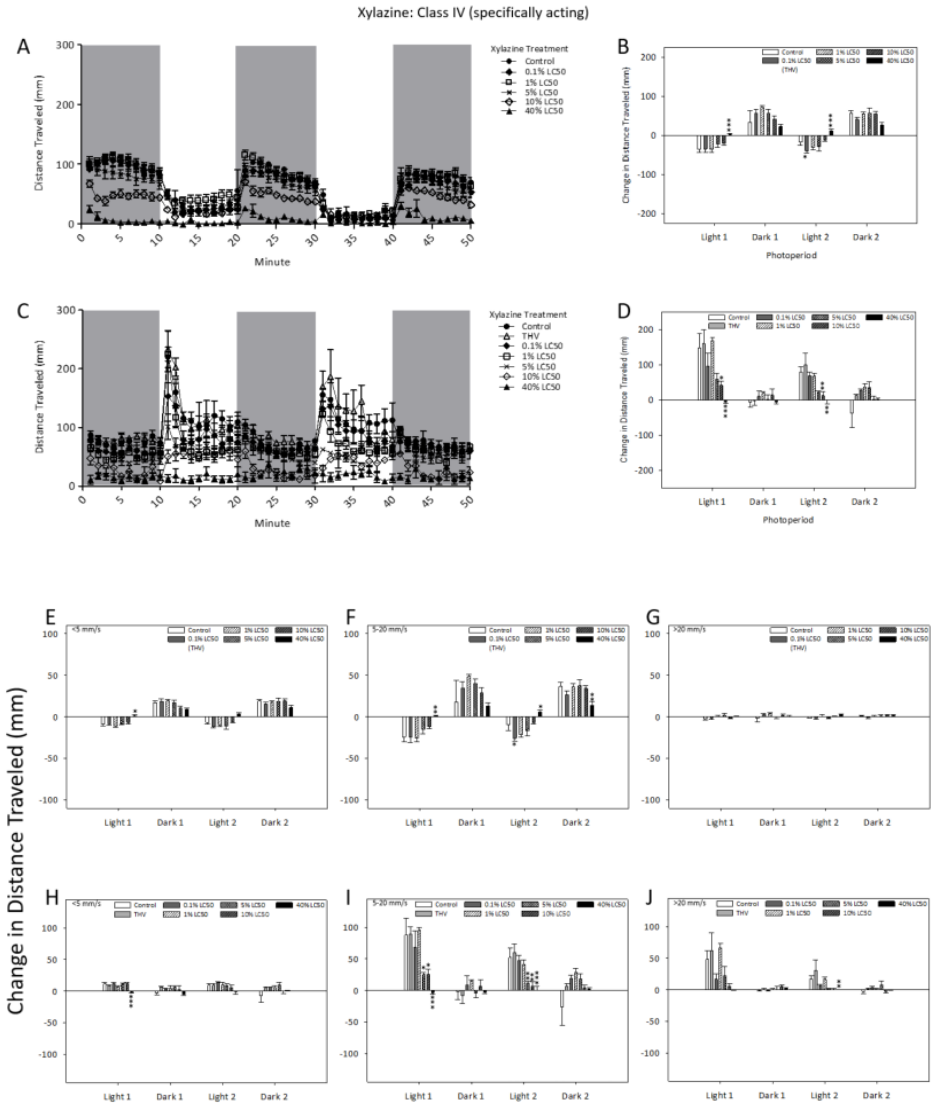
Supplementary: Figure 28: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to Citalopram. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. The vertical dashed line represents the Therapeutic Hazard Value (THV). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



Supplementary: Figure 29: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to Diazinon. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



Supplementary: Figure 30: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to pentylenetetrazole. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01. N.M.: Not Measured



Supplementary: Figure 31: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to Xyalzine. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). Two dark and two light period photomotor responses were measured. A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01. THV: Therapeutic Hazard Value

APPENDIX C

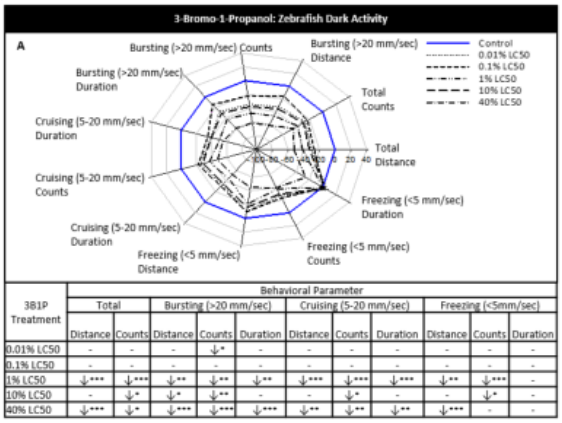
Supplemental Information for Chapter Four

Supplementary: Table 14. Nominal concentrations for each of the study compounds during zebrafish exposures.

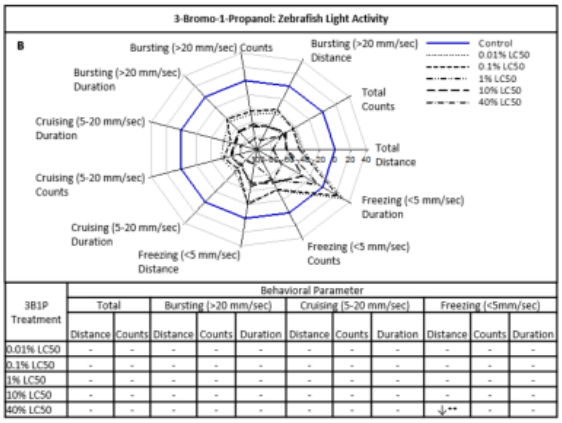
Chemical	Zebrafish Treatments (mg/L)						
	0.01% LC_{50}	0.1% LC_{50}	1% LC_{50}	5% LC_{50}	10% LC_{50}	20% LC_{50}	40% LC_{50}
3-Bromo-1-Propanol	0.02	0.16	1.58	–	15.84	–	63.36
3-chloro-1,2-propanediol	–	–	55.58	277.92	555.84	1111.68	2223.36
Dibromoacetonitrile	–	–	0.004	0.02	0.043	0.087	0.17
Glycidol	–	–	0.65	3.27	6.54	13.08	26.16
Sodium decyl sulfate	–	–	2.32	11.59	23.18	46.37	92.74
Styrene oxide	–	–	0.15	0.74	1.49	2.98	5.95
Tris(2,3-dibromopropyl) phosphate	–	–	0.03	0.16	0.31	0.62	1.24
Tris(1,3-dichloro-2-propyl) phosphate	–	–	0.03	0.14	0.27	0.54	1.09

Supplementary: Table 15. Nominal concentrations for each of the study compounds during fathead minnow exposures.

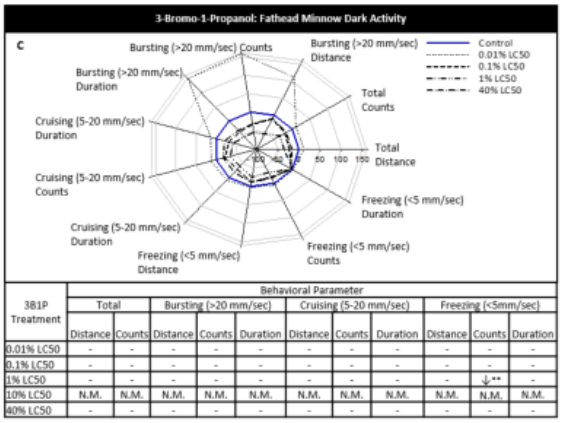
Chemical	Fathead minnow Treatments (mg/L)						
	0.01% LC_{50}	0.1% LC_{50}	1% LC_{50}	5% LC_{50}	10% LC_{50}	20% LC_{50}	40% LC_{50}
3-Bromo-1-Propanol	0.006	0.061	0.61	–	6.13	–	24.52
3-chloro-1,2-propanediol	–	–	92.35	461.75	923.50	1847.00	3694.00
Dibromoacetonitrile	–	–	0.003	0.014	0.03	0.06	0.12
Glycidol	–	–	0.654	3.27	6.54	13.08	26.16
Sodium decyl sulfate	–	–	5.02	25.09	50.19	100.37	200.74
Styrene oxide	–	–	0.13	0.629	1.26	2.51	5.03
Tris(2,3-dibromopropyl) phosphate	–	–	0.004	0.02	0.04	0.08	0.15
Tris(1,3-dichloro-2-propyl) phosphate	–	–	0.05	0.26	0.51	1.02	2.04



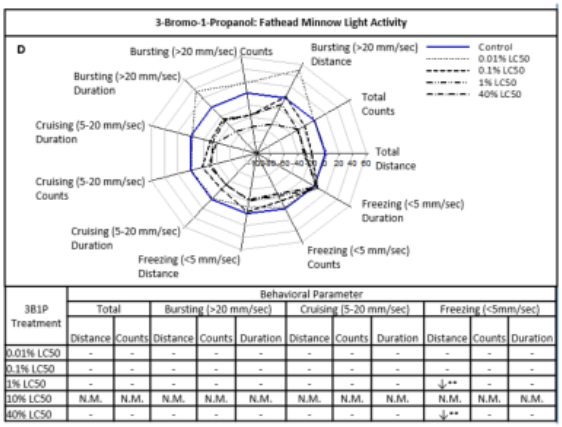
3B1P Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.01% LC50	-	-	-	↓*	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	↓***	↓***	↓**	↓**	↓**	↓***	↓***	↓***	↓**	↓***	-
10% LC50	-	↓*	↓*	↓**	-	-	↓*	-	-	↓*	-
40% LC50	↓***	↓*	↓***	↓***	↓***	↓**	↓**	↓**	↓***	-	-



3B1P Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.01% LC50	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	↓**	-	-

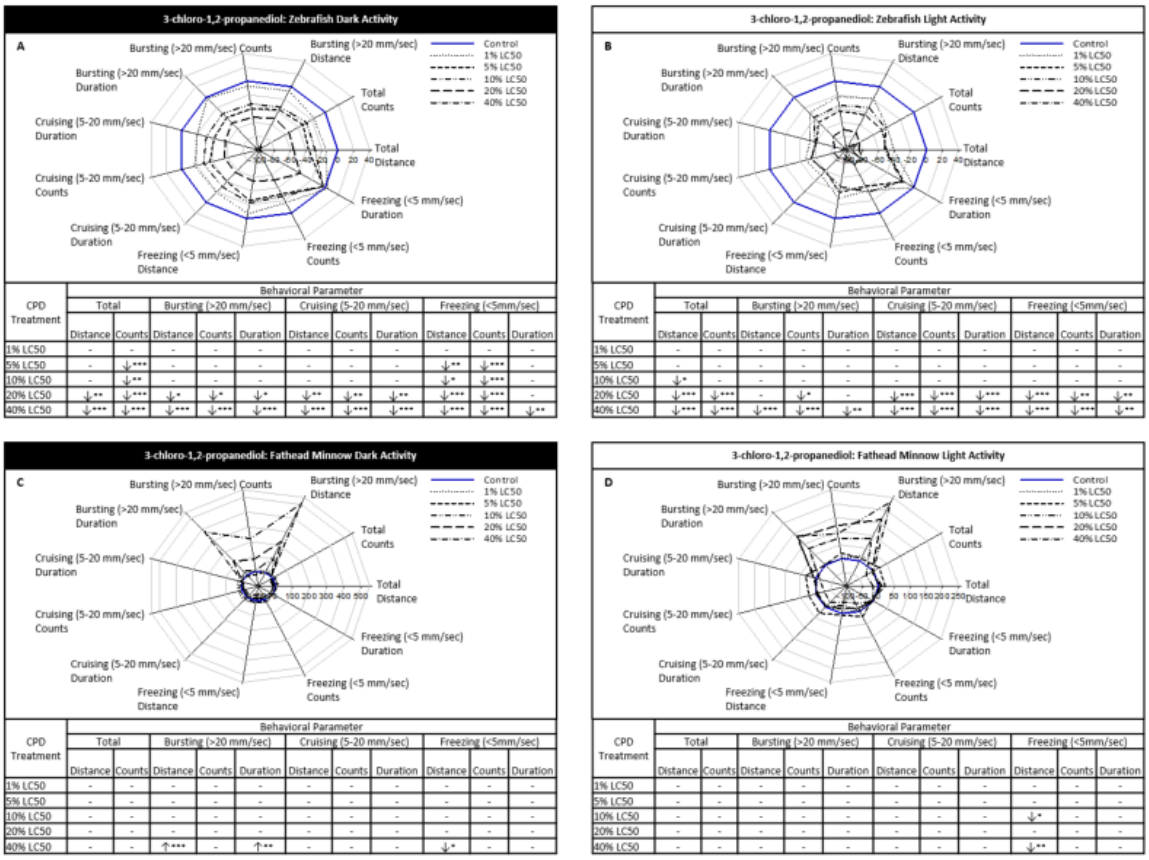


3B1P Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.01% LC50	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	-	↓**	-
10% LC50	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
40% LC50	-	-	-	-	-	-	-	-	-	-	-



3B1P Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
0.01% LC50	-	-	-	-	-	-	-	-	-	-	-
0.1% LC50	-	-	-	-	-	-	-	-	-	-	-
1% LC50	-	-	-	-	-	-	-	-	↓**	-	-
10% LC50	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
40% LC50	-	-	-	-	-	-	-	-	↓**	-	-

Supplementary: Figure 32: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to 3-Bromo-1-Propanol. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



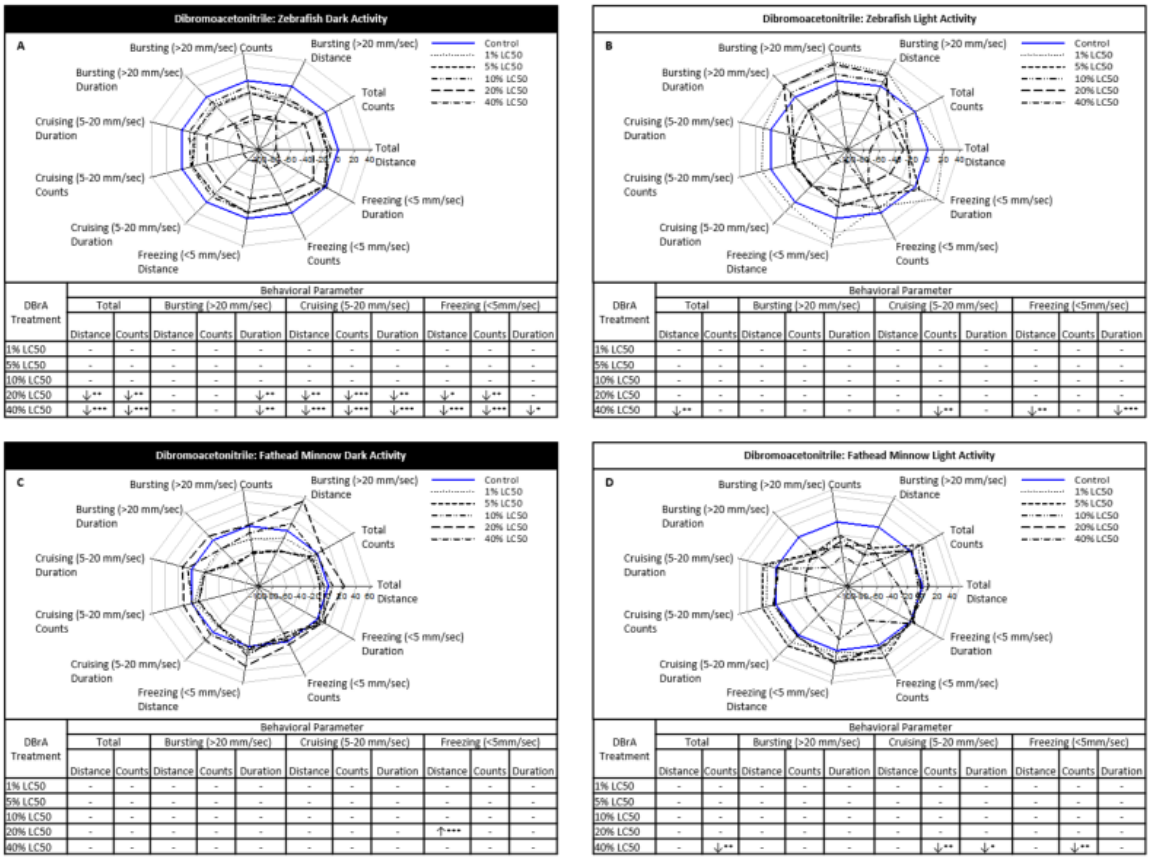
CPD Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	↓***	-	-	-	-	-	-	↓**	↓***	-
10% LC50	-	↓**	-	-	-	-	-	-	↓*	↓***	-
20% LC50	↓**	↓***	↓*	↓*	↓*	↓**	↓**	↓**	↓***	↓***	-
40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓**

CPD Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	↓*	-	-	-	-	-	-	-	-	-	-
20% LC50	↓***	↓***	-	↓*	-	↓***	↓***	↓***	↓***	↓**	↓**
40% LC50	↓***	↓***	↓***	↓***	↓**	↓***	↓***	↓***	↓***	↓***	↓**

CPD Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	↑***	-	↑**	-	-	-	↓*	-	-

CPD Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	↓*	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	↓**	-	-

Supplementary: Figure 33: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to 3-chloro-1,2-propanediol. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



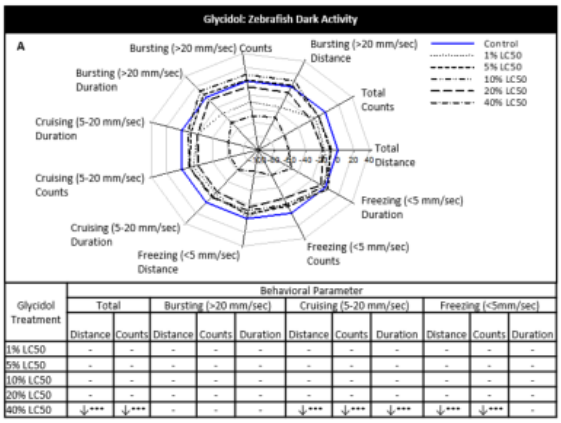
DBrA Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	↓**	↓**	-	-	↓**	↓**	↓***	↓**	↓*	↓**	-
40% LC50	↓***	↓***	-	-	↓**	↓***	↓***	↓***	↓***	↓***	↓*

DBrA Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓**	-	-	-	-	-	↓**	-	↓**	-	↓***

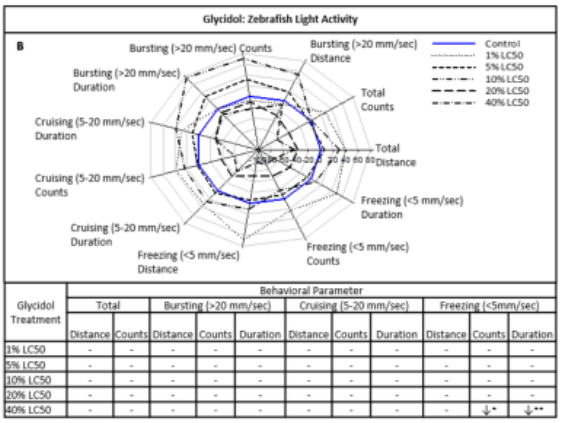
DBrA Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	↑***	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

DBrA Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	↓**	-	-	-	-	↓**	↓*	-	↓**	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

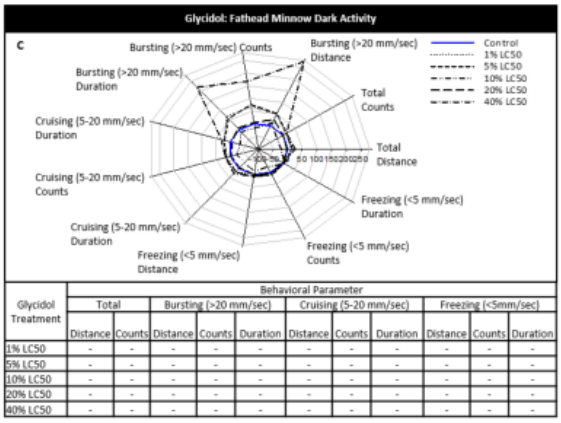
Supplementary: Figure 34: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to Dibromoacetonitrile. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



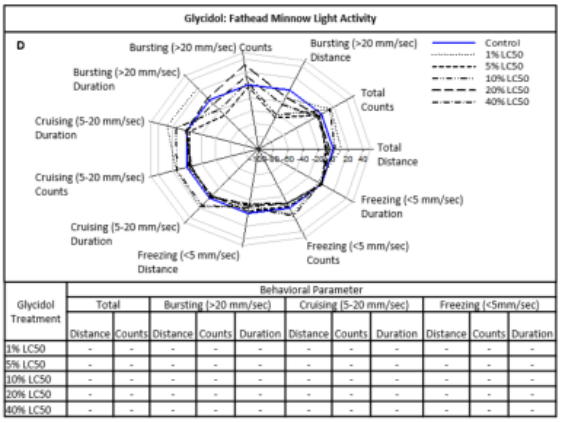
Glycidol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	-



Glycidol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	↓*	↓**

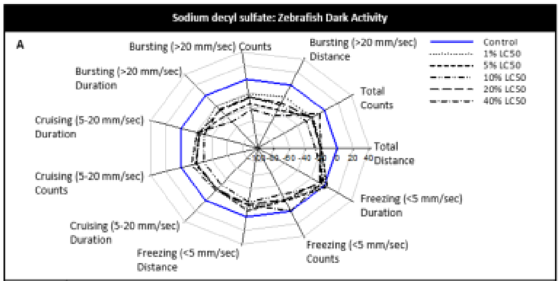


Glycidol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

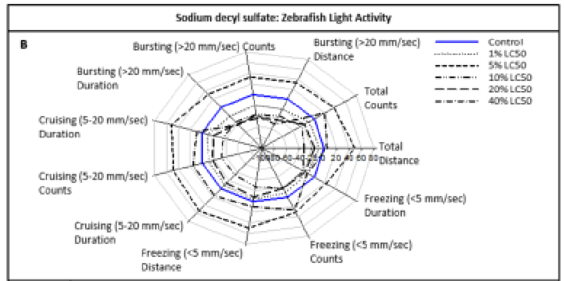


Glycidol Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

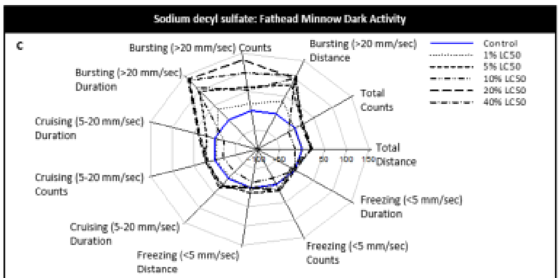
Supplementary: Figure 35: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to Glycidol. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



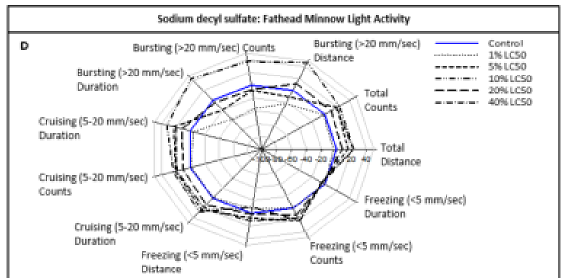
SDS Treatment	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	↓**	↓**	↓*	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	↓**	↓**	↓***	-	-	-	-	-	-



SDS Treatment	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

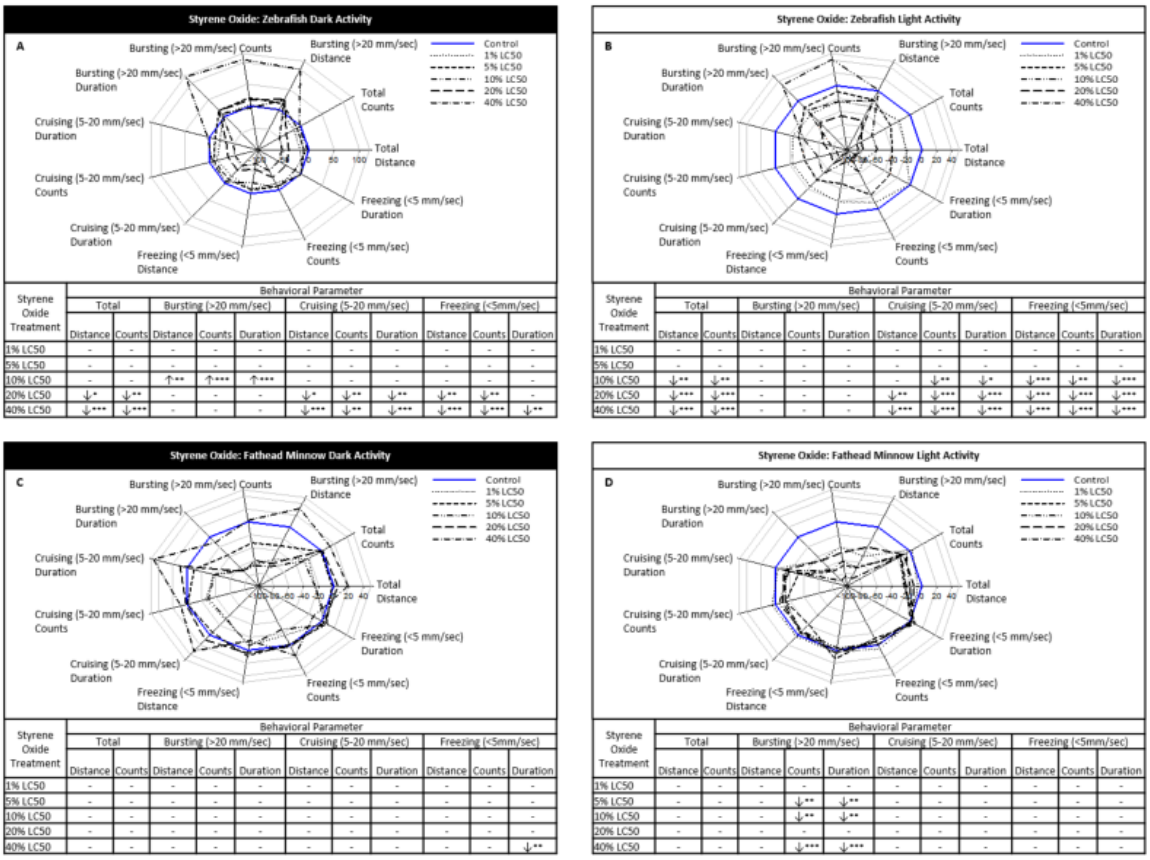


SDS Treatment	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-



SDS Treatment	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-

Supplementary: Figure 36: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to Sodium decyl sulfate. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



Styrene Oxide: Zebrafish Dark Activity

Styrene Oxide Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	-	-	-	-	-	-	-	-	
10% LC50	-	-	↑**	↑***	↑***	-	-	-	-	-	-	
20% LC50	↓*	↓**	-	-	-	↓*	↓**	↓**	↓**	↓**	-	
40% LC50	↓***	↓***	-	-	-	↓***	↓**	↓***	↓***	↓***	↓**	

Styrene Oxide: Zebrafish Light Activity

Styrene Oxide Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	-	-	-	-	-	-	-	-	
10% LC50	↓**	↓**	-	-	-	-	↓**	↓*	↓***	↓**	↓***	
20% LC50	↓***	↓***	-	-	-	↓**	↓***	↓***	↓***	↓***	↓***	
40% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	↓***	

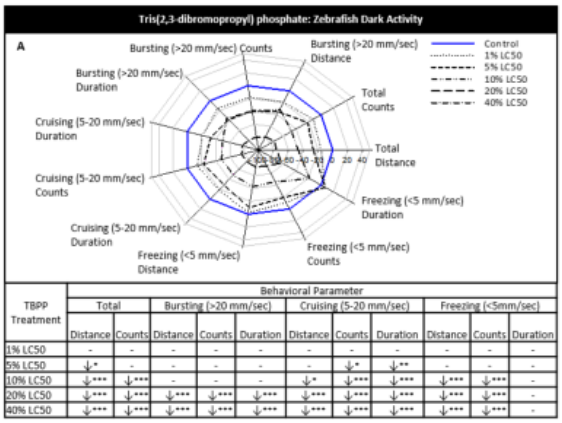
Styrene Oxide: Fathead Minnow Dark Activity

Styrene Oxide Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	-	-	-	-	-	-	-	-	
10% LC50	-	-	-	-	-	-	-	-	-	-	-	
20% LC50	-	-	-	-	-	-	-	-	-	-	-	
40% LC50	-	-	-	-	-	-	-	-	-	-	↓**	

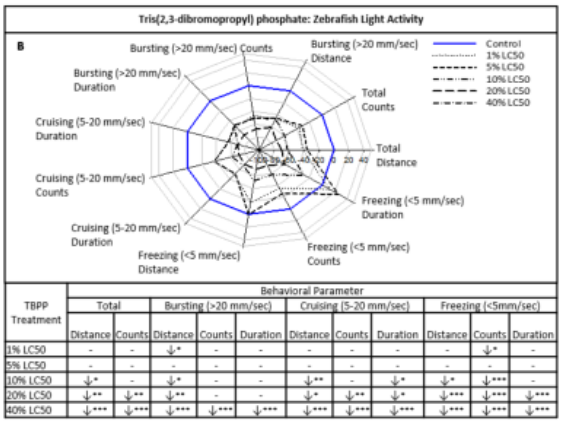
Styrene Oxide: Fathead Minnow Light Activity

Styrene Oxide Treatment	Behavioral Parameter											
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)			
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration	
1% LC50	-	-	-	-	-	-	-	-	-	-	-	
5% LC50	-	-	-	↓**	↓**	-	-	-	-	-	-	
10% LC50	-	-	-	↓**	↓**	-	-	-	-	-	-	
20% LC50	-	-	-	-	-	-	-	-	-	-	-	
40% LC50	-	-	-	↓***	↓***	-	-	-	-	-	-	

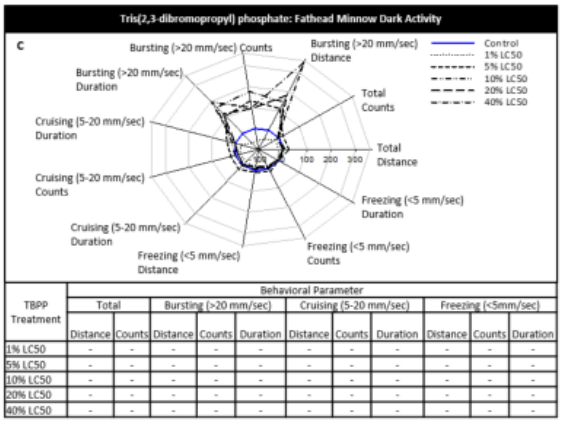
Supplementary: Figure 37: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to Styrene Oxide. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01



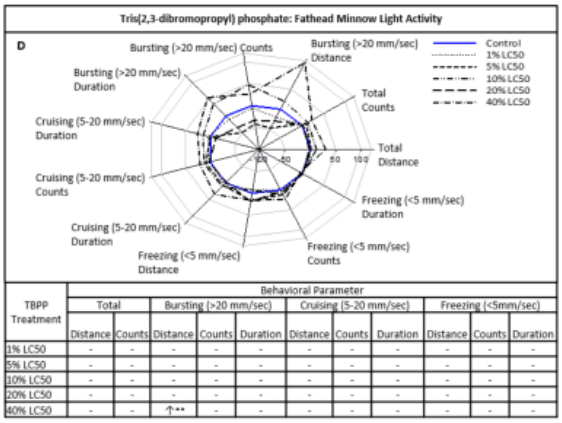
TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	↓*	-	-	-	-	-	↓*	↓**	-	-	-
10% LC50	↓***	↓***	-	-	-	↓*	↓***	↓***	↓***	↓***	-
20% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	-
40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	-



TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	↓*	-	-	-	-	-	-	↓*	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	↓*	-	↓*	-	-	↓**	-	↓*	↓*	↓***	-
20% LC50	↓**	↓**	↓**	-	-	↓*	↓**	↓*	↓***	↓***	↓***
40% LC50	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***	↓***

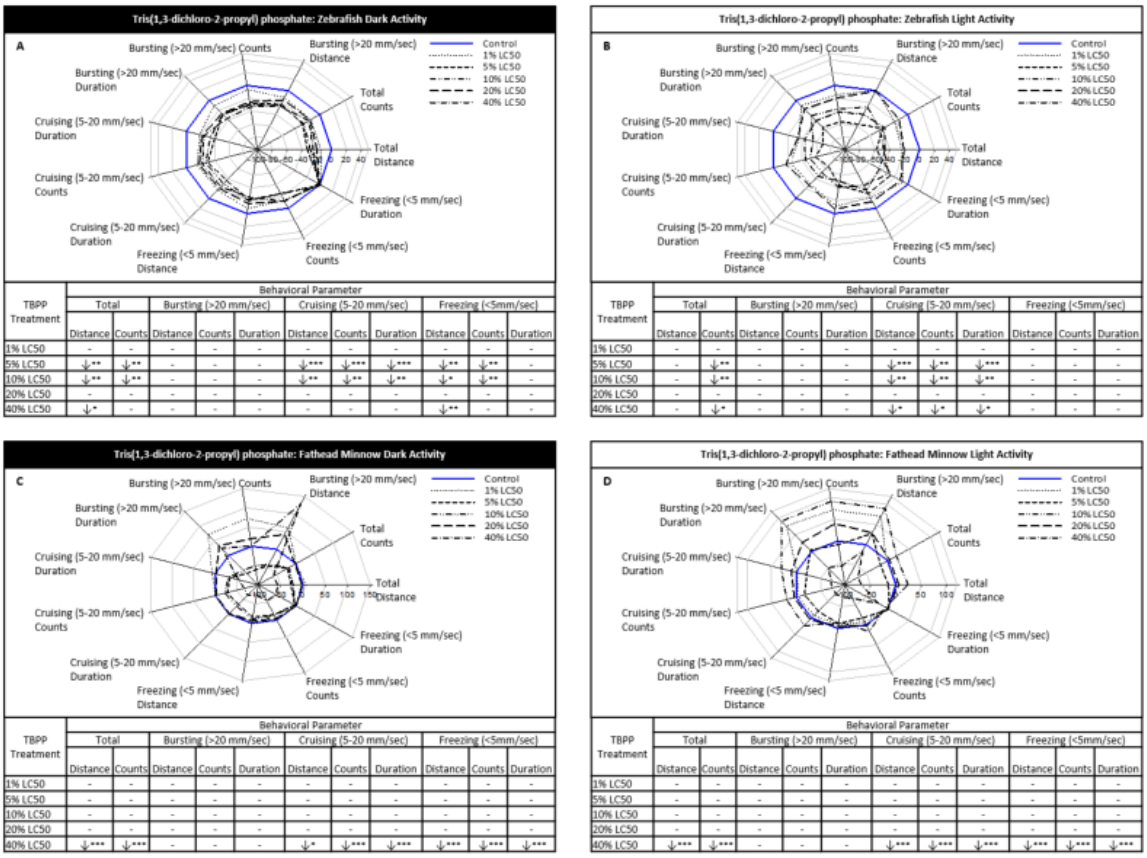


TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	-	-	-	-	-	-	-	-	-



TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	-	↑**	-	-	-	-	-	-	-	-

Supplementary: Figure 38: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to TBPP. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



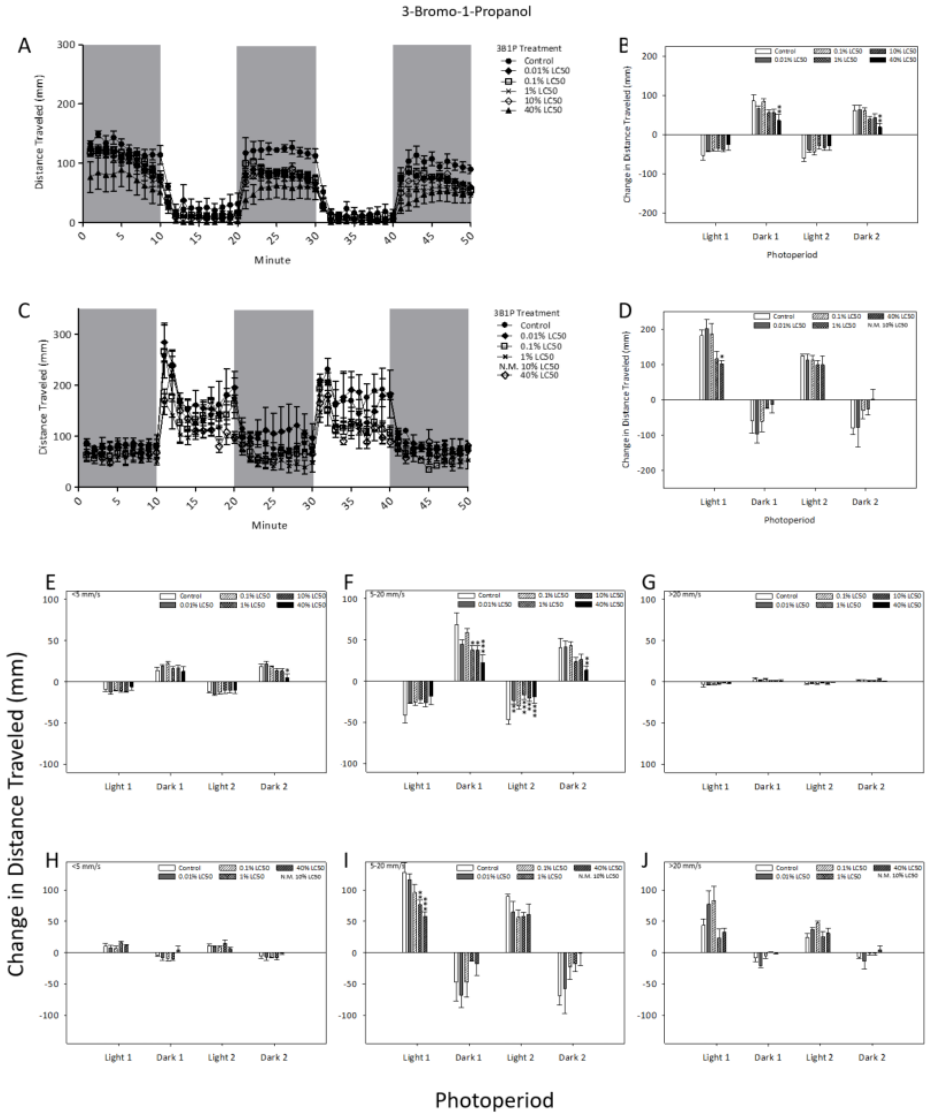
TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	↓**	↓**	-	-	-	↓***	↓***	↓***	↓**	↓**	-
10% LC50	↓**	↓**	-	-	-	↓**	↓**	↓**	↓*	↓**	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓*	-	-	-	-	-	-	-	↓**	-	-

TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	↓**	-	-	-	↓***	↓**	↓***	-	-	-
10% LC50	-	↓**	-	-	-	↓**	↓**	↓**	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	-	↓*	-	-	-	↓*	↓*	↓*	-	-	-

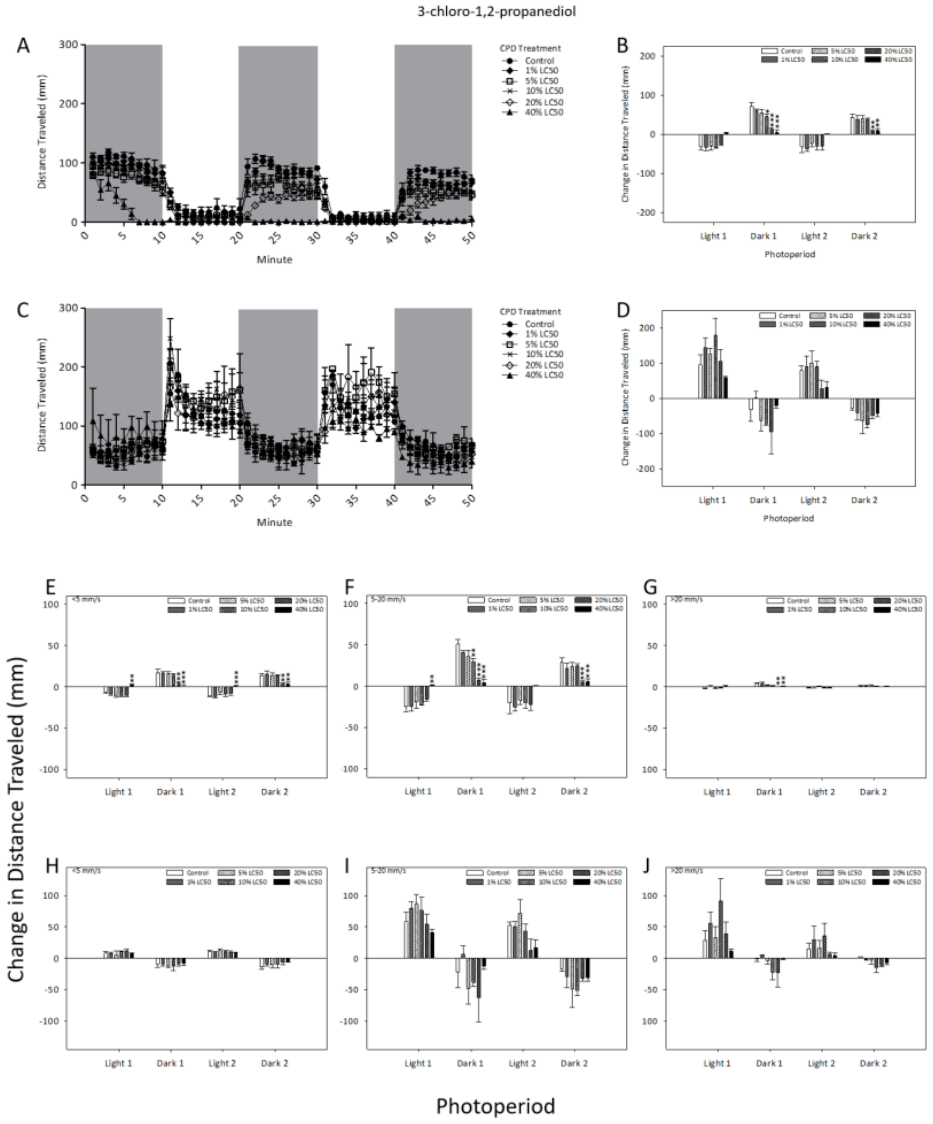
TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓***	↓***	-	-	-	↓*	↓***	↓***	↓***	↓***	↓***

TBPP Treatment	Behavioral Parameter										
	Total		Bursting (>20 mm/sec)			Cruising (5-20 mm/sec)			Freezing (<5mm/sec)		
	Distance	Counts	Distance	Counts	Duration	Distance	Counts	Duration	Distance	Counts	Duration
1% LC50	-	-	-	-	-	-	-	-	-	-	-
5% LC50	-	-	-	-	-	-	-	-	-	-	-
10% LC50	-	-	-	-	-	-	-	-	-	-	-
20% LC50	-	-	-	-	-	-	-	-	-	-	-
40% LC50	↓***	↓***	-	-	-	↓***	↓***	↓***	↓***	↓***	↓***

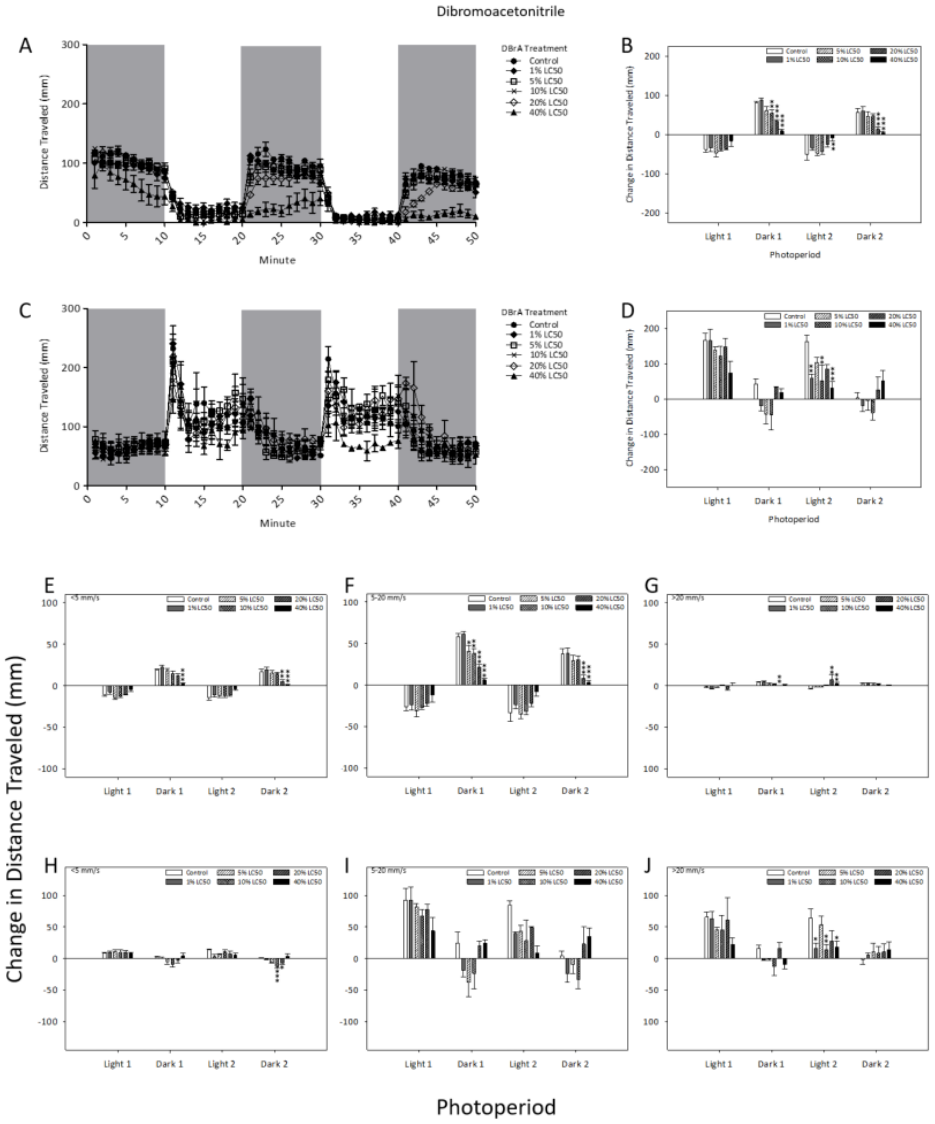
Supplementary: Figure 39: Mean zebrafish dark (A) and light (B) swimming activity compared to mean fathead minnow dark (C) and light (D) activity after 96 h exposure to TDCPP. Plotted data represents activity over two ten minute dark photoperiods and two ten minute light photoperiods for each fish model. Data is normalized to control, which is represented at the 0 axis in each figure. Behavioral parameters include distance swam, number of movements (counts), and duration of each movement across 3 speed levels, bursting (>20 mm/sec), cruising (5-20 mm/sec), and freezing (<5 mm/sec). In addition to movement patterns at each of the speed thresholds, total distance swam and total number of movements is represented. ↑ represents a significant increase in activity in comparison to control and ↓ indicates a significant decrease in activity in comparison to control. A total of 24 (N = 4 replicates of each treatment level, 6 larvae in each experimental unit) zebrafish and 12 (N = 3 replicates of each treatment level, 4 larvae in each experimental unit) fathead minnow where used in behavioral observations for each group. *p<0.10 ;**p<0.05; ***p<0.01.



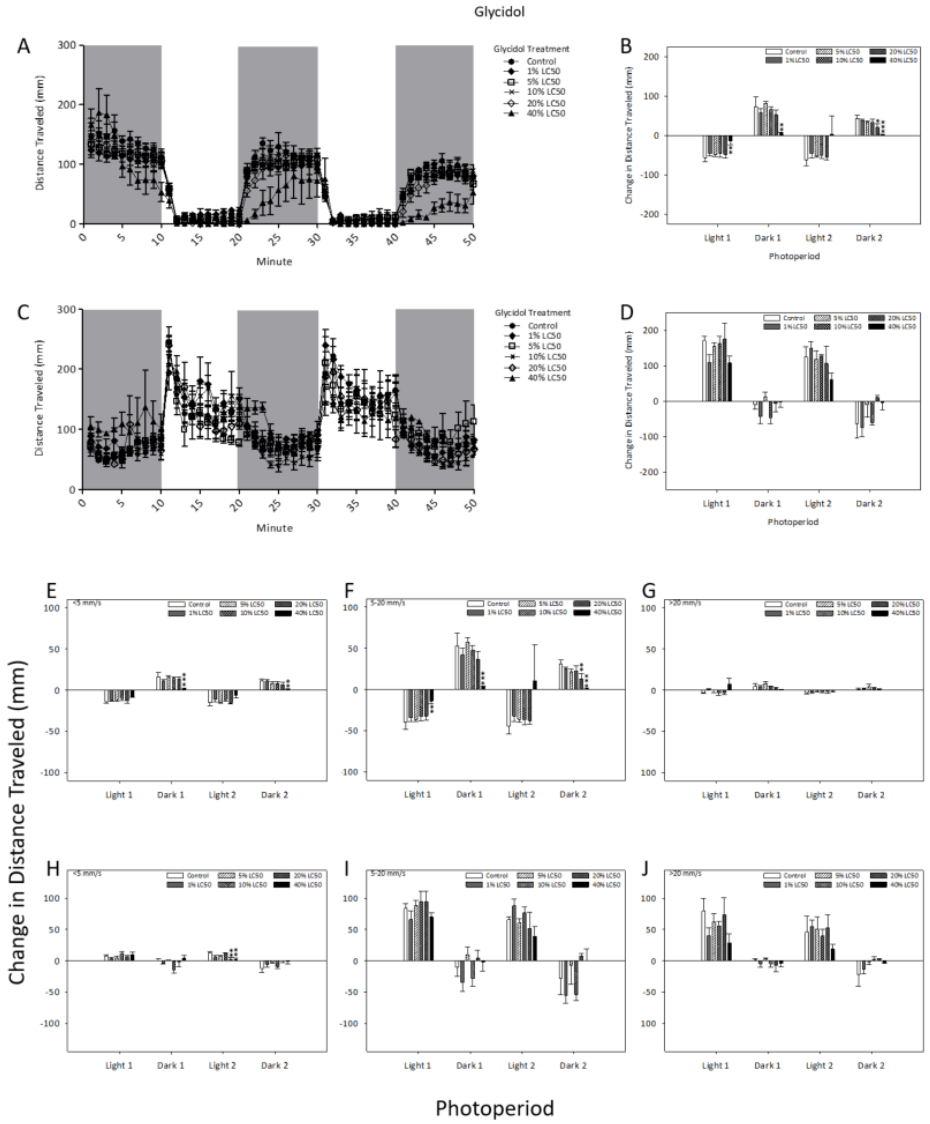
Supplementary: Figure 40: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to 3-bromo-1-Propanol. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01. N.M.: Not measured



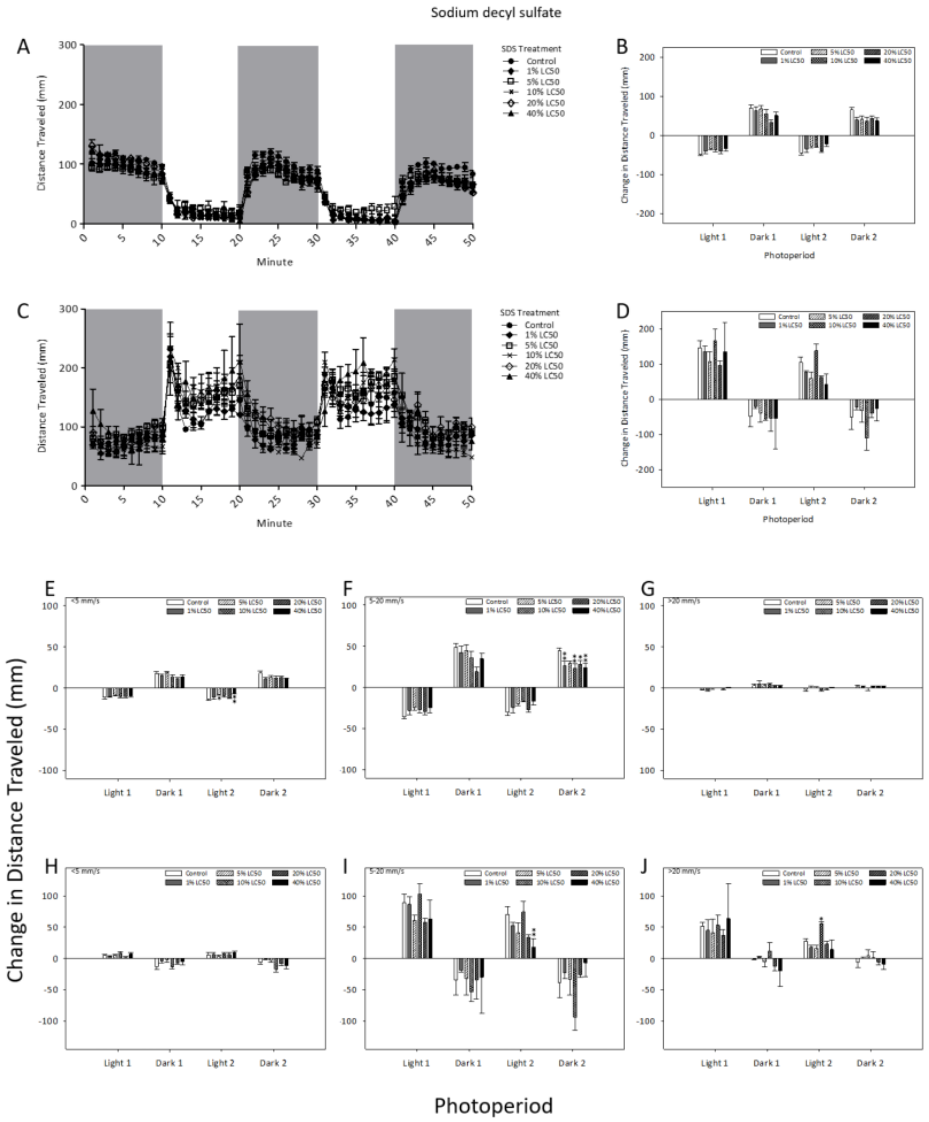
Supplementary: Figure 41: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to 3-chloro-1,2-propanediol. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



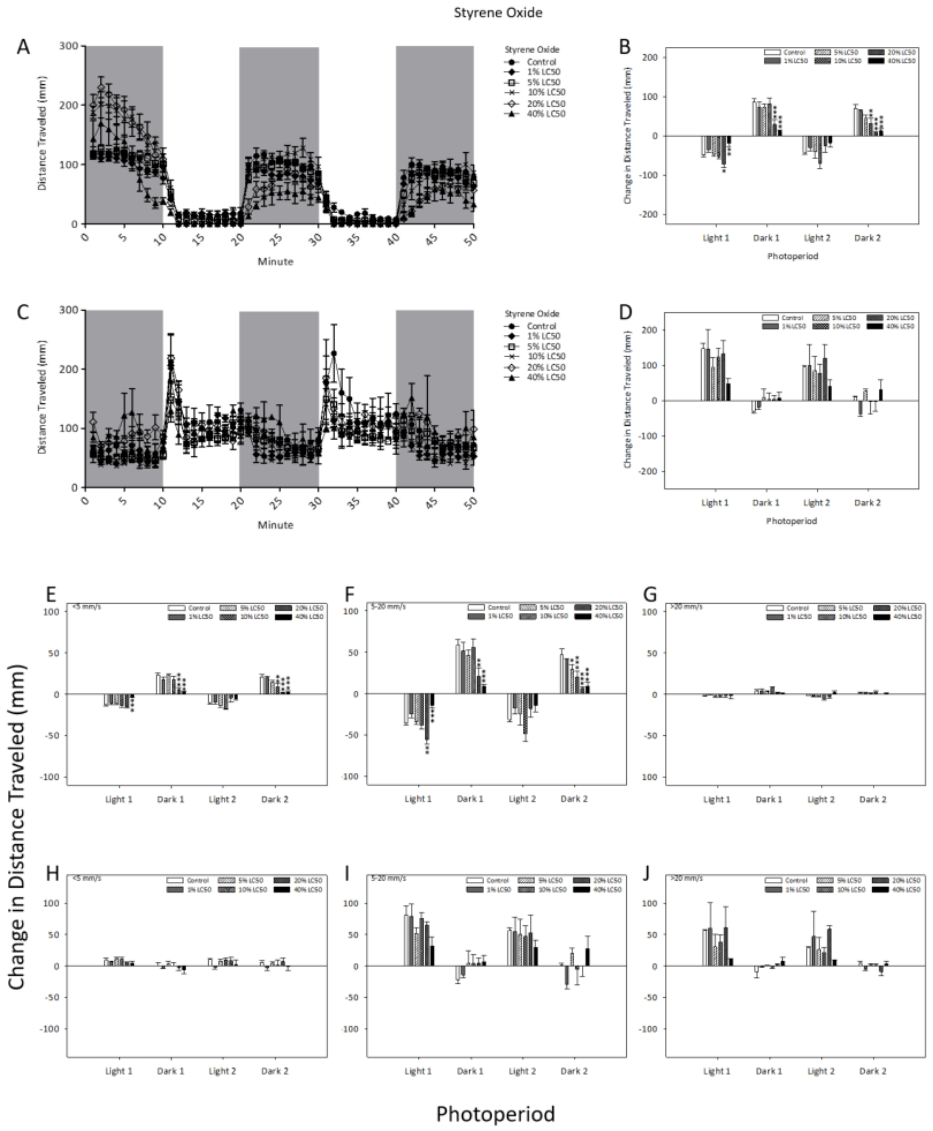
Supplementary: Figure 42: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to dibromoacetonitrile. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



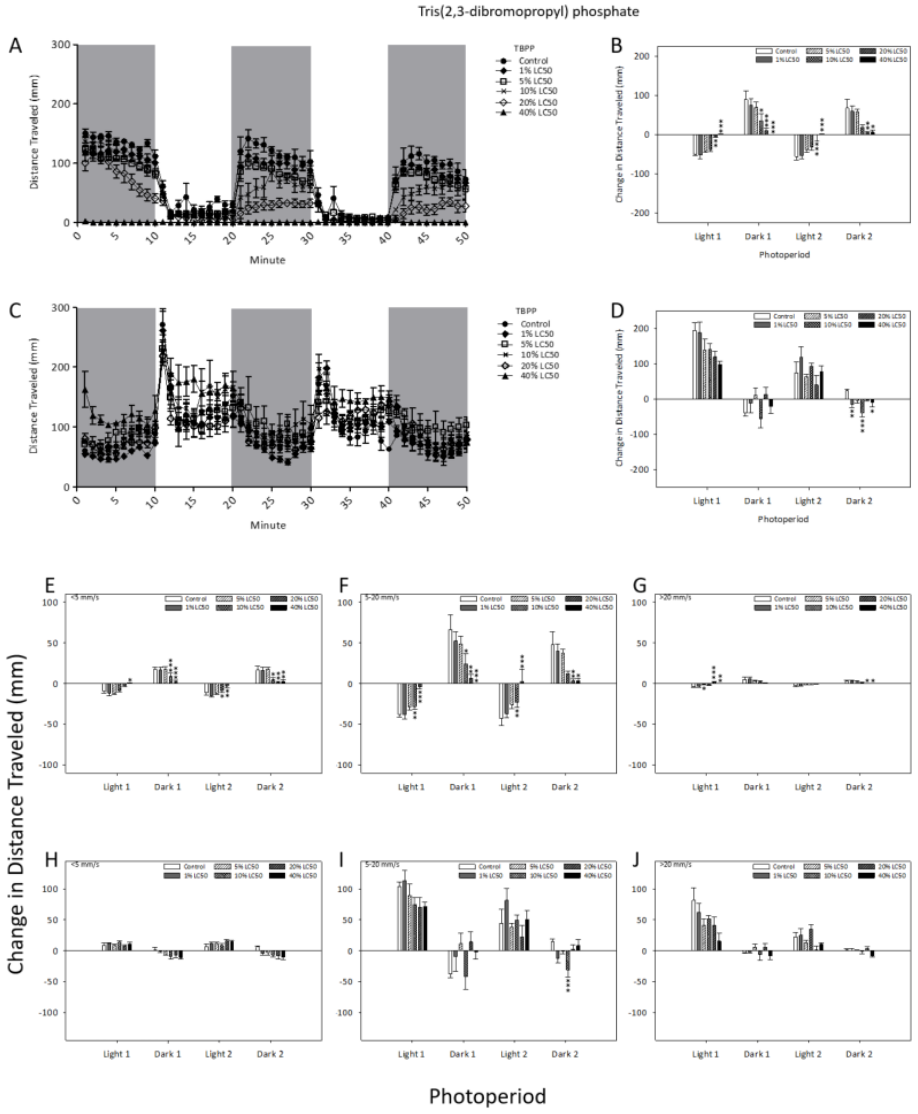
Supplementary: Figure 43: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to glycidol. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



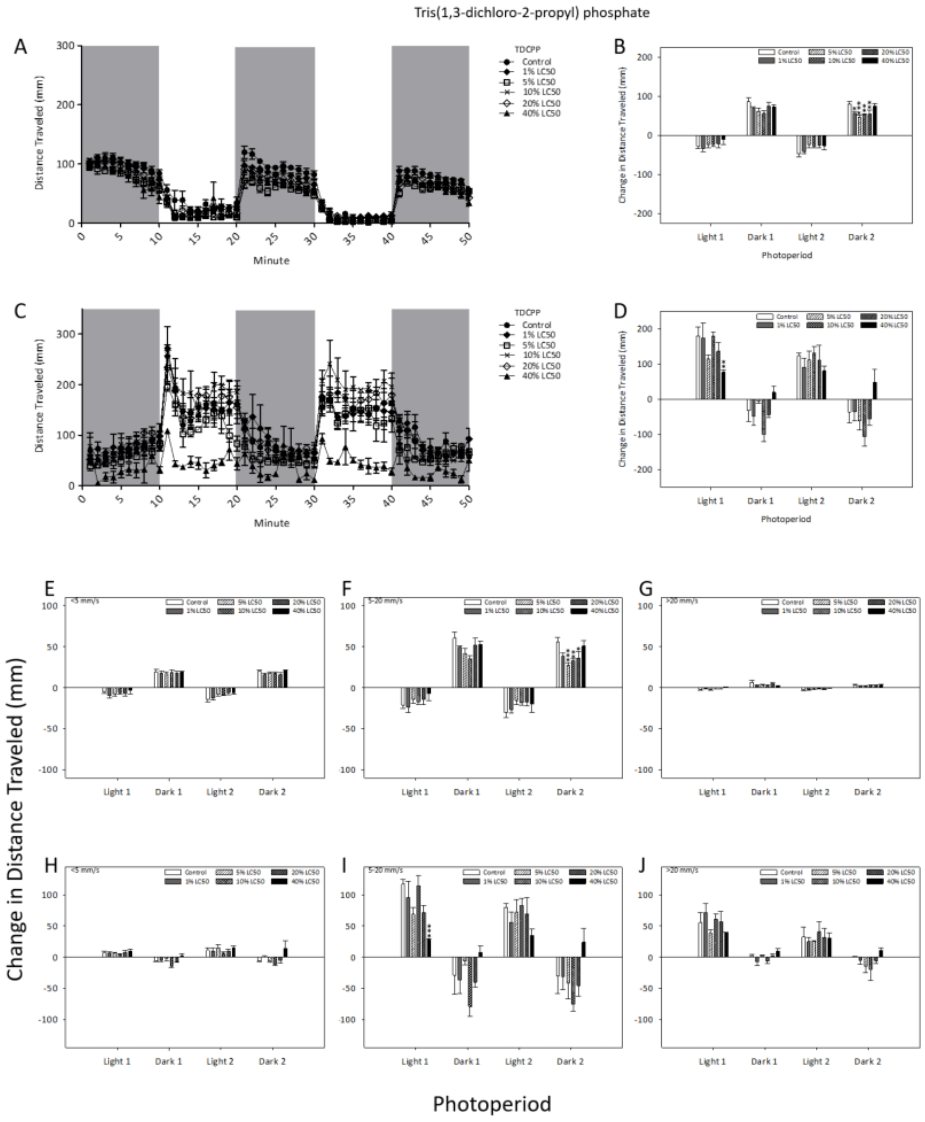
Supplementary: Figure 44: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to sodium decyl sulfate. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



Supplementary: Figure 45: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to styrene oxide. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



Supplementary: Figure 46: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to TBPP. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.



Supplementary: Figure 47: Activity of zebrafish (A and B) and fathead minnow (C and D) after 96 h exposure to TDCPP. The mean (±SE) distance swam for zebrafish (A) and fathead minnow (C) is given by dots each representing 1 minute intervals of activity. Photomotor responses of zebrafish (B) and fathead minnow (D) are measured as the change in mean (±SE) total distance traveled between the last minutes of an initial photoperiod and the first minute of the following period. Two dark and two light period photomotor responses were measured. Zebrafish (E,F, and G) and fathead minnow larvae (H,I, and J) photomotor responses were also measured across three speed thresholds (Freezing: <5 mm/s, Cruising: 5-20 mm/s, and Bursting >20 mm/s). A total of 24 (4 replicates each of 6 larvae) zebrafish and 12 (3 replicates of 4 larvae) fathead minnows were used for behavioral observation. *p<0.10 ;**p<0.05; ***p<0.01.

BIBLIOGRAPHY

Almeida JR, Oliveira C, Gravato C, Guilhermino L. 2010. Linking behavioural alterations with biomarkers responses in the european seabass dicentrarchus labrax l. Exposed to the organophosphate pesticide fenitrothion. Ecotoxicology. 19(8):1369-1381.

Alvarez D, Nicieza A. 2003. Predator avoidance behaviour in wild and hatchery-reared brown trout: The role of experience and domestication. Journal of Fish Biology. 63(6):1565-1577.

Anastas P, Eghbali N. 2010. Green chemistry: Principles and practice. Chemical Society Reviews. 39(1):301-312.

Andersen ME, Krewski D. 2008. Toxicity testing in the 21st century: Bringing the vision to life. Toxicological sciences. 107(2):324-330.

Ankley GT, Brooks BW, Huggett DB, Sumpter, P J. 2007. Repeating history: Pharmaceuticals in the environment. ACS Publications.

Ankley GT, Villeneuve DL. 2006. The fathead minnow in aquatic toxicology: Past, present and future. Aquatic Toxicology. 78(1):91-102.

Arnold KE, Brown AR, Ankley GT, Sumpter JP. 2014. Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. The Royal Society.

Aruoma OI. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American oil chemists' society. 75(2):199-212.

Association AAPH. 1999. Standard methods for the examination of water and wastewater 20th ed. Prepared and published jointly by: APHA, AWWA and WPCF.

Baraban S, Taylor M, Castro P, Baier H. 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience. 131(3):759-768.

Bean TG, Rattner BA, Lazarus RS, Day DD, Burket SR, Brooks BW, Haddad SP, Bowerman WW. 2018. Pharmaceuticals in water, fish and osprey nestlings in delaware river and bay. Environmental Pollution. 232:533-545.

Beauvais SL, Jones SB, Brewer SK, Little EE. 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (oncorhynchus mykiss) and their correlation with behavioral measures. Environmental Toxicology and Chemistry. 19(7):1875-1880.

Becker RA, Ankley GT, Edwards SW, Kennedy SW, Linkov I, Meek B, Sachana M, Segner H, Van Der Burg B, Villeneuve DL et al. 2015. Increasing scientific confidence in adverse outcome pathways: Application of tailored bradford-hill considerations for evaluating weight of evidence. Regulatory Toxicology and Pharmacology. 72(3):514-537.

Berghmans S, Hunt J, Roach A, Goldsmith P. 2007. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy research. 75(1):18-28.

Berninger JP, Du B, Connors KA, Eytcheson SA, Kolkmeier MA, Prosser KN, Valenti TW, Chambliss CK, Brooks BW. 2011. Effects of the antihistamine diphenhydramine on selected aquatic organisms. Environmental Toxicology and Chemistry. 30(9):2065-2072.

Blanchard DC, Griebel G, Blanchard RJ. 2003. The mouse defense test battery: Pharmacological and behavioral assays for anxiety and panic. European journal of pharmacology. 463(1-3):97-116.

Boxall AB, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T. 2012. Pharmaceuticals and personal care products in the environment: What are the big questions? Environmental health perspectives. 120(9):1221-1229.

Bradbury SP, Carlson RW, Henry TR. 1989. Polar narcosis in aquatic organisms. Aquatic toxicology and hazard assessment: 12th volume. ASTM International.

Brodin T, Fick J, Jonsson M, Klaminder J. 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science. 339(6121):814-815.

Brodin T, Nordling J, Lagesson A, Klaminder J, Hellström G, Christensen B, Fick J. 2017. Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish,(rutilus rutilus). Journal of Toxicology and Environmental Health, Part A. 80(16-18):963-970.

Brodin T, Piovano S, Fick J, Klaminder J, Heynen M, Jonsson M. 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Phil Trans R Soc B. 369(1656):20130580.

Brooks BW. 2014. Fish on prozac (and zoloft): Ten years later. Aquatic Toxicology. 151:61-67.

Brooks BW, Berninger JP, Kristofco LA, Ramirez AJ, Stanley JK, Valenti TW. 2012. Pharmaceuticals in the environment: Lessons learned for reducing uncertainties in environmental risk assessment. Progress in molecular biology and translational science. Elsevier. p. 231-258.

Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, La Point TW. 2003. Aquatic ecotoxicology of fluoxetine. Toxicology letters. 142(3):169-183.

Brooks BW, Steele WB. 2018. Ecotoxicological perspectives on health care and the environment. Health care and environmental contamination. Elsevier. p. 41-67.

Bruton T, Alboloushi A, De La Garza B, Kim B-O, Halden RU. 2010. Fate of caffeine in the environment and ecotoxicological considerations. Contaminants of emerging concern in the environment: Ecological and human health considerations. ACS Publications. p. 257-273.

Budick SA, O'Malley DM. 2000. Locomotor repertoire of the larval zebrafish: Swimming, turning and prey capture. Journal of Experimental biology. 203(17):2565-2579.

Burgess HA, Granato M. 2007. Modulation of locomotor activity in larval zebrafish during light adaptation. J Exp Biol. 210(14):2526-2539.

Chen T-H, Wang Y-H, Wu Y-H. 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: Implications for behavioral toxicity bioassays. Aquatic Toxicology. 102(3):162-166.

Chiffre A, Clérandeau C, Dwoinikoff C, Le Bihanic F, Budzinski H, Geret F, Cachot J. 2016. Psychotropic drugs in mixture alter swimming behaviour of japanese medaka (oryzias latipes) larvae above environmental concentrations. Environmental science and pollution research. 23(6):4964-4977.

Coish P, Brooks BW, Gallagher EP, Mills M, Kavanagh TJ, Simcox N, Lasker GA, Botta D, Schmuck SC, Voutchkova-Kostal A. 2018. The molecular design research network. Toxicological Sciences. 161(2):241-248.

Colón-Cruz L, Kristofco L, Crooke-Rosado J, Acevedo A, Torrado A, Brooks BW, Sosa MA, Behra M. 2018. Alterations of larval photo-dependent swimming responses (pdr): New endpoints for rapid and diagnostic screening of aquatic contamination. Ecotoxicology and environmental safety. 147:670-680.

Colwill RM, Creton R. 2011. Imaging escape and avoidance behavior in zebrafish larvae. Reviews in the Neurosciences. 22(1):63-73.

Connors KA, Valenti TW, Lawless K, Sackerman J, Onaivi ES, Brooks BW, Gould GG. 2014. Similar anxiolytic effects of agonists targeting serotonin 5-ht1a or cannabinoid cb receptors on zebrafish behavior in novel environments. Aquatic toxicology. 151:105-113.

Copmans D, Meinl T, Dietz C, van Leeuwen M, Ortmann J, Berthold MR, de Witte PA. 2016. A knime-based analysis of the zebrafish photomotor response clusters the phenotypes of 14 classes of neuroactive molecules. Journal of biomolecular screening. 21(5):427-436.

Corrales J, Kristofco L, Steele W, Saari G, Kostal J, Williams E, Mills M, Gallagher E, Kavanagh T, Simcox N. 2017. Toward the design of less hazardous chemicals: Exploring comparative oxidative stress in two common animal models. Chemical research in toxicology. 30(4):893-904.

Corrales J, Kristofco LA, Steele WB, Saari GN, Kostal J, Williams ES, Mills M, Gallagher EP, Kavanagh TJ, Simcox N. 2016. Toward the design of less hazardous chemicals: Exploring comparative oxidative stress in two common animal models. Chemical Research in Toxicology.

Council NR. 2007. Toxicity testing in the 21st century: A vision and a strategy. National Academies Press.

Da-Rocha MA, Puech AJ, Thiébot M-H. 1997. Influence of anxiolytic drugs on the effects of specific serotonin reuptake inhibitors in the forced swimming test in mice. Journal of Psychopharmacology. 11(3):211-218.

de Esch C, van der Linde H, Slieker R, Willemsen R, Wolterbeek A, Woutersen R, De Groot D. 2012. Locomotor activity assay in zebrafish larvae: Influence of age, strain and ethanol. Neurotoxicology and teratology. 34(4):425-433.

Diamond JM, Parson MJ, Gruber D. 1990. Rapid detection of sublethal toxicity using fish ventilatory behavior. Environmental Toxicology and Chemistry: An International Journal. 9(1):3-11.

Drummond RA, Russom CL, Geiger DL, Defoe DL. 1986. Behavioral and morphological changes in fathead minnow (pimephales promelas) as diagnostic endpoints for screening chemicals according to mode of action. Aquatic toxicology and environmental fate: Ninth volume. ASTM International.

Dzieweczynski TL, Hebert OL. 2012. Fluoxetine alters behavioral consistency of aggression and courtship in male siamese fighting fish, betta splendens. Physiology & behavior. 107(1):92-97.

Ellison CM, Piechota P, Madden JC, Enoch SJ, Cronin MT. 2016. Adverse outcome pathway (aop) informed modeling of aquatic toxicology: Qsars, read-across, and interspecies verification of modes of action. Environmental science & technology. 50(7):3995-4007.

Enoch S, Cronin M. 2010. A review of the electrophilic reaction chemistry involved in covalent DNA binding. Critical Reviews in Toxicology. 40(8):728-748.

Enoch S, Ellison C, Schultz T, Cronin M. 2011. A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. Critical reviews in toxicology. 41(9):783-802.

Farahmandfar M, Akbarabadi A, Bakhtazad A, Zarrindast M-R. 2017. Recovery from ketamine-induced amnesia by blockade of gaba-a receptor in the medial prefrontal cortex of mice. Neuroscience. 344:48-55.

Fitzsimmons PN, Fernandez JD, Hoffman AD, Butterworth BC, Nichols JW. 2001. Branchial elimination of superhydrophobic organic compounds by rainbow trout (oncorhynchus mykiss). Aquatic toxicology. 55(1-2):23-34.

Fraysse B, Mons R, Garric J. 2006. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotoxicology and environmental safety. 63(2):253-267.

Frazer A. 2001. Serotonergic and noradrenergic reuptake inhibitors: Prediction of clinical effects from in vitro potencies. The Journal of clinical psychiatry. 62:16-23.

Freidig AP, Hermens JL. 2000. Narcosis and chemical reactivity qsars for acute fish toxicity. Quantitative Structure-Activity Relationships: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis. 19(6):547-553.

Fuiman LA, Magurran AE. 1994. Development of predator defences in fishes. Reviews in Fish Biology and Fisheries. 4(2):145-183.

Giorgi O, Carboni G, Frau V, Orlandi M, Valentini V, Feldman A, Corda M. 1996. Anticonvulsant effect of felbamate in the pentylenetetrazole kindling model of epilepsy in the rat. Naunyn-Schmiedeberg's archives of pharmacology. 354(2):173-178.

Godfrey M. 1996. Ethology and sea turtle conservation. Trends in ecology & evolution. 11(10):433-434.

Godfrey MH, Barreto R. 1995. Beach vegetation and seafinding orientation of turtle hatchlings. Biological Conservation. 74(1):29-32.

Gould GG, Brooks BW, Frazer A. 2007. [3h] citalopram binding to serotonin transporter sites in minnow brains. Basic & clinical pharmacology & toxicology. 101(3):203-210.

Haefely W. 1983. Neuropharmacology of benzo-diazepines: Synaptic mechanisms and neural basis of action. The benzodiazepines: from molecular biology to clinical practice.21-66.

Harder A, Escher BI, Landini P, Tobler NB, Schwarzenbach RP. 2003. Evaluation of bioanalytical assays for toxicity assessment and mode of toxic action classification of reactive chemicals. Environmental science & technology. 37(21):4962-4970.

Hellström G, Klaminder J, Finn F, Persson L, Alanärä A, Jonsson M, Fick J, Brodin T. 2016. Gabaergic anxiolytic drug in water increases migration behaviour in salmon. Nature Communications. 7:13460.

Hermens J. 1990. Electrophiles and acute toxicity to fish. Environmental health perspectives. 87:219.

Huggett D, Cook J, Ericson J, Williams R. 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Human and Ecological Risk Assessment. 9(7):1789-1799.

Irons T, MacPhail R, Hunter D, Padilla S. 2010. Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. Neurotoxicology and Teratology. 32(1):84-90.

Johnsson JI, Abrahams MV. 1991. Interbreeding with domestic strain increases foraging under threat of predation in juvenile steelhead trout (oncorhynchus mykiss): An experimental study. Canadian Journal of Fisheries and Aquatic Sciences. 48(2):243-247.

Kalueff AV, Echevarria DJ, Homechaudhuri S, Stewart AM, Collier AD, Kaluyeva AA, Li S, Liu Y, Chen P, Wang J. 2016. Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. Aquatic Toxicology. 170:297-309.

Kato S. 2000. Perspective on “a molecular orbital theory of reactivity in aromatic hydrocarbons”. Theoretical chemistry accounts. Springer. p. 219-220.

Klaminder J, Hellström G, Fahlman J, Jonsson M, Fick J, Lagesson A, Bergman E, Brodin T. 2016. Drug-induced behavioral changes: Using laboratory observations to predict field observations. Frontiers in Environmental Science. 4:81.

Klaminder J, Jonsson M, Fick J, Sundelin A, Brodin T. 2014. The conceptual imperfection of aquatic risk assessment tests: Highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants. Environmental Research Letters. 9(8):084003.

Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, Healey D, Kim S, Werdich AA, Haggarty SJ. 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nature chemical biology. 6(3):231-237.

Kostal J, Voutchkova-Kostal A, Weeks B, Zimmerman JB, Anastas PT. 2012. A free energy approach to the prediction of olefin and epoxide mutagenicity and carcinogenicity. Chemical research in toxicology. 25(12):2780-2787.

Kretschmann A, Ashauer R, Hitzfeld K, Spaak P, Hollender J, Escher BI. 2011. Mechanistic toxicodynamic model for receptor-mediated toxicity of diazoxon, the active metabolite of diazinon, in daphnia magna. Environmental science & technology. 45(11):4980-4987.

Krewski D, Acosta D, Andersen M, Anderson H, Bailar JC, Boekelheide K, Brent R, Charnley G, Cheung VG, Green S et al. 2010. Toxicity testing in the 21st century: A vision and a strategy. Journal of Toxicology and Environmental Health, Part B. 13(2-4):51-138.

Kristofco LA, Brooks BW. 2017. Global scanning of antihistamines in the environment: Analysis of occurrence and hazards in aquatic systems. Science of the Total Environment. 592:477-487.

Kristofco LA, Cruz LC, Haddad SP, Behra ML, Chambliss CK, Brooks BW. 2016. Age matters: Developmental stage of danio rerio larvae influences photomotor response thresholds to diazinion or diphenhydramine. Aquatic Toxicology. 170:344-354.

Kümmerer K. 2010. Pharmaceuticals in the environment. Annual review of environment and resources. 35:57-75.

LaLone CA, Berninger JP, Villeneuve DL, Ankley GT. 2014. Leveraging existing data for prioritization of the ecological risks of human and veterinary pharmaceuticals to aquatic organisms. Phil Trans R Soc B. 369(1656):20140022.

Landrigan PJ, Fuller R, Acosta NJ, Adeyi O, Arnold R, Baldé AB, Bertollini R, Bose-O'Reilly S, Boufford JI, Breysse PN. 2017. The lancet commission on pollution and health. The Lancet.

Lipnick R, Watson K, Strausz A. 1987. A qsar study of the acute toxicity of some industrial organic chemicals to goldfish. Narcosis, electrophile and proelectrophile mechanisms. Xenobiotica. 17(8):1011-1025.

Little EE. 2002. Behavioral measures of environmental stressors in fish.

Little EE, Finger SE. 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology and Chemistry. 9(1):13-19.

Liu J, Liu X, Wang W, McCauley L, Pinto-Martin J, Wang Y, Li L, Yan C, Rogan WJ. 2014. Blood lead concentrations and children's behavioral and emotional problems: A cohort study. JAMA pediatrics. 168(8):737-745.

LoPachin RM, Gavin T, DeCaprio A, Barber DS. 2011. Application of the hard and soft, acids and bases (hsab) theory to toxicant–target interactions. Chemical research in toxicology. 25(2):239-251.

MacRae CA, Peterson RT. 2015. Zebrafish as tools for drug discovery. Nature Reviews Drug Discovery. 14(10):721-731.

Malaj E, Peter C, Grote M, Kühne R, Mondy CP, Usseglio-Polatera P, Brack W, Schäfer RB. 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. Proceedings of the National Academy of Sciences. 111(26):9549-9554.

Margiotta-Casaluci L, Owen SF, Cumming RI, de Polo A, Winter MJ, Panter GH, Rand-Weaver M, Sumpter JP. 2014. Quantitative cross-species extrapolation between humans and fish: The case of the anti-depressant fluoxetine. PLoS One. 9(10):e110467.

Martin CW, Fodrie FJ, Heck KL, Mattila J. 2010. Differential habitat use and antipredator response of juvenile roach (rutilus rutilus) to olfactory and visual cues from multiple predators. Oecologia. 162(4):893-902.

Martin JM, Saaristo M, Bertram MG, Lewis PJ, Coggan TL, Clarke BO, Wong BB. 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environmental pollution. 222:592-599.

Maximino C, De Brito TM, de Mattos Dias CAG, Gouveia Jr A, Morato S. 2010. Scototaxis as anxiety-like behavior in fish. Nature protocols. 5(2):209.

McDonald MD. 2017. An aop analysis of selective serotonin reuptake inhibitors (ssris) for fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 197:19-31.

McGee MR, Julius ML, Vajda AM, Norris DO, Barber LB, Schoenfuss HL. 2009. Predator avoidance performance of larval fathead minnows (pimephales promelas) following short-term exposure to estrogen mixtures. Aquatic Toxicology. 91(4):355-361.

McKim JM, Bradbury SP, Niemi GJ. 1987. Fish acute toxicity syndromes and their use in the qsar approach to hazard assessment. Environmental Health Perspectives. 71:171.

Mehta G, Hsiao AY, Ingram M, Luker GD, Takayama S. 2012. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. Journal of Controlled Release. 164(2):192-204.

Melvin SD, Wilson SP. 2013. The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. Chemosphere. 93(10):2217-2223.

Metcalfe C, Miao X-S, Hua W, Letcher R, Servos M. 2004. Pharmaceuticals in the canadian environment. Pharmaceuticals in the environment. Springer. p. 67-90.

Mikheev V, Wanzenböck J, Pasternak A. 2006. Effects of predator-induced visual and olfactory cues on 0+ perch (perca fluviatilis l.) foraging behaviour. Ecology of Freshwater Fish. 15(2):111-117.

Nakagawa S. 2004. A farewell to bonferroni: The problems of low statistical power and publication bias. Behavioral Ecology. 15(6):1044-1045.

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. 2015a. Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicological Sciences.kfv044.

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. 2015b. Advanced morphological—behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicological Sciences. 145(1):177-195.

OECD. 2013. Test no. 236: Fish embryo acute toxicity (fet) test.

Padilla S, Hunter D, Padnos B, Frady S, MacPhail R. 2011. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicology and teratology. 33(6):624-630.

Painter MM, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, Furlong ET, Schultz MM, Schoenfuss HL. 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (pimephales promelas). Environmental Toxicology and Chemistry. 28(12):2677-2684.

Pelli M, Connaughton VP. 2015. Chronic exposure to environmentally-relevant concentrations of fluoxetine (prozac) decreases survival, increases abnormal behaviors, and delays predator escape responses in guppies. Chemosphere. 139:202-209.

Peng X, Lin J, Zhu Y, Liu X, Zhang Y, Ji Y, Yang X, Zhang Y, Guo N, Li Q. 2016. Anxiety-related behavioral responses of pentylenetetrazole-treated zebrafish larvae to light-dark transitions. Pharmacology Biochemistry and Behavior. 145:55-65.

Perkins E, Garcia-Reyero N, Edwards S, Wittwehr C, Villeneuve D, Lyons D, Ankley G. 2015a. The adverse outcome pathway: A conceptual framework to support toxicity testing in the twenty-first century. Computational Systems Toxicology.1-26.

Perkins E, Garcia-Reyero N, Edwards S, Wittwehr C, Villeneuve D, Lyons D, Ankley G. 2015b. The adverse outcome pathway: A conceptual framework to support toxicity testing in the twenty-first century. Computational systems toxicology. Springer. p. 1-26.

Pope C, Karanth S, Liu J. 2005. Pharmacology and toxicology of cholinesterase inhibitors: Uses and misuses of a common mechanism of action. Environmental Toxicology and Pharmacology. 19(3):433-446.

Reif DM, Truong L, Mandrell D, Marvel S, Zhang G, Tanguay RL. 2015. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Archives of Toxicology.1-12.

Renier C, Faraco JH, Bourgin P, Motley T, Bonaventure P, Rosa F, Mignot E. 2007. Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmacogenetics and genomics. 17(4):237-253.

Richendrfer H, Pelkowski S, Colwill R, Creton R. 2012. On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behavioural brain research. 228(1):99-106.

Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, Haggarty SJ, Kokel D, Rubin LL, Peterson RT. 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science. 327(5963):348-351.

Rodríguez-Gil J, Cáceres N, Dafouz R, Valcárcel Y. 2018. Caffeine and paraxanthine in aquatic systems: Global exposure distributions and probabilistic risk assessment. Science of the Total Environment. 612:1058-1071.

Rovida C, Hartung T. 2009. Re-evaluation of animal numbers and costs for in vivo tests to accomplish reach legislation requirements for chemicals—a report by the transatlantic think tank for toxicology (t (4)). Altex. 26(3):187-208.

Ruiz-Colón K, Chavez-Arias C, Díaz-Alcalá JE, Martínez MA. 2014. Xylazine intoxication in humans and its importance as an emerging adulterant in abused drugs: A comprehensive review of the literature. Forensic science international. 240:1-8.

Russom CL, Bradbury SP, Broderius SJ, Hammermeister DE, Drummond RA. 1997. Predicting modes of toxic action from chemical structure: Acute toxicity in the fathead minnow (pimephales promelas). Environmental toxicology and chemistry. 16(5):948-967.

Ruuskanen JO, Peitsaro N, Kaslin JV, Panula P, Scheinin M. 2005. Expression and function of α2-adrenoceptors in zebrafish: Drug effects, mrna and receptor distributions. Journal of neurochemistry. 94(6):1559-1569.

Saari GN, Scott WC, Brooks BW. 2017. Global assessment of calcium channel blockers in the environment: Review and analysis of occurrence, ecotoxicology and hazards in aquatic systems. Chemosphere.

Saaristo M, Brodin T, Balshine S, Bertram MG, Brooks BW, Ehlman SM, McCallum ES, Sih A, Sundin J, Wong BB. 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proc R Soc B. 285(1885):20181297.

Saaristo M, McLennan A, Johnstone CP, Clarke BO, Wong BB. 2017. Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (poecilia reticulata). Aquatic toxicology. 183:38-45.

Santonastaso A, Hardy J, Cohen N, Fajt V. 2014. Pharmacokinetics and pharmacodynamics of xylazine administered by the intravenous or intra-osseous route in adult horses. Journal of veterinary pharmacology and therapeutics. 37(6):565-570.

Schäfer RB, Kühn B, Malaj E, König A, Gergs R. 2016. Contribution of organic toxicants to multiple stress in river ecosystems. Freshwater Biology. 61(12):2116-2128.

Scheil V, Kienle C, Osterauer R, Gerhardt A, Köhler H-R. 2009. Effects of 3, 4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (danio rerio) embryos and larvae. Ecotoxicology. 18(3):355-363.

Scheiner SM, Gurevitch J. 2001. Design and analysis of ecological experiments. Oxford University Press.

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D. 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environmental Science and Pollution Research. 15(5):394-404.

Schultz T, Carlson R, Cronin M, Hermens J, Johnson R, O'Brien P, Roberts D, Siraki A, Wallace K, Veith G. 2006. A conceptual framework for predicting the toxicity of reactive chemicals: Modeling soft electrophilicity. SAR and QSAR in Environmental Research. 17(4):413-428.

Schulz M, Iwersen-Bergmann S, Andresen H, Schmoldt A. 2012. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Critical Care. 16(4):1-4.

Sebire M, Davis JE, Hatfield R, Winberg S, Katsiadaki I. 2015. Prozac affects stickleback nest quality without altering androgen, spiggin or aggression levels during a 21-day breeding test. Aquatic Toxicology. 168:78-89.

Shumway CA. 1999. A neglected science: Applying behavior to aquatic conservation. Environmental Biology of Fishes. 55(1-2):183-201.

Sipes NS, Martin MT, Kothiya P, Reif DM, Judson RS, Richard AM, Houck KA, Dix DJ, Kavlock RJ, Knudsen TB. 2013. Profiling 976 toxcast chemicals across 331 enzymatic and receptor signaling assays. Chemical Research in Toxicology. 26(6):878-895.

Smith RJF. 1992. Alarm signals in fishes. Reviews in Fish Biology and Fisheries. 2(1):33-63.

Spulber S, Kilian P, Ibrahim WNW, Onishchenko N, Ulhaq M, Norrgren L, Negri S, Di Tuccio M, Ceccatelli S. 2014. Pfos induces behavioral alterations, including spontaneous hyperactivity that is corrected by dexamfetamine in zebrafish larvae. PloS one. 9(4):e94227.

Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere. 69(1):9-16.

Steele WB, Kristofco LA, Corrales J, Saari GN, Haddad SP, Gallagher EP, Kavanagh TJ, Kostal J, Zimmerman JB, Voutchkova-Kostal A. 2018a. Comparative behavioral toxicology with two common larval fish models: Exploring relationships among modes of action and locomotor responses. Science of The Total Environment. 640:1587-1600.

Steele WB, Mole R, Brooks BW. 2018b. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neurostimulant caffeine. Journal of visualized experiments: JoVE. (137).

Steele WB, Mole RA, Brooks BW. 2018c. Experimental protocol for examining behavioral response profiles in larval fish: Application to the neuro-stimulant caffeine. Journal of visualized experiments: JoVE. (137).

Steenbergen PJ, Richardson MK, Champagne DL. 2011. The use of the zebrafish model in stress research. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 35(6):1432-1451.

Stewart AM, Ullmann JF, Norton WH, Parker M, Brennan C, Gerlai R, Kalueff AV. 2015. Molecular psychiatry of zebrafish. Molecular psychiatry. 20(1):2.

Tierney KB. 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1812(3):381-389.

Torres-Hernández BA, Colón LR, Rosa-Falero C, Torrado A, Miscalichi N, Ortíz JG, González-Sepúlveda L, Pérez-Ríos N, Suárez-Pérez E, Bradsher JN. 2016. Reversal of pentylenetetrazole-altered swimming and neural activity-regulated gene expression in zebrafish larvae by valproic acid and valerian extract. Psychopharmacology.1-15.

Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. 2013. Multidimensional in vivo hazard assessment using zebrafish. Toxicological Sciences. 137(1):212-233.

Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. 2014. Multidimensional in vivo hazard assessment using zebrafish. Toxicological Sciences. 137(1):212-233.

US-EPA. 1993a. Methods for aquatic toxicity identification evaluations: Phase i toxicity identification procedures for samples exhibiting acute and chronic toxicity. US Environmental Protection Agency, Office of Research and Development.

US-EPA. 1993b. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, epa/600/4–90/027f.

Valenti TW, Gould GG, Berninger JP, Connors KA, Keele NB, Prosser KN, Brooks BW. 2012. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (ssri) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. Environmental Science & Technology. 46(4):2427-2435.

Valenti TW, Perez-Hurtado P, Chambliss CK, Brooks BW. 2009. Aquatic toxicity of sertraline to pimephales promelas at environmentally relevant surface water ph. Environmental Toxicology and Chemistry. 28(12):2685-2694.

van Woudenberg AB, Snel C, Rijkmans E, De Groot D, Bouma M, Hermsen S, Piersma A, Menke A, Wolterbeek A. 2014. Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs. Reproductive Toxicology. 49:101-116.

van Woudenberg AB, Wolterbeek A, te Brake L, Snel C, Menke A, Rubingh C, de Groot D, Kroese D. 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: The value of the zebrafish (danio rerio) embryotoxicity test (zet). Reproductive toxicology. 41:35-44.

Veith GD, Call DJ, Brooke L. 1983. Structure-toxicity relationships for the fathead minnow, pimephales promelas: Narcotic industrial chemicals. Canadian Journal of Fisheries and Aquatic Sciences. 40(6):743-748.

Veith GD, Mekenyan OG. 1993. A qsar approach for estimating the aquatic toxicity of soft electrophiles [qsar for soft electrophiles]. Quantitative Structure-Activity Relationships. 12(4):349-356.

Verhaar HJM, van Leeuwen CJ, Hermens JLM. 1992. Classifying environmental pollutants. Chemosphere. 25(4):471-491.

Viglino L, Aboulfadl K, Prévost M, Sauvé S. 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with lc-appi-ms/ms. Talanta. 76(5):1088-1096.

Vignet C, Parrott J. 2017. Maturation of behaviour in the fathead minnow. Behavioural processes. 138:15-21.

Voutchkova AM, Osimitz TG, Anastas PT. 2010. Toward a comprehensive molecular design framework for reduced hazard. Chemical reviews. 110(10):5845-5882.

Wang M, Chen J, Lin K, Chen Y, Hu W, Tanguay RL, Huang C, Dong Q. 2011. Chronic zebrafish pfos exposure alters sex ratio and maternal related effects in f1 offspring. Environmental Toxicology and Chemistry. 30(9):2073-2080.

Weber D. 2006. Dose-dependent effects of developmental mercury exposure on c-start escape responses of larval zebrafish danio rerio. Journal of fish biology. 69(1):75-94.

Weis JS, Smith G, Zhou T, Santiago-Bass C, Weis P. 2001. Effects of contaminants on behavior: Biochemical mechanisms and ecological consequences: Killifish from a contaminated site are slow to capture prey and escape predators; altered neurotransmitters and thyroid may be responsible for this behavior, which may produce population changes in the fish and their major prey, the grass shrimp. Bioscience. 51(3):209-217.

Winter MJ, Redfern WS, Hayfield AJ, Owen SF, Valentin J-P, Hutchinson TH. 2008. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. Journal of Pharmacological and Toxicological Methods. 57(3):176-187.

Yen J, Donerly S, Levin ED, Linney EA. 2011. Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. Neurotoxicology and Teratology. 33(6):735-741.

Zhang W, Lin K, Sun X, Dong Q, Huang C, Wang H, Guo M, Cui X. 2012. Toxicological effect of mpa–cdse qds exposure on zebrafish embryo and larvae. Chemosphere. 89(1):52-59.

Zhao J, Xu T, Yin D-Q. 2014. Locomotor activity changes on zebrafish larvae with different 2,2′,4,4′-tetrabromodiphenyl ether (pbde-47) embryonic exposure modes. Chemosphere. 94:53-61.