The Neurobehavioral and Developmental Effects of Flame Retardants on Zebrafish

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Abstract:

Flame retardants are added to a large range of consumer products—including textiles, furniture, electronics and building materials—for the purpose of preventing or slowing the the spreading of fires. Human exposure to flame retardants has been shown to occur through both ingestion of indoor air and absorption of dust particles through the skin. In recent years, concern pertaining to the health and environmental implications of certain categories of flame retardants has led to the phasing out of these chemicals and replacement with alternatives, such as organophosphate (OP) flame retardants. Thus, the present study investigates whether developmental exposure to low levels of these chemicals will result in measurable behavioral effects at early or later life stages. Zebrafish eggs are exposed to flame retardant chemicals, an OP pesticide of known neurotoxicity, or a vehicle control consisting of 0.01% solution of dimethyl sulfide oxide (DMSO) for 5 days post-fertilization. After exposure, larvae swim behavior is tested. The 6-day old larvae are then transferred to aquarium water and allowed to develop normally. The adult zebrafish are tested on a battery of assessments examining anxiety-related behavior, sensorimotor integration, predatory escape, sociability, and cognitive ability. The ultimate aim is to evaluate the safety profiles of these compounds and determine whether zebrafish high throughput behavioral assays are an effective model for characterizing neurotoxicity.

1. Background and Significance

Growing use and development of flammable plastics and electronics in the context of current strict fire safety standards has led to an increase in the incorporation of flame retardants into the manufacturing of many products. Flame retardants constitute a diverse group of chemicals that are added to a large range of consumer products—including textiles, furniture, electronics and building materials—for the purpose of preventing the chemical reactions that cause fire (Noyes et al. 2015). Chemicals associated with the production of food, drugs, and pesticides are regulated before they go into products, however there is little to no regulation regarding other industrial chemicals—specifically flame retardants (Babrauskas et al., 2013). In recent years, concern pertaining to the health and environmental implications of certain categories of flame retardants has led to the phasing out of these chemicals and subsequent replacement with alternatives. For instance, polychlorinated biphenyls (PCBs) were introduced as the first commercial flame-retardants. However, PCBs were phased out due to discoveries of their adverse health effects, and replaced by several classes of organohalogens—regardless of insufficient safety information (Hendriks & Westerink, 2015). In recent years, two classes of flame retardants have been commonly used in consumer products: brominated flame retardants (BFRs) and organophosphate-based flame retardants (OPFRs). Within these classes of compounds, there are a wide variety of structural forms—both halogenated and non-halogenated, with many structurally similar to organohalogen pesticides. Yet, despite these concerns, the safety profiles and possible human and environmental health risks—specifically the neurobehavioral toxicity—of many of the chemicals being used as flame retardants remain uncertain.

Brominated flame retardants are considered persistent organic pollutants (POP), in that they are non-combustible, subject to long-range geographic transport and resistant to degradation—which has lead to the detection of BFRs in human and environmental samples (Hendriks & Westerink, 2015). Polybrominate diphenyl ethers (PBDEs), a specific class of BFRS, were originally introduced in the 1960s and used in the production of textiles, furniture, and electronics as well as a wide range of other consumer products (Truong et al., 2014). Flame retardants are added to products, rather than being chemically bound. Consequently, overtime PBDEs—along with other classes of flame retardants—separate from these products, leading to accumulation in indoor and outdoor settings (Stapelton et al., 2009). Human exposure to PBDEs has been shown to occur through both inhalation and ingestion of indoor air and absorption of dust particles through the skin (Johnson-Restrepo & Kannan, 2009). Children and adults have been estimated to ingest approximately 100mg and 20mg of dust per day, respectively (Stapelton et al., 2009). Dust has been estimated to be the main contributor of PBDE exposure in small children (Jones et al., 2005). Due to the lipophilicity of these compounds, significant levels of PBDEs have been found in the in mothers' milk, blood, food, and adipose tissue (Schecter et al., 2006; Shaw et al., 2010; Kim et al., 2014). PBDE congeners, such as BDE-47, were estimated to be present in milk samples in concentrations of 4.2 ng/g (Toms et al., 2009). Elevated human milk concentration has been thought to provide the greatest exposure across the entire lifespan; this is especially problematic, as the period in which children are breastfed tends to coincide with critical periods of neurodevelopment (Jones et al., 2005). Further, compounds that have accumulated in pregnant women may be transferred through the placenta to the fetus, which may also lead to serious teratological and neurological consequences.

Accordingly, PBDEs have also been implicated in additional health-related contexts. Previous studies have shown that high-doses of PBDEs have led to cancer, reproductive and developmental toxicity, endocrine disruption, and adverse neurological effects (Shaw et al., 2010). Possible points of action include binding to thyroid hormone receptors and transport proteins, disruption of calcium homeostasis, and modulation of GABA and nicotinic acetylcholine receptor function (Dingemans et al., 2011). Additionally, one of the main physiological consequences of exposure to BFRs, specifically PBDEs, is apoptosis—or cell death (Souza et al., 2016). Further, biotransformation also has implications for the toxicity of these compounds. Biotransformation is an important biotic process in which organisms convert organic compounds into alternative forms (Kebamo et al., 2015). Usually, toxicants are inactivated and eliminated from the body through this process; however, in some instances, biotransformation has been shown to lead to bioactivation—where metabolites become more biologically active than the parent compound (Hendriks & Westerink, 2015). Bioactivation of polybrominated diphenyl ethers (PBDEs) has been shown to lead to the formation of metabolites that appear to add significantly to the toxicity potential of the parent compounds (Hendriks & Westerink, 2015; Macaulay et al., 2015). Both the parent compounds and metabolites of PBDEs have been detected in human samples (Dingemans et al., 2011). In particular, the metabolite hydroxylated metabolite 6-OH-BDE-47—formed through the oxidative metabolism of BDE-47—has been shown to be considerably more disruptive than the parent compound in neurotransmitter release and calcium homeostasis (Dingemans et al., 2011).

PBDEs were originally manufactured as three technical mixtures (penta-, octa-, and deca-BDE) that were intended for commercial use (Noyes et al., 2015; Usenko et al., 2011). Two of the main components in these mixtures were reported to be BDE-99 (2,2',4,4',5-

pentabromodiphenyl ether) and BDE-47 (2,2',4,4'-tetrabromodiphenyl ether) (Weber et al., 2011; Xu et al., 2013). Both congeners have been associated with neurotoxicity in humans and animal studies. Six-year old children living in households with higher concentrations of BDE-99 tended to perform less well in verbal comprehension than children with little or no exposure (Chevrier et al., 2016). Studies investigating BDE-47 and BDE-99 exposure in rodent models have also reached similar end-points, demonstrating impairments in learning, memory and other cognitive functions (Viberg et al., 2003; Eriksson et al. 2001). Taken together with similar findings, there has been a clear association between early exposure to these PBDEs congers and cognitive deficits (Chevrier et al., 2016). Subsequently, the production and use of penta- and octa-BDE in many regions have been phased out due to concerns about the toxicity and additional adverse effects (Noyes et al., 2015; Usenko et al., 2011). Although the use of PBDEs has been greatly reduced, humans and wildlife will still be exposed to PBDEs and their metabolites for many years (Dingemans et al, 2011). Their persistence is in part due to environmental exposure and bioaccumulation and also the fact that consumer products with these flame retardants are still present in many homes (Dingemans et al, 2011).

With the phasing out of many BFRs, there has been increased use in existing flame retardants, as well as increased pressure for the development of alternative formulations in order to meet flammability standards (Stapelton et al., 2009). In particular, there has been a growing reliance on the incorporation of organophosphate flame retardants (OPFRs) into consumer products. Yet, for a majority of the alternative flame retardants being produced, basic information about their chemical composition and consumer product application is often unavailable (Stapelton et al., 2009). Despite limited information about the possible adverse effects of these chemicals, emerging OPFRs are being produced in large volumes, estimated to

be on the upwards of 50 million pounds/year (Behl et al., 2015). There are a number of OPFRs that are currently present in isomeric and commercial mixtures. In comparison to PBDEs, OPFRs have been reported in equivalent, and in some cases higher, levels in indoor dust samples (Stapelton et al., 2009). Isopropylated phenol phosphate (IPP) is an example of an OPFR that is commonly used as a flame retardant in a wide variety of products. Metabolites of this compound as well as other OPFRs have been detected in urine of adults and in breast milk samples (Kim et al., 2014; Carignan et al., 2013). As previously seen with brominated flame retardants, there are many concerns surrounding possible implications of human and animal exposure to these chemicals. However, despite these concerns there still a lack of significant evidence to determine complete safety profiles for many of these compounds.

Even more alarming, many of the OPFRs that have been implemented as replacements for the phased out PBDEs are structurally similar to organophosphate pesticides. Developmental organophosphate insecticide exposure in rodents has been associated with abnormal sensorimotor response, emotional dysfunction, and cognition (Eriksson et al., 2001; Viberg et al., 2003). Subsequently, exposure during critical periods of development may seriously affect the developing nervous system. Preliminary evidence has suggested that exposure to several members of both the halogenated (such as BDE-47) and organophsophorus classes may cause disruptions in the thyroid system and developing nervous system (Chan & Chan, 2012; Jarema et al., 2015).

Although screening toxins with the use of in vitro assays can quickly and efficiently demonstrate how these compounds may lead to immediate effects on cellular function, such models do not provide information concerning complex cellular interactions and broader physiological implications. Zebrafish are an excellent animal model for studying

neurobehavioral toxicity because they provide an intermediate model—between high throughput *in vitro* screens and classical mammalian models—that incorporates both systems complexity and practical simplicity (Tran et al., 2016; Bailey et al., 2013). Zebrafish and mammalian models, such as rodents, share similar brain morphology—including cellular structure and signaling (Truong et al., 2011; Kalueff et al, 2014). Brain neurochemistry is also greatly conserved in vertebrates (Kalueff et al, 2014). Further, zebrafish embryos (and chorion) are transparent, enabling clear brain and developmental visualization. External (non-uterine) development also significantly simplifies chemical exposures. Zebrafish embryos develop rapidly, becoming fully-functioning larvae by 5 days post fertilization (dpf) and reach adulthood in approximately 3 months (Bailey et al., 2013). Zebrafish are also highly prolific—producing over a 100 embryos at a single breeding—and generally associated with low maintenance costs (Truong et al., 2014; Zhao et al., 2015). Due to their fast development and small size (approximately 2.5 cm at adulthood), zebrafish offer logistical and economical benefits in comparison to rodent models (Bailey et al., 2013). Early neurobehavioral tests, such as the larval swim assay, can be performed to assess the effects of acute exposure during development on elementary neurobehavioral function. Further, zebrafish are capable of more complex processing as development continues into adulthood. Adult zebrafish testing paradigms can provide information on more complex behavior, such as sensory motor response, emotional responses, learning and memory, social interactions, and other cognitive functions.

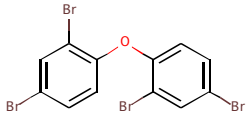
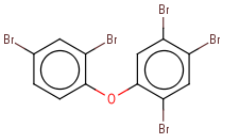
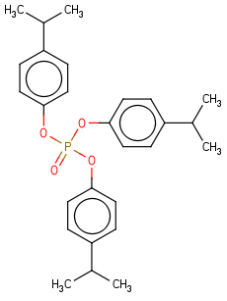
Recent evidence suggests that organophosphates and additional classes of flame retardants may have neurobehavioral and teratological consequences in zebrafish (Noyes et al., 2015, Oliveri et al., 2015). Zebrafish larvae exposed during development to chemicals from halogenated (BDE-47, BDE-99) and organophosphorus classes (IPP) have displayed abnormal

locomotor behavior in light and dark environments (Usenko et al., 2011; Jarema et al., 2015; Noyes et al., 2015). Yet, it still remains largely uncertain whether or not larval exposure to these chemicals leads to lasting long-term neurological effects. Zebrafish embryos and early larvae are ideal models for the use of high throughput behavioral assays due to their low cost and small size. However, at present few studies investigate whether or not larvae neurobehavioral assays are predictive of long term effects, enduring throughout adulthood. For one, adult zebrafish are particularly social animals, however larvae do not possess the same ability to engage in social behaviors as well as other complex behaviors (Dreosti et al., 2015). Few studies even investigate the behavioral effects of developmental exposure on adult zebrafish. Determining whether or not results from early larval testing are suggestive of later-in life deficits is a critical step in the study of toxicology and pharmacology in zebrafish.

In the present study we assessed the short and long-term neurobehavioral toxicity of developmental exposure to organophosphorous and halogenated flame retardants, specifically IPP, BDE-47, and BDE-99. Isopropylatd phenol phosphate (IPP) is classified as an organophosphate and it does not contain halogens. Both 2,2'4,4'-tetrabromodiphenyl ether (BDE-47) and 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) are considered polybrominated diphenyl ethers (PBDE) and known to be neurotoxic (Table 1). Additionally, the organophosphate pesticide chlorpyrifos (CPF)—also an established neurotoxic—was included. Our aim was to identify whether developmental exposure to low levels of these chemicals, in the absence of gross morphological effects or increased lethality, will result in measurable behavioral effects at early or later life stages. Further, we also planned to see whether or not results from larval testing are predictive of adult behavioral testing. For this purpose we exposed zebrafish embryos to each of the above chemicals separately, and evaluated the survival and

morphology of the embryos. At the early larval stage we analyzed locomotor activity in light and dark as a measure of larval behavior. The larval swim test is an important measure that provides information on short-term effects of toxins on rudimentary neurobehavioral function. Once adulthood was reached, the zebrafish that were exposed during development were tested on a battery of behavioral assessments in order to analyze anxiety-related behavior, sensorimotor response and habituation, social affiliation, and predatory stimulus escape. It was hypothesized that the developmental exposure to BDE-47, BDE-99, IPP, and CPF to show gross behavioral deficits in the larval motility assay and across the adult behavioral test battery. Additionally, we expected that the results from the larval testing to be predicative of later in life deficits.

Table 1: Table of Chemical Compounds (from NTP contract Statement of Work)

Chemical ID	**Chemical Name**	**CASRN**	**Structure**
BDE-47	2,2'4,4'-tetrabromodiphenyl ether	5436-43-1	
BDE-99	2,2',4,4',5-pentabromodiphenyl ether	60348-60-9	
IPP	isopropylated phenol phosphate	68937-41-7	

2. Methods:

2.1. Fish housing, husbandry and breeding

All experiments were conducted using AB* wild-type zebrafish strain bred from a local colony originated from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA). Fish were maintained in 3 L or 10 L flow through tanks in recirculating flowing water systems (Aquatic Habitats, Inc., Apopka, FL, USA; Aquatic Enterprises, Inc., Bridgewater, MA, USA). Juvenile and adult fish density was kept at ≤5 fish per liter. Illumination was set to a 14:10 h light: dark cycle, and water temperature was maintained at 27±1ºC. System water was a mixture of sea salt (Instant Ocean, 0.5 parts per thousand) and buffer (Seachem Neutral Regulator, 125 mg/l) in de-ionized water. Water chemistry, salinity and temperature were monitored weekly. Adult fish were fed twice daily, morning and afternoon with brine shrimp hatched in-house over 24 h (origin Brine Shrimp Direct, Ogden, UT, USA), and once daily, at noon, with a mixture of solid pellet food containing; TetraMIN Tropical Flakes (Blacksburg, VA, USA); GEMMA Micro 300 micro-pellets (Skretting USA, Tooele, Utah); Zebrafish Complete Diet (Ziegler Bros., Inc., Gardners, PA, USA). Breeding was achieved using in-tank inserts placed in the flow-through tanks approximately 12 hours prior to egg collection. Eggs were collected 1-2 h after the lights-on phase and rinsed with 10,000 x diluted solution of bleach for 1 min, followed by quick rinses with system water three times. 0-5 days post fertilization (dpf) old embryos were kept in an incubator held at 28-29 ºC, with a 14:10 light:dark cycle. From 6 dpf until 10-11 dpf, larvae were kept in 3 L tanks with a small amount of system water that was increased daily,and fed with fine-particle solid food (Golden Pearl Reef & Larval Fish Diet, 5-50 micron size) three times a day. At 10-11 dpf the tanks were started on water flow-through, and the diet was changed to

twice dailey feeding withbrine shrimp (Brine Shrimp Direct, Inc.) and once daily fine-particle solid food. At 3-4 weeks post fertilization, juvenile fish were transferred to the above detailed adult diet.

2.2 Chemical exposures

Eggs were transferred to Petri dishes and stored in the incubator until 5 h post fertilization (hpf) when they were sorted under a dissection microscope, discarding unfertilized or otherwise abnormal eggs. Eggs were then randomly and evenly distributed into glass Petri dishes filled with system water and immediately exposed according to the following protocol. The system water in each dish were replaced with Z ml of system water mixed with Z μl of 100% DMSO or 1000x toxin in 100% DMSO for a final concentration of 1x toxin and/or 0.1% DMSO. Z=number of embryos per dish. Three flame retardants (FRs) were tested; isopropylated phenyl phosphate (IPP); 2,2',4,4',5-pentabromodiphenyl ether (BDE-99); and 2,2'4,4'-tetrabromodiphenyl ether (BDE-47), and the organophosphate pesticide chlorpyrifos (CPF). The final concentrations for each chemical and the number of embryos per dish in each cohort are detailed in Table 2. Dosing was initially determined based on the available data. The goal was to ensure that the highest dose used was just below the threshold for increased lethality and overt dysmorphogenesis. The middle and lower doses were on either log or half-log scale. After initial testing, dosing was adjusted. Across all trials, 0.1% DMSO served as the control treatment. Embryos were exposed from approximately 6 hpf until 5 dpf, and the exposure solution was refreshed every 24 h. At 5 dpf, embryos were rinsed twice with fresh system water before being transferred to clean Petri dishes with non-dosed system water. Throughout the exposure period,

embryos were examined under a dissecting microscope and those exhibiting arrested development or malformations were discarded and noted.

Table 2: Chemical exposure concentrations used in the two sets of chemicals
**During all 3 cohorts in set 2, 0.3 μM of CPF was included as a positive control group.

Cohort	Toxin	Concentrations (μM)
1	IPP	0.03, 0.3, 3
1	BDE47	0.3, 3, 10
1	BDE99	0.003, 0.03, 0.3
2,3	IPP	0.01, 0.03, 1.0
2,3	BDE47	0.1, 0.3, 1.0

2.3. Larval motility assay

6-day-old larvae were placed into 96-well plates, one larvae per well in 0.5 ml system water, and then allowed to acclimate for 1 h in the incubator. Each plate represented all exposure conditions and the placement of each exposure group varied within plates. Table 3 presents information on the numbers of larvae tested in each exposure cohort. Plates were separately placed in the DanioVisionTM lightbox (Noldus, Wageningen, The Netherlands), and larval locomotion was recorded with an infrared camera. Each trial consisted of an initial 10- min acclimation period in the dark (0% illumination) followed by 2 cycles of 10 min at 100% illumination (5000 lx) and 10 min at 0% illumination. The EthoVision XT® tracking software (Noldus) was used to calculate the average distance each subject moved in cm per 10 min increments. All larval testing was conducted at 6 dpf between 10:00 AM and 5:00 PM. Following testing, larvae were either euthanized or gently transferred back to 3 L tanks as described above (section 2.1 Fish husbandry and breeding).

Table 3: Chemical exposure concentrations for larvae tested on swim motility assay.

Treatment	Cohort/DoF	# fish	Total # fish
Non-exposed	1/2-11-16	21	76
	2/2-22-16	24	
	3/3-1-16	31	
0.1% DMSO	1/2-11-16	30	84
	2/2-22-16	34	
	3/3-1-16	30	
0.3 CPF	1/2-11-16	30	82
	2/2-22-16	22	
	3/3-1-16	30	
0.01 IPP	2/2-22-16	25	54
	3/3-1-16	29	
0.03 IPP	1/2-11-16	28	80
	2/2-22-16	24	
	3/3-1-16	28	
0.003 BDE-99	1/2-11-16	35	90
	2/2-22-16	26	
	3/3-1-16	29	
0.03 BDE-99	2/2-22-16	26	53
	3/3-1-16	27	
0.3 BDE-99	1/2-11-16	31	82
	2/2-22-16	24	
	3/3-1-16	27	
0.03 BDE-47	4/5-17-16	8	8
0.1 BDE-47	2/2-22-16	23	49
	3/3-1-16	26	

2.4. Adult behavioral test battery

Developmentally-exposed adult zebrafish were tested through a series of four behavioral assays over a period of 1-2 weeks per cohort. All adult behavioral testing was conducted between the hours of 10:00 AM and 5:00 PM. Testing times were counterbalanced across all experimental groups. Table 4 presents information on the numbers of larvae tested in each exposure cohort. The fish were fed at the beginning of each testing day prior to their transfer to the testing room. In the testing room the fish were allowed to acclimate for 30-45 min before testing. The EthoVision XT® tracking software (Noldus) was used to conduct video analysis in all assays.

Table 4: Chemical exposure concentrations for fish tested on adult behavioral test battery.

Treatment	Cohort/DoF	Group	# fish	Total # fish
Non-exposed	5/6-15-16	NE	13	13
0.1% DMSO	1/2-11-16	Y	16	21
	5/6-15-16	BC	5	
0.3 CPF	1/2-11-16	V	6	13
	5/6-15-16	AV	7	
0.01 IPP	4/5-17-16	BA	7	18
	5/6-15-16	BA	11	
0.03 IPP	1/2-11-16	P	12	26
	5/6-15-16	AX	14	
0.3 IPP	1/2-11-16	R	3	3
0.003 BDE-99	1/2-11-16	S	12	28
	5/6-15-16	BD	16	
0.03 BDE-99	4/5-17-16	BB	2	8
	5/6-15-16	BB	6	
0.3 BDE-99	1/2-11-16	W	16	26
	4/5-17-16	BF	4	
	5/6-15-16	BF	6	
0.01 BDE-47	4/5-17-16	AW	3	11
	5/6-15-16	AW	8	
0.03 BDE-47	5/6-15-16	BE	9	9
0.1 BDE-47	5/6-15-16	AZ	12	12

2.5. Novel Tank dive test:

This test was used in order to assess exploration and habituation to a new environment. Testing procedure was conducted according to Oliveri et al, 2015. Briefly, individual fish were placed in a 1.5 L trapezoidal plastic test tank, filled with 10 cm of system water. A video camera was used for side-view recording of the fish. Swimming activity (distance traveled in cm/min) and average distance from the bottom of the tank (cm/min) were measured for each fish throughout the trial.

2.6. Sensorimotor Startle and Habituation Test

The purpose of this test is to assess the sensorimotor startle response and habituation to repeated stimuli of the adult fish (Figure 1). Testing procedure was conducted according to Oliveri et al, 2015. Briefly, individual fish were placed in eight translucent plastic cups for a 10-min trial. The testing cups measured 5.7 cm in diameter, each containing 40 ml of system water and were arranged in a 4 x 2 array. Fish were allowed to acclimate for a 30-sec period prior to the testing period. Then, a solenoid located beneath each dish tapped the bottom of the dishes once per minute. In total, 10 taps were delivered over 10-min. Swimming activity of the fish (distance traveled in cm/min) was calculated five seconds before and after each tap for each fish separately.

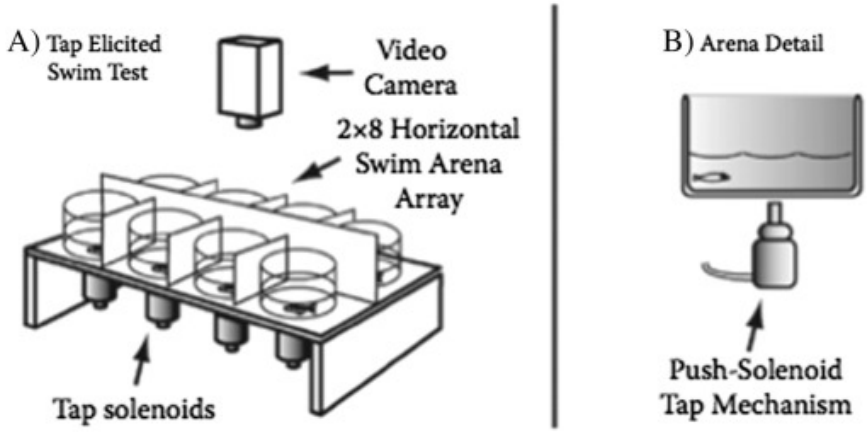


Figure 1: Diagram of experimental set-up for the tap test, which measures startle response. Fish are placed in each of the 8 cylindrical chambers. The startle response, given at one minute intervals, is a tap delivered from a push-solenoid. The camera captures the 10 minute trial, which is imported into EthoVision to measure swimming distance. Adapted from Bailey et al., 2015.

2.7. Shoaling Assay:

The shoaling test is a measure of social affiliation. Zebrafish tend to swim in groups or shoals. Typically, as zebrafish approach a 'shoal', or a group of conspecifics, they participate in a dart/pause locomotor behavior beside the group—the behavior is known as shoaling. Testing

procedure was conducted based on Oliveri et al, 2015 with modifications. In order to enhance social tendency, each fish was first isolated for 30-min in a 1.5 L tank surrounded by opaque dividers before being placed in the testing tank. The testing tank was a 50 cm long 30 cm wide acrylic tank. Black partitions were inserted into the tank to create two lanes each measuring 50 cm long and 11.5 cm wide. A computer screen was place on each side of the tank (long side). Two fish were individually placed in each lane. During the first two minutes of the trial the computer monitors displayed a background of static ovals that resembled the size and color patterning of adult zebrafish. Following the first two minutes, a video of a shoal of zebrafish started playing on one of the monitors for 5 min. Swimming activity (distance traveled in cm/min) and distance from the side of the tank corresponding to the screen playing the video were calculated.

2.8. Predatory Escape Test:

The purpose of this test was to assess escape and avoidance behavior in response to a predator-like stimulus. Fear responses are important behavioral reactions and may also have clinical relevance. Adult fish were placed into the testing tank, which was positioned between two computer monitors. The testing tank set-up was the same as described above in 2.7. Shoaling Assay. Two fish were individually placed in each lane. The test was a total of 9 minutes, with a 1-min acclimation period and 4 rounds of a 2 minute testing cycle that was displayed on one of the monitors. The testing cycle consisted of 1-min presentation of a small circle in the center of the screen (1.3 cm in diameter) that gradually grew to a larger circle (30.5 cm in diameter) then disappeared and grew again, followed by a 1-min period of a blank white screen. Two color dots were used: a blue dot that grew slowly (4s) and a red dot that grew quickly (1s) and alternated

between rounds. The growing dot is meant to mimic the appearance of a predator approaching the fish in order to assess escape and avoidance behavior in response to a predator-like stimulus. Swim speed and distance from the screen were both measured and recorded with Ethovision®. The difference between the distance from the tank wall during the minute of stimulus presentation and the distance from the wall during the following minute of the blank screen represents the escape/avoidance response.

2.9. Statistical analysis

The results of the larval and adult testing were analyzed with two-way ANOVA with Dunnett's post-hoc comparison to the 0.1% DMSO control. ANOVA was performed in GraphPad Prism 7 (GraphPad Software, Inc., version 7.00). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Survival at 6 days post fertilization (dpf)

The viability and survival of the embryos were tracked from 5 h post fertilization to 6 days post fertilization, throughout the developmental exposures and are presented in Table 4. Exposure treatments to 3 μM IPP, 3 μM BDE-47, 10 μM BDE-47, 1 μM BDE-47, 0.3 μM BDE-47 caused major reduction in survival and the exposure levels were adjusted accordingly (Table 5).

Table 5: Larvae survival data at 6 dpf across three different cohorts.

	Group identity and exposure concentration (μM)	**% Survival**
1	DMSO	75
	0.3 CPF	75
	0.03 IPP	70
	0.3 IPP	67.5
	3 IPP	-
	0.3 BDE47	82.5
	3 BDE47	-
	10 BDE47	-
	0.003 BDE99	87.5
	0.03 BDE99	-
	0.3 BDE99	77.5
	Non-exposed	
2	DMSO	90
	0.3 CPF	55
	0.01 IPP	82.5
	0.03 IPP	82.5
	0.1 IPP	82.5
	0.01 BDE47	75
	0.03 BDE47	-
	0.1 BDE47	-
	0.003 BDE99	80
	0.03 BDE99	80
	0.3 BDE99	85
	Non-exposed	95
3	DMSO	92.5
	0.3 CPF	85
	0.01 IPP	82.5
	0.03 IPP	77.5
	0.1 IPP	77.5
	0.01 BDE47	75
	0.03 BDE47	-
	0.1 BDE47	-
	0.003 BDE99	87.5
	0.03 BDE99	75
	0.3 BDE99	75

3.2. 6 dpf larval motility assay

A main effect of light condition on locomotion was observed, demonstrating that the larval fish swam more in the dark phases than in the light phases. This effect was observed

regardless of treatment. Comparisons made across all three exposure cohorts indicated several main effects (Figures 3, 4 and 5). The ANOVA revealed a main effect of CPF exposure on locomotor activity during both dark phases ($p < 0.001$). Subsequent post-hoc analyses showed that larvae exposed to 0.3 μM of CPF swam less than the control fish during the initial acclimation period and both dark phases (Figure 2).

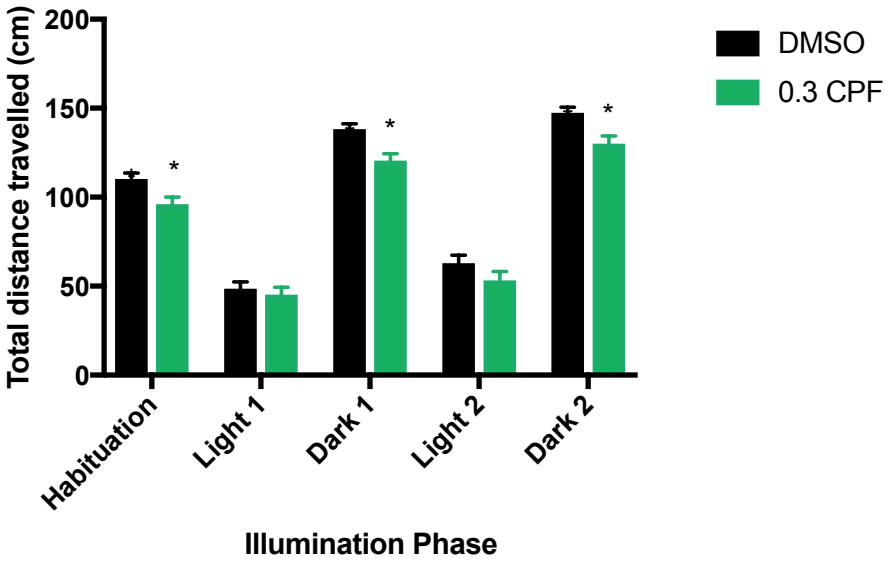


Figure 2: Larval Activity in control groups. Total distance traveled (cm) during the light and dark phases of the 6dpf larval activity assay across the controls. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

The ANOVA indicated a main effect of BDE47 exposure on larval locomotor activity in both dark phases and both light phases ($p < 0.0001$). Further post-hoc analyses revealed that larvae exposed to 0.1 μM BDE-47 showed a global reduction in swimming activity across all phases compared to controls (Figure 3). Dunnett's comparison revealed a significant ($p < 0.05$) increase in swimming behavior for larvae exposed to 0.3 μM of BDE-99 compared to controls (Figure 4). There was also a main effect of IPP exposure on locomotion in both dark phases ($p < 0.005$). Post-hoc analyses showed larvae exposed to 0.1 μM IPP swam faster than the control fish during the dark phases (Figure 5).

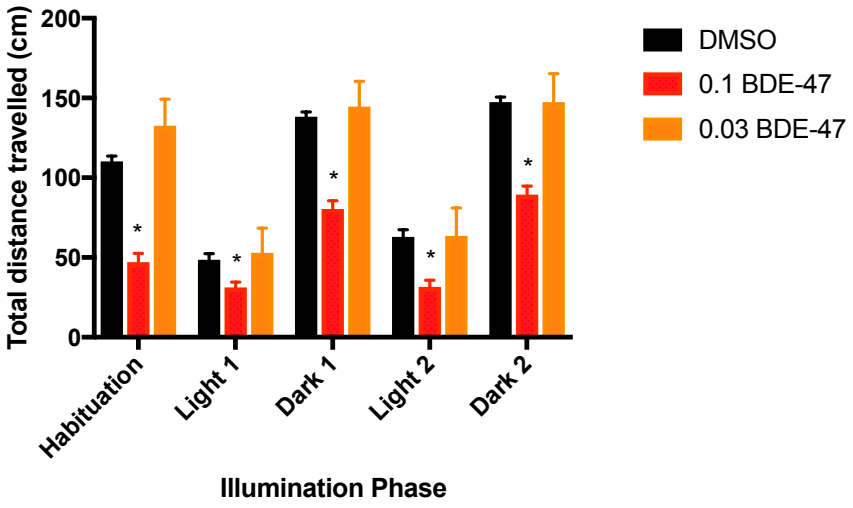


Figure 3: Larval Activity following BDE-47 exposure. Total distance traveled (cm) during the light and dark phases of the larval activity assay across the BDE-47 treatment groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

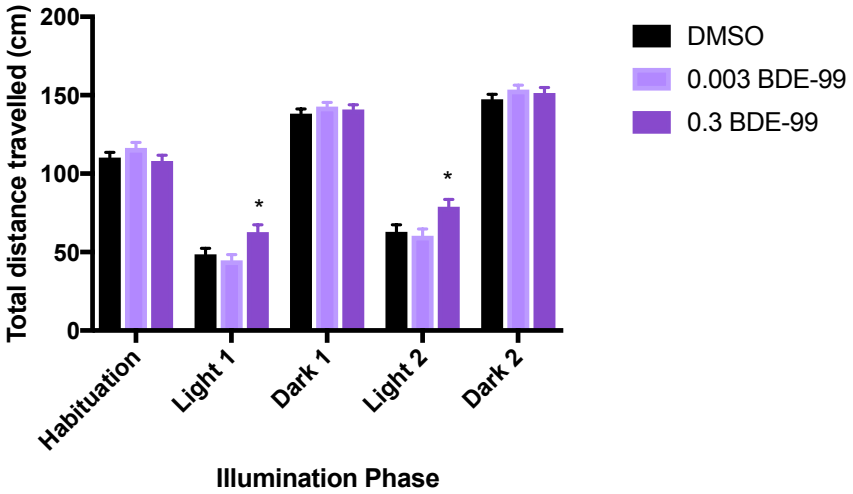


Figure 4: Larval Activity following BDE-99 exposure. Total distance traveled (cm) during the light and dark phases of the larval activity assay across the BDE-99 treatment groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

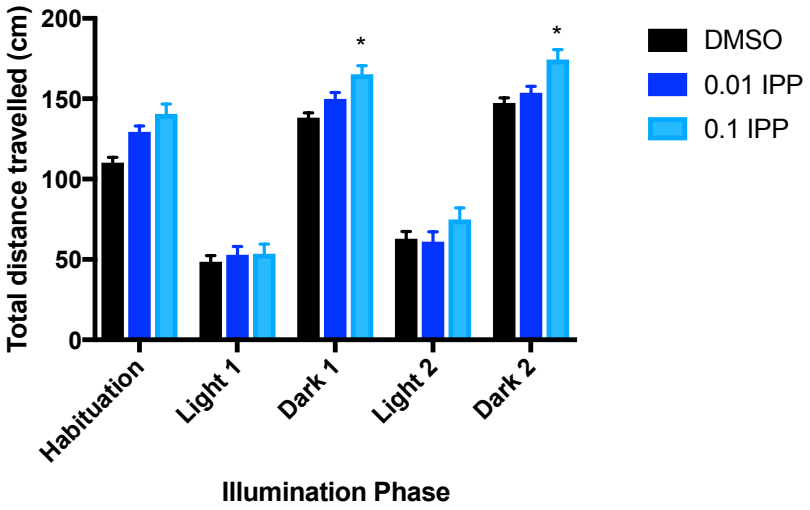


Figure 5: Larval Activity following IPP exposure. Total distance traveled (cm) during the light and dark phases of the larval activity assay across the IPP treatment groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

3.3. Novel tank test

A main effect of minute on distance from the bottom of the tank was observed across all groups ($p < 0.0001$). For most of the groups, the fish gradually swam farther from the bottom as the trial progressed, as is typically seen during this test. The ANOVA revealed a main effect of CPF exposure on mean distance from the bottom the tank ($p < 0.0001$). Subsequent post hoc analyses showed that fish exposed to 0.3 μM CPF, on average remained at a larger distance from the bottom during the first three minutes of the task compared to the control group exposed to 0.1% DMSO (Figure 6).

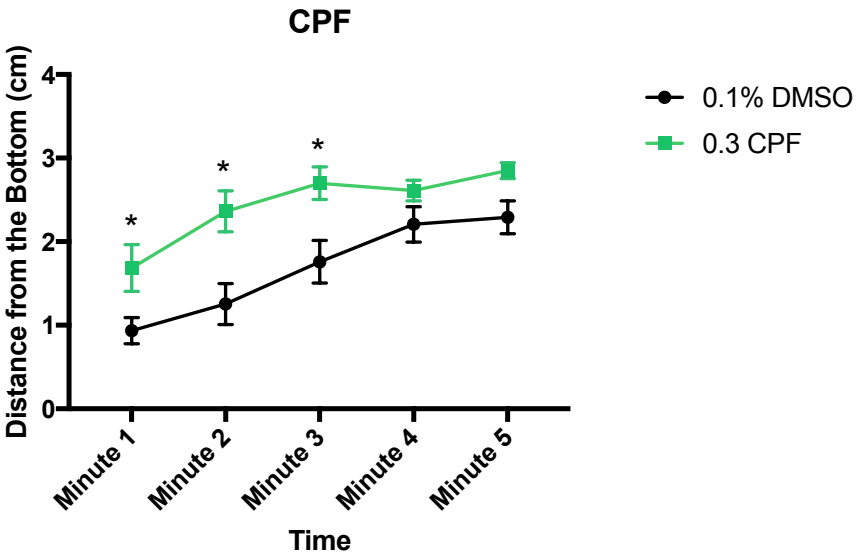


Figure 6: Novel Tank test. Mean distance from the bottom of the tank is plotted for the positive (CPF) and negative (0.1% DMSO) control groups across the five minutes of the novel tank exploration trial. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

There was a significant interaction of minute X IPP exposure on distance from the bottom ($p < 0.0001$). Dunnett's comparison revealed that fish exposed to 0.01 μM IPP remained further from the bottom during the first two minutes of the test compared to control fish (Figure 7). A main

effect of IPP exposure on distance traveled per minute was also found ($p < 0.05$). However, further post-hoc analysis did not reveal any significant differences at specific time points.

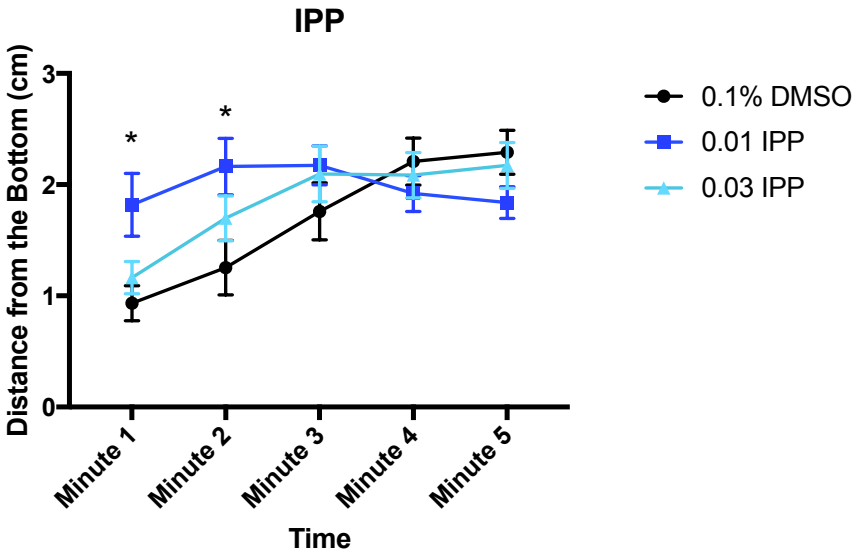


Figure 7: Novel Tank test. Mean distance from the bottom of the tank is plotted for the IPP exposure group across the five minutes of the novel tank exploration trial. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

There was a main effect of BDE-47 exposure on the average distance from the bottom of the tank ($p < 0.05$). There was also a significant interaction of minute X BDE-47 exposure on distance from the bottom ($p < 0.001$). Post hoc analyses showed that on average, fish exposed to 0.01 μM BDE-47 spent the first minute further from the bottom compared to controls. Additionally, fish exposed to 0.1 μM BDE-47 remained further from the bottom of the tank for the first three minutes, compared to controls (Figure 8). No significant effects of BDE-99 exposure on the average distances from the bottom of the tank were found (Supplementary Material, Appendix A).

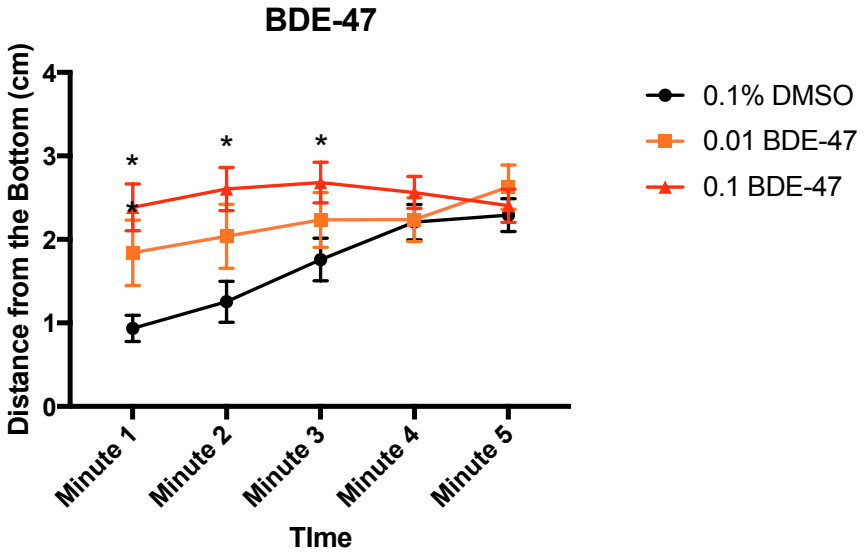


Figure 8: Novel Tank test. Mean distance from the bottom of the tank is plotted for the IPP exposure group across the five minutes of the novel tank exploration trial. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

Dive recovery represents the difference between the average location (mean distance from the bottom) of the fish in the tank from minutes 2 to 5 and the average location of the fish in minute 1. There was a main effect of IPP and BDE-47 exposure on dive recovery ($p < 0.05$). Dunnett's post-hoc comparison revealed that fish exposed to 0.01 μM IPP and 0.1 μM BDE-47 experienced a smaller dive recovery (Figure 9).

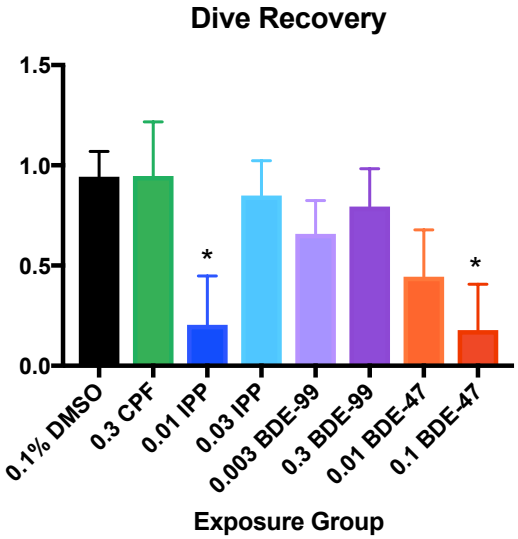


Figure 9: Novel Tank test. Dive recovery across all treatment groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

3.4. Startle habituation

There were no significant main effects of chemical exposure on pre-tap (base activity levels) distance traveled. There was no main effect of IPP exposure on post-tap swim behavior. However, Dunnett's comparison showed that fish exposed to 0.01 μM IPP swam significantly ($p < 0.05$) less than controls following the second, third, and fourth taps (Figure 10A). The ANOVA revealed a main effect of IPP exposure on the difference in distance traveled before and after the tap stimulus ($p < 0.05$). Subsequent analysis revealed that there was a smaller difference between pre and post tap behavior during the second, third and fourth taps for fish exposed to 0.01 μM IPP (Figure 10B).

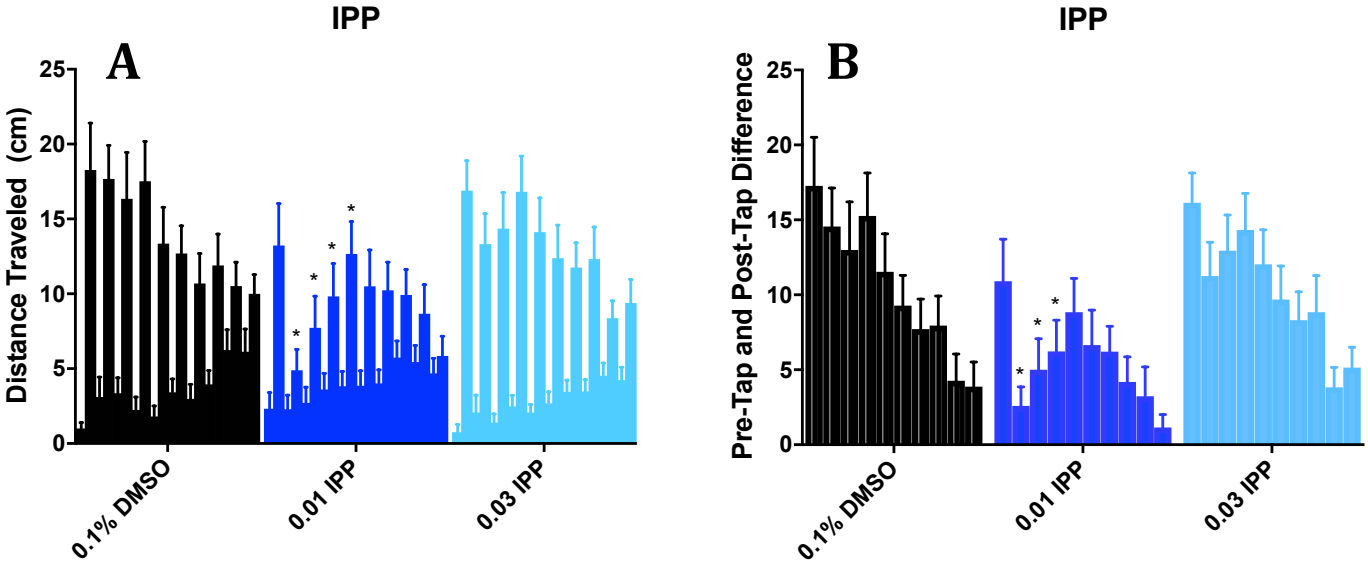


Figure 10: Startle Habituation Assay. A: Total distance traveled in the 5 seconds before and after each tap stimulus for IPP exposure group. B: Difference in distances traveled before and after the tap stimulus for the IPP exposure group. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

There was a main effect of BDE-47 exposure on distance traveled post tap stimulus ($p < 0.01$). Dunnett's comparison revealed that fish exposed to 0.01 BDE-47 swam less following the first, second, third, and fourth taps in comparison to controls. Dunnett's comparison also showed that

fish exposed to 0.1 μM BDE-47 swam significantly ($p < 0.05$) less than controls following taps three and four (Figure 11A). The ANOVA also revealed a main effect of BDE-47 exposure on the difference in distance traveled before and after the tap stimulus ($p < 0.01$). Follow-up analysis revealed that there was a smaller difference between pre and post tap behavior during the first five taps for fish exposed to 0.01 μM BDE-47 and during the fourth tap for fish exposed to 0.1 μM BDE-47 (Figure 11B).

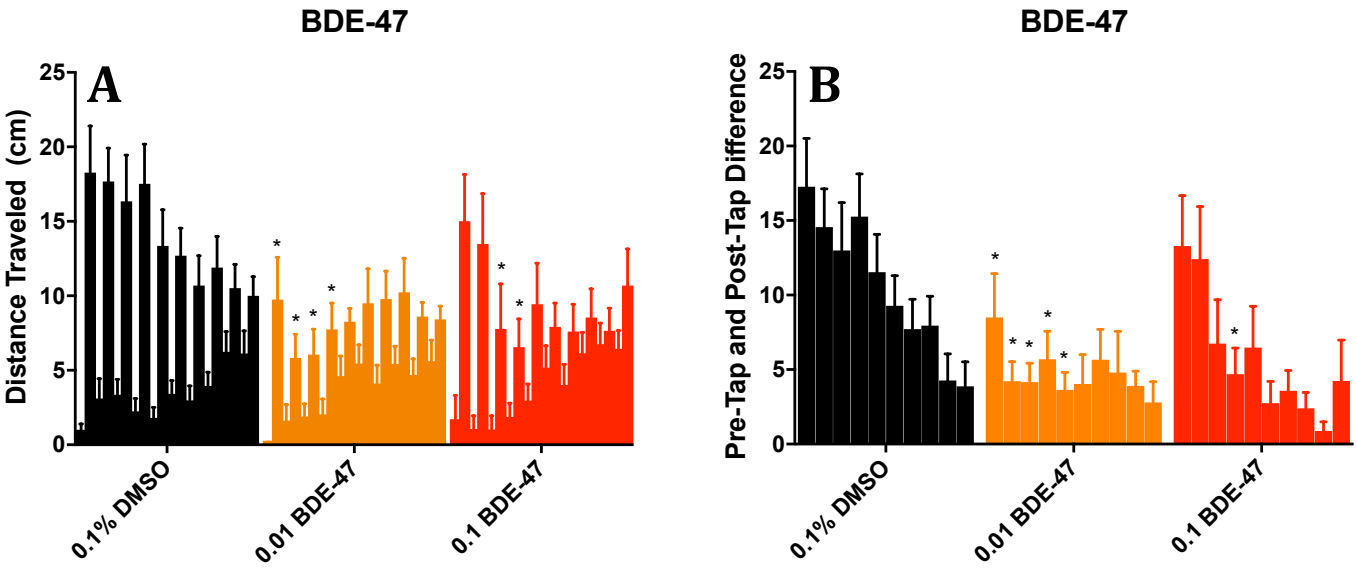


Figure 11: Startle Habituation Assay. A: Total distance traveled in the 5 seconds following each tap stimulus for BDE-47 exposure group. B: Difference between distances traveled pre-tap and post-tap for BDE-47. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

There was no main effect of BDE-99 exposure on post-tap swim behavior. However, dunnett's comparison revealed that fish exposed to 0.003 μM BDE-99 swam significantly ($p < 0.05$) less than controls following the first, second, and fourth taps and that fish exposed to 0.3 μM BDE-99 swam significantly ($p < 0.05$) less than controls following the second and fourth taps (Figure

12A). There was a significant ($p < 0.01$) interaction of tap X BDE-99 exposure on the difference in distance traveled before and after the tap stimulus (Figure 12B).

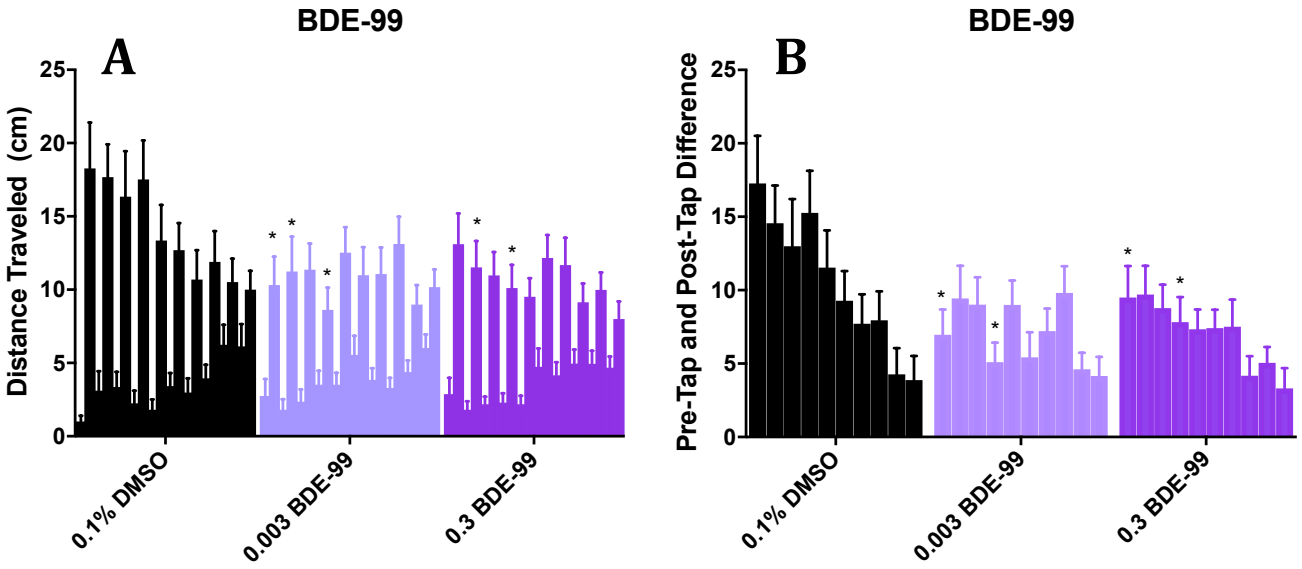
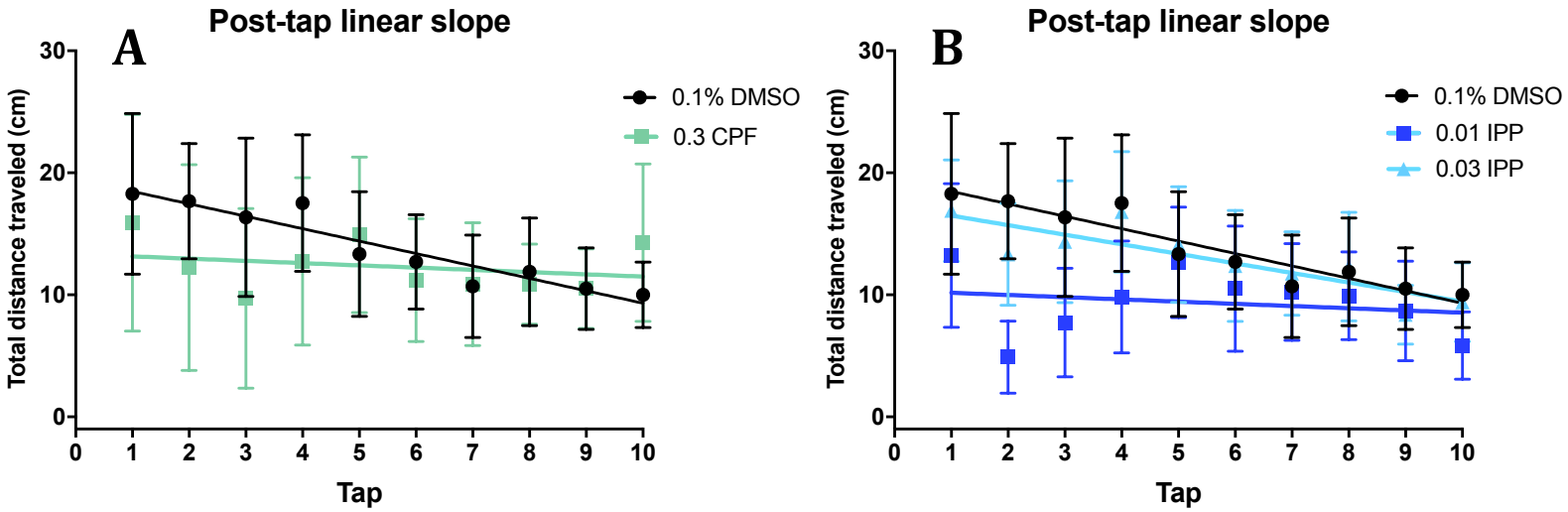


Figure 12: Startle Habituation Assay. A: Total distance traveled in the 5 seconds following each tap stimulus for BDE-99 exposure group. B: Difference between distances traveled pre-tap and post-tap for BDE-99. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

Linear regression analyses were performed on the post-tap distance traveled measurements to see whether or not the habituation curves of each treatment group differed significantly from the control group (Figure 13). The analyses revealed that 0.3 μM CPF, 0.01 μM IPP, 0.003 μM BDE-99, 0.3 μM BDE-99, 0.01 μM BDE-47 all had slopes that were significantly ($p < 0.05$) different than the slope for the fish treated with 0.1% DMSO. The results from the linear regression analyses are reported in Table 5.

Cohort	0.1% DMSO	*0.3 µM CPF* *	*0.01 µM IPP* *	0.03 µM IPP	*0.003 µM BDE-99* *	*0.3 µM BDE-99* *	*0.01 µM BDE-47* **	0.1 µM BDE-47
Slope (95% CI)	-1.511 to -0.5235	-0.8052 to 0.4372	-0.6316 to 0.2694	-1.22 to -0.3467	-0.3983 to 0.3694	-0.6944 to -0.0164	4.797 to 9.665	-0.9847 to 0.08421
Y-int (95% CI)	16.43 to 22.56	9.483 to 17.19	7.556 to 13.15	14.57 to 19.99	8.538 to 13.3	10.47 to 14.67	-infinity to -8.15	8.625 to 15.26
F		4.303	6.052	0.9261	10.2	5.075	11.6	2.213
DFn		1	1	1	1	1	1	1
DFd		306	366	437	466	226	296	306
P		*0.0389* *	*0.0144* *	0.3364	*0.0015**	*0.0248* *	*0.0008* **	0.1379

Table 5: Linear Regression Analysis for Startle Habituation Assay. Each treatment group was compared with 0.1% DMSO to determine whether or not the lines were significantly different. An "*" indicates a significant difference from control ($p < 0.05$). An "**" indicates a significant difference from control
($p < 0.001$).



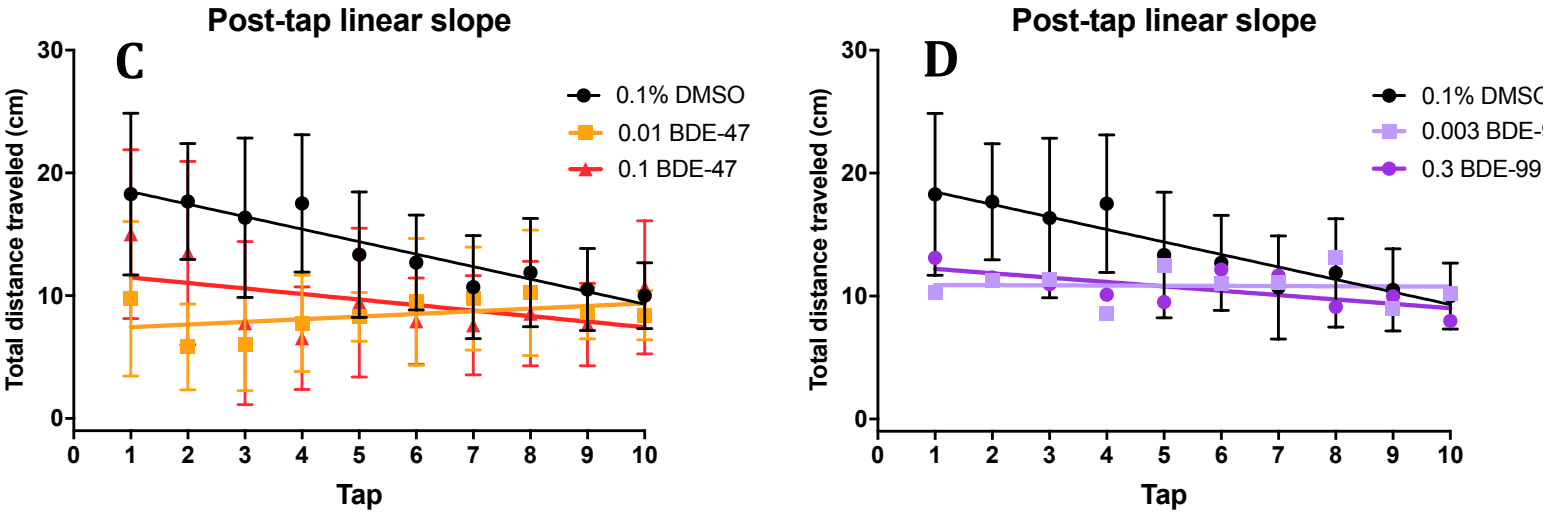


Figure 13: Startle Habituation Assay. The following graphs detail the linear regressions for the distance moved following the stimulus across the exposure groups. A: Linear regression comparison between CPF and controls. B: Linear regression comparison between IPP exposure group and controls. C: Linear regression comparison between BDE-47 exposure groups and controls. D: Linear regression comparison between BDE-99 exposure groups and controls.

3.5. Social affiliation

A main effect of time on distance from the side of the tank corresponding to the screen playing the video was observed. On average, the fish remained closer to the wall during the first two minutes the video was played in comparison to the two minutes before the video was played. Further analysis did not detect any significant effects of chemical exposure on the linear trend of distance from the shoal over time (Figure 14). No significant effects on locomotor activity were found (data not shown).

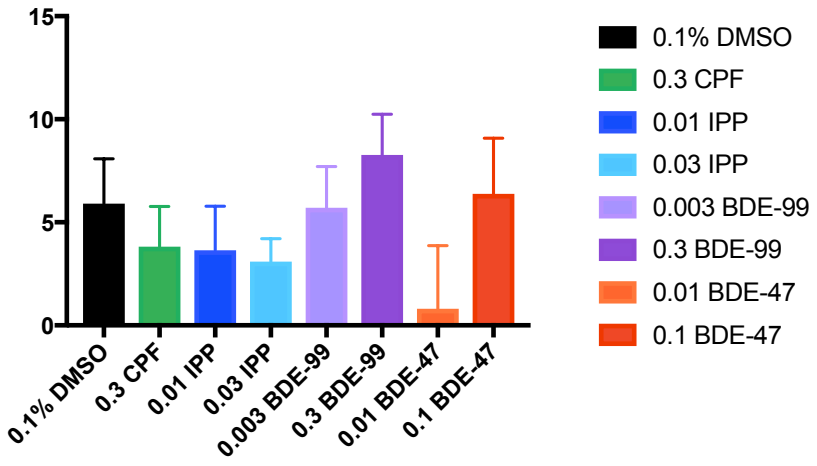
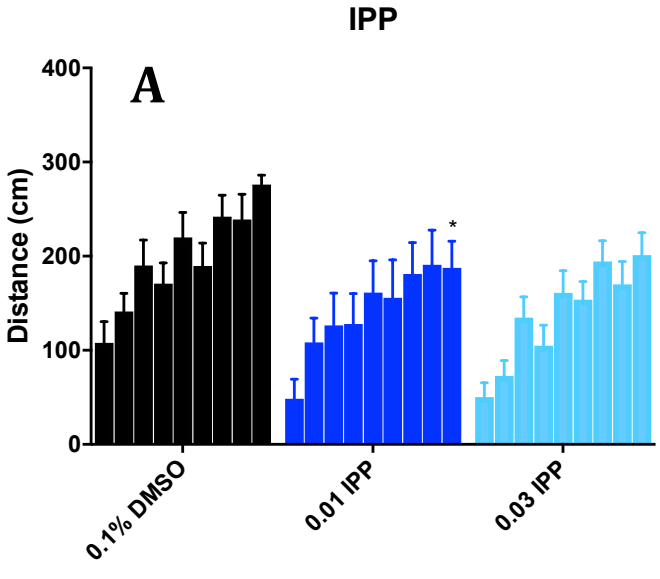


Figure 14: Shoaling Assay. Difference between distance from the screen before the video was played and the average distance from the screen during the first two minutes of the video. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

3.6. Predator escape

There was a main effect of treatment on distance traveled per minute for all three-exposure groups—IPP, BDE-47, and BDE-99 ($p < 0.05$, Figure 15). Dunnett's comparison revealed that fish exposed to 0.01 μM IPP swam less than controls during minute 9 (Figure 15A). Further, fish exposed to 0.01 μM BDE-47 swam less than controls during minutes 3, 4, 5, 6, 8, and 9 (Figure 15B). Dunnett's comparison also revealed that fish exposed to 0.003 μM BDE-99 swam less than controls during minutes 3, 4, 7, 8, and 9 (Figure 15C).



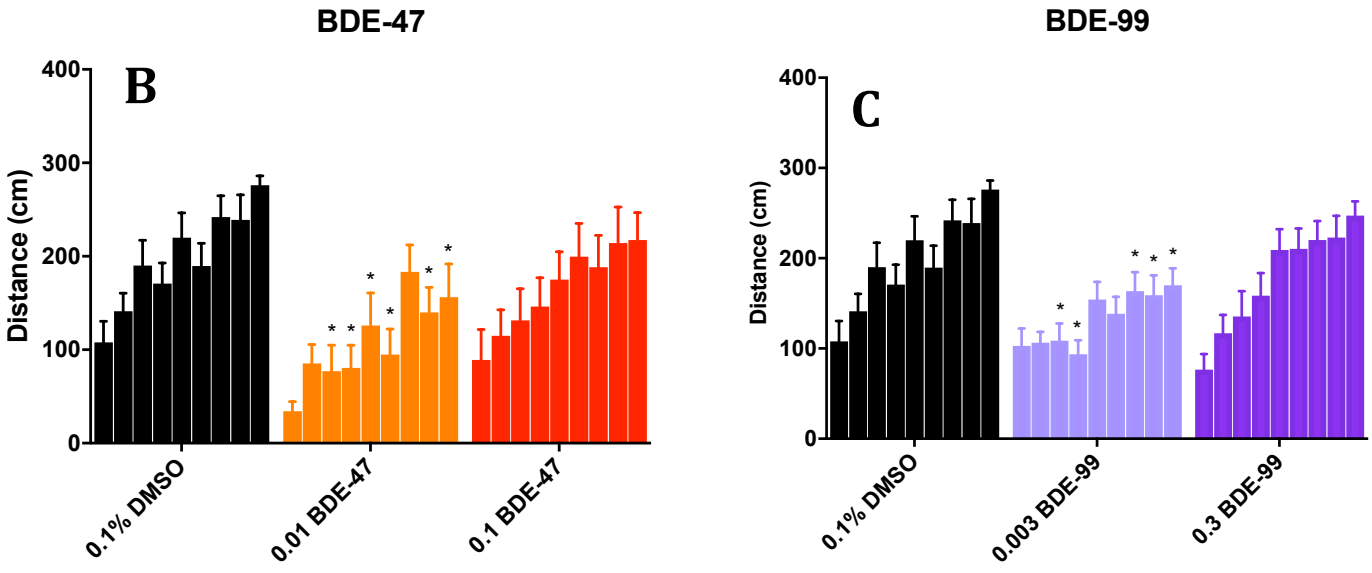


Figure 15: Predator Escape/Avoidance Assay. Total distance traveled during each minute of the 9 minute trial across IPP (A), BDE-47 (B), and BDE-99 (C) exposure groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

There was also a main effect of treatment on distance from side in which the predator simulation was presented—the growing red and blue dots—for BDE-47 and BDE-99 ($p < 0.01$). Subsequent post-hoc analyses revealed that fish exposed to 0.01 μM BDE-47 remained further from the side the video was played on than controls during minutes 3 (no stimulus; NS) and 9 (NS). Fish exposed to 0.1 μM BDE-47 remained closer to the video side during minute 1 (NS) (Figure 16A). In addition, fish exposed to 0.003 μM BDE-99 remained further from the side than controls during minute 3 (NS). Fish exposed to 0.3 μM BDE-99 remained closer to the side than controls during minute 1 (NS) (Figure 16B). Flee response is calculated as the difference in average distance from the tank side between trial minutes in which the dot stimulus was presented and minutes in which there was no stimulus (NS). There were no significant effects of treatment on flee found. However, on average the fish showed a larger flee response when represented with the red stimulus in comparison to the blue stimulus (Figure 16C).

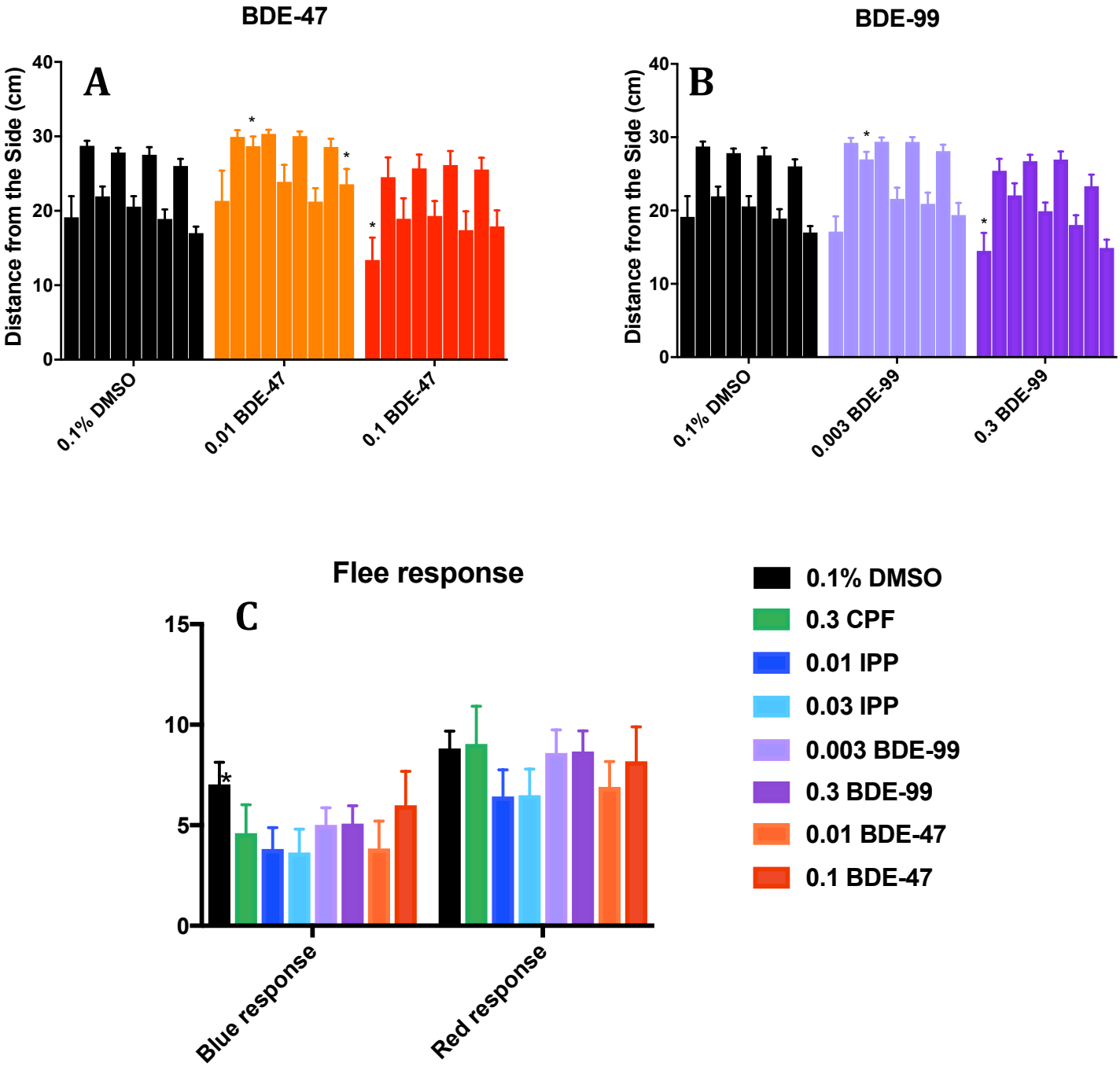


Figure 16: Predator Escape/Avoidance Assay. A, B: Distance from the side of the tank where the predator stimuli are displayed for each minute during the trial for the BDE-47 (A) and BDE-99 (B) exposure groups. C: Flee response for both the blue and red stimuli across all exposure groups. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).

4. Discussion

Despite the widespread use of flame-retardants and consequently human exposure, there still remain major gaps concerning the safety profiles of these chemicals, specifically, the possible neurotoxicity. The translatability of flame retardant neurotoxic effects between animal (rodent) models and humans presents the opportunity to use a lower-vertebrate model—such as the zebrafish—in order to more efficiently asses the safety of these new chemicals. The ultimate goal of the study was to develop a high throughput screen that can be used to assess the neurobehavioral risks specific toxicants pose. Subsequently, these results provide insight into whether early-life exposure to brominated and organophosphorus flame retardants causes neurobehavioral effects at early or later stages and whether or not results from larvae testing may be predictive of adult behavioral impairment.

In the present study, the highest non-acute concentrations of all of the chemicals tested significant produced behavioral effects in the 6 day old zebrafish larvae. Developmental exposure to the flame retardants also perturbed behavior in at least one—and in some cases several—of the adult behavioral assays. The main effects on adult behavior were seen in novel tank, tap, and predator avoidance tests. Further, the chemicals tested produced widespread behavioral effects at concentrations in the very low mircomolar range, far below overt toxicity.

In particular, locomotor activity was decreased in the larval assay for the CPF (0.3 μM) and BDE-47 (0.1 μM**,** 0.3 μM). These results are consistent with previous findings of lower swimming rates in larvae exposed to these chemicals (Dishaw et al., 2014; Jarema et al., 2015; Noyes et al, 2015). Conversely, larvae exposed to IPP (0.1 μM) showed an increase in swimming behavior during the dark phases. Jarema et al. (2015) also noted hyperactivity in the dark phases

with IPP exposure groups. Previous findings (Oliveri et al., 2015) have suggested that developmental exposure to OPFR may cause hyperactivity throughout the lifetime. However, we did not see any additional evidence to support this claim. Larvae exposed to BDE-99 (0.3 μM) also showed increased locomotion during the light phases. However, these results are not consistent with previous studies, which observed hypoactivity in the dark phases (Noyes et al., 2015). However, these findings may be due to differences in the strain of fish (5D strain), dosing concentrations, or the illumination cycles. Lastly, these effects were observed after exposure to low doses of flame retardants that did not result in significant physical malformations.

The battery of adult behavioral assays further demonstrated the cognitive impact of developmental exposure to these flame retardants. In particular, one trend to emerge from the results is that low level exposure to BDE-47, BDE-99, and IPP during development can lead to reduced response to anxiety-provoking conditions and adverse stimuli. During the novel tank diving test, both IPP (0.01 μM) and BDE-47 (0.1 μM) exposure groups demonstrated an altered temporal pattern of exploration. Typically, fish remain close to the bottom in the initial minutes of the task and then gradually begin to explore the new environment, increasing their distance from the bottom of the tank. When fish are placed in a novel environment, it is considered an anxiety-provoking situation—consequently, they initially remain close to the bottom of the tank. However, with the adult fish developmentally exposed to IPP and BDE-47 they spent the initial minutes further from the bottom than the control fish. Consequently, these fish showed a reduced dive reaction. Similar end points were reached in animal models, where the behavioral profiles of rodents exposed to BDE-99 were suggestive of reduced anxiety-like responses (Branchi et al., 2002; Branchi et al., 2005).

Further, adult fish that had been developmentally exposed to BDE-47 (0.01 μM, 0.1 μM), BDE-99 (0.003 μM, 0.03 μM) and IPP (0.01 μM) exhibited reduced startle response and habituation during the first part of the tap test. Startle response provides a measure of sensory and motor integration and is typically associated with a prompt habituation curve after repeated trials (Eddins et al., 2010). Habituation is considered a form of non-associative learning and can provide insight into neuroplasticity. The fish in these exposure groups also showed a smaller difference between pre and post-tap swimming behavior. Linear regression analysis revealed significant differences in the slopes of the post-tap swimming behavior. However, the reduced responses may also account for these differences in slopes. Overall, these results are indicative of reduced response to the aversive stimuli for fish developmentally exposed to BDE-47, BDE-99, and IPP.

In addition, throughout the adult behavior testing there seemed to be a non-monotonic dose response for the IPP exposure group—with behavior impairment seen at the lower dose—0.01 μM—but no significant effects in the higher dose—0.03 μM. These results are not atypical in toxicology studies. One potential explanation could be that IPP is acting as an endocrine disrupter, which act through different modes of action, often acting on many physiological systems. Endocrine disrupting chemicals (EDCs) have been frequently associated with non-monotonic dose-response (NMDR) relationships (Lagarde et al., 2015). Further, endocrine disruptors can lead to a variety of adverse consequences, including neurobehavioral impairments (Weiss 1998). However, the present study only considered two doses. More substantial research investigating the effects of a wide range of doses, as well as the possible mechanism of action of IPP is needed before conclusions can be drawn.

A small number of studies have proposed zebrafish larval testing as a means of screening toxicants (Usenko et al, 2011; Jarema et al., 2015; Noyes et al., 2015; Oliveri et al., 2015). However, only the present study—along with Oliveri et al. (2015)—consider both larval and adult testing. This is significant as the present results suggest that both larvae and adult testing may be needed in order to more comprehensively understand the neurobehavioral consequences of developmental exposure to toxicants. For BDE-47, BDE-99, and IPP only the higher doses showed effects on behavior in the larval testing. However, when adult testing was performed, behavioral impairments were seen in both the lower and higher doses. Evidently, larval testing alone may not be sufficient to determine the full extent of the neurobehavioral consequences of developmental exposure to flame retardants. Although these systems may be disrupted early in life, cognitive deficits may not be seen until full development, as zebrafish display much more complex behaviors once they reach adulthood.

By utilizing a multiple behavioral paradigms the present study plans to broadly assess cognitive function following exposures rather than singly focusing on cellular effects. The specific tests are important for providing insight into how each flame retardants affects the different neurobehavioral systems. Further, the adult behavioral assays help determine whether the neurobehavioral pathology persists into adulthood. In contrast to prior research, the present study used lower level dosages to compare effects across a spectrum of concentrations in order to determine whether or not deficits are dose-dependent. The study's focus on lower doses is more ethologically relevant when considering human exposure levels. Further, the lower-dose paradigm can potentially indicate more subtle effects.

Future research is planned to expand to more sets of chemicals using the same proposed research plan. The results from the present study—as well as those obtained from different

chemical sets—will be used as a complex screen in to help determine which flame retardants should be further tested in mammalian models. Further testing in rodent models may provide insight into the underlying neurobiological mechanisms of these behavioral effects. Additionally, acute exposures as well as exposures during other critical periods of development may provide additional information on the consequences of exposure to these chemicals. Human exposure during neurodevelopment may lead to serious teratological and neurological consequences. With the increased reliance and development of new flame retardant alternatives, it remains critical to evaluate the complete safety profiles and fully understand all possible adverse health effects for these compounds.

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Supplementary Material:

Appendix A:

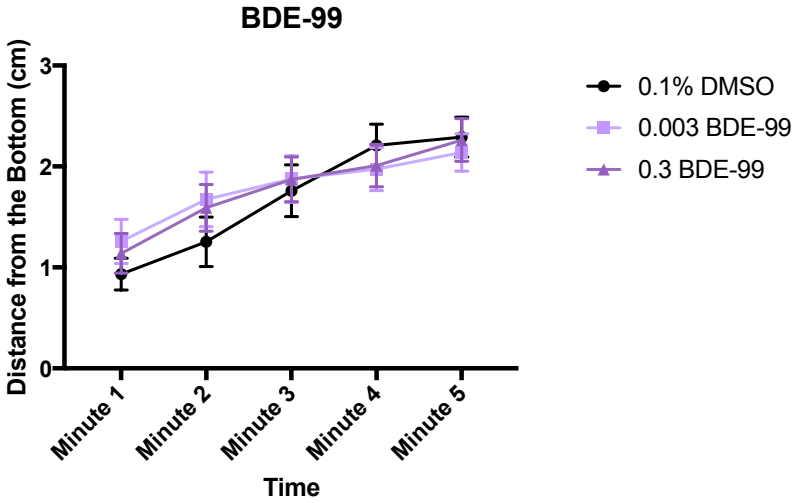

Figure 8: Novel Tank test. Mean distance from the bottom of the tank is plotted for the IPP exposure group across the five minutes of the novel tank exploration trial. Error bars represent SEM. An "*" indicates a significant difference from control ($p < 0.05$).