MATERNAL TRANSFER AND TOXICITY PATHWAYS OF HEXABROMOCYCLODODECANE IN THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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ABSTRACT

Hexabromocyclododecane (HBCD) is a persistent organic pollutant (POP) that undergoes maternal transfer and hinders development and growth of early-life stages of fish. However, there is limited understanding of the maternal transfer kinetics and subsequent molecular mechanisms that drive the embryotoxicity of HBCD. The purpose of this study was to (1) characterize the accumulation of dietary HBCD (11.5, 36.4, 106 mg/kg, ww [wet weight]) in adult fathead minnows (FHM) and the subsequent maternal transfer kinetics to eggs, and (2) link transcriptomics responses to apical and physiological effects in larvae exposed through maternal transfer at seven- and 14-days post-fertilization (dpf), respectively. Maternal transfer kinetics displayed similar egg-to-muscle ratios (EMR) in the low and medium treatment groups (1.65 and 1.27, respectively). However, the high treatment group deviated from other treatments with an EMR of 4.2, potentially due to reaching diffusion and/or lipid saturation limits. A positive correlation was observed between egg HBCD concentration and time of exposure. Larvae sampled at 7dpf revealed dysregulation of pathways involved in membrane integrity (inhibition of calcium channel) and metabolic processes (downregulation of amino acid, glucose, and lipid biosynthesis), while the larvae reared for 14 days exhibited a significant decrease in survival at the highest treatment condition. These results indicate that maternal transfer of HBCD is of concern in fish, which may act through indirect mechanisms involving the inhibition of membrane transport leading to disruption in metabolic processes, collectively resulting in energy depletion and subsequently mortality. This study is part of the EcoToxChip project (www.ecotoxchip.ca). The data derived will be used to inform the development of EcoToxChips, which are qPCR arrays that aim to predict apical endpoints of ecological and regulatory relevance for three model species and three native species for eight model chemicals.

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LIST OF ABBREVIATIONS

α	alpha
A1	anti-apoptotic protein A1
ADD	average daily dose
ANOVA	analysis of variance
anpepb.2	alanyl (membrane) aminopeptidase b, tandem duplicate 2
AO	adverse outcome
AOP	adverse outcome pathway
Apaf-1	apoptotic protease activating factor 1
Apf-1	aspartyl protease family protein 1
ATP	adenosine 5'-triphosphate
ATRF	Aquatic Toxicology Research Facility
β	beta
Bad	Bcl-2-associated death promoter
BAF	bioaccumulation factor
Bax	Bcl-2-associated protein
BCE	before the common (or current) era
Bcl-2	B-cell lymphoma 2
Bcl-Xs	B-cell lymphoma-extra small
BFRs	brominated flame retardants
Bid	BH3-interacting domain death agonist
BMF	biomagnification factor
BP	biological process
Br	bromine
Ca^{2+}	calcium
cacna1fa	voltage-dependent L-type calcium channel subunit α-1D
CAR	constitutive active/ androstane receptor
CAT	catalase
CCAC	Canadian Council of Animal Care
CEPA	Canadian Environmental Protection Act

chia.2	chitinase, acific.2
Cl	chlorine
CXR	chicken xenobiotic-sensing orphan nuclear receptor
cyp2p7	cytochrome P450, family 2, subfamily 2, polypeptide 7
CYP450	cytochrome P450
DEG	differentially expressed genes
DNA	deoxyribonucleic acid
dpf	days post-fertilization
dph	days post-hatch
dpyda.1	dihydropyrimidine dehydrogenase α, tandem duplicate 1
dw	dry weight
eif2ak4	eukaryotic translation initiation factor 2 α kinase 4, general control non-repressed 2
ELS	early life stage
EMR	egg to muscle ratio
EPS	expanded polystyrene foam
Eq	equation
EtOH	ethanol
F	fluorine
F1	first generation
FA	fatty acids
FC	fold change
FHM	fathead minnows
FRs	flame retardants
g	gram
GO	Gene Ontology
GS-Px	glutathione peroxidase
GSI	gonadosomatic index
GTP	guanosine triphosphate
H	hydrogen
H+	hydrogen ion

H_2O_2	hydrogen peroxide
HBB	hexabromobenzene
HBCD	hexabromocyclododecane
HIPS	high impact polystyrene
HPLC	high-performance liquid chromatograph
I	iodine
K	condition factor
KE	key event
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilogram
K_{ow}	n-octanol/water partition coefficient
L	liter
lc	lipid corrected
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	level of detection
LSI	liver somatic index
lw	lipid weight
MDA	malondialdehyde
Mdm2	mouse double minute 2 homolog
MF	Molecular Function
mg	milligram
mL	milliliter
MoA	mechanism of action
MRM	multiple reaction monitoring
mRNA	messenger RNA
NADH	nicotinamide-adenine dinucleotide + hydrogen
ng	nanogram
NIH	National Institute of Health
nM	nano Molar
^oC	degrees Celsius
OECD	Organisation for Economic Co-operation and Development

OH^+	hydroxide ion
p53	tumor protein p53
padj.	p-value-adjusted
PBDEs	polybrominated diphenyl ethers
PBT	persistent, bioaccumulative and toxicological
PCBs	polychlorinated biphenyls
pcolcea	procollagen C-endopeptidase enhancer α
plbd1	phospholipase B domain containing 1
POPs	persistent organic pollutants
PXR	pregnane X receptor
QC	quality control
qPCR	quantitative polymerase chain reaction
RBT	rainbow trout
RIN	RNA integrity number
RNA	ribonucleic acid
ROS	reactive oxygen species
SCCPs	short-chain chlorinated paraffins
SEM	standard error of mean
slc25a10b	solute carrier family 25, member 10
SOD	super oxide dismutase
SPV	sulfophosphovanillin
$t_{1/2}$	half-life
T3	triiodothyronine
T4	thyroxine
TBARS	thiobarbituric acid reactive substances
TBBPA	tetrabromobisphenol A
TCA	tricarboxylic acid cycle
TD	toxicodynamic
TF	transcription factor
TK	toxicokinetic
TNF	tumor necrosis factor

TQ-S	triple-quadrupole
µg	microgram
UGT	glucuronylstransferase
UNEP	United Nations Environment Programme
US-EPA	United States Environmental Protection Agency
wt	weight
ww	wet weight
XPS	extruded polystyrene foam
ZF	zebrafish

*****This thesis followed ZFIN Zebrafish Nomenclature Conversions (zfin.atlassian.net) for naming genes/ transcripts. Gene nomenclature: full gene names and gene symbols are in lowercase and italics.

NOTE TO THE READER

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Postdoctoral Research guideline for a manuscript-style thesis. As a result of the manuscript-style formatting, there is some repetition of content between the presented chapters. Chapter 1 is a general introduction of the thesis topic. Chapters 2 and 3 are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 4 contains the overall discussion and conclusion of the thesis. Chapter 2 has been published in *Environmental Toxicology & Chemistry* and Chapter 3 is in preparation for submission. Author contributions are provided following the preface of each chapter. References cited in each chapter are combined and listed in the "References" section of this thesis. Supplementary materials from Chapter 2 and 3 have been included in the "Supplemental Data" section at the end of this thesis.

CHAPTER 1: GENERAL INTRODUCTION

Aquatic ecosystems serve as diverse habitats for a plethora of species; however, they are a sink for many anthropogenic contaminants, which may threaten the health of these systems. More than 144 000 chemicals are in use today (ECHA, 2017), and together with the ever-increasing number of new chemicals that are being developed and used by society, the chemical load in aquatic systems continues to rise. In an attempt to reduce the number of toxic chemicals, many countries have established guidelines/ regulations (i.e., EPA; Environmental Protection Agency; Toxic Substances Act of 1976, CEPA; Canadian Environmental Protection Act of 1999; List of Toxic Substances, UNEP; United Nations Environment Programme; Stockholm Convention on Persistent Organic Pollutants) for the production, use, and disposal of various compounds. Toxic compounds may be classified into different groups based on their structure, use, physical property, radiological property, and/ or other factors (ATSDR, 2008). One group of chemicals of particular concern are persistent organic pollutants (POPs). POPs are environmental pollutants that are prone to persist, bioaccumulate, and biomagnify; they are often very lipophilic and can be difficult to eliminate or metabolize (Elskus et al., 2005). As such, the Stockholm Convention has classified 22 chemicals or groups of chemicals as persistent organic pollutants (POPs); these chemicals are in the process of/ are banned or have maximum concentration benchmarks for production/ use/ export. These lipophilic compounds are of concern as they may enter the environment at low concentrations, but over time the chemicals may accumulate in organism(s) to levels inducing adverse effects (Carpenter, 2011; Law et al., 2014). This is of further concern in oviparous organisms (i.e., fish) as they can depurate these high levels of contaminants to their offspring (González-Doncel et al., 2017; Haldén, 2014; Nyholm et al., 2008).

One class of chemicals that has been adopted into the UNEP Stockholm Convention on POPs are halogenated flame retardants (FRs) due to their toxicity, relatively high lipophilicity, and ubiquitous use and prevalence in the environment (Sharkey et al., 2020). In addition, halogenated flame retardants have a greater propensity to persist in the environment than non-halogenated compounds (Doble & Kumar, 2005). As such, they are considered one of the predominant sources of organic environmental pollutants (Shaw et al., 2010). Brominated flame retardants (BFRs), including hexabromocyclododecanes (HBCDs), are a prime example of

halogenated flame retardants that have been widely used and ubiquitously present in the environment; in addition, they have been reported as toxicants of concern in fish and other aquatic organisms (Babrauskas et al., 2014). However, there are many knowledge gaps regarding HBCD toxicity in fishes, specifically with regard to their maternal transfer potential and the subsequent mechanism of action (MoA) of toxicity after maternal transfer.

One of the major concerns with lipophilic compounds such as HBCD is their potential for maternal transfer; research comparing the sensitivity to environmental pollutants in oviparous fish at different life stages has shown early life stages (ELS) as being more sensitive than adult organisms (Russell et al., 1999). Exposure to environmental pollutants through maternal transfer has been linked to adverse effects on larval development; however, as deposition in individual embryos is not uniform there is a large variation in developmental toxicities that may occur (Janz 2012). This lack of uniformity in toxicant deposition and the time-consuming, laborious nature of executing maternal transfer studies has resulted in significant knowledge gaps in objectively describing the toxic effects of many POPs, including Hexabromocyclododecane (HBCD) through maternal transfer. Understanding the rate of maternal transfer allows greater interpretation of the accumulation potential in adult organisms and the approximate concentrations subsequent generations may be exposed to.

The rate of exposure is an important factor to consider, as it may impact the toxicokinetic and toxicodynamic properties of a chemical, therefore studies utilizing maternal transfer and mechanistic research may be better equipped to evaluate the toxicity of lipophilic compounds. Mechanistic data provides valuable insight into molecular changes (i.e., gene, transcript, protein, metabolite), and with the use of bioinformatics, these changes may be integrated to showcase altered cellular functions (Hahn, 2011). Mechanistic data provides a more informed approach to hazard identification and dose response assessment than classical toxicity testing; data may be utilized to develop endpoints of interest and characterize toxicants acting through a similar MoA (Haber et al., 2001). By categorizing chemicals based on MoA, chemical assessments may be ranked based on level of potential hazard and prioritized for testing, thereby increasing the rate of chemical screenings (Ankley et al., 2010; Basu et al., 2019). Therefore, the focus of this research was to bridge the knowledge gaps in our current understanding of the toxicity of maternally transferred HBCD and to characterize the mechanism by which this chemical affects

normal development, particularly in the F1 generation of a commonly used lab species, the fathead minnow (FHM; *Pimephales promelas*).

1.1 Flame Retardants

Flame retardants (FRs) are chemicals used to reduce the flammability of many of today's manufactured items (i.e., furniture, electronics and electronic devices, building and construction materials, transport products). They have been in use dating back as far as 360 BCE (vinegar on timber used to build fortifications) and have been produced for commercial applications since the 1730s (patent no. 551) (Covaci et al., 2006; Marvin et al., 2011). Over the years there has been a significant turnover of different types of FRs on the market due to environmental and human health concerns that have emerged from the exposure to these compounds. However, due to their lipophilic properties, many FRs that are out of production persist in the environment (Venier et al., 2015) and are ubiquitous in various environmental matrices such as air, suspended particulates in water, sediment, soil, and organisms.

1.1.1 History and status of FRs

The first recorded, patented FR was a mixture of alum, borax and ferrous sulphate created by Obadjah Wyld in 1735 to treat wood, paper, and textiles. As the occurrence of fires became more prevalent, new methods of fire protection were being investigated, as was observed in 1820 when Gay-Lussac proposed the use of a mixture of ammonium salts (phosphoric, sulfuric, and hydrochloric acid) to reduce the occurrence of fires in French theatres (Giraud et al., 2016). Over time, the development of these fire-resistant compounds became a large market for chemical companies resulting in the production of four distinct categories of FRs: halogenated, phosphorous-containing, nitrogen-containing, and inorganic, each suppressing or inhibiting the spread of fire by physical or chemical action. For example, phosphorous or nitrogen-containing FRs inhibit the spread of fire by cooling, diluting or coating the substrate (i.e., physical action), thereby reducing the supply of oxygen to the flame, this is also termed solid-phase interaction (van der Veen & de Boer, 2012). In contrast, halogenated FRs undergo chemical action by trapping H^+ and OH^+ radicals, thereby removing the fuel source for the combustion process, termed vapor pressure inhibition (Salmeia et al., 2015). As the need and use of FRs rose and the

apparent adverse effects linked to each FR were identified a slow turnover of FRs was observed over the course of time.

One of the most prevalent groups of FRs in recent history were polychlorinated biphenyls (PCBs); their high flashpoint (resistant to catching fire), resistance to thermal degradation, high thermal conductivity, and low electrical conductivity made them ideal FRs (Domínguez et al., 2011). PCBs were discovered in 1881, and by the late 1920s they were commercially used in dielectric and coolant fluids in electrical equipment. The first indications towards PCB as contaminants of concern occurred in 1964 when an unknown elution peak was observed during gas-liquid chromatographic separation of an environmental sample of organochlorine pesticides, which was later identified as PCBs (George et al., 1988). This observation resulted in a series of studies that revealed that PCBs were widely distributed in the environment across the world. The toxic properties of PCBs were investigated when outbreaks of poisonings were observed in humans and domestic animals from contaminated food. In 1976, PCBs were under regulation by the Toxic Substances Control Act of 1976 and in 1979 the United States Environmental Protection Agency (US-EPA) banned the manufacture of PCBs, with stringent regulations on disposal and distribution due to their potential for carcinogenicity, bioaccumulation, persistence in the environment, and immunotoxicity (George et al., 1988). Since the ban of PCB production, new FRs entered the market as replacements, most notably, polybrominated diphenyl ethers (PBDEs). PBDEs are similar in structure to PCBs and also exist in many congener forms. The three main formulations are deca-BDE, octa-BDE, and penta-BDE. PBDEs have been commercially produced since the 1970s and were used mainly in electronic circuits, plastics, and textiles. Similar to PCBs, widespread distribution, bioaccumulation, persistence, and toxicity including carcinogenicity, deficiencies in neural responses, and thyroid hormone disorders were observed in organisms exposed to PBDEs, resulting in the ban of some congeners including octa- and penta-BDE in 2004 and voluntary phase out of deca-BDE by the end of 2012 (Lee & Kim, 2015).

The concern about the hazardous properties of certain FRs not only arises from the parent compounds but also from degradation products; compounds containing aromatic rings may produce dioxin-like derivatives, especially during production, exposure to heat, or recycling (Stubbings & Harrad, 2014). Another factor increasing the toxicity of BFRs is the number of bromine atoms; a lesser number of bromine atoms in a molecule suggests increased toxicity as a

result of higher volatility and water solubility (i.e., easier to absorb as it is a smaller molecule) (Cantón et al., 2006). Abiotic or biotic degradation results in the loss of bromine atoms of higher brominated congeners, which can increase their bioavailability and toxicity (Covaci et al., 2006). Metabolic processes can also yield toxic metabolites, which can bind with greater affinity to transport proteins or other molecules, mimic endogenous hormones, or impact neurotransmission and receptor activity (Zhang et al., 2014). This increase in toxicity was observed for PBDEs, where hydroxylated metabolites of PBDEs displayed greater developmental neurotoxicity than the parent compounds (Dingemans et al. 2011). Due to their persistent, bioaccumulative and toxicological (PBT) properties, regional, national and international regulations have been placed on transport, storage, and disposal of select FRs resulting in decreased concentrations in the environment over time (Airaksinen et al., 2014; Rawn et al., 2017). Yet, novel or current FRs have continued use, which may result in potential adverse effects to wildlife and humans due to their unknown hazard potential.

Over the past decades FR use has increased as a result of safety regulations such as fire safety standards for furniture, textiles, electronics, and building products. In 2017, the global market for FRs reached $6.8 billion US with Asia-Pacific accounting for more than 50% of the shares (Grand View Research, 2017). However, due to increased environmental and health concerns, residual concentrations in sediments (Combi et al., 2016), soils (Guazzoni et al., 2011), fish (Airaksinen et al., 2014), and human breast milk (Rawn et al., 2017), many FRs are being categorized as persistent organic pollutants (POPs). Currently, PCBs, PBDEs, hexabromobenzene (HBB), short-chain chlorinated paraffins (SCCPs), and hexabromocyclododecane (HBCD) are categorized as POPs and are either banned or being phased out (Stockholm Convention, 2015). Of the four FR groups (inorganic, phosphorous-containing, nitrogen-containing, and halogenated) the most prevalent in the environment and most widely utilized today are halogenated FRs (Birnbaum & Staskal, 2004). The most commonly used halogens are iodine (I), bromine (Br), chlorine (Cl), and fluorine (F); however, brominated products currently dominate the FR market (Cantón et al., 2006).

1.1.2 Brominated Flame retardants

Globally, BFRs dominate the market due to cost effectiveness (less amount required to prevent fire), limited or no interference with the physical properties of the polymer, and stability

(i.e., high decomposition temperature) (Domínguez et al., 2011). Although the stability of BFRs is beneficial for the product, it is this property that presents a significant cause for concern in the environment. Within the BFR family there are three subgroups: reactive, polymeric and additive, which is based on how the halogen is incorporated into the polymer. Reactive BFRs incorporate bromine by covalently binding to the polymer. These BFRs are more stable than additive BFRs and less likely to be released into the environment. Polymeric BFRs incorporate bromine into the backbone of the polymer and are the most stable of the three and the least bioavailable group. Additive BFRs incorporate bromine by mixing with other components of the polymer (i.e., HBCD, TBBPA [tetrabromobisphenol A]), therefore they are less costly to produce than the reactive and polymeric-type BFRs (Cantón et al., 2006). The disadvantage with additive-type BFRs, however, is that they have the greatest potential to enter the environment. BFRs may enter the environment as a result of leaching and volatilization during manufacturing, use, and or disposal (i.e., combustion and recycling of waste products) (Segev et al., 2009). It is suggested that the main mechanism of release of BFRs is through volatilization (gas-phase emission) resulting in elevated concentrations in dust (Schripp et al., 2010); however, other physical processes (i.e., abrasion or weathering of products) may account for particle-derived concentrations (Webster et al., 2009).

As traditional halogenated FRs, such as PBDEs, were being phased out in the late 2000s, the production of alternative compounds, such as HBCD, grew in demand and use in the late 1990s, early 2000s (ITT, 2015). However, following the phase out of PBDEs, environmental and health screenings found significant evidence that replacement BFRs, including HBCD, shared similar environmental concerns of persistence, bioaccumulation, and toxicity to aquatic and terrestrial organisms (USEPA, 2010). Although HBCD is in the process of being phased out, its widespread use early on resulted in its ubiquitous presence in the environment and still remains an environmental toxicant of concern today (Huang et al., 2020).

1.1.3 Hexabromocyclododecane (HBCD)

HBCD is an additive-type BFR used primarily in the building and construction industry for thermal insulation panels as expanded or extruded polystyrene foam (EPS and XPS, respectively). Additional uses of HBCD include textile coating on upholstered furniture and car seats and in certain plastics (high impact polystyrene; HIPS) used in electrical and electronic

appliances (Domínguez et al., 2011). In production and use from the 1960s, HBCD was the predominantly used cycloaliphatic BFR (Marvin et al., 2011) making it ubiquitous in various environmental matrices (US NRC, 2000). Concentrations of HBCD have been documented in air (0.011 ng/m^3; Hoh and Hites, 2005 – 1070 ng/m^3; Remberger et al., 2004), soil (140 ng/g dry weight (dw); Remberger et al., 2004 – 89 600 ng/g dw; Dames & Moore, 2000a), sediment (0.012 ng/g dw; Marvin et al., 2006 – 2430 ng/g dw; Guerra et al., 2009), water (0.003 ng/L; Law et al., 2006 – 15 800 ng/L Dames and Moore, 2000b) and biota (0.46 ng/g lipid weight (lw); Johnson-Restrepo et al., 2008 - 6800 ng/g lw; Morris et al., 2004) primarily at or in the vicinity of sites of production and disposal. However, traces of HBCD have also been reported in remote regions, including the Arctic (> 0.001 ng/m^3 in air; Xiao et al., 2010, >0.6 ng/g lw in marine biota; Tomy et al., 2008). Presence of HBCD in remote areas as well as in dated sediment samples (Minh et al., 2007) revealed that HBCD undergoes long-range transport and is persistent in the environment. Not only is HBCD an environmental concern due to its persistence; it is of concern to human and animal health due to its toxicological properties.

Exposure to HBCD can occur through inhalation, ingestion, and dermal exposure. Concentrations of HBCD have been documented in human breast milk (0.1 ug/kg – 20 ug/kg lw), blood (0.16 ng/g – 856 ng/g lw) and adipose tissue (1-12 ug/g kg lw) (Cantón et al., 2006; Covaci et al., 2006; Weiss and Bergman, 2006; Antignac et al., 2008). The main concerns that have risen from exposure to HBCD are impacts on reproduction, development, and thyroid homeostasis (Darnerud, 2003). Although levels of HBCD in both the environment and in biological fluids/ tissues are typically detected below the Federal Environmental Quality Guidelines for HBCD in Canada (water = 0.56 μg/L; sediment = 1.6 mg/kg dry weight (dw); mammalian wildlife diet = 40 mg/kg ww), they were shown to pose significant adverse effects on aquatic life. Research indicates HBCD concentrations increase as the trophic level increases (Harrad et al., 2009); this suggests higher order, or predatorial animals (i.e., fish and birds) (DeBoer et al., 2002) are indirectly affected by HBCD as it biomagnifies up the food chain (Haukås et al., 2009).

As a result of the persistence of HBCD, its long-range transport, bioaccumulation and adverse environmental and health effects, the UNEP (United Nations Environment Programme) Stockholm Convention on Persistent Organic Pollutants (POPs) categorized HBCD as a POP in their 2009 screening assessment and on November 2015 the UNEP implemented a global ban on

the production, use, import and export of HBCD, with complete phase-out by 2024. Similarly, HBCD importation, manufacture, use, sale and offer for sale is prohibited in Canada under CEPA (Canadian Environmental Protection Agency). Regardless, significant concentrations of this compound are still found in the environment due to its persistent and bioaccumulative properties as discussed above.

1.1.3.1 Chemical and physical properties

HBCD ($C_{12}H_{18}Br_6$) is a white or off-white powder at room temperature with a molecular weight of 641.7g/mol (UNEP, 2015). It consists of 16 stereoisomers which are subject to isomerization during production or natural means in the environment (Marvin et al., 2011). Industrial HBCD is composed of three main diastereomers (alpha; α, beta; β, and gamma; γ), each of which has different physiochemical properties (Table 1.1). Due to these differences in physiochemical properties between each diastereomer, it is difficult to predict the behaviour of industrial mixtures of HBCDs in the environment. The most consistent trend to emerge is the preferential bioaccumulation of α-HBCD in the tissues (Law et al., 2006; Tomy et al., 2004); the specific rationale is not clear but a number of factors may be influencing this preferential bioaccumulation. One hypothesis is the varying solubilities of each diastereomer (Table 1.1), with α-HBCD showing the highest solubility. Characteristically, highly soluble compounds are less likely to bioaccumulate as they are more likely to be metabolized and eliminated. However, compounds of moderate lipophilicity and moderate solubility have the potential for rapid diffusion through both aqueous and lipid phases (Müller & Nendza, 2007), thereby increasing the uptake rate of a specific diastereomer. This prompts the question of whether water-borne exposures, utilizing concentrations greater than the solubility of HBCD, are reliable measures of accumulation and toxicity as both β and γ forms will precipitate in water and then become unavailable to organisms (Marvin et al., 2011). Other factors that may impact preferential bioaccumulation include kinetics (uptake, biotransformation, and depuration) and *in-vivo* biotransformation from β- or γ-HBCD to α-HBCD (Roosens et al., 2010).

The high K_{ow} of HBCD results in its accumulation in organisms, and its lipophilic properties facilitate accumulation in sediment, and organic phases (i.e., liver, muscle, eggs, milk) and can persist in these matrices for extended periods of time. The half-lives within each organic phase can vary as well; few studies have found that a greater lipid content is correlated with

greater concentrations of HBCD and a slower rate of depuration (Haukas et al., 2009; Szabo et al., 2010). This is of concern due to the bioaccumulation and biomagnification factors of HBCD; the bioaccumulation factor (BAF) is the rate of absorption, metabolism, and elimination of a chemical whereas the biomagnification factor (BMF) is the "ratio of the chemical concentration to its diet at steady state" (Arnot & Gobas, 2006). BAFs of HBCD range from 3.7 to 6.1 (Marvin et al., 2011), which is at or above the CEPA (Canadian Environmental Protection Act; 1999) regulatory standard set by Environment and Climate Change Canada (BAF < 3.7); this informs HBCDs propensity to accumulate. The biomagnification factor (BMF) of HBCD ranges between 0.1 and 11, which was observed in the study conducted by Tomy et al. (2004) assessing the increase in stereoisomer-specific accumulation up the trophic levels.

The widespread use and physiochemical properties of HBCD have resulted in its distribution across various matrices, and its potential to bioaccumulate and biomagnify urges further studies on the long-term effects of exposure.

Table 1.1 Physiochemical properties of industrial HBCD and its three main diastereomers (α, β, and γ-HBCD).

	Industrial mix	α-HBCD	β-HBCD	γ-HBCD	Reference
Chemical formula	$C_{12}H_{18}Br_6$	$C_{12}H_{18}Br_6$	$C_{12}H_{18}Br_6$	$C_{12}H_{18}Br_6$	Sigma Aldrich (2022)
Molecular mass (g/mol)	641.70	641.70	641.70	641.70	Sigma Aldrich (2022)
Boiling point (ºC; at 760 mmHg)	>190	na	na	na	Sigma Aldrich (2022)
Melting point (ºC)	188 172-184	na 179-181	152-158 170-172	na 207-209	Sigma Aldrich (2022) ECHA (2008)
Log K_{ow}	5.6 5.62	5.07 5.8	5.12 5.8	5.47 6.3	MacGregor & Nixon (1997) ECC (2008), EPA (2014), Sigma Aldrich (2022)
Water solubility (µg/L; 20ºC)	65.6 (sum)	48.8	14.7	2.08	Hunziker et al. (2004)
Soil $t_{1/2}$ (days)	≥ 182	na	na	na	Canada (2000)
Water $t_{1/2}$ (days)	≥ 182	na	na	na	Canada (2000)
Air $t_{1/2}$ (days)	≥ 2	na	na	na	Canada (2000)
Sediment $t_{1/2}$ (days)	≥ 365 101 (aerobic)	na	na	na	Canada (2000) ECHA (2008)

	66 (anaerobic)				ECHA (2008)
BCF	na	na	na	23 988 (fish)	Arnot & Gobas (2003)
	8 974 (RBT)			na	Drottar et al. (2001)
	18 100 (FHM)				Veith & Defoe (1979)
BAF	3.7-6.1	na	na	na	Marvin et al. (2011)
	na			275 423 (fish)	Arnot & Gobas (2003)
BMF	na	9.2 (RBT)	4.3 (RBT)	7.2 (RBT)	Law et al. (2006)
	0.1-11	na	na	na	Marvin et al. (2011)

g = gram; mol = molar; ºC = degrees Celsius; µg = microgram; L = liter; BCF = bioconcentration factor; BAF = Bioaccumulation factor; BMF = biomagnification factor K_{ow} = water-octanol coefficient; K_{oc} = sediment/soil absorption/desorption coefficient; $t_{1/2}$ = half-life; RBT = rainbow trout; FHM = fathead minnow; na = not available

1.1.3.2 Distribution of HBCD in the environment

HBCD is ubiquitous in the environment, especially in the Northern Hemisphere (Marvin et al., 2011); baseline concentrations have been reported in biotic (> 0.01ng/g lw; Tomy et al., 2004) and abiotic (i.e., water ~ 0.003, soil > 1.7 ng/g dw, air > 0.001 ng/m^3, sediment > 0.04 ng/g dw) matrices (Law et al., 2006; Yu et al., 2008; Xiao et al., 2010). The physiochemical properties of HBCD have resulted in its widespread distribution across various matrices (i.e., air, suspended particulates in water, and sediment) presenting a challenge in determining persistence in the environment. In the 2011 Screening Assessment Report on Hexabromocyclododecane by Environment and Health Canada, presence of HBCD was predominantly in soil and sediment, although it may be altered depending on the matrices of release (i.e., released into air, water, or soil). It was suggested if 100% of HBCD was released into the air only about 0.002% would be partitioned to air, 2.1% to water, 10.6% to sediment and 87.3% to soil. In contrast HBCD released via the water would be partitioned only to water (17%) and sediment (83%) (EQC, 2003).

Commercial HBCD is primarily composed of three diastereomers, α, β, and γ-HBCD, with γ-HBCD accounting for approximately 70% (Marvin et al., 2011). However, diastereomer

composition differs greatly between abiotic and biotic matrices as well as across trophic levels. Composition in the environment tends to be a mixture of the three diastereomers with γ-HBCD accounting for the majority in sediment and soil (Harrad et al., 2009, Haukås et al., 2009). In contrast, diastereomer composition in the atmosphere favours α-HBCD (Yu et al., 2008). The specific rationale is not clear; however, a potential factor may be due to variation in degradation, rearrangement, and biodegradation rates in the environment. Furthermore, increased concentration of γ-HBCD may be associated with continuous emission from point sources as γ-HBCD biodegrades at a faster rate than α-HBCD (Son et al., 2015). Diastereomer concentrations in biota tend to consist mainly of α-HBCD (Davis et al., 2005); the greater the metabolic complexity of an organism the concentration of α-HBCD (Tomy et al., 2008). In addition, biomagnification factors (BMF) differ greatly among the diastereomers; data suggests α-HBCD has a greater potential for biomagnification than β, and γ-HBCD (Law et al., 2006). This preferential accumulation may be the result of structure symmetry; α-HBCD is more symmetrical than β, and γ-HBCD forms (Law et al, 2005; Janak et al., 2005).

In addition to metabolic capacity, BMF and symmetry, Zhang et al. (2014) reported varying bioisomerization efficiencies in four tissue types (viscera, muscle, skill, and gill) depending on the route of exposure. The major routes of exposure of HBCD are oral or inhalation; leaching from products or e-waste and burning of products containing FRs (i.e., electronics and electronic waste) will result in the chemical entering air, water, soil and sediment, which can then be inhaled, absorbed via gills or skin or ingested orally. Bioisomerization efficiency may be lower with waterborne exposure and greater selectivity may be observed in tissues with longer HBCD retention times (Zhang et al., 2014).

As mentioned previously, HBCD's lipophilic nature results in accumulation in tissues, soil, and dust. The half-lives differ between diastereomers; however, the overall trend is approximately 182 days in water and soil and 365 days in the sediment (Table 1.1). This range in half-life makes it difficult to predict the nature of HBCD in the environment as well as the potential toxic effects associated, another area lacking information.

1.1.3.3 Adverse effects

The first recorded adverse case of BFRs was seen in Michigan, 1973: a mix-up between FireMaster (FR) and NutriMaster (nutritional supplement for dairy cows) produced by one

company resulted in accidental feeding of PBB to thousands of dairy cows. The outcome of this accident was decrease in milk production, malnourishment, and abnormal hoof development (Venier, Salamova, and Hites, 2015). HBCD has been characterized as very toxic to aquatic organisms (acute toxicity in plants and chronic in invertebrates) and toxic to mammals and birds (Environment Canada & Health Canada, 2011). Exposure to HBCD can impact endocrine function, growth, survival, reproduction and development (Environment Canada & Health Canada, 2011).

Thyroid axis disruption is the most prevalent finding with exposure to HBCD; thyroid hyperplasia and decreased thyroxine (T4) levels are consistent across animal classes, and have been observed in birds, rats, and fish (ACC, 2001). Other BFRs display similar impacts on thyroid homeostasis; the proposed mechanism is the induction of phase II enzyme UDP-glucuronylstransferase (UGT). UGT is the major excretory pathway for T4 by biliary excretion (Palace et al., 2008); induction of UGT would increase the excretion of T4, and therefore, decrease plasma T4. This induction of UGT is likely induced by upstream interaction with xenobiotic activated receptors pregnane-X receptor (PXR) and constitutive androstane receptor (CAR) or chicken xenobiotic-sensing orphan nuclear receptor (CXR; avian equivalent). Although this mechanism has not been definitively confirmed there is evidence to suggest thyroid hormone dysregulation is caused by changes to hepatic metabolism, which in turn may impact lipoprotein synthesis and cholesterol and fatty acid homeostasis (van der Ven et al., 2006). Activation of PXR/ CAR/ CXR may also be linked to cytochrome P450 (CYP) induction, although CYP induction is not a consistent outcome of HBCD exposure. A study observing the diastereomer-specific effects of HBCD in RBT (rainbow trout; *Oncorhynchus mykiss*) found inhibition of CYP1A activity (Palace et al. 2008), whereas in another RBT study high concentrations of HBCD reported induction of CYP1A activity (Ronisz et al., 2004). In contrast, a study conducted by Germer et al. (2006) on rats observed no activation of CYP1A activity. Thyroid hormones are also vital for neuronal development; it was reported that exposure to HBCD can induce nerve cell activity and hypo-reactivity in mice (Eriksson et al., 2006). It is also evident that HBCD is of particular concern during early development as it can alter the development of the brain (Saegusa et al., 2009) and be maternally transferred (Ema et al., 2008; Nyholm et al., 2008).

The impacts of aqueous exposure to HBCD on growth, survival and reproduction in zebrafish (*Danio rerio*) appear to occur at concentrations > 0.5 mg/L (Deng et al., 2009), as exposure to 0.0001 mg/L – 0.1 mg/L displayed no effect on survival (Wu et al., 2013). However, there is uncertainty in these findings as many of the ELS exposures were waterborne, and as mentioned in previous sections the true concentration the organisms are exposed to is highly uncertain as a result. Avian exposure (injections) to concentrations ranging from 0.22 mg/mL – 50 mg/mL has reported impacts on hatching success (50 mg/mL only; Crump et al., 2010) and co-exposure (3.27 ± 0.68 and 15.61 ± 2.43 ng/g ww dietary HBCD) with PBDE (DE-71) has shown to have impacts on survival and reproduction, characterized by thinning of the eggshell and hatching success (Fernie et al., 2009). In mammals (rats), dietary exposure to HBCD has been related to decreased pup weight (Brandsma et al., 2009); however, no other species have shown similar effects.

As mentioned above, abnormal development is of concern with regard to exposure to HBCD, either due to exposure at a young age or maternally transferred; it can adversely impact an individual or a population. The greatest concern surrounds aquatic organisms as they are more prone to be exposed directly (i.e., wastewater treatment plants effluents) and indirectly (i.e., feeding on lower trophic organisms and maternal transfer).

1.1.3.4 Toxicity in fish

HBCD exposure has reported multiple sublethal effects in adult fish (i.e., thyroid hormone dysregulation, metabolism, oxidative stress, and reproduction) and acute toxicity (i.e., developmental deformities and mortality) in fish embryos (Palace et al., 2008; Deng et al., 2009). However, the specific molecular mechanism of action by which HBCD elicits its toxicity is still unclear. Recent studies have suggested HBCD may induce oxidative stress and apoptosis in fish (Deng et al., 2009; Ronisz et al., 2004), which adversely link to development and survival.

Oxidative stress occurs when an organism's ability to defend against radicals and other reactive oxygen species (ROS) is compromised due to intrinsic processes (i.e., mitochondrial respiration, peroxisome metabolism, enzymatic synthesis of nitric oxide, etc.) and/or extrinsic processes (i.e., xenobiotics, radiation, heat, etc.). It creates an imbalance between pro-oxidants and cellular antioxidants, which can result in damage to lipids, proteins and DNA (Timbrell, 1991). Superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radicals are the three main

ROS of concern; however, defense mechanisms such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) remove these ROS from the body before excess build up (Boelsterli 2007). During oxidative stress these defense mechanisms are compromised and unable to withstand xenobiotic attack, resulting in downstream adverse effects such as oxidation of lipids, proteins and DNA. ROS may be produced by the induction of CYP450 enzymes or Fenton reactions (Lemaire & Livingstone, 1993). Induction of CYP450 enzymes is typically in place as a detoxification mechanism; however, a chemical with low turnover rates can uncouple the CYP450 detoxification pathway and produce superoxide anion radicals and H_2O_2. Fenton reaction is a metal-catalyzed reaction that converts H_2O_2 (a product of superoxide conversion by SOD) to hydroxyl radicals. Lipophilic chemicals, such as HBCD, threaten the organism's capacity to eliminate ROS by disrupting electron flow in membranes; CYP450 systems are embedded in the membrane and components of the system are driven by the electrical gradient. The uptake of these lipophilic compounds can uncouple the CYP450 systems and produce ROS.

Early life stage exposure to HBCD has been linked to oxidative stress in fish. Zebrafish studies displayed a dose-dependent increase in protective nonenzymatic and enzymatic antioxidants and ROS at concentrations greater than 0.05 mg HBCD/L (Deng et al., 2009). The molecular mechanism of action is still unclear; however, findings by Hu et al. (2009) in zebrafish suggest uncoupling of membrane bound CYP450 pathways to be a key factor in inducing oxidative stress after exposure to HBCD. Another study by Mariussen and Fonnum (2003) displayed disruption to cellular membrane (i.e., plasma membrane) integrity and function at concentrations greater than 0.5 mg/ L, which resulted in significant induction of oxidative stress in zebrafish embryos. Although these findings do suggest a link between HBCD and oxidative stress it should be noted that all exposures were aqueous, and therefore, a certain level of uncertainty follows the concentrations at which effects were observed. In addition, considerable amounts of solvent likely have been required to dissolve HBCD, potentially contributing to the adverse effects reported and changing exposure dynamics. Regardless, induction of oxidative stress has been linked to abnormal development resulting in malformations (Deng et al., 2009; Yamashita, 2003) and delayed hatching (Wu et al., 2013) in zebrafish embryos making it a key endpoint of concern with HBCD exposure. Oxidative stress is also a trigger of apoptosis (Boelsterli 2007; Tan et al., 2018), which has been observed with exposure to HBCD during early development.

Apoptosis, or programmed cell death, is an active, energy-consuming process that is essential during embryogenic development, after birth and during adulthood of all multicellular organisms. It is a well-researched topic, and the apoptotic pathway is evolutionarily conserved among species (Yamashita, 2003). Induction of apoptosis can occur through two pathways; 1) an extrinsic pathway that is triggered by extracellular ligands (i.e., xenobiotic) binding to plasma membrane "death receptors", or 2) by an intrinsic pathway that is triggered by intracellular stressors (i.e., growth factor withdrawal, DNA damage, oxidative stress). The extrinsic pathway triggers death receptors such as Fas ligand (FasL) and tumor necrosis factor (TNF), which in turn recruit amplifying caspases that then activate effector caspases (i.e., caspase-3 and 9). The intrinsic pathway is mediated by the mitochondria; upon receiving a stress signal, pro-apoptotic proteins (i.e., Bax, Bad, Bid, Bcl-X_S) are activated and anti-apoptotic proteins (i.e., Bcl-2, Bcl-X_L, Mcl-1, A1) are inactivated, which creates a destabilization of the mitochondrial membrane releasing apoptotic factors (i.e., cytochrome c) that trigger proteolytic caspases (Boelsterli, 2007). These proteins are regulated by the expression of specific transcription factors (TF), and one major TF is p53. P53 is a pro-apoptotic gene that is often described as the tumor suppressor gene; it signals downstream genes and proteins to initiate apoptosis and is a vital aspect of embryo development. P53 activates pro-apoptotic genes (i.e., FAS, PUMA, Bax) and anti-apoptotic genes (i.e., Bcl-2, Bcl-X_S), and the decision to undergo apoptosis or not is dependent on the sum of these signals (Chen and Wong, 2009). Research done by Al-Mousa and Michelangeli (2014) suggested that exposure to TBBPA and HBCD induces cell death through the intrinsic pathway.

A study by Deng et al. (2009) showed upregulation of pro-apoptotic genes p53, Bax, Puma, Apaf-1, caspase-3 and 9 and downregulation of anti-apoptotic genes Mdm2, Bcl-2 after exposure to 1.0 mg HBCD/L. The logical progression would be to predict that p53 plays a vital role in mediating apoptosis as it mediates the activation of many of the downstream proteins involved in the apoptotic process; as such, a study done by Langheinrich et al. (2002) found p53 must be activated for apoptosis to occur in zebrafish embryos. Few studies have tried to discern the molecular mechanism of action of HBCD-induced apoptosis; Deng et al. (2009) proposed the production of ROS may activate p53, which begins the cascade of adverse effects.

Li et al. (2007) and Chen and Wong (2009) observed a link between ROS and apoptosis in human cancer cells. As apoptosis is a highly conserved process, a similar interaction may be

occurring in fish species. It has been found that ROS-induced apoptosis is one of the reasons for early abnormal development during embryogenesis (Yabu et al., 2001) suggesting oxidative stress coupled with apoptosis may be the two key drivers in HBCD toxicity in early life stages of fish; however, further research needs to be done to provide adequate evidence. Along with the uncertainties associated with waterborne exposures, there is a limited number of studies that attempted to discern the molecular mechanism of HBCD in inducing oxidative stress and the subsequent apoptotic effects, therefore, further research needs to be conducted.

1.2 Maternal Transfer and Toxicokinetic

Embryonic development in oviparous organisms is largely dependent on maternal transfer of nutrients, specific hormones, and other components (i.e., immune factors such as lectins and lysozymes). The key nutrients required for the first days of survival are passed on by the mother to the egg in the yolk sac by way of maternal transfer (Wang et al., 2012). Maternal transfer can also present as a potential route of toxicant exposure to ELS of oviparous organisms, particularly fish. Research has shown that ELS of oviparous fish are more sensitive to chemical contaminants than during adulthood (Russell et al., 1999). During early development, most organisms are unable to mount significant immune responses (Wang et al., 2012) and are therefore more vulnerable to environmental stressors than their adult counterparts. In addition, the metabolic functions in early life stage organism are not fully functional, increasing their sensitivity to chemicals that are typically eliminated/ detoxified in older/ adult organism (Couillard et al., 2004). Factors increasing the sensitivity of young organisms include differential sensitivity of organ systems during early development (Ozoh, 1979), shorter time for the chemical to reach target sites in the body due to size differences, and greater uptake of toxicants as skin is quite permeable during early development (Mohammed, 2013). As a result, the offspring are exposed to toxicants during a crucial time of development (embryogenesis) (Wu et al., 2013), which can compromise their development and survival.

Impacts on hatching success, growth, and survival have been observed in birds, fish and frogs exposed to lipophilic organic pollutants (Hall & Oris, 1991; Huestis et al., 2009; Fernie et al., 2009). Maternal transfer of polychlorinated biphenyl (PCBs) and polybrominated diphenyl ethers (PBDEs) have been reported in both laboratory and field exposed fish (Miller, 1993, Nyholm et a., 2008). HBCD is another lipophilic pollutant that has been characterized to be

maternally transferred in fish and birds (Nyholm et al., 2008; Nyholm et al., 2008); however, the dynamics of this transfer are poorly understood and require further research. Generation of such information is particularly important because dietary exposure and maternal transfer are two of the main exposure routes of concern for HBCD in fish.

Toxicodynamics (TD) is an area of research focused on the mechanism of action of toxicants relative to concentration and time of exposure while toxicokinetics (TK) is the study of a toxicant's absorption, distribution, metabolism, and excretion (ADME), or its disposition in an organism (Klassen and Watkins, 2010). In other words, TK and TD describe what happens to the chemical within an organism and what a chemical does to an organism, respectively. Based on previous research, adverse effects on growth, survival, and development were identified in ELS of fish exposed to waterborne HBCD. However, there is very limited information on the toxicity of maternally transferred HBCD, and the TD and TK involved. Research done by Nyholm et al. (2008) in zebrafish revealed that HBCD requires approximately 28 days to reach maximum levels in maternal tissue and approximately 7 days to transfer from maternal tissue to eggs. Previous research on other lipophilic organic pollutants suggests that concentrations in eggs are within the range of maternal tissue concentrations rather than concentrations found in the diet or water (Russell et al., 1999). Russell et al. (1999) described the lipid-based concentration ratio in the fugacity model (model that summarizes the processes involved in chemical partitioning in environmental matrices and biota), which predicted the egg to fish ratio to be close to 1.0 with a 95% confidence interval of 0.56 and 2.5; however, other factors (i.e., lipoprotein capacity) may shift the egg to fish ratio from the proposed model.

Nyholm et al. (2008) suggested that changes in egg to fish concentration ratios may be due to selective transport processes during vitellogenesis; a process when nutrients (vitellogenin derived egg yolk) are deposited, mainly as lipoproteins, from maternal tissue to the oocyte (Hara et al., 2016). These lipoproteins, similar to other transporters within the body, can be selective as well as become saturated. For example, PCBs have been shown to be carried by mainly high-density lipoproteins whereas methylsulfonyl-PCBs may be transported by high and low-density lipoproteins alike (Norén et al., 1999). This suggests BFRs, similar to HBCD, may undergo selective transport during vitellogenesis and result in egg to fish concentration ratio that differ from the fugacity model described above. In the same study, Nyholm et al. (2008) saw HBCD concentrations that were greater in the eggs than in maternal tissue as well as greater transfer of

BFRs in fish exposed to the low dose rather than the high dose. The potential theory being HBCD transport via lipoproteins were saturated and could transport a maximal concentration (Tocher, 2003) as was seen in PCBs mentioned above, although further research is required to fully understand the toxicokinetics and toxicodynamics of maternal transfer of HBCD.

1.3 Environmental risk assessment

Environmental risk assessment provides information on the probability of an adverse effect on the environment due to anthropogenic activity (i.e., chemicals), which is used to inform regulatory and industry standards and decisions (Birnbaum et al., 2016). In 1983 a 4-step framework for risk assessment was developed by the National Research Council (US NRC, 1983); it included hazard identification, dose response assessment, exposure assessment, and risk characterization. This tiered system screens for chemicals of concern based on the concentration in the environment, adverse apical effects (i.e., growth, mortality, reproduction, development, pathology) in relation to concentration, and impact on the ecosystem (i.e., what animals and plants may be affected) (Birnbaum et al., 2016). ERAs provide information, such as threshold values, potential adverse effects, and uncertainties for each chemical in an effort to reduce hazardous chemical exposure to humans and wildlife. However, there are major shortfalls with the current testing methods. Dose response and exposure assessments are primarily based on animal tests that are very costly, take a long time, are of ethical concern, and are resource intensive; this inevitably results in a bottleneck situation with the number of chemicals needing to be tested exceeding the rate of assessments that can be completed (Basu et al. 2019).

Advancements in science and technology as well as increased interest in environmental stewardship and demands for more ethical testing have led to efforts in developing alternative testing strategies (Erhirhie et al., 2018) driven by the "3 Rs" principle (reduce, refine, replace) (Russel & Burch, 1959). New technologies (i.e., toxicogenomics, high-throughput technologies, bioinformatics) and alternatives to animal testing (i.e., *in silico*, *in vitro*, embryo-based tests) integrate current scientific literature and understanding of biology with molecular data with the eventual goal being to forecast apical outcomes of regulatory relevance.

1.3.1 Toxicity pathways and ELS exposures

Toxicity pathways are defined as "cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects" (US NRC, 2007). Understanding the molecular and cellular interactions that occur with chemical exposure and the link to subsequent apical responses (i.e., mortality, growth inhibition) will enable more targeted testing by categorizing and prioritizing chemicals based on the severity of effects on key biological pathways (Basu et al., 2019). These conceptual frameworks link a molecular initiating event (MIE; the first interaction of a chemical with a biological molecule) to higher organizational level responses, which are termed key events (KEs), that can lead to an adverse outcome (AO) (Ankley et al., 2010; Liu et al., 2019). A fully realized pathway from MIE to AO is termed an adverse outcome pathway (AOP). AOPs, unlike toxicity pathways, are chemical agnostic, and can be used to categorize chemicals of similar mechanism of action (Ankley et al., 2010), thereby streamlining risk assessment of environmental chemicals based on their mechanism of action (MoA) to better meet current needs of regulatory agencies and industry to assess the safety of the large number of chemicals used by society.

Derivation and validation of toxicity pathways requires extensive testing; the use of alternative animal tests such as *in vitro* assays and ELS exposures with oviparous organisms are prime candidates. Embryo-based exposures utilize organisms that do not feed independently (i.e., dependent on their yolk sac), and according to legislations in North America, Europe and several other jurisdictions these organisms are not protected and may be used as a substitute for acute toxicity tests (Batt et al., 2005; EU, 2010; Strähle et al., 2012; UK, 1993). These short-term exposures would be more cost effective and less time consuming than full-scale adult or juvenile exposures. However, there are some reservations regarding embryo exposures due to the potential protective mechanism of the eggshell/chorion that can result in low absorption of some chemicals with high molecular weight, lower neurotoxic effects observed in embryos than in adults (Klüver et al., 2015), and differences in biotransformation capacity (Klüver et al., 2015; Knöbel et al., 2012). However, utilizing maternal transfer as the route of exposure eliminates any concerns associated with low absorption.

1.3.2 Toxicogenomics

Toxicogenomics is defined by the National Institutes of Health (NIH) as the utilization of omics technologies (i.e., genomics, transcriptomics, proteomics, metabolomics) to study the effects of anthropogenic and naturogenic stressors on human health (US NRC, 2007). In recent years, these technologies have been increasingly applied in ecotoxicology research (Ankley et al. 2006). Toxicogenomics technologies show great promise as a screening tool to address some of the issues associated with conventional environmental risk assessments; they enable the grouping of chemicals based on disruption of similar molecular responses or similar MoAs, quantify toxicity endpoints that may otherwise be unattainable with conventional testing strategies, and have the potential to identify key molecular events prior to the onset of adverse effects (Health Canada, 2018). The major molecular profiling technologies of toxicogenomics are genomics, epigenetics, transcriptomics, proteomics, and metabolomics (Tennant, 2002), all of which must be coupled with bioinformatics (i.e., advanced mathematical and computational tools) for data analysis and interpretation.

Integration of omics technologies (genomics, transcriptomics, proteomics, and metabolomics) allows for the development and improvement of our current understanding of biological effects of toxicant exposure; it enables derivation of comprehensive biological response networks and the subsequent apical response as a result of chemical exposure (Liu et al., 2019). The most advanced and widely used technology in support of toxicogenomic studies are transcriptomics, while both proteomics and metabolomics are limited due to greater complexity in measurement and interpretation as there are multiple modified and diverse forms of proteins and metabolites that may exist from one transcript (US NRC, 2007). Therefore, genomics and transcriptomics technologies are more readily available for rapid profiling of toxicant exposure. It should be noted, though, that both proteomics and metabolomics technologies are advancing rapidly with the rapid evolution of mass spectrometry (MS) and NMRS (nuclear magnetic resonance spectroscopy) technologies, and thus, the measurement and identification of diverse proteins and metabolites are improving.

The Central Dogma of Molecular Biology states that the genetic material of all living beings is encoded by unique DNA sequences, which are transcribed to RNA and translated to proteins (Dunkler et al., 2011). Genomics is the study of inherited genes; the entire or partial genome of various species (i.e., humans, rats, mice, zebrafish, rainbow trout, FHM, *Xenopus sp.*)

have been annotated to date (The *Danio rerio* Sequencing Project, *Xenopus tropicalis* Genome Assembly 4.1), and the number of species being annotated is increasing as interest in omics research grows (Ankley et al., 2006, 2010). Using annotated genomes, changes in gene regulation (i.e., up- or down-regulated) can be quantified by qPCR (quantitative polymerase chain reaction) or RNAseq (ribonucleic acid sequencing of the partial or entire transcriptome of an organism) technologies (Arivaradarajan & Misra, 2018). The benefit of utilizing RNAseq techniques stems from the ability to identify well annotated sequences (as with qPCR) as well detect novel transcripts, in the form of messenger RNA (mRNA). Transcriptomics provides a snapshot of the mRNA expressed in a cell, tissue, or organism at a specific time point (Aizat et al., 2018). Transcriptomics data is quantified by read count, which is an estimation based on the expression level and length of the transcript, therefore, some form of normalization is required to reduce selection bias (Young et al., 2010). Interpretation of transcriptomics data should be done with some caution as mRNA expression does not absolutely correlate with protein expression. The mRNA undergoes post-translational changes (i.e., post-transcriptional, translational, protein degradation); therefore, the final protein abundance can differ from the expressed transcripts (Arivaradarajan & Misra, 2018). Despite these limitations, the use of transcriptomics data remains an informative tool; the identification of dysregulation of certain genes and their enrichment along complex molecular pathways has been found to be a good indicator of subsequent biological change (Lowe et al., 2017).

1.4 Project rationale and hypotheses

The aim of this master's research project was to 1) investigate the kinetics of dietary HBCD in adult fish as well as their progeny, and 2) observe the effects of maternally transferred HBCD in the F1 generation to derive the specific toxicity pathways by which this chemical elicits adverse effects in fish using the model species, *Pimephales promelas*. Specifically, this study investigated the accumulation kinetics of HBCD in adult fish exposed to dietary HBCD and the maternal transfer kinetics in the F1 generation. The second aspect of this study was to investigate molecular response patterns after short term exposure of maternally exposed embryonic FHMs to graded concentrations of HBCD and assess whether these can be used to predict classical apical adverse effects observed after sub-chronic exposure later in life. The goal of my thesis was to gain a better understanding of the molecular events involved in initiating

adverse effects of HBCD, namely survival, condition factor, and development in larval fish following a realistic exposure scenario using maternal transfer.

The chapter-specific objectives and hypotheses were as follows:

Objective 1:

Characterize maternal transfer and apical and physiological effects of dietary HBCD exposure in parental fathead minnows (FHMs)

Sub-objectives

1. Characterize tissue specific uptake and maternal transfer of HBCD in/ from adults exposed through the diet to their gametes.

   H_0: HBCD concentration in adult FHMs exposed to graded concentrations of dietary HBCD will display no statistically significant concentration-dependent or tissue-specific uptake.

2. Describe the accumulation kinetics of HBCD in eggs exposed through maternally transferred HBCD.

   H_0: HBCD concentration in FHM embryos will show no statistically significant concentration-dependent increase as a function of time and concentration.

3. Describe physiological and histological effects in adult FHMs exposed to dietary HBCD.

   H_0: Exposure to graded concentrations of HBCD via maternal transfer will not result in statistically significant concentration-dependent apical effects in adult FHMs.

Objective 2:

Integrating omics analysis with apical outcomes of maternally transferred HBCD in early life stage FHM

Sub-objectives

1. Describe physiological and histological effects in FHM larvae maternally exposed to HBCD.

   H_0: Exposure to graded concentrations of HBCD via maternal transfer will not result in a statistically significant concentration-dependent effect on apical and physiological endpoints in FHM larvae.

2. Conduct whole transcriptome analyses to identify key gene expression signatures in FHM larvae exposed to graded concentrations of HBCD via maternal transfer.

   H_0: Exposure of FHM embryos/ larvae to graded concentrations of HBCD via maternal transfer does not result in statistically significant changes across the whole transcriptome.

3. Integrate physiological and histological endpoints with omics endpoints to characterize the toxicity pathway(s) of HBCD in *P. promelas*.

   H_0: Exposure to graded concentrations of HBCD via maternal transfer does not display any statistically significant correlation among apical, physiological and omics endpoints.

CHAPTER 2: MATERNAL TRANSFER AND APICAL AND PHYSIOLOGICAL EFFECTS OF DIETARY HEXABROMOCYCLODODECANE EXPOSURE IN PARENTAL FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

Preface

This chapter aimed to characterize the accumulation kinetics of HBCD in adult fathead minnows (FHMs) and the subsequent maternal transfer to the F1 generation. In addition, morphometrics, fecundity, and histopathology was conducted on the parental organisms. Adult FHMs were exposed to dietary HBCD for 49 days; muscle and egg samples were taken to evaluate the accumulation potential in adult fish and egg-to-muscle ratio to quantify maternal transfer potential.

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2.1 Abstract

Hexabromocyclododecane (HBCD) is a persistent organic pollutant (POP) that has been characterized as an endocrine disruptor, undergoes maternal transfer, and hinders development and growth in oviparous organisms. This study examined the apical effects of dietary HBCD (11.5, 36.4, 106 mg/kg, ww) on adult fathead minnow (FHMs) exposed for 49 days and the subsequent accumulation and maternal transfer kinetics in adult tissue and eggs, respectively. Exposed adults displayed a significant increase in egg production in the medium treatment group, however, no other significant effects were noted. Maternal transfer of dietary HBCD had a similar egg-to-muscle ratio (EMR) in the low and medium treatment groups (1.65 and 1.27 [ww], respectively). However, the high treatment group deviated from other treatments with an EMR of 4.2 (ww), potentially due to differences in total lipid content in food and/or reaching diffusion/ lipid saturation limits in adult tissue resulting in lower accumulation in the adult muscle tissue. A positive correlation was observed between egg HBCD concentration and time of exposure, which indicate that maternal transfer of HBCD is of concern in fish, and further studies should be conducted to fully elucidate the potential adverse effects that may be observed in early life stage (ELS) of oviparous organisms.

2.2 Introduction

Brominated flame retardants (BFRs) are industrial chemicals that are commonly used as flame-retardants in consumer products due to their cost effectiveness, minimal-to-no interference with the physical properties of polymers, and stability (high decomposition temperature) (Domínguez et al., 2011). Hexabromocyclododecane (HBCD) has been one of the most prominent BFRs with high application rates dating back to the 1960s, and it was the most widely used BFR during the early 2000s (Kodavanti & Loganathan, 2014; Marvin et al., 2011). HBCD is an additive-type flame retardant, primarily used in polystyrene foams for insulation, with additional applications in textiles and electrical and electronic equipment. Compounds incorporated as additives into products, such as HBCD, present significant concern as they can inadvertently leach into the environment during the manufacturing, use, and disposal of products (0.0025 – 36 mg/kg dw in Netherland landfill leachates; Morris et al., 2004); in addition, its halogen moiety increases its potential to persist in the environment (Segev et al., 2009). HBCD is highly hydrophobic with a $\log K_{ow}$ of 5.6 (EPA, 2014), and thus, has the propensity to

743 accumulate in dust, soil, sediment, organisms, and suspended particulates in water (Environment
744 Canada & Health Canada, 2011; Remberger et al., 2004), where it can remain for long durations
745 of time. HBCD has been detected in various environmental and biological matrices, both at
746 point sources, remote locations (Covaci et al., 2006), and in dated sediment samples (Minh et al.,
747 2007), suggesting its potential to bioaccumulate and undergo long-range transport.
748 Toxicologically, HBCD has been reported to impact reproduction, development, survival, and
749 thyroid homeostasis in terrestrial and aquatic species (Darnerud, 2003). Due to these properties,
750 HBCD was categorized as a persistent organic pollutant (POP) by the United Nations
751 Environment Programme (UNEP) Stockholm Convention on POPs, and a reduction in
752 production, import, and use were issued (Sharkey et al., 2020). However, despite the reduced
753 use/ production of HBCD it continues to be globally detected with increasing trends in aquatic
754 environments (Drage et al., 2015; Guo et al., 2017; Parvizian et al., 2020; Vorkamp et al., 2018),
755 and represents an ongoing environmental issue.
756 Current findings from studies assessing the toxicity of HBCD have displayed disruption
757 of thyroid function, quantified by a decrease in thyroxine (T4) and an increase in
758 triiodothyronine (T3) levels in adult fish (Halldorson et al., 2008; Kuiper et al., 2007; Palace et
759 al., 2010), and significant increase in malformation rate and reduction in survival in early life
760 stage (ELS) fish (Deng et al., 2009). Downstream endocrine effects included impaired somatic
761 growth, reproduction, or ability to tolerate changes in the environment (Kuiper et al., 2007).
762 Furthermore, maternal transfer of HBCD was observed from adult fish to the subsequent F1
763 generation in zebrafish (Nyholm et al., 2008; Wang et al., 2012). This is of concern in oviparous
764 fishes as prolonged exposure to low concentrations of HBCD may lead to high internal
765 concentrations in the parental generation (Eljarrat & Barceló, 2018), as well as the resulting
766 embryos. The parallel transfer of hydrophobic organic compounds has been observed in other
767 BFRs, such as polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA),
768 where concentrations in the offspring were equivalent or within the same range as in maternal
769 tissues (Nyholm et al., 2008; Xian et al., 2008). Similarly, other hydrophobic organic compounds
770 (i.e., organochlorines) have displayed egg-to-maternal tissue concentration ratios (EMRs) of 1.0
771 with a variation factor of 2 (Russell et al., 1999). Embryonic development in oviparous
772 organisms is facilitated by the maternal transfer of nutrients, hormones, and other components
773 (i.e., immune factors such as lectins and lysozymes). However, the concurrent transfer of

chemical contaminants may disrupt normal development and adversely impact the survival of the embryos. It is well documented that ELSs of fish are more sensitive to contaminant exposure than adults (Russell et al., 1999) and exposure of offspring during the sensitive embryonic life stage may hinder normal development, growth, survival, and ultimately population dynamics. It may be that HBCD exposure is more potent in the ELS of fish species, and therefore, it is necessary to identify the accumulation kinetics of HBCD from the diet to the adults, in addition to quantification of the maternal transfer potential.

Although our knowledge on the maternal transfer and toxicity of HBCD in fathead minnows is limited, the current environmental concentrations in Canada considered with HBCD's persistence and bioaccumulation risk quotients suggest that HBCD may pose an ecological threat (Environment Canada & Health Canada, 2011). In particular, there are concerns about the potential embryotoxicity of HBCD, given its potential to bioaccumulate and be maternally transferred to embryos. Therefore, the objectives of this study were to 1) quantify accumulation in adult fish dietarily exposed to HBCD; 2) characterize the maternal transfer of HBCD from adult fish to their progeny; 3) describe the accumulation kinetics of maternally transferred HBCD in the eggs; 4) observe apical and physiological effects in the adults.

2.3 Materials & methods

2.3.1 Experimental organism source

Adult fathead minnows (FHMs) were obtained from an in-house culture at the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre (SK, Canada). Adult fish were maintained in 500 L Min-o-Cool Systems® with continuous water flow and controlled temperature (25 ± 2 °C). The fish were fed chironomid larvae, *ad libitum*, twice a day. All fish, embryos, and larvae were reared following the Canadian Council of Animal Care (CCAC) experimental fish guidelines, and all experiments were approved by the Animal Research Ethics Board at the University of Saskatchewan (protocol #20160090).

2.3.2 Diet formulation

HBCD spiked food was prepared following the protocol by Nyholm et al. (2008) with a few modifications. In brief, dosing solutions were made using 100% ethanol (EtOH; #P016EAAN, Commercial Alcohols) and 1,2,5,6,9,10-HBCD (95% purity; 11% α-HBCD, 9%

β-HBCD, and 79 % of γ-HBCD, Sigma-Aldrich), similar to commercial HBCD formulations. Twenty grams of ground, freeze-dried chironomids were mixed with 20 mL of dosing solution containing known quantities of HBCD for two hours. The solvent was then slowly evaporated in a fume hood at room temperature (24-48 hrs) and the dosed food was then stored in the dark at 4°C. The nominal concentrations of the diet were 10, 33, and 100 mg of HBCD/ kg of food. The lowest treatment condition is representative of globally detected concentrations in animal tissues (8 mg/kg lw in teleosts sampled in Norway, Sweden, and South Africa; Environment Canada & Health Canada, 2011) while the two greater concentrations were selected to elicit molecular and physiological effects that were assessed in a parallel study. Diet concentrations were confirmed by LC-MS/MS at SGS-AXYS (BC, Canada) as described in Section 2.5.

2.3.3 Reproductive adult exposure and sampling

Adult FHMs (>6 months) were exposed to one of four treatment conditions (0.0599 [solvent control], 11.5, 36.4, 106 mg of HBCD/ kg of food wet weight [ww]) for 49 days following OECD guideline 229 (2012), with minor modifications. Fish were exposed in 20 L tanks in breeding groups of five fish per tank (2 males and 3 females) with two breeding tiles. Five replicate tanks were tested per treatment group. Exposures were conducted under static renewal conditions (75% water change every day) with continuous aeration (>80% dissolved oxygen), controlled temperature (25 $\pm$ 2°C), and a photoperiod of 16 hrs light: 8 hrs dark. Water quality was assessed five days a week (one replicate/ day) by measuring temperature, dissolved oxygen, pH, ammonia, nitrite, nitrate, hardness, and alkalinity (S1). A 14-d acclimation period was used to initiate breeding and to introduce the freeze-dried control diet. Following acclimation, a seven-day period was used to determine baseline fecundity (defined as the number of eggs produced based on the number of females in the tank); the average fecundity during the baseline period was used to assign treatment conditions (i.e., randomized block design) as follows: average fecundity for each tank was ranked from best to worst; treatment conditions were assigned such that each treatment had an equal distribution of breeding performance (i.e., four best-performing breeding tanks were randomly assigned to the first replicate across all treatment conditions, then the second-best four tanks were assigned as the second replicate, and so on). Fish were fed a diet of HBCD-spiked food at 5% bw/ day, three times a day. Eggs were collected daily and weekly to quantify cumulative egg production and quantify chemical

concentration in the F1 generation, respectively. Additionally, eggs exposed for 21 days were subsampled for total lipid analysis. At the end of the exposure, minnows were euthanized, and their standard lengths and weights were recorded to calculate the condition factor (K; Eq. 1). A Subset of adults were fixed for thyroid histology (see below), and muscle tissue was sampled for quantification of HBCD concentration. Additionally, maternal muscle tissue exposed for 7 days was subsampled for total lipid analysis. Livers and gonads were weighed to calculate liver somatic and gonadal somatic indices (LSI: Eq. 2; GSI: Eq. 3).

Pooled samples of FHM eggs (1 g/ treatment [ww]; *n*=1 for each time point) and adult FHM muscle tissue (0.3 g/ replicate, separated by sex; n=1-5) were stored in 5 mL amber vials and kept at -20^{o}C until analytical confirmation of HBCD concentration. Individual whole adult minnows were incised midline from anus to the operculum and fixed in Cal-Ex for 48 hrs and then preserved in 70% EtOH for histopathology analysis.

2.3.4 Analyses of HBCD in tissues and diet

Samples of adult muscle tissue, food, and eggs were analyzed for three diastereomers of HBCD (alpha-, beta-, and gamma-HBCD) at SGS-AXYS Analytical Services Ltd. Samples were homogenized and Soxhlet-extracted for 16 hrs using dichloromethane. To calculate recovery and extraction loss, isotopically labeled surrogate standards ($^{13}C_{12}$-alpha-HBCD, $^{13}C_{12}$-beta-HBCD, and $^{13}C_{12}$-gamma-HBCD) were added to the samples before extraction. Prior to instrumental analysis, samples were chromatographically cleaned using a gel permeation and a Florisil column, respectively. HBCD isomers were isolated using a Waters Acquity UPLC® HSS T3 C18 column (1.8 µm, 30 x 2.1 mm) maintained at 35^{o}C, using a mobile phase composed of 1:1 acetonitrile: methanol (A) and HPLC grade water (B); the elution gradient following (time, %A): 0.0, 50%; 0.2, 50%; 0.4, 75%; 2.5, 95%; 4.3, 95%; 4.4, 50%; 5.5, 50%. HBCD diastereomer separation was done using a UPLC coupled to a TQ-S Xevo/Xevo Triple Quadrupole Micro MS/MS with negative ion electrospray, multiple reaction monitoring (MRM) modes (Waters Acquity, Canada). The flow rate was set to 0.45 mL/min. Instrument linearity was calibrated with a seven-point calibration curve using 1/x weighted linear calibration and responses relative to the isotopically labeled standards. Quality control (QC) measures were implemented for each batch of samples by running laboratory blanks, which were used to correct data, in parallel to the samples.

Total lipid content was measured in adult female (lipid corrected paternal concentrations were derived from female total lipid content) muscle tissue (day 7), food, and eggs (day 21) to correct concentrations based on lipid content. Samples (n = 4) were quantified using the microcolorimetric sulfophosphovanillin (SPV) method as described by Grimard et al. (2020). In brief, samples were homogenized in chloroform: methanol (2:1), purified in saline, and quantified by the addition of sulphuric acid and SPV reagent. Absorbance was measured at 525 nm using a multi-well plate reader (POLARstar Optima, BMG Labtech, Ortenberg, Germany). A 6-point standard curve (0.00, 0.65, 1.3, 2.6, 5.4, 10.4 mg/mL; r^2 = 0.9775) utilizing cod liver oil (CAS 8001-69-2, Sigma Aldrich) in 2:1 (v/v) chloroform:methanol was used to calculate percent lipid content.

2.3.5 Apical endpoints

Adult FHMs were assessed for daily and cumulative egg production, and at the end of the experiment for mass, standard length, condition factor, GSI, LSI, and percent mortality. Males and females were analyzed separately to account for differences in size and sex-specific metabolic capacity. Condition factor (Eq 1), GSI (Eq 2), and LSI (Eq 3) were calculated for individual fish and the mean for each replicate was compared to that of the solvent controls. ADD (Average Daily Dose; Eq 4), percent accumulation, BMF (Biomagnification Factor; Eq 5), and EMRs (Eq 6) were calculated based on U.S. EPA's Exposure Assessment Tools and the Exposure Factors Handbook (US EPA, 2011). Percent mortality, cumulative egg production, ADD, BMF, and EMRs were calculated for each replicate and compared to the solvent control.

$$Condition\ Factor\ (K) = \frac{100 * fish\ weight\ (g)}{standard\ length\ (cm)^3} \quad (1)$$

$$GSI(\%) = \frac{gonad\ weight\ (g)}{total\ fish\ weight\ (g)} \times 100 \quad (2)$$

$$LSI(\%) = \frac{liver\ weight\ (g)}{total\ fish\ weight\ (g)} \times 100 \quad (3)$$

$$ADD\ (\frac{mg}{kg}/d) = \frac{HBCD\ concentration\ in\ food\ (mg/kg) \times Ingestion\ Rate\ (kg/day)}{Weight\ (kg)} \quad (4)$$

$$BMF = \frac{HBCD\ concentration\ in\ tissue(mg/kg)}{HBCD\ concentration\ in\ food\ (mg/kg)} \quad (5)$$

$$EMR = \frac{HBCD\ concentration\ in\ eggs\ (mg/kg)}{HBCD\ concentration\ in\ adult\ females\ (mg/kg)} \quad (6)$$

2.3.6 Histology

At the end of the adult exposure, 1 male and 1 female per tank were euthanized and fixed for histology. A mid-ventral incision was made through the body wall, and the whole-body samples were immersed in Cal-Ex™ II Fixative/Decalcifier (Fisher Chemical™) for 48 hrs, then transferred to 70% ethanol for storage. Only the control and high treatment condition samples were further processed for analysis of potential thyroid effects. For these samples, the lower jaw was removed and placed in a histocassette. They were dehydrated in graded alcohols, cleared in xylene, and infiltrated with molten paraffin using a Belair RVG/1 Vacuum Tissue Processor (University of Saskatchewan Histology Core Facility). The jaws were embedded in paraffin blocks for step-sectioning (5 µm thick) in frontal plane through the follicle-containing region at the base of the gill arches. The sections were placed on glass slides and stained with Harris' hematoxylin and eosin.

Each individual was assessed based on the overall appearance of thyroid tissue across multiple sections and multiple fields of view. Males and females were assessed separately. The thyroid tissue was assessed for the presence of alterations in the HBCD-exposed high treatment group relative to controls, based on Schmidt and Braunbeck (2011): follicle appearance (total number, size, shape), hyperemia, colloid appearance (homogeneity, colloid depletion/vacuolation), and epithelial cells (cell height, stratification, cell crowding).

2.3.7 Statistics

Data on standard length, mass, condition factor, GSI, LSI, cumulative egg production, and percent mortality were analysed for significant differences among treatment conditions and the controls. All data was tested for normality of residuals and homogeneity of variance using Shapiro-Wilk test and Bartlett's test, respectively. Normally distributed data was analyzed using a one-way ANOVA and, if significant differences or trends were observed, Tukey's multiple comparison test followed by Welch's t-test was used to determine any dose-dependent effects.

Data that failed homoscedasticity test were log-transformed and re-analysed as mentioned above or analysed using one-way ANOVA on ranks (Kruskal Wallis H test) followed by Dunnett's multiple comparison test to identify dose-dependent effects.

2.4 Results

2.4.1 Concentrations of HBCD in diet and tissues

HBCD concentrations were detected in the diet, muscle tissue (maternal and paternal), and eggs (Table 2.2) and ADD, percent accumulation, BMF, and EMRs were calculated from the measured concentrations of eggs and muscle tissue (Table 2.3). HBCD concentrations in adult muscle tissue exhibited an approximate 4- (maternal) and a 10-fold (paternal) increase between the low and medium treatment groups, with a marginal increase between the medium and high treatment groups. The calculated BMF and percent accumulation (based on ADDs) displayed a similar pattern; however, they were not significantly different from the control due to high variation among replicates. Mean measured concentrations in the eggs were proportional to HBCD concentrations in food. FHM eggs maternally exposed to HBCD showed first-order saturation kinetics (r^2 = 0.93, 0.99, 0.99) for low, medium, and high treatment conditions (Figure 1). All treatment conditions were at or near steady state by day 28 and displayed a time-dependent increase in concentration (p = 0.0189, 0.0434, 0.0323 in low, medium, and high treatments, respectively). Lipid corrected EMRs ranged between 1.27 – 2.73, whilst wet weight EMRs ranged between 1.27 – 4.20 (Table 2.3).

Table 2.1 Measured total lipid (mean ± SEM) content in adult female FHM muscle (day 7), and eggs (day 21). Each sample had five biological replicates and three technical replicates. Statistically significant differences within tissue type are indicated by $^{a, b, c}$ and * indicates across tissue type (Tukey's multiple comparison test, $p < 0.05$).

Treatment	Food (%)	Adult Muscle Tissue (%)	Eggs (%)
Solvent	$14.7 \pm 0.095^{a*}$	$4.66 \pm 0.103^{a*}$	$2.43 \pm 0.077^{a*}$
Low	$14.5 \pm 0.169^{a*}$	$2.77 \pm 0.035^{b*}$	$1.76 \pm 0.007^{b*}$
Medium	$15.1 \pm 0.291^{a*}$	$1.73 \pm 0.051^{c*}$	$2.56 \pm 0.053^{a*}$
High	$11.7 \pm 0.203^{b*}$	$1.36 \pm 0.019^{c*}$	$2.09 \pm 0.017^{ab*}$

Table 2.2 Measured (mean ± SEM) concentrations (mg/kg) of hexabromocyclododecane in food, FHM muscle (maternal and paternal), and egg samples. A subsample (1.0 g) of food used to feed adult FHMs was taken for each treatment condition at the start of the exposure and analyzed along with the remaining samples. Adult muscle samples were collected on day 49. The egg samples were collected between day 25 and day 46; each sample was a composite, collected from five replicates within a treatment group at a specific time point. Lipid corrected muscle and egg concentrations were calculated from total lipid analysis subsamples taken on day 7 (maternal only) and day 21, respectively. Level of detection for each sample was calculated from the slope of the calibration curve and its standard deviation.

Matrix	Treatment	Concentration (ww; mg/kg)	Concentration (lc; mg/kg)
Food	*Solvent*	0.0599	0.409
	Low	11.5	79.2
	Medium	36.4	242
	High	106	909
Muscle – Maternal	*Solvent*	<LOD	<LOD
	Low	0.349 ± 0.159	12.6 ± 5.73
	Medium	1.39 ± 0.763	80.1 ± 43.9
	High	1.40 ± 0.371	103 ± 31.5
Muscle – Paternal	*Solvent*	<LOD	<LOD
	Low	0.104	3.75
	Medium	1.14 ± 0.507	65.7 ± 29.2
	High	1.34	98.9
Eggs – Day 28	*Solvent*	<LOD	<LOD
	Low	0.488	27.7
	Medium	2.39	93.1
	High	6.37	305
Eggs – Day 35	*Solvent*	<LOD	<LOD
	Low	0.626	35.5
	Medium	1.60	62.4
	High	3.42	164

Eggs – Day 42	*Solvent*	<LOD	<LOD
	Low	0.614	34.8
	Medium	1.33	51.9
	High	7.89	378
Eggs – Mean	*Solvent*	<LOD	<LOD
	Low	0.576 ± 0.0441	32.7 ± 2.51
	Medium	1.77 ± 0.316	102 ± 12.4
	High	5.89 ± 1.31	282 ± 24.3

LOD = level of detection, ww = wet weight, lc = lipid corrected, n (food) = 1 replicate, n (muscle) = 1-5 replicates (maternal = 5, paternal = 1-2), n (eggs) = 1 pooled sample/ treatment

Table 2.3 Calculated (mean ± SEM) Average Daily Dose (ADD), BMF in female adult muscle tissue (Biomagnification Factors), and EMR (egg-to-muscle ratio), wet weight and lipid corrected (lc), of dietary exposed HBCD in adult FHM muscle and maternally transferred egg samples. Adult muscle samples were collected on day 49 and eggs were collected between day 25 and day 46; each sample was a composite, collected from five replicates within a treatment group at a specific time point.

	ADD (mg/kg-day)	***BMF***	***EMR***
Solvent	<LOD	<LOD	<LOD
Low	0.575	0.030 ± 0.014 (ww) 1.10 ± 4.67 (lc)	1.65 (ww) 2.59 (lc)
Medium	1.82	0.038 ± 0.021 (ww) 2.20 ± 6.94 (lc)	1.27 (ww) 1.27 (lc)
High	5.3	0.013 ± 0.003 (ww) 0.974 ± 1.24 (lc)	4.20 (ww) 2.73 (lc)

LOD = level of detection, ADD = average daily dose, BMF = biomagnification factor, ww = wet weight, lc = lipid corrected, n (muscle) = 5 replicates, n (eggs) = 1 pooled sample/ treatment

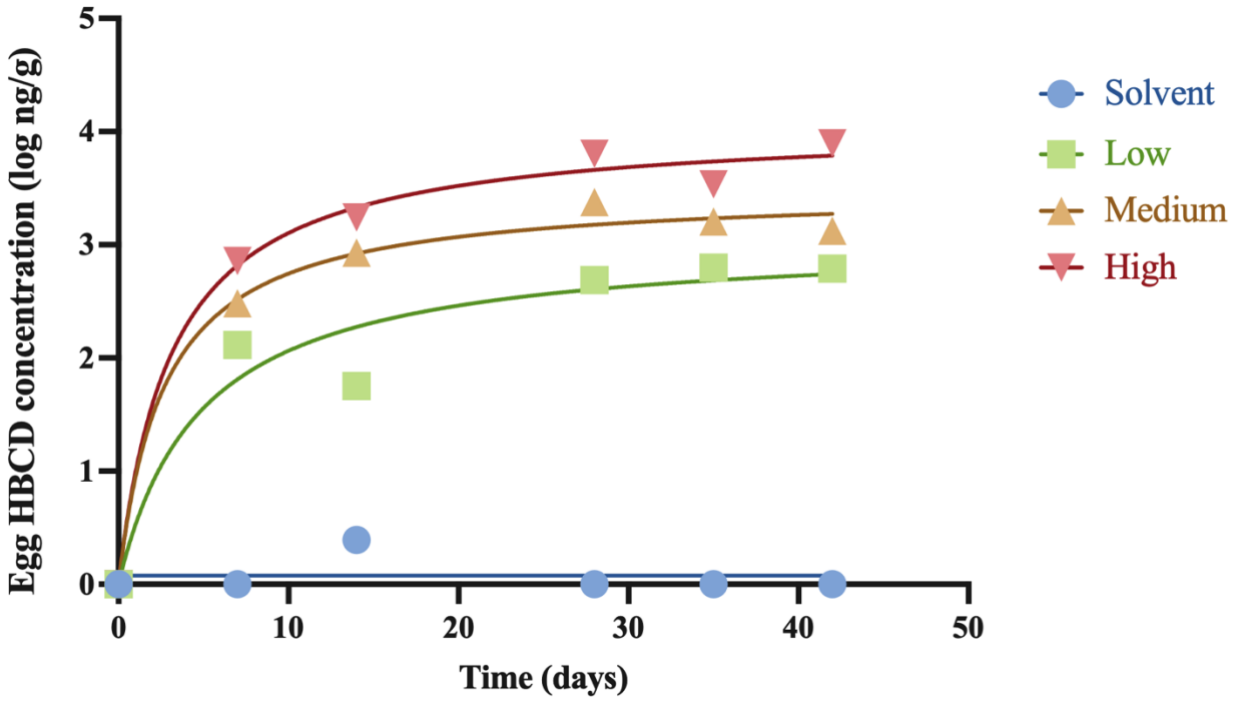

Figure 2.1 Concentrations of HBCD (log ng/g ww) in eggs maternally exposed during the 49 days of exposure. Concentrations below LOD were plotted as 0.

2.4.2 Adult apical endpoints

There was no statistically significant effect on morphometric parameters in adult FHM exposed to HBCD (Figure 2). However, male mortality was high in the control and low treatment conditions; this may have impacted fecundity as now a single male was the sole breeder for the tank. A significant increase in cumulative egg production was observed in the medium treatment group ($H = 15.32$, $p = 0.0016$) when compared to the solvent control (Figure 3).

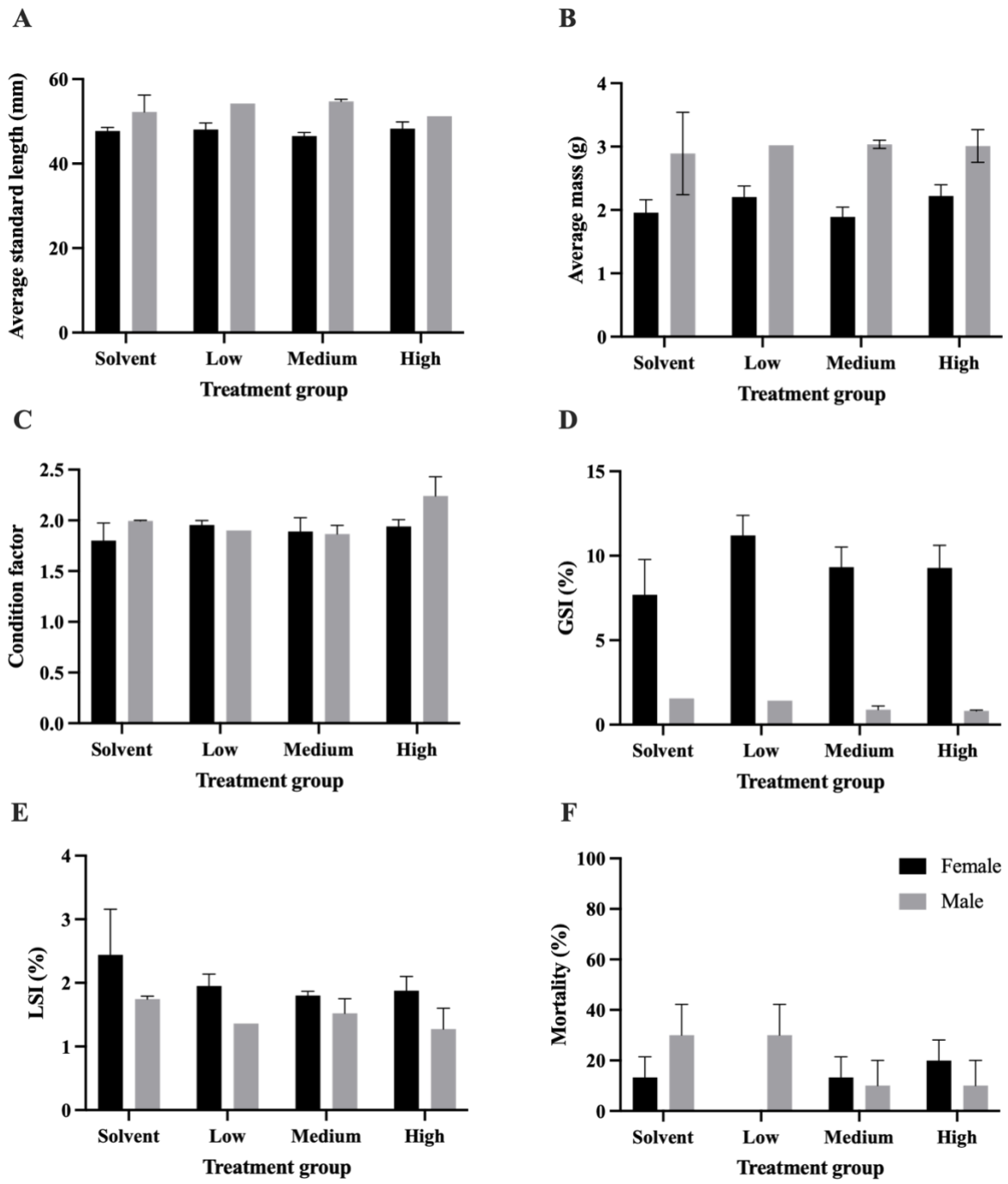

Figure 2.2 Somatic parameters measured in adult FHMs exposed to HBCD and EtOH control, including A) average standard length, B) average mass, C) condition factor, D) GSI, E) LSI, and F) percent mortality. Error bars represent the standard error of the means, no statistically significant differences ($p = 0.05$) were observed.

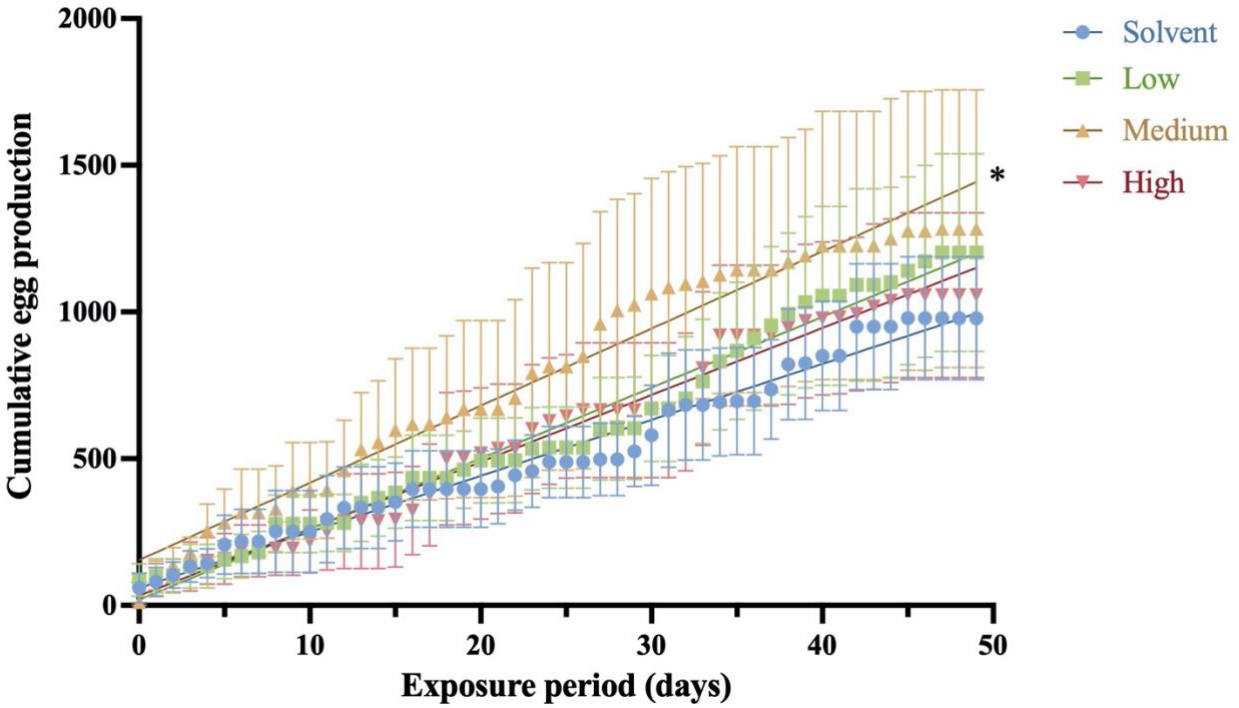

Figure 2.3 Cumulative egg production in adult FHMs exposed to HBCD treatment groups and EtOH control. (*) indicates a statistically significant difference from the solvent control ($p < 0.05$).

2.4.3 Histopathology

There was no apparent effect of the high treatment group on the appearance of the thyroid tissue in either males or females. The number, size, and shape of follicles were consistent between treatment conditions; follicles ranged from small and ovoid to large and irregular (S2.2). Thyroid epithelial cells were squamous to low cuboidal with no evidence of hypertrophy or hyperplasia. The colloid showed no indication of depletion, vacuolation at the surface, or heterogeneity and was uniform between treatment conditions.

2.5 Discussion

2.5.1 HBCD and apical responses in adult FHMs

Dietary exposure to HBCD had no impact on the health of adult FHMs, as was evident in the lack of statistically significant effects observed in apical endpoints and thyroid

histopathology. Although BMFs in adult tissues displayed no significant difference across treatment groups, there was greater accumulation in the lipid corrected adult tissues (BMFs > 1 indicate greater accumulation in the organism). However, cumulative egg production was significantly increased in the medium treatment. Previous studies in juvenile rainbow trout exposed to low concentrations of HBCD (5 – 29.14 ng/g) reported similar findings, where no significant effects in weight, length, condition factor, and survival were observed (Halldorson et al. 2008; Palace et al. 2010). Studies observing fecundity in nematodes, daphnids, rats, and birds described a decrease in viable eggs or young (Chen et al., 2019; CMABFRIP, 1998; Ema et al., 2008; Fernie et al., 2011), contradictory to the findings of the current study. The increase in production observed in our study is difficult to rationalize. However, based on previous literature, two hypotheses have been brought forth: change in the thyroid axis and hormesis.

Changes in thyroid hormone levels may be observed as an increase in weight and cell activity in the liver and thyroid (Van der Ven et al., 2006); however, no significant changes were observed in the LSI or adult thyroid tissue histopathology in the current study. Exposure to HBCD has been reported to decrease circulating T4 and increase T3 and TSH levels in RBT (Halldorson et al., 2008; Hamers et al., 2006), which has been observed to induce egg production (Richter et al., 2007). However, as no thyroid hormone responses were measured during the current study it remains speculative whether the observed increase in fecundity was due to disruption of thyroid hormone homeostasis. Although analysis of thyroid hormones was not within the scope of the present study, further investigation into circulating hormone levels would be necessary to clarify the potential effects of HBCD on the thyroid axis in adult FHMs.

The second hypothesis for the increase in cumulative egg counts is HBCD-induced hormesis. Hormesis is a biphasic or triphasic response characterized by a stimulatory or beneficial effect at low concentrations and an inhibitory or toxic effect at high concentrations (Mattson, 2008). Studies with HBCD observing apical, biological, and molecular endpoints have described such responses (Bertucci et al., 2020; Wang et al., 2016; Wu et al., 2013). Wu et al. (2013) observed higher hatching rates with water-borne exposure to HBCD and noted a significant increase in hatching rate at the low concentration (2 nM) compared to the control. The mechanism behind this hormetic effect is still unclear and requires further exploration; however, the observed changes in fecundity imply stimulation of the reproductive capacity of the

female at low concentrations, which suggests HBCD may have a sex-specific effect in adult fathead minnows.

2.5.2 HBCD and accumulation kinetics in adult tissue and eggs

Maternal transfer of nutrients in oviparous organisms plays a vital role in embryonic development; however, it can also be a significant depuration mechanism in adults and a route of toxicant exposure to subsequent generations, as demonstrated in the current study. It has been well characterized that oviparous fish are more sensitive to chemical exposure during ELS than adulthood (Russell et al., 1999). In the current study, HBCD concentrations in adult muscle and egg tissues displayed similar accumulation patterns up to the medium treatment. The adults exposed to the high treatment had muscle tissue concentrations comparable to the medium treatment group, while HBCD levels in the embryos increased up to 4 times greater (ww based) than in adults. The accumulation kinetics of HBCD across different tissues have displayed higher levels in the liver, eggs, and viscera when compared to muscle tissue in freshwater fishes (Haukås et al., 2009; Xian et al., 2008). This may be due to preferential distribution to highly perfused, central compartments (i.e., heart, brain, kidney), followed by distribution to less perfused, peripheral compartments (i.e., adipose, muscle, skin), as is observed with other orally absorbed compounds (Ahmed 2015; Stadnicka et al., 2012; Szabo et al., 2010; Xian et al., 2008). Nevertheless, differences in perfusion rate do not explain the difference in accumulation pattern observed in the treatment groups between the two life stages; therefore, further exploration is necessary, as accumulation patterns were almost identical for the two lower doses and differed considerably for the high treatment group.

The traditional model used to describe bioaccumulation kinetics with dietary exposure assumes continuous or constant food uptake, which would indicate a steady-state, whole-body concentration. However, uptake kinetics of hydrophobic organic compounds (i.e., PAHs) seem to follow a discrete process, where the frequency of encountering a food source and average food intake varies, resulting in peaks and dips in whole-body concentrations over time (hours and days; Wang et al., 2019). As more information is generated on the bioaccumulation of hydrophobic compounds, the classic toxicokinetic model shifts to account for factors that may alter the assumed accumulation pattern. These factors may include lipid diffusion, lipid content, and metabolic capacity.

The first factor follows the hypothesis that the absorption and elimination of hydrophobic compounds follows a lipid diffusion gradient within intestinal tissues (Geusau et al., 1999; Schlummer et al., 1998). As such, it may be that the balance between absorption and elimination shifts in favour of elimination at maximal dietary concentrations. However, the maximal concentration may be altered by pre-exposure to the contaminant, which may result in a decrease in the uptake rate (Doi et al., 2000). Although there was no pre-exposure period in the current study, the notion of preconditioning may be applied to chronic exposures, such that an extended duration of exposure to a toxicant may result in the saturation of tissues (Hong et al., 2017), thereby reducing the uptake rate, similar to pre-exposure outcomes. In the current study, fish exposed for 49 days to 106 mg HBCD /kg of food may have saturated tissue concentrations prior to/ by the final sampling date, resulting in a decrease in final tissue concentrations as the diffusion gradient may have shifted to favour elimination. This suggests lipid diffusion may play an influential role in modulating the dietary uptake of hydrophobic compounds and potentially alter uptake/elimination dynamics. It would be interesting to observe the accumulation patterns between pre-exposure conditions in an acute study versus a chronic study (with no pre-exposure conditioning) to identify if similar kinetics would be observed.

The second factor, lipid content, has been shown to positively correlate with the accumulation of hydrophobic compounds (Price, 2017). It has been observed that the higher the initial percentage of lipid in an organism, the greater the initial uptake and overall accumulation of hydrophobic compounds (Price 2017). Although, multiple factors may impact this correlation, including individual lipid compartments reaching a steady-state and the primary source of lipid utilized in the synthesis of vitellogenin, a yolk precursor protein (Mann & Mill, 1979; Wootton, 1979). Tissue lipid content fluctuating as a result of the compound not obtaining a steady state may result in varying accumulation patterns and reduce any potential treatment effects related to lipid content. As such, a total lipid content analysis was conducted in food, adult muscle tissue (day 7), and eggs (day 21) to observe any variation in lipid content (Table 2.1); the results displayed significant differences in the total lipid content as a function of tissue type and treatment group. The total lipid content in adult muscle tissue was negatively correlated with HBCD concentration in the adult tissue, where initial lipid content decreased significantly as concentration increased. A decrease in total lipid content with increasing HBCD concentration may indicate alterations to storage lipid mobilization, as has been observed in

oysters exposed to PCB, resulting in reduced whole body triacylglyceride levels (Capuzzo et al. 1989). This shift in storage lipid mobilization could increase HBCD concentration and may impact downstream pathways such as membrane-mediated solute transport, cell signaling, bioenergetics, and overall membrane integrity and function of the organism (Capuzzo & Leavitt 1988). The decrease in lipid content observed in the adult tissue may have been due to lipid mobilization, however as there was no consequential increase in HBCD concentration (in the high treatment group) it may be that the organism was able to eliminate HBCD at a substantial rate. The second factor potentially impacting the correlation of lipid content and accumulation of hydrophobic compounds is the type of lipid utilized for vitellogenesis. Iteroparous fish (multiple reproductive cycles) utilize the diet as the primary source of lipids for vitellogenesis (Mann & Mills, 1979; Wootton, 1979); therefore, lipid content in the organisms may have a less significant role in the accumulation of hydrophobic compounds. This was observed in goldfish exposed to compounds with log Kow values greater than 4.5 and less than 6.3, where there was no significant impact on the accumulation of compounds as a function of lipid content (Gobas et al., 1993). Interestingly, lipid content in the food displayed similar total lipid percentages, with the exception of the high treatment condition. Dietary lipid content was approximately 20% lower in the high treatment condition compared to the other treatments It is unclear what may have caused the difference in total lipid content in the food; it may be that the chironomids used in each diet had varying lipid content due to potential batch differences on the side of the supplier as very large quantities of chironomids were required; however, further validation is required to confirm the hypothesis. Differences in lipid content in the food may impact palatability and food consumption, these factors may be indicated by condition factor. As there was no evident food aversion or change in condition factor across treatment conditions, it was concluded food lipid content did not impact both the factors. Although food lipid content did not impact condition factor, it may have significantly contributed to the lower HBCD concentration in the high treatment adult tissue samples. The synthesis of vitellogenin in FHMs may be primarily derived from dietary lipids, a decrease in the lipid content in the high treatment condition may equate to the decrease in HBCD accumulation observed in the adult muscle tissue. Therefore, further research in the area of dietary lipid content and the relationship with HBCD accumulation in tissues should be considered a priority.

The third factor to consider is metabolic capacity, as biotransformation enzyme activity has been observed to increase with age (Couillard et al., 2004), comparison between life stages may not indicate the effects of a toxicant, rather the effects of changes in life stage. However, changes in biotransformation activity may impact the accumulation potential of hydrophobic compounds in adult organisms (Kropf et al., 2016). Commercial HBCD mixtures are composed of three diastereomers (alpha; α, beta; β, and gamma;γ); the γ-HBCD form is most prominent in commercial mixtures and abiotic substrates, while α-HBCD form predominates in biota (Covaci et al., 2006). However, the tissue concentrations in adults (and eggs) displayed a higher γ-HBCD proportion (67 – 74%) in comparison to the other two diastereomers in all treatment conditions (S3). Previous studies observing the kinetics and biotransformation of HBCD observed γ-HBCD is rapidly bioisomerized and cleared (Fournier et al., 2011; Erratico et al., 2022). This may explain the reduced accumulation observed in the high treatment condition. As the current study did not measure metabolic capacity this hypothesis cannot be validated; however, it may be beneficial to analyze differences in enzyme activity with exposure to HBCD to clarify if it impacts accumulation potential.

2.5.3 HBCD and maternal transfer

Based on the tissue concentrations, the calculated EMRs displayed significant maternal transfer in all treatment groups. Interestingly, the EMRs for the low and medium treatment groups corroborate the previously described ratio of 1.0 with a variation factor of 2 (Russell et al., 1999; Nyholm et al., 2008). However, the high dose (EMR of 4.20) deviated from the ratio, likely a result of the overall lower concentrations of HBCD in the adult muscle tissue, in addition to egg concentrations following similar trends as in the diet. The difference in accumulation observed in the highest treatment appears to be a result of reaching tissue diffusion limits, tissue lipid saturation, differences in lipid content in the food, and potential preferential bioisomerization/ elimination as discussed above; therefore, the difference in EMR observed in the highest treatment cannot be exclusively a result of maternal transfer kinetics, but rather a combination of the accumulation kinetics in the adult tissue and the subsequent depuration/ transfer to the embryos.

The factors described above illuminate the complexity of HBCD kinetics in adult fish, and the substantial transfer observed to the eggs suggests a significant cause for concern in the

ELS of oviparous fish. The study also verifies the considerable variation in tissue HBCD concentrations reported in aquatic wildlife. For example, Canadian environmental concentrations reported in teleosts ranged between <0.030 and 92 ng/g (lipid wt.) (Law et al., 2006; Tomy et al., 2004), whereas global concentrations (including Norway, Sweden, South Africa) ranged from <0.00020 – 8.0 mg/kg (lipid wt.) with potential for trophic magnification (i.e., lake trout > alewife, sculpin, smelt), as was observed in the pelagic food web in Lake Ontario (Environment Canada & Health Canada, 2011; Tomy et al. 2004). Although the concentrations reported in the current study well exceed environmental concentrations observed in Canadian ecosystems, they are within observed global concentrations.

2.5.4 Conclusion and future perspectives

Dietary exposure to HBCD displayed no significant morphological or histological apical effects in adult FHMs; however, cumulative egg production was significantly increased in the medium treatment group. HBCD accumulation kinetics displayed similar patterns of uptake in adult tissue up to the medium treatment condition, potentially due to reaching tissue diffusion and/ or saturation limits, variations in food lipid content, and preferential biotransformation and elimination. Maternal transfer kinetics displayed a one-to-one transfer, as is expected with hydrophobic compounds. Overall, further research into thyroid hormone levels, biotransformation capacity, and toxicokinetics in adult fish are needed to fully divulge the observed difference in egg production, tissue accumulation, and maternal transfer of HBCD in oviparous organisms. In particular, the high transfer of HBCD to offspring may be cause for concern due to the potential effects in the often more sensitive ELS.

CHAPTER 3: EMBRYO/ LARVAL TOXICITY AND KEY GENE EXPRESSION SIGNATURES OF MATERNALLY TRANSFERRED HEXABROMOCYCLODODECANE (HBCD) IN FATHEAD MIINNOWS (*PIMEPHALES PROMELAS*)

Preface

This chapter aimed to study the effects of maternally transferred HBCD in the F1 generation. Fathead minnow (FHM) larvae maternally exposed to HBCD were reared in clean facility water for seven- and 14-days post fertilization for transcriptomics and apical and physiological analysis, respectively. Transcriptomic responses were assessed to identify key molecular signatures that may be predictive of apical outcomes. Apical and physiological responses were quantified by morphometrics, histopathology, and oxidative stress test (TBARS assay).

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3.1 Abstract

Hexabromocyclododecane (HBCD) is a brominated flame retardant that presents a threat to aquatic organisms as it can be maternally transferred and has been reported to hinder development and survival in early-life stage (ELS) fish. However, little is currently known about the molecular mechanisms that drive the toxicity of HBCD. This study examined the apical and molecular response patterns following exposure to maternally transferred HBCD in fathead minnow (*Pimephales promelas*) larvae at seven (whole transcriptome) and 14 (apical and physiological) days post-fertilization (dpf). Transcriptomics analysis revealed dysregulation of pathways involved in membrane integrity (inhibition of calcium channel) and metabolic processes (downregulation of amino acid, glucose, and lipid biosynthesis), while the larvae reared for 14 days exhibited a significant decrease in survival in the highest (100 mg/kg) treatment condition. These results indicate that maternal transfer of HBCD is of concern in fish and exposed progeny may lead to the inhibition of membrane transport and disruption in metabolic processes, collectively resulting in energy depletion and subsequent mortality.

3.2 Introduction

Persistent organic pollutants (POPs) are a class of non-ionizable and mainly non-polar compounds that are hydrophobic and often recalcitrant to metabolism. POPs present a unique concern as they can remain in the environment for long periods after their release and they have the potential to bioaccumulate in aquatic organisms (Rimayi et al., 2022; Yin et al., 2017). Many halogenated aromatic compounds are classified as POPs, including hexabromocyclododecane (HBCD), which has been detected globally in water (0.003 – 15 800 ng/L; Law et al., 2006; Dames & Moore, 2000b), sediment (0.012 – 2430 ng/g dry weight [dw]; Marvin et al., 2006; Guerra et al., 2009), soil (140 – 89 600 ng/g dw; Remberger et al., 2004; Dames & Moore, 2000a), and organisms (0.46 – 8000 ng/g lipid weight [lw] in various fish species; Johnson-Restrepo et al., 2008; Environment Canada & Health Canada, 2011). HBCD is an additive-type, brominated flame retardant (BFR), formerly used in polystyrene foams for insulation materials, as well as in textiles, electrical, and electronic equipment. Its relatively high log K_{ow} (5.6) and additive-type incorporation into products has led to the inadvertent leaching of the parent compound into the environment during its manufacture and disposal (EPA, 2014). In addition to

the propensity of HBCD to accumulate, it has been reported to impact reproduction, development, survival, and thyroid homeostasis in terrestrial and aquatic species (Darnerud, 2003); as a result HBCD was categorized as a POP with restrictions placed on production, use, and export (Environment Canada & Health Canada, 2011). Although HBCD concentrations are trending down or leveling off in North America and Europe, concentrations in Asia remain significantly high and are continually monitored (Wang et al., 2018).

HBCD can bioaccumulate over time to yield high internal concentrations in organisms (Eljarrat & Barceló, 2018); this can become detrimental in oviparous vertebrates such as fishes since highly lipophilic compounds are maternally transferred to the embryo (Miller, 1993; Nyholm et al., 2008). During gamete maturation, nutrients, hormones, and other components (i.e., immune factors such as lectins and lysozymes) are deposited into the egg by the mother; however, chemical contaminants can also undergo parallel transfer. During early development, these contaminants can then be mobilized from the yolk, and adversely affect the normal development and survival of the embryos during this sensitive life stage (Russell et al., 1999). Studies have demonstrated that HBCD exposure can result in abnormal embryonic/ larval development and decreased survival (Deng et al., 2009; Wu et al., 2013), however, as these studies were waterborne exposures, a level of uncertainty remains and due to the low water solubility of HBCD. Due to the scarcity of studies and information on accumulation kinetics of HBCD, research observing alternate exposure routes is necessary to understand HBCDs toxic potential in aquatic oviparous organisms.

As mentioned above, early life stage (ELS) exposure to HBCD can result in adverse effects on development and survival; these apical endpoints have been hypothesized to be downstream effects of induction of oxidative stress and apoptosis in ELS fish (Deng et al., 2009; Wu et al., 2013; Yamashita, 2003). Exposure to concentrations of 0.05 mg HBCD/L (dimethyl sulfoxide; DMSO carrier solvent) was reported to increase the expression of nonenzymatic and enzymatic antioxidants and reactive oxygen species (ROS) (Deng et al., 2009). The molecular mechanism of action of oxidative stress following exposure to HBCD was hypothesized to be associated with the uncoupling of the membrane-bound Cytochrome P450 (CYP450) enzyme, potentially as a result of disruption of membrane integrity (Mariussen & Fonnum, 2003; Hu et al. 2009), although this has not been well characterized to date. HBCD-induced oxidative stress can trigger apoptosis, which has been observed previously during early development in zebrafish (Su

et al., 2019). A study by Deng et al. (2009) supported these findings; exposure to high HBCD concentrations (1.0 mg HBCD/L, DMSO carrier solvent) in zebrafish resulted in upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes. Although studies have reported effects of HBCD on select molecular endpoints in ELS fish, the sequence of molecular and biological events resulting in adverse outcomes is still unclear. Understanding the cellular response pathway of HBCD that instigate and/or cause adverse biological outcomes in ELS fish would enable better interpretation of the biological implications and toxicity, relevant to risk assessment, not only for HBCD but also for other compounds with a similar mechanism of action (MoA).

The use of next generation sequencing platforms (i.e., transcriptomics) presents an opportunity to derive non-target molecular data that may be used to identify and characterize disruptions in specific biological pathways of interest (Kleinstreuer et al., 2016). These data may be used to inform and develop specific molecular toxicity pathways for chemicals acting through a certain molecular initiating event (MIE), which is defined as the first interaction of a chemical with a biological molecule in an organism. Because our knowledge of the toxicity of HBCD in ELS fish is limited, derivation of its molecular toxicity pathways may not only provide evidence in support of the hypothesized MoA and potential subsequent adverse outcomes but also shed light on areas requiring further research. As such, the objectives of this study were to 1) describe the apical and physiological effects in fathead minnow (FHM) larvae maternally exposed to HBCD, 2) conduct whole transcriptome analyses to identify key gene expression signatures in FHM larvae in response to HBCD, and 3) link apical, physiological, and histological observations with transcriptomics data to develop/ construct a toxicity pathway model of HBCD in maternally exposed FHM larvae.

3.3. Material & Methods

3.3.1 Experimental Organisms

All animals used in the study were reared following the Canadian Council of Animal Care (CCAC) experimental fish guidelines and approved by the Animal Research Ethics Board of the University of Saskatchewan (protocol #20160090). FHM embryos were obtained from adult fish exposed to HBCD through diet (Malala Irugal Bandaralage et al, 2022). In brief, adult

FHMs (> 6 months) were fed a diet of one of four treatment conditions (solvent control [hereafter referred to as control] = 100% ethanol – evaporated, low = 11.5, medium = 36.4, high = 106 mg/ kg wet weight [ww]) following OECD guideline 229 (2012) with slight modifications (extended for 49 days to allow the compound to be at or near steady state). Diets were formulated by dissolving HBCD in 100% ethanol (#P016EAAN, Commercial Alcohols) which were mixed with freeze-dried chironomids. The solvent was then slowly evaporated in a fume hood at room temperature (24-48 hrs) and stored in the dark until feeding. The low treatment condition was representative of global concentrations in teleost tissues (8 mg/kg lw in Norway, Sweden, and South Africa; Environment Canada & Health Canada, 2011), and the two other concentrations were selected to elicit molecular and physiological effects. Fish were fed a diet of HBCD-spiked food (freeze-dried chironomids) at 5% body weight (bw)/day, *ad libitum*. Fertilized eggs were obtained between day 25 and 42 days of exposure and F1 replicates were matched to the adult replicates.

3.3.2 ELS Exposure & Sampling

The F1 generation was raised until 14 days post fertilization (dpf) following OECD guideline 210 (2012) with minor modifications. In brief, fertilized embryos (20 embryos/replicate for transcriptomics; 30-35 embryos/replicate for apical and physiological endpoints) at the high blastula stage were transferred to glass Petri dishes and reared under static renewal conditions (50% water renewal per day). Treatment groups consisted of five replicates per endpoint, collected over three days, to ensure sufficient numbers and high-quality egg collection. At 7 dpf, larvae were transferred to hatching cups (PVC pipes lined with mesh windows and base) in 10L tanks and maintained in a flow-through system (four tank renewals/ day) of clean facility water. Larvae were fed live *A. salina* nauplii three times per day (*ad libitum*). All organisms were maintained at a temperature of 25 $\pm$ 2 ^{o}C and a photoperiod of 16 hrs light, 8 hrs dark. Water quality was tested daily (one replicate/ day) by measuring temperature, dissolved oxygen, pH, ammonia, nitrite, nitrate, hardness, and alkalinity (S3.1).

Larvae sampled at 7 dpf (transcriptomic analysis) and 14 dpf (morphometric measurements, histology, and biochemical analyses [oxidative stress test – MDA]) were not fed 24 hours prior to sampling. Five individual larvae per tank were subsampled randomly for

histology analysis and the remainder of the apical samples were flash-frozen in liquid nitrogen and stored at -80ºC for oxidative stress test (TBARS assay).

3.3.3 Quantification of HBCD in Diet and Tissue

HBCD concentrations in eggs were analyzed by SGS-AXYS Analytical Services Ltd. as described previously (Malala Irugal Bandaralage et al, 2022). In brief, the average concentrations in the maternally exposed eggs were measured in control, low, medium, and high treatment conditions in composite samples from 28, 35, and 42 days (n = 1 pooled sample per time point) of exposure (S3.3). Measured concentrations in the control, low, medium, and high treatment groups were <LOD, 0.576 $\pm$ 0.0441, 1.77 $\pm$ 0.316, and 5.89 $\pm$ 1.89 mg/kg (ww), respectively (S3.2).

3.3.4 Apical & Physiological endpoints

Larvae sampled at 14 dpf were euthanized using an overdose of buffered MS-222 (ethyl13-aminobenzoate methanesulfonate; 700mg/L). Larvae were assessed for apical endpoints at 14 dpf; measurements included length, weight, frequency (presence/ absence) of skeletal deformities (Lemly, 1997), and mortality. The length was assessed by measuring the total length (nose to the tip of the tail) of each larva to the nearest 0.01 mm. Deformities observed included kyphosis, lordosis, and scoliosis. Condition factor (Eq 1) and percent deformity (Eq 2) were calculated for individual fish and the mean for each replicate was compared to that of the controls (n = 5).

$$Condition\ Factor\ (K) = \frac{100 * fish\ weight\ (g)}{standard\ length\ (cm)^3} \qquad (1)$$

$$Deformity\ (\%) = \frac{\#\ of\ larvae\ deformed}{total\ \#\ of\ fish} \times 100 \qquad (2)$$

Histology was conducted on whole-body samples, which were processed and embedded following the methodology described by Alcaraz et al. (2021). In brief, five larvae per tank were placed in individually labeled histocasettes, fixed in Cal-ExTM II Fixative/ Decalcifier (Fisher ChemicalTM, Canada) for 48 hours, and then decanted twice with 70% EtOH before storage in

70% EtOH. Control and high treatment groups (n =10 per treatment group; 2 fish per replicate) were used for Tier I histological assessment to screen if any effects could be identified. Whole-body ELS samples were dehydrated in graded alcohols, cleared in xylene, and infiltrated with molten paraffin using a Belair RVG/1 Vacuum Tissue Processor. Individual samples were embedded in paraffin blocks for serial sectioning (sectioned through the entire body cavity), mounted on glass slides, and stained with Harris' hematoxylin and eosin. Thyroid and liver histology for embryos was attempted; however, the samples were too small, and the life stage was too early for meaningful interpretation of the thyroid tissue (S3.4).

The oxidative stress biomarker, Malondialdehyde (MDA), was measured in the 14 dpf tissues using a TBARS (thiobarbituric acid reactive substances) Assay kit (KGE013, R&D Systems Inc, MN, USA), following the manufacturer's protocol. In brief, three whole-body samples were pooled per replicate group for control, low, and medium dose groups (n = 4; the high treatment group was not included due to significant mortality) and homogenized in 300-500 µL (dependent on the weight of tissue) of Tris-Triton solution. Initial optical density was measured at 532 nm at time 0 and was read every 30 minutes for 3 hours on a Spark® Multimode Microplate Reader (TECAN, Switzerland). The final concentration of TBARS content was normalized with a blank and evaluated against the control samples.

3.3.5 Transcriptomics & enrichment analyses

Transcriptomics samples were flash-frozen in liquid nitrogen and stored at -80^{o}C until analysis. Transcriptomic analyses were done on pools of larvae (n = 20) from each replicate tank (n = 5 per treatment group) from the control, low, and medium treatment conditions. Total RNA was extracted from pools of whole larvae using the Qiagen RNeasy Plus Universal Mini Kit (Qiagen, Germany) in a QIAcube instrument (Qiagen), following the manufacturer's protocol. Total RNA quantification and integrity were assessed using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and a 2100 Bioanalyzer (Agilent Technologies), respectively. Samples with a RIN $\geq$ 8 were sent to McGill Applied Genomics Innovation Core (MAGIC) at McGill University (formerly Genome Quebec Innovation Centre at McGill University; QC, Canada) for library preparation and sequencing. Libraries were prepared using NEBNext Ultra Directional kit with poly(A) magnetic isolation module (New England BioLabs, MA, USA). Average fragment size of double-stranded DNA libraries was assessed on a

LabChip GX (PerkinElmer, MA, USA) instrument. High quality double-stranded DNA libraries were sequenced using a 200-cycles, paired-end (2 ×100) sequencing cartridge in a NovaSeq S4 (Illumina, CA, USA).

Raw fastq files (available through NCBI GEO Acc # GSE212295) were assessed for quality, trimmed to a Phred score ≥30 and a minimum length of 35 bases using Trim Galore!, then pseudo-aligned to the FHM reference transcriptome (NCBI Acc# GCA_016745375.1; Martinson et al., 2021) using Kallisto (Bray et al., 2016) in the EcoToxXplorer Galaxy server (https://galaxy.ecotoxxplorer.ca/). Transcript counts were then filtered to remove features with less than 20 counts per million in at least five samples. Differential expression analysis was done using the DESeq2 R package (Love et al., 2014) and genes were considered significantly differentially expressed at a false discovery rate (FDR; Benjamini-Hochberg) ≤ 0.05 relative to the control treatment group.

Enrichment analyses were conducted in g:Profiler (Raudvere et al., 2019) using databases for *Danio rerio* (map based on similar FHM gene symbols; Gene Ontology and KEGG). Significantly dysregulated genes with FDR ≤ 0.05 were assessed for enriched, with statistical domain based on all known and unknown genes, and with a g:SCS significance cut-off threshold of p-value < 0.05.

3.3.6 Statistics

Data on morphological (mortality, deformity, and condition factor) and biochemical outcomes (oxidative stress) were tested for normality of residuals and homogeneity of variance using Shapiro-Wilk test and Bartlett's test, respectively. Normally distributed data were analyzed using a one-way ANOVA, and if significant differences or trends were observed, Tukey's multiple comparison test was used to determine any dose-dependent effects. Data that failed the homoscedastitiy test were log-transformed and re-analyzed as mentioned above or analyzed using one-way ANOVA on ranks (Kruskal Wallis H test) followed by Dunnett's multiple comparison test to observe any significant dose-dependent effects. Data were considered significantly different if p-value < 0.05.

3.4 Results & Discussion

3.4.1 Apical & biochemical responses

FHM larvae, maternally exposed to HBCD, did not display any significant effects on fish condition (quantified by length and weight) or percent deformity (S3.5 A, B); however, mortality significantly increased in the high treatment group (Figure 3.1) relative to the control. Similarly, previous high level (0.05 – 1.0 mg/L, with DMSO carrier solvent) aqueous HBCD exposures in fishes resulted in a significant increase in mortality (Deng et al., 2009; Du et al., 2012). Increased malformation rate has also been observed following HBCD exposure in fish (Hong et al., 2014), counter to the findings in the current study. The observed lack of deformities may be due to differences in exposure route and species; it might be possible that maternally transferred HBCD binds to the yolk due to the high lipophilicity of the compound and is slowly released during yolk-sac absorption (approximately 13 dpf). This in turn may have allowed FHMs to eliminate the toxicant more efficiently than zebrafish and medaka in which deformities were observed. Additionally, there may have been survivorship bias, where high mortality in the high treatment group could have incidentally removed deformed individuals from the experiment prior to the apical takedown.

The previously proposed mechanism of action for the developmental toxicity following HBCD exposure was the production of reactive oxygen species (ROS) and subsequent apoptosis (Du et al., 2012). The presence of ROS is indicative of oxidative stress in an organism and can be quantified by measuring ROS or ROS intermediates (i.e., MDA, TBARS) or antioxidant enzymes (i.e., superoxide dismutase, catalase, glutathione-dependent peroxidase, and glutathione reductase). No significant change was observed in TBARS (S3.6) content across treatment conditions, but an increasing trend was observed in the low treatment. The observed trend in the low treatment may have been due to HBCD exposure or the assay's potential to overestimate MDA content by measuring non-MDA substances (Valenzuela, 1991); therefore, measuring antioxidant enzymes' activity (i.e., SOD, CAT, GSH) may be an alternative method to verify if the observed increase was associated with HBCD exposure or a systemic error. To assess if HBCD induced apoptosis, histopathology was conducted on the control and high treatment livers (S3.7). The liver parenchyma was dense and compact in appearance, with typical-appearing spherical hepatocyte nuclei and central nucleoli for both tested groups; however, no apparent difference between treatment conditions was observed. Similar to the lack of deformity

observed, survivorship bias and limited bioavailability due to maternal transfer may have impacted oxidative stress test and histology outcomes, therefore, a level of uncertainty remains as to what the main cause of mortality may be with HBCD exposure. However, the findings from the study suggest that HBCD-induced oxidative stress may not be the main cause of mortality and deformity; instead, it may be that HBCD exposure elicits apical effects (i.e., mortality) through a diffuse network of molecular effects as discussed below.

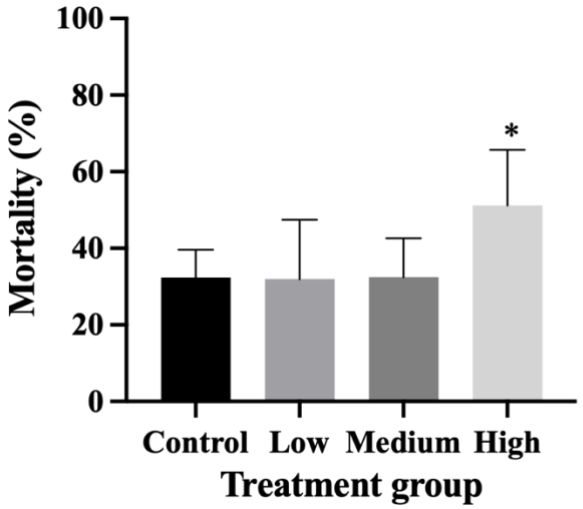

Figure 3.1 Mortality (%) in FHM larvae exposed to HBCD treatment groups and control. Error bars represent the standard error of the means, (*) indicates a statistically significant difference relative to the control ($p < 0.05$).

3.4.2 Differential gene expression and enrichment analysis

Transcriptomics analyses resulted in 56 and 76 differentially expressed genes (DEGs; FDR < 0.05) relative to controls in the low and medium treatment conditions, respectively (Figure 3.2). A total of 11 genes were commonly dysregulated between treatment groups (Table 3.1); the majority of these genes indicated perturbation to membrane function (Bradford et al., 2022), such as *cacna1fa* (voltage-dependent L-type calcium channel), *eif2ak4* (Eukaryotic translation initiation factor 2 α kinase activity), *anpepb.2* (alanyl (membrane) aminopeptidase activity), *dpyda.1* (dihydropyrimidine dehydrogenase activity), *plbd1* (phospholipase activity), and *cyp2p7* (cytochrome P450 family 2 activity). Enrichment analyses identified 28 and 6 significantly enriched GO terms/ KEGG pathways in the low and medium treatment groups, respectively. Key terms and pathways identified included oxidoreductase activity (observed in

both treatment conditions), carbohydrate and amino acid metabolism, and ion binding capacity (S3.8).

HBCD exposure in ELS fish has been linked with oxidative stress and apoptosis (Deng et al., 2009); the molecular MoA triggering these events has been hypothesized to be the disruption of membrane integrity, energy imbalance, and uncoupling of membrane-bound CYP450-mediated substrate oxidation (Wang et al., 2016). The current exposure resulted in significant induction of the *cacna1fa* gene, localized to voltage-gated calcium channel complexes in peripheral neurons and cardiac cells that may also be present in all excitable and non-excitable cells (Tsien et al., 1991). An increase in intracellular calcium has been observed in human liver cells after exposure to HBCD, which was linked with the suppression of energy metabolism by limiting amino acid and glucose uptake and synthesis (Wang et al., 2016). In the current study, we observed a similar trend on genes involved in the amino acid metabolism, with downregulation of *anpepb.2* and *eif2ak4* as well as GO term tryptophan metabolism and KEGG pathway biosynthesis of amino acids. The downregulation of intracellular amino acids and the subsequent inhibition of protein biosynthesis may lead to adverse neurological effects (Al-Mousa & Michelangeli, 2012; Dingemans et al., 2009), although behavioural or similar neurological endpoints were not assessed in the current study. In addition, this degenerative effect may be better observed at later life stages and merits further investigation. Further disruption to energetics was observed with the downregulation of pyruvate metabolism (product of glucose metabolism [i.e., glycolysis]) and the *plbd1* gene (maintains phospholipid and fatty acid homeostasis). In combination, the two responses may result in a decrease in ATP (adenosine 5'-triphosphate) production, further inhibiting amino acid and glucose transport across the membrane (Wang et al., 2016).

Similar to other BFRs, HBCD has shown the potential to uncouple CYP enzymes (Du et al., 2015); the theorized MoA is that the substrate (i.e., BFR) binds tightly to the active site of the enzyme but is not rapidly metabolized, instead, the substrate remains in the active site generating ROS, which may then be released from the active site or be retained and inactivate the enzyme (Narasimhulu, 2010). In the current exposure, *cyp2p7* was significantly dysregulated. Although the physiological and endogenous functions of *cyp2p7* in fish are poorly understood, it has been observed that CYP 1, 2, 3, and 4 families are involved in xenobiotic and fatty acid metabolism (Goldstone et al., 2010) as well as predicted to be involved with oxidoreductase activity (Uno et

al., 2012). HBCD was shown to be metabolized/ detoxified by CYP enzymes (Crump et al., 2008; Du et al., 2015; Germer et al., 2006; Zegers et al., 2005) based on induction of specific CYPs (CYP1A1), in contrast to our observed inhibition (FC -1.32 and -1.45 in the low and medium treatments, respectively). In addition, Du et al. (2015) reported HBCD as both gene- and diastereoisomer-specific when interacting with CYPs. Therefore, it may be that HBCD exposure in ELS FHMs downregulates *cyp2p7* as a result of its specificity in addition to potentially uncoupling its activity; evidence of enzyme uncoupling may be interpreted by downregulation of catalytic activity (as is observed in enrichment analysis; S3.8), however, further evidence is necessary to validate this hypothesis. Furthermore, magnitude of changes was low in some cases, and it is uncertain whether these would translate into biologically meaningful outcomes.

The induction of L-type voltage-gated Ca^{2+} channels may have contributed to disruption of membrane integrity and subsequent HBCD toxicity. The influx of mitochondrial calcium has been associated with ROS due to the stimulation of calcium-sensitive matrix dehydrogenases; these matrices are key sites of NADH (reduced form of nicotinamide-adenine dinucleotide) production for the respiratory chain and subsequently, mitochondrial ATP and ROS production (Cardenas et al., 2010). In addition to mitochondrial dysfunction, influx of calcium has been linked to cytotoxicity, oxidative stress, and apoptotic/ necrotic/ or autophagic cell death (Dingemans et al., 2009; Londoño et al., 2010; Orrenius et al., 2011). Although induction of pro-apoptotic genes (i.e., dynamins with GTPase activity) has been observed in previous HBCD exposures (Al-Mousa & Michelangeli, 2012; Rasinger et al., 2014; Wang et al., 2016), the current study displayed downregulation of genes involved in anti-apoptotic functions, *GIMAP8* (LOC100005864) at FCs of -1.49 and -1.69 in the low and medium treatment conditions, respectively. However, no significant apoptotic/ anti-apoptotic terms/ pathways were found to be enriched. Based on the findings from the current exposure, oxidative stress coupled apoptosis does not appear to be the driver of HBCD toxicity in larvae exposed maternally, but rather, both effects appear to be downstream effects of disruption of membrane integrity and energetics. However, it cannot be excluded that localized oxidative stress and associated apoptosis occurred as only whole larvae were analyzed.

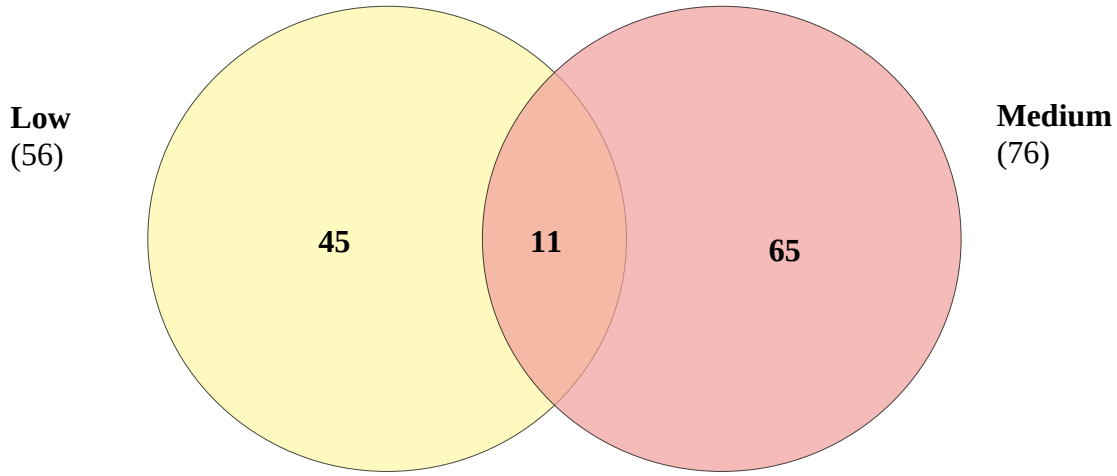

Figure 3.2 Intersection of DEGs in low (yellow) and medium (pink) treatment groups. 11 dysregulated genes were commonly shared between low (56) and medium (76) treatment groups (FDR < 0.05).

Table 3.1 Summary of commonly dysregulated genes (DEGs; FDR < 0.05) in maternally exposed FHM larvae in low (0.576 ± 0.0441 mg/ kg ww) and medium (1.77 ± 0.316 mg/kg ww) HBCD treatment groups.

Gene ID	Name	Description	Fold Change	
			Low	Medium
cyp2p7	Cytochrome P450, family 2, subfamily 2, polypeptide 7	Predicted to have heme-binding activity, oxidoreductase activity, xenobiotic metabolism. Localized to cytoplasm and intracellular membrane-bounded organelle.	-1.32	-1.45
plbd1	Phospholipase B domain containing 1	Predicted to have phospholipase activity, involved in lipid catabolic process, and expressed in yolk syncytial layer.	-1.34	-1.38

eif2ak4	Eukaryotic translation initiation factor 2 α kinase 4, general control non-repressed 2	Predicted to have ATP binding activity and (serine/ threonine-) protein kinase activity, regulating translation in response to stressors (i.e., amino acid and purine deprivation, etc.).	-1.22	-1.30
anpepb.2	Alanyl (membrane) aminopeptidase b, tandem duplicate 2	Predicted to have metalloaminopeptidase activity (peptide binding activity, zinc ion binding activity), peptide catabolic processes, and proteolysis.	-1.90	-1.72
slc25a10b (previously *slc25a10*)	Solute carrier family 25, member 10	Predicted to be involved in mitochondrial transport and localized to integral component of membrane and mitochondrial membrane.	-1.33	-1.18
pcolcea	Procollagen C-endopeptidase enhancer α	Predicted to localize to extracellular region.	1.54	1.36
dpyda.1	Dihydropyrimidine dehydrogenase α, tandem duplicate 1	Predicted to have iron-sulfur cluster binding activity.	-1.24	-1.28
chia.2	Chitinase, acidic.2	Predicted to have chitin binding activity, chitinase activity, and chitin catabolic process. Predicted to be localized to cytoplasm and extracellular region.	1.62	1.33
cacna1fa	Voltage-dependent L-type calcium channel subunit α-1D	Predicted to have high voltage-gated calcium channel activity, involved in synapse	2.49	2.45

		organization, and localized to voltage-gated calcium channel complex.		
LOC100005864	GTPase IMAP family member 8-like	Predicted to have anti-apoptotic effects in the immune system and involved in infections (i.e., T lymphocyte roles in mammals).	- 1.49	-1.69
GGNBP2	Gametogenetin-binding protein 2	Predicted to be involved in oxidoreductase activity and protein binding (phosphyrylation and kinase activity). Predicted to be localized to membrane, cytoplasmic vesicle, nucleus.	- 1.27	- 1.33

DEG = Differentially expressed genes, FDR = dales discovery rate, ATP = adenosine 5'-triphosphate, GTP = guanosine triphosphate.

[Transcript] data for this paper was retrieved from the Zebrafish Information Network (ZFIN), University of Oregon, Eugene, OR 97403-5274; URL: http://zfin.org/; [28/03/22].

3.4.3 Biological insights through transcriptomics data integration

Exposure to toxicants may not always elicit visible apical damage; instead, subtle changes to cell function are more likely, and may be a more sensitive indication of toxicity when apical effects are absent. This may in turn increase the susceptibility of the tissue/ organism to (other) stressors, potentially resulting in apical effects. In other fish species, aqueous HBCD exposures in ELS have been observed to induce oxidative stress and the consequential triggering of apoptotic genes at concentrations of 0.1 - 1.0 mg/L (Deng et al., 2009). These two mechanisms have been linked with developmental toxicity resulting in deformity and/or mortality of the organism (Chen et al., 2019; Yamashita, 2003). The current HBCD exposure in FHM larvae suggests that membrane integrity could have been affected by way of disrupting calcium homeostasis; downstream enrichment analysis revealed pathways/ terms involved in oxidative stress and imbalances in amino acid and carbohydrate metabolism. These molecular signatures have been previously reported in fish exposed to HBCD and were suggested to be indicative of apical and physiological effects, predominantly, an increase in deformity, mortality, and ROS production at concentration ranges of 0.1 – 1.0 mg/L (Deng et al., 2009; Du et al., 2012; Hong et al., 2014). In the current exposure, mortality was the one apical endpoint significantly affected by HBCD exposure (highest concentration), with no significant impacts on deformity or oxidative stress (measured as TBARS concentration), however, as mentioned above, survivorship bias may have affected the quantification of latter endpoints (deformity and oxidative stress). It is difficult to conclude if the differences in effects observed were due to alternative mechanistic pathways, other factors (i.e., exposure route, length of exposure, concentration), species differences, or a combination thereof. As FHM larvae were exposed to HBCD maternally, and then transferred to clean water post-fertilization, HBCD may have been bound to the yolk, resulting in the slow release of HBCD during the absorption of the yolk. This may have reduced the toxicant load at any given time compared to aqueous exposure to HBCD and allowed the larvae to metabolize and eliminate HBCD from the body prior to any serious injury to cells/ organs/ organism, preventing the manifestation of any deformities or physiological indications of oxidative stress. The observed mortality in the high treatment may be due to a combination of energy depletion and poor metabolic proficiency to reduce toxicant load; however, further research is necessary to elucidate the specific mechanism by which

HBCD triggers a toxic response in FHM larvae when exposed maternally (i.e., metabolomics analyses to observe any changes in energetics).

HBCD has been reported to alter hormone response pathways; however, unlike other BFRs (i.e., PBDEs) that act directly on endocrine-related pathways and hormones, HBCD acts indirectly by altering calcium ion homeostasis, which may affect neural signaling along hormone response pathways related to energy homeostasis (Tilley & Fry, 2015). In the current exposure scenario, exposed FHMs may have experienced excessive production of oxygen radicals in the lower concentrations (as was observed with expression of pathways/ terms involved in oxidative stress in the low and medium treatment conditions), and as a protective response, downregulated metabolic pathways to reduce excess radical production arising from metabolism in the higher treatment groups (Lee et al., 2020). However, this change in cell function may have caused energy depletion and increased the susceptibility of the organism to HBCD exposure, resulting in the observed increase in mortality in the high treatment condition. As such, it may be that HBCD exposure in FHMs does not trigger physiological oxidative stress responses by classical induction of pro-oxidants and decrease of antioxidants, but by modulating metabolic processes leading to energy depletion and the inability to mount a significant defensive response.

3.4.4 Final thoughts and future perspectives

FHMs maternally exposed to HBCD displayed no significant effect on deformity, condition factor, or histology; however, mortality was significantly increased in the high treatment condition. Transcriptomics analysis in the low and medium treatments displayed significant dysregulation of genes that may be indicative of perturbation of membrane functions, notably calcium homeostasis, while enrichment analysis indicated oxidative stress and disruption of energetics. The study indicate HBCD may act through indirect mechanisms involving the inhibition of membrane transport (i.e., inhibition of calcium channel activity) leading to disruption in energetics (i.e., downregulation of carbohydrate and amino acid metabolism), collectively resulting in energy depletion and subsequent mortality (in high treatment). However, as findings were assessed at the gene expression level, further confirmation utilizing functional assays would be needed to validate this hypothesis. Although a toxicity pathway could not be constructed, the data derived in collaboration with previous and future studies may be utilized to establish a complete toxicity pathway and potentially an AOP for compounds

acting through a similar MoA. Most importantly, what can be inferred from the current study is that HBCD can be maternally transferred to embryos, where it elicits a potentially different mechanism of toxicity than previously suggested by waterborne studies. In addition, maternal transfer of HBCD appears to follow a 1:1 transfer (Malala Irugal Bandaralage et al., 2022; Nyholm et al., 2008) and observed concentrations are within the range of global concentrations observed in adult fishes (i.e., < 0.0012 – 6.8 mg/kg ww in brown trout in UK; Allchin & Morris, 2003). Although these concentrations may not adversely affect adult fish, they could present substantial concern for ELS fish. As maternal transfer is a more realistic exposure pathway for lipophilic compounds, this study may provide greater insight into the toxicity of HBCD experienced in natural environments.

CHAPTER 4: GENERAL DISCUSSION

4.1 Project rationale

HBCD is a persistent organic pollutant (POP) with a propensity to remain in environmental and biological matrices long past its production and use (Law et al., 2014; Parvizian et al., 2020). In particular, it presents cause for concern in oviparous fishes as it may be maternally transferred to sensitive early-life stages (ELS) (Eljarrat & Barceló, 2018; Nyholm et al., 2008). Previous studies observing the effects of HBCD have demonstrated impacts on thyroid homeostasis, reproduction, development, and survival (Darnerud et al., 2003; Du et al., 2012; Deng et al., 2009; Hong et al., 2014; Wu et al., 2013). However, one significant concern with the research conducted to date to assess the hazards of HBCD in aquatic receptors is that it has predominantly employed aqueous routes of exposure; as HBCD has low water solubility, alternative routes of exposure (i.e., dietary and maternal transfer) are needed to provide more realistic exposure routes (Barber, 2009; Usenko et al., 2016) comparable to environmental conditions. Differences in route of exposure may result in considerable variation in accumulation kinetics and subsequently, any adverse effects which may be observed (Environment Canada & Health Canada, 2011; Law et al., 2006; Tomy et al., 2004). Due to the limited research on maternal transfer and accumulation kinetics in larvae with dietary exposure to HBCD and the specific molecular toxicity pathway leading to the previously observed embryotoxicity in ELS fish, the aims of this research were two-fold: 1) to characterize the accumulation and maternal transfer potential of HBCD in adult FHMs (fathead minnows) and their F1 generation, and 2) to develop a molecular toxicity pathway model for HBCD exposure in FHM larvae. The former would provide a greater understanding of the toxicokinetics of HBCD under conditions more realistically representing environmental exposures. The latter would be beneficial in linking early molecular signatures with apical endpoints of regulatory relevance, thereby supporting, and streamlining conventional environmental risk assessment as discussed in Chapter 1.

The objectives of Chapter 2 were to 1) quantify the accumulation of HBCD in adult FHMs after dietary exposure; 2) characterize the maternal transfer of HBCD from adult fish to their progeny; 3) describe the accumulation kinetics of maternally transferred HBCD in the eggs; and 4) observe apical and physiological effects in the adults. Although HBCD exposure in adults disturbed fecundity, no other apical endpoint was significantly affected. However, the major

findings from this chapter originate from the accumulation patterns observed. Both adult muscle and egg tissues displayed similar accumulation patterns up to the medium treatment condition. A divergence in accumulation was observed in the high treatment condition, with the adult tissue reaching diffusion/ lipid saturation limits whilst egg concentrations continued to increase, reaching concentrations four times (ww based) greater than in the adults. Accumulation in the eggs showed a positive correlation between concentration and time of exposure, reaching a steady state in all treatment conditions by approximately day 28. The findings from this chapter suggest HBCD may not be directly toxic to adult fish populations, however, the consequence of exposure may be heavily observed in their progeny due to the high body burden (EMRs ranging from 1.27-2.73, lipid corrected) and greater sensitivity seen in ELS organisms (Russell et al., 1999). As such, Chapter 3 looked at the molecular, apical, and physiological effects of maternally transferred HBCD in the subsequent F1 generation to further explore the potential impact on the subsequent generation.

The objectives of Chapter 3 were as follows: 1) describe the apical and physiological effects in FHM larvae maternally exposed to HBCD; 2) conduct whole transcriptome analyses to identify key gene expression signatures in FHM larvae; and 3) integrate apical, physiological, and histological outcomes with whole transcriptomics to characterize the molecular toxicity pathways of HBCD in maternally exposed FHM larvae. The major findings from this chapter displayed HBCD exposure in ELS FHMs significantly reduced survival (high treatment) and perturbed genes related to membrane integrity (i.e., inhibition of *cacna1fa*, *cyp2p7*) and pathways/ terms involved in metabolic processes (i.e., carbohydrate and amino acid metabolism). The disruption to membrane integrity via calcium channel inhibition and downregulation of CYP450 has been previously reported with HBCD exposure and linked to cytotoxicity, oxidative stress, and apoptotic/ necrotic/ or autophagic cell death (Dingemans et al., 2009; Londoño et al., 2010; Orrenius et al., 2011); however, only a trend was observed (low treatment) in the TBARS assay conducted. Additionally, transcriptomics analysis in the current study suggested potential effects on intracellular calcium levels, which have been linked with disruption of metabolic processes such as lipid, amino acid, and carbohydrate metabolism/ synthesis (Wang et al, 2016). However, no downstream apical effects relating to these molecular disturbances (i.e., neurological and developmental effects (Al-Mousa & Michelangeli, 2012; Dingemans et al., 2009)) were observed in the current study. The results from this chapter indicate the toxic

potential of HBCD may vary according to exposure route; previously conducted aqueous exposures have reported significant induction of oxidative stress and apoptosis with downstream effects on survival and deformity (Deng et al., 2009; Wu et al., 2013; Yamashita, 2003). Although, indications of oxidative stress and downregulation of a single anti-apoptotic enzyme was observed, downstream apical effects were limited to mortality. These results indicate the need to utilize exposure routes relevant to the compound and future studies should consider the physio-chemical properties of the chemical of interest prior to investigating potential adverse effects.

The link between exposure concentration and adverse effects may be impacted by the route of exposure, as was observed in Chapter 3. Maternal transfer studies provide valuable insight into the dynamics of lipophilic compounds in both adult organisms and their (often-times) more sensitive F1 generation. In addition, they present a more realistic exposure route than aqueous exposures that often require utilizing high solvent concentrations to dissolve the compound and/or are unable to fully dissolve the compound. The result of either or both shortfalls are uncertainties in the exposure concentration as well as observed adverse effects (i.e., if the induction of a specific pathway is due to the toxicant or a result of the high solvent concentration in conjunction with the chemical of interest), both of which are vital factors in determining the potential toxicity of a compound.

The presently utilized maternal transfer exposure method demonstrated HBCD toxicity may be lower than the previously described aqueous exposures. It may be that maternally transferred HBCD is more efficiently metabolized and eliminated due to the slow-release absorption of the yolk sac. In contrast, aqueous exposures may induce a greater insult on the organism as the entire dose is available at one time and thereby a higher concentration than with maternal transfer exposures. Furthermore, the presence of a solvent required to bring lipophilic chemicals such as HBCD in solution for aqueous exposures is likely to both alter uptake dynamics and cause physiological effects. In addition to apical endpoints, the results from Chapter 3 suggest that mortality (and potentially deformity) may not be a response to oxidative stress-induced apoptosis, as has been theorized by Deng et al. (2009), but an indirect effect of subtle cellular changes. It may be that HBCD exposure disrupts membrane transport (i.e., inhibition of calcium channel activity), leading to excess oxygen in the organism (i.e., induction of oxidative stress pathways), leading to disruption in energetics to reduce production of excess

oxygen (i.e., downregulation of carbohydrate and amino acid metabolism pathways), resulting in energy depletion and subsequent mortality. However, as the final objective of deriving a toxicity pathway for HBCD was not fully realized, the data derived in Chapter 3 may be utilized to further our understanding of HCBDs molecular response patterns and assist with developing a toxicity pathway for HBCD and compounds acting through a similar mechanism of action.

4.2 Alternatives to animal testing and new approach methodologies to prioritize chemical testing

Current ERAs utilize large-scale animal tests to quantify the aforementioned apical endpoints; this can be costly, time and resource intensive, and is of significant ethical concern. The outcome of all these factors is a lag in the assessment process as the number of chemicals needing to be assessed greatly outweighs the rate at which assessments can be completed. Advancements in science, technology, and increased interest in environmental stewardship are driving 21st century toxicity testing. New technologies (i.e., toxicogenomics, high-throughput technologies, bioinformatics) and alternatives to animal testing (i.e., *in silico*, *in vitro*, embryo-based tests) show great promise in prioritizing chemicals by categorizing chemicals acting through a similar mechanism of action (MoA) and reducing associated costs, resources, time, and animals (Ankley et al., 2010; Basu et al., 2019; Knapen et al., 2015).

In order to generate toxicity pathways or adverse outcome pathways, additional research is still necessary for poorly studied chemicals, particularly novel compounds, broad-acting chemicals (i.e., not acting through a specific mechanism of action), and/or lipophilic compounds. The current study demonstrates a potential alternative method to annotate toxicity pathways; the use of embryos/ larvae prior to independent feeding (i.e., 7dpf [days post-fertilization]) reduces ethical concerns as these organisms are not protected under current animal ethics legislation in many regions including North America and Europe (Batt et al., 2005; EU, 2010; Strähle et al., 2012). In addition, as the ELS of fish are often more sensitive to toxicant exposure, the Fish, Early-Life Stage (FELS) toxicity test (OECD 210) may be utilized to predict full fish life cycle outcomes (Villeneuve et al., 2014), thereby reducing the need for large-scale adult fish testing. The correlation between molecular changes and endpoints of regulatory relevance were made with the 14dph (days post-hatch) larvae, assessing apical and physiological endpoints (i.e.,

mortality, condition factor, histology, oxidative stress test), to demonstrate the potential of transcriptomics data coupled with early sampling (i.e., 7dpf) as a method to prioritize chemical assessments. This prioritization would allow for industry/government to reduce costs, resources, and time necessary for assessment of the growing number of chemicals today while reducing the reliance on live animal testing with regulated life stages (i.e. post swim up fish).

The current study utilizing transcriptomics and subsequent enrichment analyses in 7dpf larvae indicated disruption of membrane function (primarily inhibition of calcium channel activity) and metabolic processes (i.e., downregulation of carbohydrate and amino acid metabolism); these changes in cell function suggests the organisms may be experiencing mitochondrial dysfunction, oxidative stress, and/or cytotoxicity (Dingemans et al., 2009; Londoño et al., 2010; Orrenius et al., 2011). However, the link to apical endpoints were lacking, such that a marginal increase in oxidative stress as determined by the TBARS assay was the sole endpoint observed in relation to the molecular changes observed. Instead, what was observed was an increase in mortality in the highest treatment condition; when connecting to early molecular changes, transcriptomics data suggests maternally transferred HBCD inhibits membrane transport, leading to disruption in metabolic processes, which cumulatively result in depletion in energy, which may have resulted in the observed mortality at 14dph. As the derivation of a toxicity pathway for maternally transferred HBCD in FHM larvae was not fully realized in the current study, further studies observing maternally transferred HBCD would be beneficial in discerning toxic potential of other broad-acting lipophilic compounds. Conducting these large-scale studies can be labour- and time-intensive, with significant ethical concerns associated with the number of animals necessary to conduct such assays. However, they provide necessary insight into the toxicokinetic and toxicodynamic of lipophilic compounds, which tend to behave dissimilar to more hydrophilic substances. Although alternative methods (i.e., *in silico* models, AOPs, embryo-based toxicity testing prior to independent feeding) are being presented, they require further validation. As such, the current study may not only be utilized in informing/ validating our current understanding of the toxicokinetic and toxicodynamic properties of HBCD in adult and F1 generations of oviparous fish but also demonstrate the potential for the use of ELS toxicity testing as a viable alternative to adult fish toxicity tests in annotating toxicity pathways in fishes.

4.3 Limitations of current work and recommendations for future research

During this research project, certain limitations were faced due to time, financial, and logistical restrains; major limitations and recommendations for future research are presented below.

One of the major findings from Chapter 2 was the significant increase in egg production observed in the medium treatment condition; one of the hypotheses formulated included changes to the thyroid axis, however, as no hormone analysis was conducted this could not be verified. Thyroid hormones play a vital role in the body as a principal metabolic regulator, impacting growth, metamorphosis, reproductive events, and overall maintenance of homeostasis as environmental conditions fluctuate (Rabah et al., 2019). Maternal T3 administration prior to spawning has been shown to increase oocyte growth (Soyano et al., 1993) and reduce mortality during early development and growth phases (Tachihara et al., 1997). In relation, mortality in the F1 generation displayed significant increase in the high treatment condition. Previous HBCD exposures have linked oxidative stress and apoptosis as the mechanism of action for ELS mortalities, however, changes in maternal thyroid hormone levels may play a significant role in development and survival. As, such it may be beneficial to measure thyroid hormone levels to observe any impacts on the thyroid axis as previous exposures to HBCD reported decrease in circulating T4 and increase in T3 and TSH levels (Halldorson et al., 2008; Hamers et al., 2006).

The second noteworthy finding from Chapter 2 was the difference in tissue accumulation between life stages in relation to HBCD concentration; adult tissue concentrations appeared to reach a maximum by the medium treatment condition, while egg concentrations continued to increase as a function of time and exposure concentration. The bioaccumulation of dietarily exposed hydrophobic organic compounds have been observed to follow a more discrete accumulation pattern, with peaks and dips in whole-body concentrations due to the variations in the frequency of encountering a food source and average food intake (Wang et al, 2019). However, as this was a laboratory study food availability should remain stable. Instead, factors such as lipid diffusion (Geusau et al., 1999; Schlummer et al., 1998), lipid content (Price 2017), and metabolic capacity (Kropf et al., 2016) may have altered HBCD toxicokinetics in the adult tissues. Chronic exposure to HBCD may have saturated adult tissues, resulting in a shift in the lipid diffusion gradient. As such, future studies may benefit from observing the accumulation

kinetics of the adult tissue throughout the exposure to observe if and when diffusion limits are reached. The second factor, lipid content in the organism and their diet, are important factors in the uptake of hydrophobic compounds (Mann & Mills, 1979; Wang et al., 2019). The observed decrease in lipid content in the high treatment organisms (quantified using maternal tissue exposed for 7 days) and diet, raises the question whether the differences in HBCD accumulation are consequences of one or both of the aforementioned lipid content discrepancies. Therefore, future studies may benefit from quantifying lipid content in the organism for the duration of the exposure and attempt to maintain a constant lipid content in the diet. The third factor discussed was the impact of metabolic capacity; previous HBCD exposures have reported γ-HBCD is rapidly bioisomerized and cleared (Fournier et al., 2011; Erratico et al., 2022). Therefore, analyzing differences in enzyme activity (i.e., CYP450 enzymes) in relation to the stereoisomer may aid in determining the bioaccumulation and elimination of HBCD in adult FHMs.

One of the major limitations faced in this research project was the lack of cohesion between apical and molecular endpoints. Although survival was adversely affected, and molecular signatures indicating oxidative stress and energy imbalances were observed, histology and biochemical analysis of oxidative stress displayed no significant difference among treatment groups. This may be in part due to the fact that transcriptomics data is a snapshot of the total mRNA expressed in an organism, however, as not all template mRNA will be translated into proteins, there is a discrepancy between measured transcripts and translated proteins involved in cellular functions and eliciting an apical response (Aizat et al., 2018). Therefore, future research studies attempting to discern HBCD's toxicity pathway may find it useful to conduct proteomics analysis to verify the molecular changes that may be observed.

4.4 Summary & conclusion

The present research described in this thesis has provided information regarding the bioaccumulation of HBCD in adult tissues, maternal transfer to the F1 generation, and the apical and molecular disturbances in the F1 generation. Similar to other hydrophobic organic compounds, HBCD bioaccumulation exhibited diffusion/ lipid saturation limits at the high treatment condition and a one-to-one transfer to the F1 generation. This suggests HBCD is not a direct threat to adult fishes but could hinder the development and survival of ELS organisms due

to the high body burden at such a sensitive life stage. These findings may be used to further develop toxicokinetic models for hydrophobic compounds acting through a similar mechanism.

The findings from Chapter 3 demonstrated the potential impact of maternally transferred HBCD in ELS FHMs; increase in mortality, induction of oxidative stress responses and energy imbalances. Although no conclusive biological insights could be made between the apical and molecular responses to construct a toxicity pathway for HBCD, the data derived may be used to establish/ validate a complete toxicity pathway in the future. In addition, this research is part of a large, collaborative research project, the EcoToxChip project (EcoToxChip.ca), which aims to develop qPCR arrays (entitled "EcoToxChip") to aid in current risk assessment practices and chemical management programs (Basu et al., 2019). The project aims to link molecular signatures (i.e., transcriptomics and proteomics) to apical outcomes in eight model chemicals in fish, amphibian, and avian species (three model and 3 native species). The findings from Chapter 3 were used to inform the selection of genes for the fathead minnow EcoToxChip. The research presented in both chapters delivers substantial information to the limited, but growing literature on HBCD exposure in fish. The study highlighted the importance of studying relevant exposure routes for any toxicant of interest. Lipophilic compounds, such as HBCD, may be better studied using dietary/ maternal transfer studies due to differences in the toxicokinetics that may be observed when compared to aqueous exposure routes, which may affect the toxicity of the compound. Since its induction into the POPs list, there has been an increase in literature quantifying HBCD concentration in various organisms; however, HBCDs toxicity pathway has not been fully developed, and certain knowledge gaps remain to be further discussed and researched.

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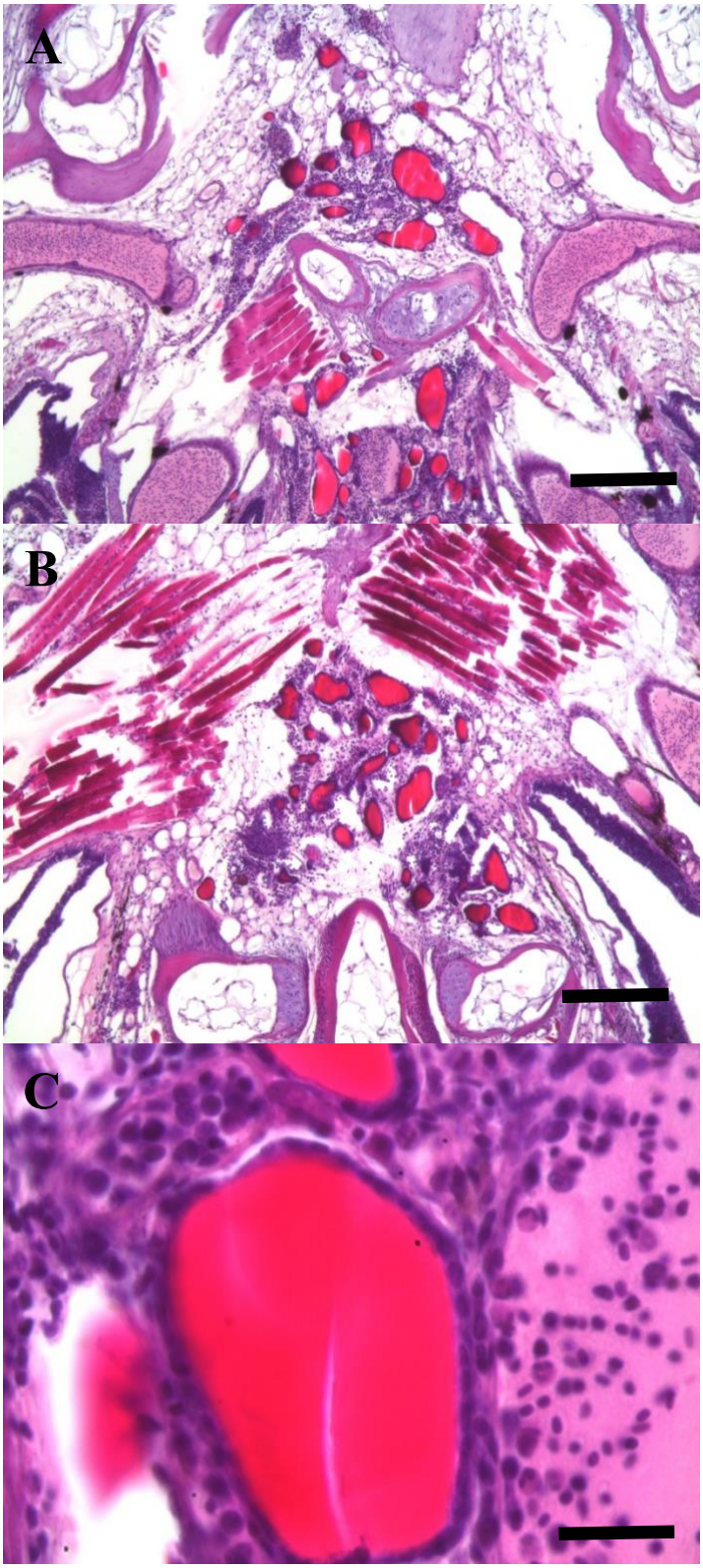
SUPPLEMENTAL DATA

S2.1 Mean (± SEM) water quality parameters measured over the FHM adult HBCD exposure period (including the acclimation and baseline periods). Water quality (temperature, pH, DO and conductivity) and water parameters (ammonia, nitrite, nitrate, hardness, and alkalinity) were measured at least twice a week (5 – 10 aquaria/dose group).

	Treatment			
	Solvent	***Low***	***Medium***	***High***
Temperature (ºC)	26.0 ± 0.29	25.9 ± 0.27	26.2± 0.31	26.0 ± 0.27
pH	7.93 ± 0.06	7.91 ± 0.06	7.94 ± 0.07	7.95 ± 0.06
Dissolved Oxygen (%)	81.8 ± 3.64	79.4 ± 4.36	79.6 ± 4.94	80.4 ± 3.99
Total Ammonia (ppm)	0.42 ± 0.08	0.40 ± 0.07	0.31 ± 0.05	0.43 ± 0.28
Nitrate (ppm)	8.7 ± 3.13	8.9 ± 3.11	9.6 ± 3.09	10.1 ± 3.44
Nitrite (ppm)	0.90 ± 0.32	0.90 ± 0.31	0.84 ± 0.31	0.86 ± 0.34
Hardness (ppm)	187.8 ± 4.48	190.8 ± 3.57	191.0 ± 3.56	190.2 ± 3.52
Alkalinity (ppm)	130.7 ± 7.92	129.6 ± 8.58	128.0 ± 7.71	128.7 ± 8.11
Conductivity (µS/cm)	566.2 ± 22.4	561.9 ± 19.9	553.3 ± 26.8	550.9 ± 35.6

DO = dissolved oxygen, ppm = parts per million, µS = micro-Siemens, cm = centimeter



S2.2 Photomicrographs of thyroid tissue from the adult FHMs exposed to dietary HBCD (Hematoxylin and Eosin stain) for 49 days, including (A) solvent control female, (B) high

treatment condition female, (C) solvent control male thyroid follicle. Bar A, B=200 microns, C=20 microns.

S2.3 Measured (mean ± SEM) concentrations (mg/kg ww) of hexabromocyclododecane in food, FHM muscle (maternal and paternal), and egg samples based on isomer profile. A subsample (1.0 g) of food used to feed adult FHMs was taken for each treatment condition at the start of the exposure and analyzed along with the remaining samples. Adult muscle samples were collected on day 49. The egg samples were collected between day 25 and day 46; each sample was a composite, collected from five replicates within a treatment group at a specific time point. Level of detection for each sample was calculated from the slope of the calibration curve and its standard deviation.

	Isomer	Solvent (mg/kg)	Low (mg/kg)	Medium (mg/kg)	High (mg/kg)
Food	*Alpha-HBCDD*	0.00213	1.30	4.17	11.5
	Beta-HBCDD	0.00151	0.873	2.80	8.84
	Gamma-HBCDD	0.00563	9.30	29.4	86.0
Maternal tissue	*Alpha-HBCDD*	<LOD	0.458 ± 0.035	1.59 ± 0.14	2.20 ± 0.11
	Beta-HBCDD	<LOD	0.041± 0.008	0.225 ± 0.034	0.123 ± 0.010
	Gamma-HBCDD	<LOD	1.25 ± 0.12	5.15 ± 0.60	4.69 ± 0.26
Eggs	*Alpha-HBCDD*	<LOD	0.505 ±0.029	1.33 ±0.05	4.12 ± 0.20
	Beta-HBCDD	<LOD	<LOD	0.193 ± 0.015	0.612 ± 0.045
	Gamma-HBCDD	<LOD	1.22 ± 0.03	3.80 ± 0.26	13.0 ± 1.07

LOD = level of detection, ww = wet weight, n (food) = 1 replicate, n (muscle) = 1-5 replicates (maternal = 5, paternal = 1-2), n (eggs) = 1 pooled sample/ treatment

S3.1 Mean (± SEM) water quality parameters measured over the FHM larvae HBCD exposure period. Water quality (temperature, pH, DO and conductivity) and water parameters (ammonia, nitrite, nitrate, hardness and alkalinity) were measured daily (5 tanks/dose group).

	Treatment			
	Control	***Low***	***Medium***	***High***
Temperature (ºC)	23.9 ± 0.25	24.2 ± 0.26	24.6 ± 0.33	24.1 ± 0.18
pH	7.91 ± 0.04	7.91 ± 0.04	7.89 ± 0.04	7.92 ± 0.05
Dissolved Oxygen (%)	88.6 ± 2.97	87.8 ± 2.86	88.3 ± 2.67	87.9 ± 2.69
Total Ammonia (ppm)	0.12 ± 0.06	0.07 ± 0.05	0.04 ± 0.04	0.06 ± 0.05
Nitrate (ppm)	5.28 ± 1.04	4.92 ± 0.89	4.52 ± 0.79	4.53 ± 0.81
Nitrite (ppm)	0.18 ± 0.03	0.17 ± 0.03	0.17 ± 0.03	0.17 ± 0.03
Hardness (ppm)	185 ± 4.14	189 ± 3.29	184 ± 3.51	188 ± 3.80
Alkalinity (ppm)	134 ± 3.26	136 ± 2.75	135 ± 2.93	136 ± 4.90
Conductivity (µS/cm)	487 ± 25.3	501 ± 26.8	502 ± 27.7	503 ± 28.1

DO = dissolved oxygen, ppm = parts per million, µS = micro-Siemens, cm = centimeter

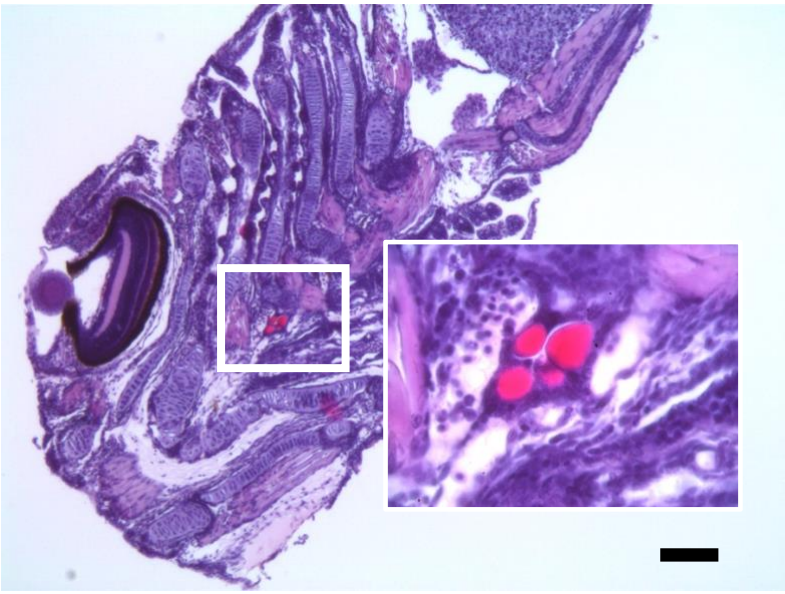
S3.2 Measured (mean ± SEM) concentrations (mg/kg) of hexabromocyclododecane in egg samples; concentrations were analyzed in ww, and lipid corrected based on total lipid content in maternally exposed eggs. Eggs were collected between day 25 and day 46; each sample was a composite, collected from five replicates within a treatment group. Limit of detection for each sample was calculated from the slope of the calibration curve and standard deviation.

	Mean Measured Concentration (mg/ kg, ww)	**Mean Measured Concentration (mg/kg, lc)**
Control	<LOD	<LOD
Low	0.576 ± 0.0441	32.7 ± 2.51
Medium	1.77 ± 0.316	102 ± 12.4
High	5.89 ± 1.31	282 ± 24.3

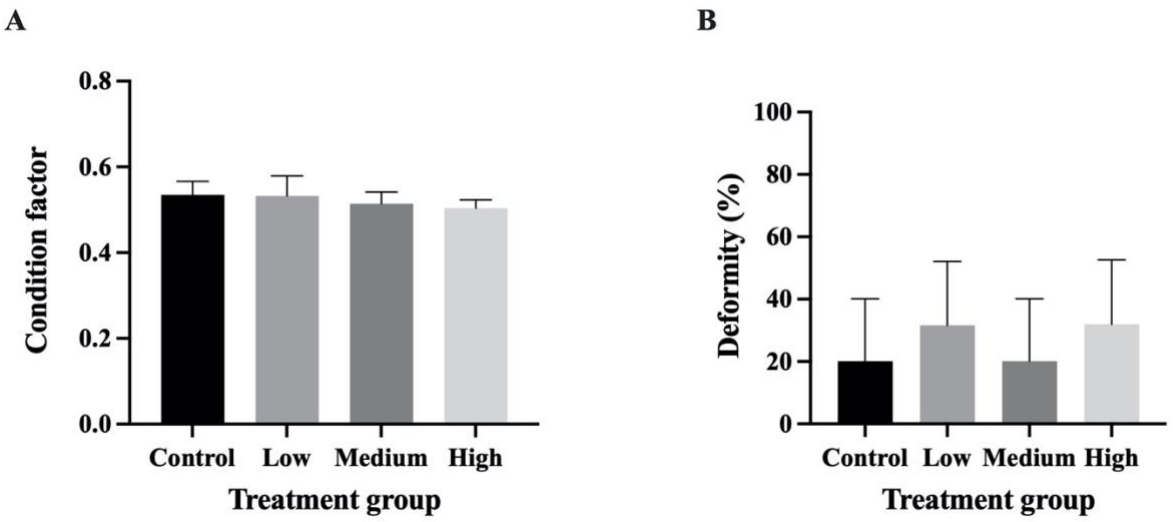
LOD = limit of detection (0.00228 - 0.0826 mg/kg, respectively), n= 3 samples/ treatment.

S3.3 Diet and Tissue Validation

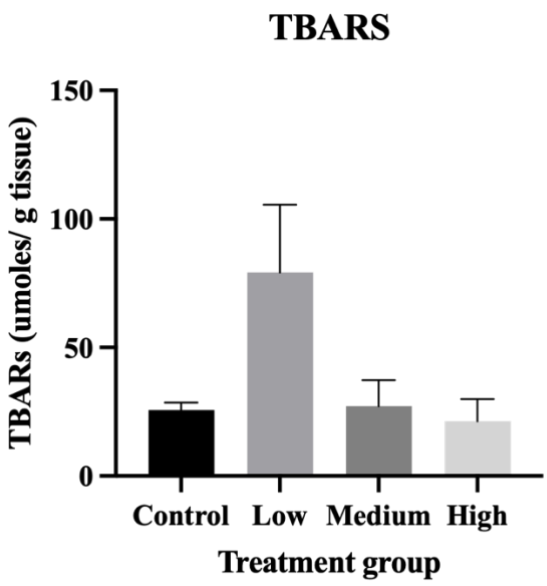
HBCD concentrations in the diet and eggs were processed and analyzed by SGS-AXYS Analytical Services Ltd. as described previously (Malala Irugal Bandaralage et al, submitted). In brief, samples were homogenized, Soxhlet-extracted and analyzed on a UPLC coupled to a TQ-S Xevo/Xevo Micro MS/MS with negative ion electrospray, multiple reaction monitoring (MRM) modes (Waters Acquity, Canada). Recovery and extraction losses were calculated using isotopically labeled surrogate standards (13C12-alpha-HBCD, 13C12-beta-HBCD, and 12C12-gamma-HBCD). Instrument linearity calibration was done with a seven-point calibration curve using 1/x weighted linear calibration and responses relative to the isotopically labeled standards. Quality control (QC) measures were implemented for each batch of samples by running laboratory blanks in parallel to the samples.



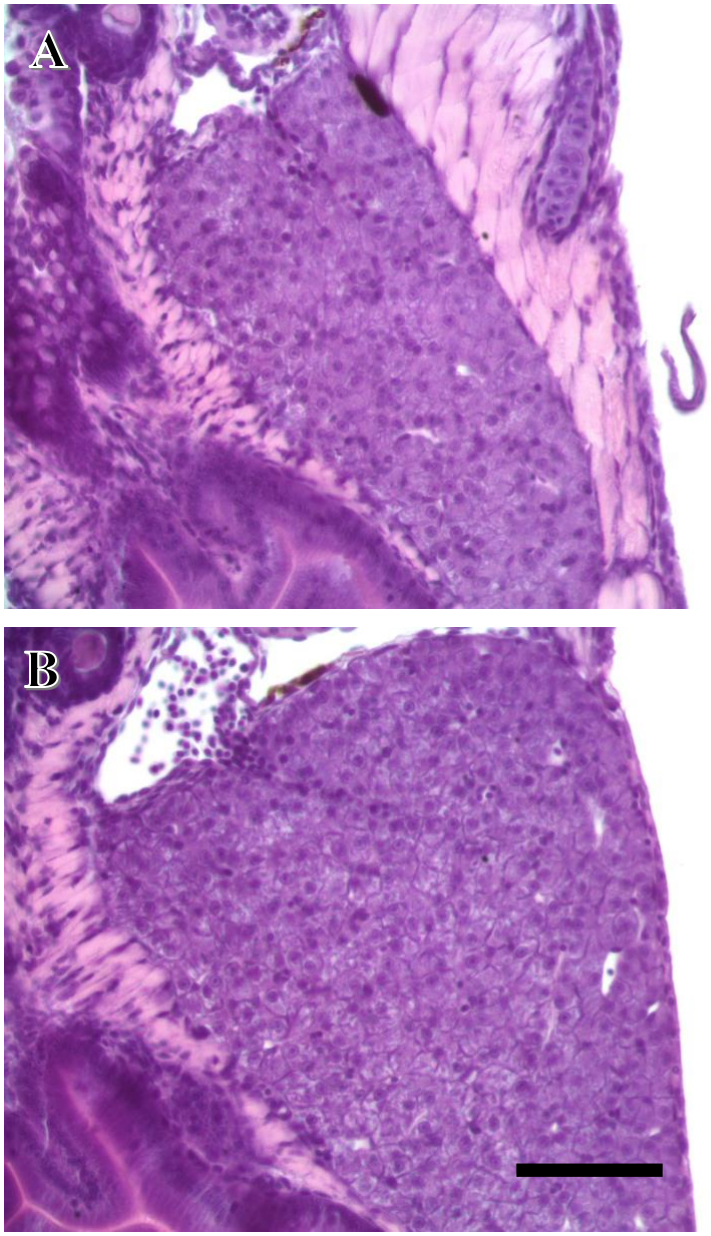
S3.4 Photomicrograph of a larval FHM from the maternal HBCD exposure. A small cluster of developing thyroid follicles can be seen at the base of the gill arches. Cuboidal thyroid epithelial cells are in a spheroid arrangement surrounding the eosinophilic colloid. Thyroid tissue was only detected in a single individual from the exposure study. Bar=100 microns.



S3.5 Morphometric and apical parameters measured in FHM larvae exposed to HBCD treatment groups and EtOH control, including A) condition factor and B) deformity (%). Error bars represent the standard error of the means, (*) indicates a statistically significant difference relative to the control ($p < 0.05$).



S3.6 Biochemical analysis measuring TBARS in 14 dpf FHM larvae exposed to HBCD and the control. Error bars represent the standard error of means.



S3.7 Photomicrographs of FHM larvae including, (A) Control and (B) High dose. Livers contained typical appearing hepatocytes, with variable degrees of vacuolation. Bar=50 microns.

S3.8 Overrepresented upregulated (UP) and downregulated (DOWN) GO terms and KEGG pathways in FHM larvae maternally exposed to low (0.576 ± 0.0441 mg/ kg) HBCD. Significantly dysregulated genes were filtered for FDR > 0.05.

	Term name	Adjusted *p*-value	Intersection size	Source
UP	*KEGG root term*	0.0146688	7	KEGG
	Metabolic pathways	0.01822528	4	KEGG
DOWN	*Catalytic activity*	0.00015551	19	GO:MF
	Binding	0.00021633	27	GO:MF
	Small molecule metabolic process	0.00031913	9	GO:BP
	Cellular catabolic process	0.00052143	9	GO:BP
	Catabolic process	0.00159956	9	GO:BP
	Ion binding	0.00216942	16	GO:MF
	Metabolic pathways	0.00303052	7	KEGG
	Organic substance catabolic process	0.00467127	8	GO:BP
	Citrate cycle (TCA cycle)	0.00478314	2	KEGG
	Organic acid metabolic process	0.00584386	6	GO:BP
	Tryptophan metabolism	0.01139649	2	KEGG
	Pyruvate metabolism	0.01139649	2	KEGG
	Metal ion binding	0.01340772	11	GO:MF
	Cation binding	0.01655807	11	GO:MF
	Oxidoreductase activity	0.02382585	6	GO:MF
	Nucleobase-containing small molecule metabolic process	0.0256599	5	GO:BP
	Biosynthesis of amino acids	0.03280424	2	KEGG

KEGG = Kyoto Encyclopedia of Genes and Genomes; GO = Gene Ontology; MF = Molecular Function; BP = Biological Process; FDR = false discovery rate; FHM = fathead minnow; mg = milligram; kg = kilogram; HBCD = hexabromocyclododecane