

“And once the storm is over, you won’t remember how you made it through, how you managed to survive. You won’t even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won’t be the same person who walked in. That’s what this storm’s all about.” — Haruki Murakami

MOLECULAR CHARACTERIZATION OF DIELDRIN IN THE ZEBRAFISH

HYPOTHALAMUS

by

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ABSTRACT

Dieldrin is a legacy pesticide that has multiple modes of action (MOA) that include being an estrogen receptor agonist, GABA receptor antagonist, and a chemical that disrupts mitochondrial function. There is also evidence that dieldrin exposure is significantly associated with an increased risk for neurodegeneration in humans. The objective of this thesis was to clarify the effects of dieldrin in the hypothalamus, the major neuroendocrine region of the brain, in the zebrafish (*Danio rerio*). Zebrafish were fed pellets containing 0.03, 0.15, or 1.8 µg/g dieldrin for 21 days and a global gene expression analysis was performed to characterize cellular processes and pathways affected by dieldrin. Signaling pathways associated with T-cell receptors and several interleukin receptors were significantly down regulated in both the 0.15 and 1.8 µg/g treatment groups. Moreover, transcripts related to mitochondrial function were significantly down-regulated in the hypothalamus following dieldrin treatments. The dysregulation of immune related transcripts raises the possibility that altered immune system function may be a mechanism underlying dieldrin induced neurotoxicity and neurodegeneration.

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List of Symbols, Nomenclature or Abbreviations

A2m; alpha-2-macroglobulin
Akt; protein kinase B
ANOVA; analysis of variance
AP-1; activating protein 1
ATF; activating transcription factor
ATP; adenosine triphosphate
ATP5a1; ATP synthase, H⁺ transporting, mitochondrial F1 complex alpha subunit 1, cardiac muscle
ATP5b; ATP synthase, H⁺ transporting, mitochondrial F1 complex, beta polypeptide
Ca²⁺; calcium ion
CGC; cerebellar granule cells
CN; cortical neurons
CNS; central nervous system
Cox17; cytochrome C oxidase copper chaperone
Cox6a1; cytochrome c oxidase subunit VIIa polypeptide 1
CREB; cAMP response element binding protein
CREB; CREB-binding protein
CRM; certified reference material
Cytc, cytosolic cytochrome
DA; dopamine
DAT; dopamine transporter
DDT; dichlorodiphenyltrichloroethane
DEGs; Differentially-expressed genes
ELK-SRF; ETS domain-containing protein-serum response factor
F11; coagulation factor XI
FDR, false discovery rate
FOXO; forkhead box protein O
g d.w.; grams dry weight
GABA; gamma-aminobutyric acid
GABAAR; GABAA receptor
GEO; Gene Expression Omnibus
ggact; gamma-glutamylamine cyclotransferase, tandem duplicate 2
GO; gene ontology
GSE; Gene Series Expression
GSEA; Gene set enrichment analysis
gstcd; glutathione S-transferase, C-terminal domain containing
HeLa; human cervical carcinoma cell line
IL; interleukin
L-DOPA; L-3,4-dihydroxyphenylalanine
LMB; Largemouth bass
Irrk2; leucine-rich repeat kinase 2
LSD; lysergic acid diethylamide
MBs; method blanks

MPTP; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS-222; tricaine methanesulfonate
MSs; method spikes
Na⁺-K⁺ ATPase; sodium-potassium adenosine triphosphate
NADH; nicotinamide adenine dinucleotide
ND1; NADH dehydrogenase 1
ND4; NADH dehydrogenase 4
NFATC; nuclear factor of activated T-cells
NF-κB; nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA; N-methyl-D-aspartate
OCPs; organochlorine pesticides
PAGE; Parametric analysis of gene set enrichment
park2; parkin
park7; parkinson protein 7
PCB 209; polychlorinated biphenyl 209
PD; Parkinson's disease
pink1; PHEN induced putative kinase 1
R; receptor
RIN; RNA Integrity Number
ROS; reactive oxygen species
Scye1; small inducible cytokine subfamily E member 1
Sdhaf2; Succinate dehydrogenase complex assembly factor 2
slc20a1; solute carrier family 20, member 1a
slc25a10; solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
slc30a1b; solute carrier family 30 (zinc transporter), member 1b
slc4a1; solute carrier family 4, anion exchanger, member
slc6a5; solute carrier family 6 (neurotransmitter transporter, glycine), member 5
SNEA; subnetwork enrichment analysis
STAT; signal transducers and activator of transcription
TH; tyrosine hydrolase
Tlr2; toll-interleukin-1 receptor interacting protein II
UNEP; United Nations Environment Programme
Vtg; vitellogenin

MOLECULAR CHARACTERIZATION OF NEUROTOXINS IN THE ZEBRAFISH
NEUROENDOCRINE BRAIN

1. Introduction:

1.1. Neuroendocrine disruption

Disruptions in the neuroendocrine brain by environmental chemicals (i.e. neuroendocrine disruption) is less studied compared to other endocrine organs (such as thyroid, adrenals, and gonads) but is important as the central nervous system (CNS) controls all endocrine systems downstream. In a recent review of neuroendocrine disruption, León-Olea et al. (2014) defined neuroendocrine disruptors as “*exogenous substances found in the environment that alter normal neuroendocrine function and result in an adverse effect on the organism or population*” (p.168). Classes of exogenous substances include, but are not limited to, pharmaceuticals, personal care products, industrial effluents, and pesticides. Each chemical has multiple mechanisms of action that include the disruption of neurotransmitter or neurohormone systems within the brain, including the hypothalamus. Research suggests that industrial effluent, pesticides from agricultural run-off, and pharmaceuticals affect the neuroendocrine system in non-target organisms (reviewed in León-Olea et al., 2014). Neuroendocrine disruptors can elicit effects by agonistic (i.e. stimulatory) or antagonistic (i.e. inhibitory) actions on neurons that produce neuropeptides, neurotransmitters or neurohormones (Waye & Trudeau, 2011). Disruptions in these neurotransmitter systems and altered synthesis and release of neuro-hormones and neuropeptides are associated with an array of neuroendocrine, endocrine and neuro-psychological human diseases such as depression (Dunlop & Nemeroff, 2007), anxiety (Möhler,

2006), epilepsy (Möhler, 2006) and Parkinson's disease (Remy et al 2005) to name but a few. Thus, aquatic contaminants can pose a significant risk to neuroendocrine function.

1.2. The major tissues of the neuroendocrine system

Physiological processes are controlled by higher brain centres in the CNS. The two major tissues in the fish CNS that regulate pituitary hormone release are the telencephalon and the hypothalamus (Canosa, et al. 2007). While the telencephalon contains the preoptic area, an area rich in neuroendocrine cells, the hypothalamus is the primary control center that integrates environmental signals within the endocrine systems, regulating processes such as metabolism (Frankish, et al. 1995), energy balance (Horvath, et al. 2001), circadian rhythms (Saper et al. 2005), and sleep (Machluf et al. 2011). Cells producing neuropeptides are located within the hypothalamus (Blechman et al. 2011). In zebrafish (*Danio rerio*), there are a number of orthologs to mammalian hypothalamic peptides including oxytocin (Unger & Glasgow, 2003), arginine-vasopressin (Eaton et al. 2008), corticotrophin releasing hormone (Chandrasekar et al. 2007), thyrotropin-releasing hormone (Diaz et al. 2002), somatostatin (Blechman et al. 2007), hypocretin (Faraco et al. 2006; Kaslin et al. 2004) and growth hormone-releasing hormone (Wu et al. 2008), among others.

Neurotransmitters also play a significant role in regulating neuroendocrine function in the CNS and can regulate, directly or indirectly, different neuropeptides. Gamma-aminobutyric acid (GABA) neurons have been identified in the hypothalamus of zebrafish and more specifically, in the stratum album centrale and stratum periventriculare (Kim et al. 2004). GABA cells are also found as large clusters of neurons in the olfactory bulb, sub-pallium and preoptic area, and

smaller multiple nuclei in the hypothalamus (Wullimann & Rink 2002). . Therefore, similar to mammals, the zebrafish hypothalamus contains GABA

1.3. Dieldrin as an environmental pollutant

Dieldrin [(1*R*, 4*S*, 4*aS*, 5*R*, 6*R*, 7*S*, 8*S*, 8*aR*)-1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4*a*, 5, 6, 7, 8, 8*a*-octahydro-6,7- epoxy-1, 4:5, 8-dimethanonaphthalene] is an organochlorine legacy pesticide that was used globally as a highly effective insecticide for soil dwelling insects and pests that damage wood such as termites and wood borers (de Jong, 1991). Dieldrin was also used in developing countries to control insect vectors for diseases such as malaria, yellow fever, Chagas disease, Oroya fever, African sleeping sickness, river blindness, and filariasis (de Jong, 1991). Despite its effectiveness, the chemical has been restricted or banned in many parts of the world due to adverse effects in non-target species (Meister, 1989, Martyniuk et al. 2013, Sonne et al. 2014). By 1995, dieldrin was banned in more than 70 countries (Pesticide Action Network North America, 1995) and in 1997, the Governing Council of the United Nations Environment Programme designated dieldrin as a persistent organic pollutant (UNEP, 1997). More recently, the USA Government ranked dieldrin 18th (of 31) on the list of priority chemicals in the 2013 U.S. Agency for Toxic Substances and Disease Registry, a list that describes those contaminants most concerning for human health. Sediment cores from the Great Lakes in the US show contamination from dieldrin beginning in the 1940s, peaking in the 1970s and recently decreasing slightly (Jorgenson, 2001).

Dieldrin has been detected worldwide at varying levels in water, sediments, and organisms. In Canadian freshwater lakes, the highest concentration of dieldrin measured in river and stream

sediments was 86 ng/g sediment (Environment Canada, 1998). In sugarcane soils in the Herbert region of Australia, dieldrin ranged from 0.028-22.2 ng dieldrin /g dry weight (Cavanagh et al. 1999) while concentrations in sediment in the Lauritzen channel, San Francisco Bay were as high as 748 µg/dry kg (Swartz et al. 1994). This site was in close proximity to a former dieldrin and dichlorodiphenyltrichloroethane (DDT) production plant. In fish such as common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys nobilis*), and yellow catfish (*Peltobagrus fluvidraco*) in Baiyangdian lake, (China), lipid concentrations of dieldrin were 98, 117, 266, and 432 ng/g lipid weight, respectively (Hu et al. 2009) while in herring, in the northern part of the Baltic Sea (*Clupea harengus*), body, burden levels of dieldrin were 120 ng/g lipid (Strandberg et al. 1998). In Bow Lake, Banff National Park, Canada, Mountain whitefish and Lake trout accumulated levels of dieldrin up to 0.7 and 2.3 ng/g wet weight, respectively (Campbell et al. 2000). These examples demonstrate that dieldrin is still present at relatively high concentrations in the environment as well as in the tissues of organisms. Thus, even though dieldrin has been banned from most developed countries since the 1970-80s, dieldrin is still present in our environment today and levels can be relatively high in many areas of the world.

1.4. Dieldrin: Mode of action

Dieldrin is an endocrine disruptor that has multiple modes of action. The pharmacological mode of action of dieldrin is to antagonize (or block) GABA_A receptors (Ikeda et al. 1998). In mice, CNS primary cultures treated with dieldrin resulted in disrupted expression of GABA_A receptor subunits (GABA_AR) (Pomés et al. 1994). Dieldrin at concentrations of 0.2 µM and 3.0 µM in cerebellar granule cells inhibited GABA_A and glycine receptor function, respectively

(Vale et al. 2003). A 50% inhibition of GABA_A receptor subunit 2 in the channel catfish (*Ictalurus punctatus*) brain was observed after treatment with 0.59 μM of dieldrin (Carr et al. 1999). As a consequence of GABA_AR inhibition by dieldrin, NMDA receptors were found to be internalized from the outer cell membrane to inside the cell in cerebellar granule cells and cortical neurons (Babot et al. 2007). These alterations could explain cognitive deficits observed in animals chronically exposed to dieldrin (Carlson & Rosellini, 1987). Another example of an indirect effect by dieldrin comes from studies in mice, where it was observed that dopamine transporter (DAT) protein and mRNA levels increased in the ventral mesencephalon after the mice were fed 3 μg/g dieldrin (Richardson et al. 2006). These studies show that dieldrin affects the GABAergic systems of the brain by inhibiting the activation and expression of the GABA_AR.

A second mode of action for dieldrin appears to be related to the reproductive axis. For example, dieldrin has been shown to inhibit the binding of 17β-estradiol to the estrogen receptor in mouse primary cultures of cortical neurons (CN) and cerebellar granule cells (CGC) (Briz et al. 2011). In both the CGC and CN, dieldrin caused a concentration-dependent inhibition of [³H]-E₂ binding (Briz et al. 2011). Moreover, the same study showed that protein kinase B (Akt) phosphorylation, which is a major intracellular signaling pathway associated with estrogen receptor dependent, non-genomic effects of estrogens, is enhanced after 5 h exposure to dieldrin in CN and CGC. A non-genomic response to 17β-estradiol is an induction of intracellular Ca²⁺; dieldrin also results in an increase in intracellular Ca²⁺ (Wozniak et al. 2005). In cervical cancer cells (HeLa) transfected with estrogen α and β receptors, dieldrin significantly activated the α receptor but not the β receptor, suggesting that it can activate specific isoforms of the receptor (Lemaire et al. 2006). Lastly, evidence that dieldrin can act as an estrogen is based on studies in

largemouth bass (LMB, *Micropterus salmoides*) where dieldrin increased *vtg* expression, an estrogen-responsive transcript, in the liver (Garcia-Reyero et al. 2006).

In addition to being an endocrine disruptor and a GABA_A antagonist, dieldrin has also been shown to have secondary effects on mitochondrial function. In immortalized rat mesencephalic cells, dieldrin increased reactive oxygen species (ROS) generation and the release of cytochrome C into the cytoplasm, and causes increased caspase-3 activity (Kanthasamy et al. 2008). The release of cytochrome C from the mitochondria into the cytoplasm by dieldrin initiates the process of apoptosis and these endpoints are indications of mitochondrial dysfunction and cell death. In PC12 cells, dieldrin decreased mitochondrial activity and mitochondrial membrane potential at concentrations as low as 30 µM in addition to increasing ROS (Kitazawa et al. 2001). The mitochondrial membrane and caspase 3/7 activity of N27 DA neuronal cells treated with either 25 µM dieldrin or 25 µM lindane (another organochlorine pesticide), showed mitochondrial membrane disruption (Sharma et al. 2010). In the blue gill fish (*Lepomis macrochirus*) brain, Na⁺-K⁺ ATPase enzymes, proteins which are localized to the mitochondrial membrane, were inhibited by 40% after treatment with 20.8 µM dieldrin (Yap et al. 1975). Thus, mitochondria and proteins related to mitochondrial function are impaired by dieldrin.

1.5. Dieldrin as a human health concern

Due to its ubiquitous distribution and persistence, dieldrin remains a human health concern. Dieldrin has been associated to human neurological disorders such as Parkinson's disease (PD). In mammals, dieldrin has been shown to induce Parkinsonian symptoms, including tremors, transient dyskinesia, and body rigidity (Sharma et al. 1976; Wagner & Greene, 1978; Heinz et al. 1980). Dieldrin has also been measured in the serum and at levels between 0.52- 0.92 µg/g lipid in the substantia nigra of PD patients (Corrigan et al. 2000;

Parkinson's Disease Society Brain Bank). There was a significant association between dieldrin and the occurrence of PD in a Finnish population compared to 2001-2002 US National Health and Nutrition Examination Survey populations (Weisskopf et al. 2010). More recently, Chhillar et al (2013) found significantly higher levels of dieldrin in subjects showing symptoms of PD in a population in India. In an Indian population, 9.3% of the control subjects with no PD symptoms (90.7 % with no detection of dieldrin) had a detectable concentrations of dieldrin ranging from 0.96 – 4.2 ng dieldrin/mL blood and 61.43 % of the Parkinson's patients with concentrations ranging from 2.1 – 28.63 ng/mL (Chhillar et al. 2013). Thus, epidemiological evidence suggests that increased dieldrin levels in the body are associated with an increased risk to PD.

1.6. Effects of Dieldrin in non-target aquatic organisms

Adverse biological responses in the CNS have also been reported in teleost fishes exposed to dieldrin . After intraperitoneal injection of 10 µg dieldrin/g into female LMB, GABA levels increased 20-30% in the hypothalamus and cerebellum (Martyniuk et al. 2010b). Dieldrin treatments at levels ranging from 25 µM to 50 µM in zebrafish increased aridine orange staining in the brain, which is a general indicator of neurotoxicity (Ton et al. 2006). Zebrafish embryos exposed to 100 µM dieldrin had reductions in both optic tectum size and projections to the posterior commissure (Ton et al. 2006) which are regions of the brain containing DA and GABA neurons and nuclei (Kim et al. 2004). Dieldrin also significantly decreased α -tyrosine hydrolase (TH)-positive cells (a catecholaminergic neuron) in the larvae zebrafish brain, which is related to tremors (Ton et al. 2006). Therefore, dieldrin is a good model chemical to understand how exposures to pesticides that act by antagonizing the GABA_A receptor are related to human neurological diseases, and studies with dieldrin can provide important baseline data for

comparisons to other GABA_A antagonists such as fipronil, which is one of the newer replacements for legacy pesticides such as dieldrin.

1.7. Omics responses to dieldrin

In recent years, global transcriptome and proteome profiling has been conducted in the fish neuroendocrine region to better understand the effects of neuroactive pollutants (Wong et al. 2013, Weber et al. 2013). Commercial oligonucleotide arrays (i.e. microarrays) are synthesized *in situ* on platforms in strands of 60 oligonucleotides. Each strand represents a probe with a unique transcript (Lipshutz et al. 1999). Microarray analyses allow for the assessment of tens of thousands of mRNA in parallel over the organism transcriptome. The transcriptome provides insight into the underlying mechanism of action of a particular chemical and how it may cause effects at other levels of organization. In teleost fishes, microarrays have been used to elucidate the mode of action of herbicides such as linuron (Ornostay et al 2013), and can generate insight into cell processes that are altered by neuroactive pharmaceuticals such as fluoxetine, venlafaxine and carbamazepine (Thomas et al. 2012). Genes related to circadian rhythm were affected in the whole brain of male zebrafish exposed to 273 µg/L diazepam, and embryos exposed to 273 µg/L diazepam significantly increased time spent in locomotion (Oggier et al. 2010).

Global proteome and transcriptome profiling to study the effects of dieldrin in the neuroendocrine brain has been limited to three studies in the LMB (Martyniuk et al. 2010a; 2010b; 2013), thus there are preliminary data on the transcriptomic responses to this pesticide in the neuroendocrine brain but not in a genomic model animal such as the zebrafish. Martyniuk et al. (2013) showed that dopamine receptor D1 signaling was up regulated while GABA_A receptor

and dopamine receptor D2 signaling were down-regulated at the level of the transcript after dieldrin was fed to LMB (~3 µg dieldrin/g feed). In the same study, transcripts related to ROS and Ca²⁺ levels were also increased in LMB hypothalamus after feeding treatments with dieldrin (Martyniuk et al. 2013). Gene ontology terms enriched in the LMB hypothalamus after dieldrin treatments included zinc ion binding, DNA repair, and ubiquitin cycle (Martyniuk et al. 2010a). In a second independent study, genes related to the ubiquitin-proteasome pathway were increased by 86% in LMB after a single intraperitoneal injection of 10 µg dieldrin/g (Martyniuk et al. 2010b).

It is interesting to note that transcripts for mitochondrial proteins were differentially expressed in LMB hypothalami after dieldrin treatments (Martyniuk et al. 2010a), suggestive of mitochondrial dysfunction. In a proteomics study, Martyniuk et al. (2010a) observed a significant increase in ATP synthase, H⁺ transporting, mitochondrial F1 complex, beta polypeptide (ATP5b), ATP synthase, H⁺ transporting, mitochondrial F1 complex alpha subunit 1, cardiac muscle (ATP5a1), and cytosolic cytochrome (CytC) in LMB hypothalami after treatment (Martyniuk et al. 2010a). A limitation of the available transcriptome studies for dieldrin is that only ~9000 transcripts were examined, as the LMB genome is not completely sequenced. Other fish, such as the zebrafish (*Danio rerio*), can provide a more complete model as the genome of the zebrafish is more completely annotated.

1.8. Zebrafish as a vertebrate model

1.8.1. General

The primary animal models for biomedical research are small rodents because they have highly conserved physiology with humans. More recently, the zebrafish (*Danio rerio*) has

become an important vertebrate model for chemical toxicity (as reviewed in Hill et al. 2005), bridging the gap between aquatic and mammalian models. The increasing popularity of zebrafish as a model organism is the result of its numerous beneficial attributes. Zebrafish do not typically exceed 5 cm in length, allowing them to be kept at high density in the laboratory and to be easily managed (Löhr et al. 2011). Zebrafish also exhibit a short, well-documented developmental period and are fully mature within three months (Kimmel et al. 1995). Zebrafish have also gained popularity as an animal model due to their high fecundity (100-500 eggs every 2-3 days), transparent embryo and larval stages, synchronized fertilization and development of eggs, environmental durability, and the possession of endocrine systems that are conserved with humans (Löhr et al. 2011). Thus, for mechanistic studies on developmental effects of aquatic pollutants, the zebrafish is an excellent model for experimentation. Zebrafish are also an emerging model for research areas that include neurological diseases (Best & Alderton, 2008; Xi et al. 2011), cardiac disease (Bakkers, 2011), obesity (Tinguad-Sequeira et al. 2011), and cancer (Teittinen et al. 2012). Zebrafish have many advantages over mammalian models, including low maintenance costs, short generation time, and large egg clutch sizes; however, a disadvantage is that they are not sexually dimorphic and have a small body/tissue size (reviewed in Hill et al. 2005).

1.8.2. Zebrafish as a model for neurodegeneration

Zebrafish, due to the fact that their genome is fully sequenced and there is a large bank of genetic mutations, have become a genetic model for several human diseases including PD. Several orthologs of major Parkinson's related genes have been discovered in zebrafish, including *parkinson protein 7 (park7)*, *leucine-rich repeat kinase 2 (lrrk2)*, *parkin (park2)* and

PHEN induced putative kinase 1 (*pink1*) (Bai et al. 2006; Baulac et al. 2009; Sheng et al. 2010; Anichtchik et al. 2008). When *park7* is mutated, a rare autosomal-recessive early onset PD occurs (Bonifati et al. 2003) in humans. The *park7* transcript is heavily involved in mitochondrial protection against oxidative stress, a primary mechanism for neurodegeneration (Bai et al. 2006). Increased mitochondrial oxidative stress may be one mechanism by which dieldrin is related to PD. The zebrafish *park7* protein has 83% similarity in amino acid identity to human *park7* (Bai et al. 2006). Interestingly, knockdown of *park7* in zebrafish does not lead to a decrease in DA neurons which is a hallmark of PD, but rather DA neurons become more sensitive to oxidative stress (Baulac et al. 2009). Deletion of β -transducin domain of *lrrk2* by morpholino injection significantly decreased DA neurons and locomotor defects in zebrafish (Sheng et al. 2010). Parkin mutations are a common autosomal-recessive for early onset of PD. *Parkin* encodes for E3 ubiquitin ligase, which is involved in the ubiquitin proteasome degradation system and plays a role in mitochondrial function. *Parkin* is upregulated during exposures to other pesticides such as rotenone (a mitochondrial inhibitor) (Fett et al. 2010) and is prone to misfolding, thus becoming insoluble and forming aggregates (Winklhofer et al. 2003). Another common autosomal-recessive early onset PD mutation is *pink1* which encodes for a ubiquitously expressed protein with an N-terminal mitochondrial targeting motif. Zebrafish *pink1* has 54% amino acid sequence identity to humans (Anichtchik et al. 2008). Knockdown of *pink1* by morpholino did not alter DA neurons in the ventral diencephalon in zebrafish but caused locomotor dysfunction such as impaired response to tactile stimuli and reduced swimming behaviour (Xi et al. 2010). Thus, zebrafish are an informative model for the relationship of genetic variability to PD and PD-like symptoms, and they have allowed

researchers to study in more detail the development of PD in adult organisms (Bretaud et al. 2004).

1.8.3. Neurobehavioural assessment in zebrafish

Memory, learning, and locomotion serve as phenotypic endpoints for neurological dysfunction. These endpoints can be useful when assessing the effects of pesticides on organisms. For Alzheimer's disease, memory and learning play an important role in the assessment of progression of the disorder (Walsh & Selkoe, 2004). The use of model organisms for the assessment of memory has long fallen to mammalian models (i.e. monkeys, rats and mice). In the mammalian literature, the use of tests that include the T-maze for memory and learning is abundant (Tolman et al. 1946; Blodgett et al. 1947; Elmore et al. 2012) but in recent years, the T-maze has been applied to zebrafish (Darland et al. 2001; Colwill et al. 2005; Grossman et al. 2010; Echevarria et al. 2011). Levin et al. (2007) applied a novel tank test to assess anxiety and locomotion in zebrafish. A novel tank is one that the fish has not inhabited previously. Once in the new tank, a fish will dive and stay at the bottom until they become habituated to the novel environment and start to explore. If anxious, the individual will take a longer time to explore the environment. The novel tank test has been used with zebrafish for the assessment of nicotine, buspirone, chlordiazepoxide, diazepam and ethanol (Bencan et al. 2009; Egan et al. 2009; Grossman et al. 2010). Behavior is an important whole animal endpoint that can be associated with neurological impairment.

1.9. Statement of need, objectives, and hypotheses

Despite previous studies reporting on the effects of dieldrin in the CNS of fish, there remain two readily identifiable knowledge gaps: (1) For global analysis of molecular pathways

in the hypothalamus, no species with a completely known genome has yet been used and (2) there have been no behavioural assessments conducted following dieldrin treatments in fish, despite the fact that this pesticide is related to neurodegenerative diseases. Thus, a more complete transcriptomics assessment was performed in this study using zebrafish, and there was the incorporation of behavioural tests to learn more about the whole animal impacts of pesticide exposures.

The research objectives of this thesis were to (1) characterize molecular pathways affected by dieldrin in the zebrafish hypothalamus to better understand the impact on the neuroendocrine system and (2) use a novel tank test to characterize changes in behaviour as a result of dieldrin exposure. The following null hypotheses were tested:

H₀: Dieldrin will not accumulate in tissues of whole zebrafish. The rationale for this hypothesis is dieldrin has been seen to accumulate in many species and this will be the first time for dieldrin measurement in zebrafish.

H₀: Dieldrin will not alter the expression of genes related to its mode of action in the hypothalamus. The rationale for this hypothesis was that these processes are related to the major modes of action for this pesticide.

H₀: Dieldrin will not alter normal exploratory behaviour in zebrafish. This hypothesis was formulated based on the understanding that GABA_A receptor antagonists are used as anti-anxiety drugs, and fish in a novel tank are expected to more willingly explore new environments if treated with a chemical that has anti-anxiety properties. This research will examine the relationship between dieldrin exposure and human neurological disorders, using zebrafish as a surrogate for human health.

2. Methods

2.1. Feed preparation

Twelve milligrams of dieldrin was dissolved into 4 mL of olive oil and was mixed for 1 hour (stock solution). A serial dilution was performed by adding 0.5 mL of the stock solution into 4.5 mL of fresh oil to generate three treatment feeds (10 times dilution series). Corey Aqua Feed Optimum extruded fish pellets (Corey Feed Mills Ltd., Fredericton, NB, Canada) were coated with either the 4 mL olive oil (control) or one of the three dieldrin preparations. Experimental feed was prepared by mixing 400g of pellets in a rotating glass jar and adding 1 mL of each oil treatment every hour (3 mL oil total). Control feed was prepared in the same way but with 3 mL of oil only. The nominal feed concentrations that were prepared were 0, 0.0225, 0.225 and 2.25 μg dieldrin / grams dry weight (g d.w.) feed to generate a control feed (no dieldrin), and a low, medium, and high dose feed, respectively. The measured feed concentrations (Section 2.4 describes the analytical methods) were 0, 0.03, 0.15, and 1.8 μg dieldrin/g d.w. feed. These concentrations were chosen to generate an environmentally relevant body burden level with the 2.25 $\mu\text{g}/\text{g}$ d.w. feed also a lower concentration was obtained (1.8 μg dieldrin/g d.w. feed). Initial calculations were based on a study by Muller et al. (2004), which reported that feeding 3 $\mu\text{g}/\text{g}$ to LMB for 30 days resulted in whole body burdens of 0.5 $\mu\text{g}/\text{g}$ wet weight. The two lower doses were generated to target body burdens of 0.10 – 0.50 $\mu\text{g}/\text{g}$, a level that approximates concentrations detected in patients with Parkinson's disease (Weisskopf et al. 2010). From this point forward in the thesis, treatments will be referred to by their measured feed dose.

2.2. Experimental design:

Reproductive adult zebrafish (4-6 months of age) were obtained from Mirdo Inc. (Montréal, QC, Canada). Zebrafish were housed at the Canadian Rivers Institute (Saint John, NB, Canada) in 20 L tanks. After an acclimation period of 3 weeks, zebrafish were divided into 9 tanks per experimental group giving a total of 36 tanks, each with active carbon within the tank as an extra precaution to minimize any exposure to water-borne dieldrin. Five fish were added to each tank, with the goal of maintaining a 3 to 2 female to male sex ratio. Zebrafish in designated tanks were fed 0.065 g of the control or one of the three contaminated feeds twice a day representing a daily intake of 0.13 g (approximately 4% of fish body weight per tank). Feeding took place at 10 am and 4 pm each day. Every three days, approximately half of the water in the experimental tanks was removed and replaced with fresh water. Faeces and uneaten food were removed by siphon daily to minimize exposure to dieldrin by leaching into the water. Water parameters (mean \pm standard deviation) measured daily during the experiment were as follows; temperature = 25.5°C (\pm 1.5, n=78), dissolved oxygen = 84.1% (\pm 8.4, n=78), and pH = 6.54 (\pm 0.57, n=78).

After 21 days, two zebrafish from each tank were subjected to behavioural testing employing the novel tank test (Section 2.3). Following the novel tank test, animals were immediately euthanized in a sodium bicarbonate buffered solution of MS-222 (250 mg/L; Sigma-Aldrich, St. Louis, MO, USA) and their spinal cords were severed. The hypothalami of all male and female zebrafish within a single tank were dissected and pooled into one biological replicate for each sex (i.e. one male and one female pool). Thus, each treatment contained 9 biological replicates for both male and female hypothalami. The remaining fish carcass was used for dieldrin measurements in whole body.

2.3. Behavioural testing:

2.3.1. Experimental Design

The novel tank test was used to assess anxiety and locomotion (Levin et al., 2007; Bencan et al., 2009; Egan et al., 2009; Grossman et al., 2010; Rosemberg et al., 2012). For these experiments, methodology was adapted from Rosemberg et al. (2012). After the 21 day exposure, individual zebrafish were placed in a 1.5 L trapezoidal tank (Figure 1) (Aquabiotech Inc., Quebec). Two fish per experimental tank were tested in the novel tank apparatus resulting in a total of 18 fish per treatment. The number of female zebrafish assessed using the novel tank test was 11, 12, 10, and 9 for 0, 0.03, 0.15, and 1.8 μg dieldrin/g treatments, respectively. The number of male zebrafish used in the novel tank test was 7, 6, 8, 9 for 0, 0.03, 0.15, 1.8 μg dieldrin/g, respectively. Zebrafish were randomly selected from each of the experimental tanks as sex could not be determined until fish were dissected. A total of 72 zebrafish were tested for anxiety and locomotion. Tanks were divided into three equal horizontal portions, marked by a virtual dividing line (Figure 1). Zebrafish swimming behaviour was recorded using a SONY Handycam DCR-SR45 for 3 min. at a frame rate of 30 frames/sec. and behavioral parameters (latency to first exit from bottom, time spent in bottom, time spent in middle, and time to first enter top) were automatically measured using video-tracking software (ANY-maze®, Stoelting CO, USA). All fish were handled carefully to avoid undue stress when moving the animals from the experimental tank to the novel tank. The novel tank was filled with 1300 mL of clean water for each trial. All trials were performed in the same room to ensure uniform illumination and water quality between trials.

2.3.2. Vertical exploration:

Zebrafish in an open novel tank tend to dive to the bottom and will slowly start to explore the upper portion. This behavior reflects habituation to the novel environment (Wong et al 2010; Rosemberg et al 2011). Vertical activity is defined by time spent and the number of crosses out of the bottom portion of the tank, and increased time in the top and middle portions of the tank is indicative of reduced anxiety levels (Levin et al 2007; Egan et al 2009; Mathur & Guo 2011). The variables measured in this study included latency to first exit from the bottom (in seconds), total time spent in the bottom, time spent in middle, and time taken to first entry into the top portion of the tank. Statistical analysis was performed using a two-way ANOVA with sex and treatment as independent variables (GraphPad Prism® V6). If there was a significant interaction term, a one-way ANOVA was performed to determine which of the independent variables resulted in the effect. The Tukey's post-hoc test was performed if differences were detected by ANOVA. Alpha was set to $\alpha = 0.05$.



Figure 1: Apparatus used for the novel tank test. A 1.5 L tank (Aquabiotech Inc., Quebec) was virtually divided into a top, middle and bottom portion. The height of the water remained consistent throughout the experiments at 100 mm with a total volume 1300 mL of water.

2.4. Determination of dieldrin in feed and zebrafish

2.4.1. Extraction, Lipids, Cleanup and Fractionation

The amount of dieldrin in feed pellets and the whole body burden of dieldrin in fish were quantified using the same method. Three glass vials for each treatment were filled with whole bodies of zebrafish with hypothalamus removed (about ~15 fish per vial) and were taken for dieldrin measurements. The tissue was freeze dried for 48 hrs, percent moisture was calculated, and the tissue was homogenized using a mortar and pestle. Approximately 2 g of dry zebrafish homogenate (or 10 g of dried feed) was weighed into an accelerated solvent extraction cell (ASE300: Dionex, Sunnyvale, CA, USA) and then filled with Ottawa Sand (Fisher Scientific). The purpose of the sand in the method spike was to ensure that the cells were full to allow for an efficient extraction. The sample was spiked with 100 μ L of surrogate containing PCB 209 (AccuStandard, New Haven, CT, USA). Samples were extracted using an accelerated solvent extractor (model ASE 300, Dionex, Sunnyvale, CA, USA) with a mixture of 50:50 dichloromethane (DCM)/hexane at 1500 psi and 125°C for 10 min. The solvent was released into the collection bottle and then the instrument was set to 1500 psi was repeated a second time. The sample was then flushed with 60% of the vessel volume and then purged with nitrogen for 300 sec. to remove trace sample from the vessel and lines.

The extract was concentrated with a Büchi Rotavapor R200 (Büchi Labortechnik AG, Flawil, Switzerland) and further concentrated with a N-Evap™ 112 nitrogen evaporator (Organomation Associates Inc., West Berlin, MA, USA) to a volume of 6

mL of 50:50 DCM:hexane. A 1 mL aliquot of the extract was removed and used to determine the percent lipid gravimetrically. The other 5 mL was passed through a pre-calibrated automated gel permeation column (GPC: PrepLinc; J2 Scientific, Columbia, MO, USA) packed with Bio-Beads S-X3 (J2 Scientific, Columbia, MO, USA). The sample was eluted through the column with 50:50 DCM:hexane to remove lipids from the sample. The collected sample was concentrated with a Büchi Rotavapor R200 and further concentrated with a N-Evap™ 112 nitrogen evaporator to 1 mL. The 1 mL of extract was added to a packed column of 8 g of 1.2 % deactivated Florisil (Fisher Scientific, Ottawa, ON, Canada) and anhydrous sodium sulfate (Fisher Scientific, Ottawa, ON) with HPLC grade water (Fisher Scientific, Ottawa, ON, Canada). Samples were then eluted with a series of non-polar to polar solvents. First, 32 mL of hexane was eluted and collected as Fraction 1. Following this, 48 mL of 15:85 dichloromethane and hexane was eluted to the column, and collected in a separate flask as Fraction 2. Lastly, 70 mL of 50:50 dichloromethane:hexane was eluted and collected in a third flask as Fraction 3. Fraction 1 contained the surrogate PCB 209 while Fraction 2 contained dieldrin. Approximately 1 mL of isooctane (Caledon Laboratory Chemicals, Georgetown, ON, Canada) was added to each fraction and then the entire fraction was concentrated to a volume of approximately 1 mL using the Büchi Rotavapor R-200 and the N-Evap™ 112 nitrogen evaporator. Each fraction then had 10 µL of pentanitrobenzene (AccuStandard®, Inc, New Haven, CT, USA) added as an internal standard for internal calibration. Final sample concentrations were adjusted to compensate for the 1 mL removed for % lipid determination.

2.4.2. Quantification GC-ECD

Individual fractions were analyzed on an Agilent 6890N GC with a ^{63}Ni Electron capture detector (ECD) (Agilent Technologies, Mississauga, ON, Canada). Exactly 1 μL of sample was injected onto a HP-5 60 m x 0.25 mm x 0.25 μm column (Agilent Technologies, Mississauga, ON, Canada). The internal calibration technique was used for quantifying dieldrin with pentanitrobenzene as the internal standard (USA EPA Method 8081B). The chromatograms were evaluated and quantified using the Enhanced ChemStation MSD ChemStation version D.03.00.611 (Agilent Technologies, Mississauga, ON, Canada).

2.4.3. Quality Assurance/Quality Control

Method blanks (MBs and method spikes (MSs)) were assessed with each batch of 10 samples. The method spikes, which consisted of 10 g of Ottawa sand, were processed in a similar manner to the samples. Method spikes were comprised of 100 μL surrogate containing PCB 209 and 100 μL of dieldrin standard. Method blanks were comprised of 100 μL of surrogate only. Percent recoveries for PCB 209 and dieldrin were determined. All extracts were analyzed by high-resolution gas chromatography with electron-capture detection using a 60-m 0.25-mm inside diameter DB-5 column (Hewlett-Packard, Palo Alto, CA, USA) with H_2 carrier gas to ensure absolute dieldrin was quantified. Quantitation and peak identification was done using Varian Instruments Star software (Palo Alto, CA, USA).

Surrogate recoveries in feed ranged from 81% to 128% with no correlation to the level of dieldrin (data not presented). A certified reference material (CRM) was analyzed with samples and recovery of dieldrin ranged from 78% to 125% while method

spike recovery ranged from 76% to 99.5%. The detection limit was assigned to be the lowest point in the calibration curve (0.001 µg / g) and this was considered to be the reporting limit. Control feed contained no detectable dieldrin and values were set to the limit of detection of the assay (0.001 µg dieldrin/ g d.w. feed) whereas mean dieldrin in feed was 0.03, 0.15, 1.8 µg dieldrin/g d.w. feed for the low, medium and high dose, respectively. The treatments were significantly different from the control group ($F_{3,5} = 997.5$; $p < 0.0001$) following a Dunnett's post hoc test (Figure 2A).

Recovery of surrogate (PCB209) ranged from 66.8% to 124.4% with no correlation between dieldrin concentration in the zebrafish and recovery percentage of surrogate. The CRM recovery, method spike, and method blank for body burden samples were 103.2%, 105.1% and 126.9% (N=1 for each), respectively. The reporting limit was assigned based upon the lowest point in the calibration curve (0.0011 µg /g) multiplied by 5 as there was 5 times less tissue per weight sample than in the feed samples. The detection limit of the assay was therefore 0.0055 µg /g. The measured dieldrin in zebrafish was below the limit of detection for control samples, and was 0.011 in low, 0.15 in medium and 1.8 in high µg dieldrin / g dry weight (Figure 2B). The reporting limit for the instrument was set at 0.001 µg / g for feed and 0.005 µg/g for body burden.

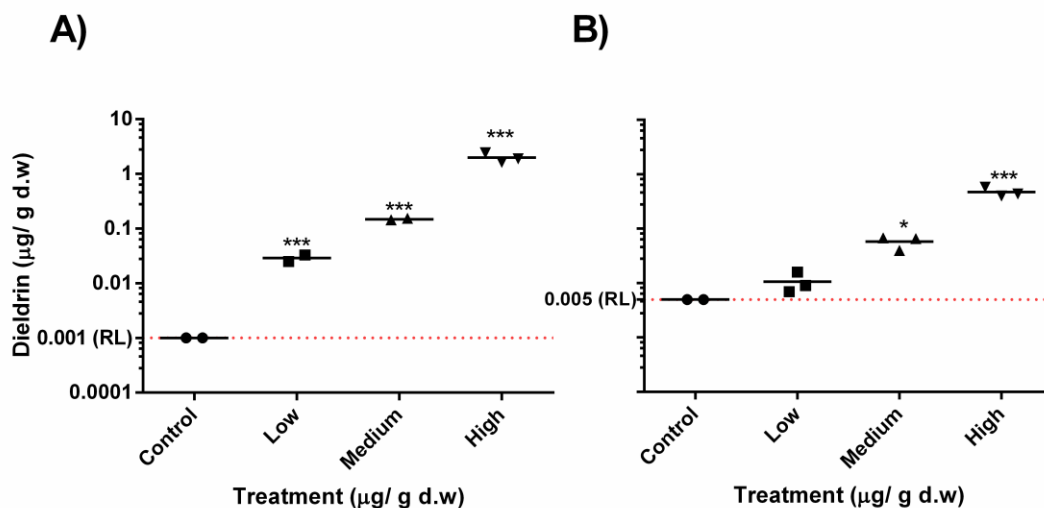


Figure 2: (A) Dieldrin concentration in fish pellets (μg dieldrin/ g food (dry weight)) in control, low, medium, and high treatment groups after preparation. Each treatment group was different from controls ($p < 0.0001$, $N = 2-3$ per group). Dieldrin was below the reporting limit (RL) in control pellets ($\text{RL} = 0.001 \mu\text{g/g}$). (B) Concentrations of dieldrin in zebrafish after 21 days of feeding ($N = 2-3$ per group). Body burdens of zebrafish in the lowest treatment were not significantly different from controls whereas carcasses from the medium ($p < 0.05$) and highest ($p < 0.001$) dose were significantly different from control fish. The reporting limit for dieldrin was $0.005 \mu\text{g/g}$. $p < 0.0001 = ***$; $p < 0.001 = **$; $p < 0.05 = *$. Data are presented as log₁₀ transformed data.

2.5 Microarray analysis

2.5.1. RNA extraction:

RNA was extracted using the Qiagen RNeasy® Mini Kit (Qiagen). All buffers are proprietary to the supplier and the identity of reagents and their concentrations have not been released, thus these are referred to as per the kit. To each biological replicate, 350 μ L of Buffer RLT was added and the tissue pool was homogenized using a Omni-VWR tissue homogenizer. After homogenization, 350 μ L of 70% ethanol was added and mixed well by pipetting. Total lysate was transferred to an RNeasy Mini spin column and centrifuged at 9000 g for 15 s. Following this, the flow-through was discarded. The collection tube of the spin column was replaced and 700 μ L of Buffer RW1 was added and the columns centrifuged at 9,000 g for 15 s. The flow-through was discarded and 500 μ L of Buffer RPE was added to the columns, and the columns were centrifuged at 9,000 g for 15 s. After discarding flow-through, a second wash of 500 μ L of Buffer RPE was added to the spin column and centrifuged at 9,000 g for 2 min and flow through was discarded. Following this, samples were centrifuged for an additional 1 min at 9,000 g with a new collection tube. The RNA was eluted by adding 20 μ L of RNase-free water directly onto the spin column membrane, followed by centrifugation for 1 min at 9,000 g. Samples were then measured for quantity and quality using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

2.5.2. RNA quantity and quality using Nanodrop and 2100 Bioanalyzer

The quality (i.e. integrity) of the RNA samples was evaluated using the Agilent RNA 6000 Nano Assay Protocol (Agilent) and 2100 Bioanalyzer, which generates a RNA Integrity Number (RIN, Schroeder et al., 2006). Briefly, 550 μL of RNA 6000 Nano gel matrix (Agilent) was added to a spin column in order to be filtered. The gel matrix was centrifuged for 10 minutes at 15,000 g at room temperature. After equilibrating the RNA 6000 Nano dye concentrate and RNA 6000 Nano dye marker for 30 min at room temperature, 1 μL of the concentrate was added to a 65 μL aliquot of the filtered RNA 6000 Nano gel matrix. The solution was then vortexed and centrifuged for 10 min. at 13,000 g at room temperature. An RNA 6000 Nano chip was placed on the chip priming station and 9 μL of matrix/concentrate dye mix was placed into the designated well. The syringe plunger was positioned to 1 mL and the chip priming station was closed. The plunger was lowered until clipped into place, and the plunger remained in that position for 30 sec. The clip was then released and slowly pulled back into the 1 mL position on the syringe. This was followed by the addition of 5 μL of marker, followed by adding 1 μL of each sample and an RNA ladder into the designated wells on the microchip. The chip was then vortexed for 1 minute in an IKA vortexer (Agilent) at 2400 rpm, and then placed in the 2100 Bioanalyzer. The mean ($\pm\text{SD}$) RNA Integrity Number (RIN) was 9.0 (± 0.7) for female hypothalami pools and samples ranged in RIN values from 7.5 – 9.7.

2.5.3. Gene expression analysis using a ZF microarray

Microarray analysis was performed on hypothalami collected from females in 0 μg dieldrin / g (N=5), 0.15 μg / g (N=5), and 1.8 μg / g (N=6) groups; the 0.03 $\mu\text{g}/\text{g}$ dieldrin treatment was not used for microarray analysis as the level of dieldrin in the fish was not significantly different from control. Microarray analysis was performed according to manufacturer's protocols (Agilent Low RNA Input Fluorescent Linear Amplification Kit and Agilent 60-mer oligo microarray processing protocol, Agilent). The Agilent Zebrafish platform (V3, Catalog ID: G2519F-02647, Agilent) was used to measure the hypothalamic transcriptome and hybridization followed established protocols. Following 17 hours of hybridization, slides were removed from the incubation oven and washed according to the protocol. An ozone-barrier slide cover was placed on each slide prior to scanning. Microarray slides were scanned by Agilent DNA Microarray Scanner with SureScan high-resolution technologies. Raw expression data along with *tif* images were extracted by Agilent Feature Extraction Software (v10.7.3.1). All microarray data reported in this study follow established guidelines "Minimum Information About a Microarray Experiment (MIAME)" (<http://www.ncbi.nlm.nih.gov/geo/info/MIAME>) and are located in the Gene Expression Omnibus (GEO, Gene Ontology Consortium, 2000).

Differentially-expressed genes (DEGs) were identified by first importing raw intensity data into JMP® Genomics (v6). Intensity data were normalized using locally weighted linear regression (LOWESS) with a smoothing factor of 0.2 to account for technical variability across slides (Smyth & Speed, 2003; Berger et al., 2004). The arrays were quality control checked using a distribution analysis that plots the intensity

distributions of each microarray slide to ensure these distributions are relatively equal. Box plots of normalized intensity also showed that the intensity distribution was not different across slides (data not shown). Normalized intensity data were then filtered based on the limit of detection of the microarrays and any value falling below the limit of detection of 2.08 was assigned a value of 2.08. The limit of detection was determined by calculating the mean of all the dark corners on the microarrays. This accounts for any background signal on the slides. A one-way ANOVA, followed by a multiple test correction using the non-permutation based Benjamini and Hochberg method (Benjamini & Hochberg, 1995) was used to determine which transcripts significantly differed in the hypothalamus.

The number of gene probes differentially expressed at both $p < 0.05$ and those differentially expressed after a post-hoc correction are reported. All p-values reported as “p=” are unadjusted p-values and p-values reported as “FDR p =” are p-values adjusted by a false discovery rate. FC stands for fold change. All expression data were deposited into Gene Expression Omnibus, an open source repository for transcriptomics data.

A hierarchical cluster analysis was performed using differentially expressed probes ($p < 0.05$) from the 0.15 $\mu\text{g} / \text{g}$ and 1.8 $\mu\text{g} / \text{g}$ treatments. The Fast Ward’s method was applied (Milligan, 1980) to cluster normalized expression data in JMP v6. The Fast Ward’s method calculates the distance between two clusters using ANOVA sum of squares added from all variables. At each generation of the cluster, the within-cluster sum of squares is minimized over all partitions obtainable by merging two clusters from the previous generation. Under the assumptions of multivariate normal

mixtures, spherical covariance matrices, and equal sampling probabilities, the Ward's method joins clusters on each level of the hierarchy using maximum likelihood (Milligan, 1980).

2.5.4. Parametric analysis of gene set enrichment

Bioinformatics methods improve analysis of gene expression datasets by developing new computational pipelines and algorithms. Gene ontologies, which are a standardized vocabulary of biological terms, are commonly used to describe effects of chemical treatments on molecular function, cellular location, and biological processes (The Gene Ontology Consortium 2000). Gene set enrichment analysis (GSEA) is a bioinformatics technique that uses gene ontology or other gene classification and a Fisher exact test to determine if there are differences in the proportion of differentially expressed genes within a list compared to that expected by chance. Gene set enrichment analysis (Subramanian et al 2005) improves insight into cell processes altered by environmental pollutants, such as neuroactive pharmaceuticals (Thomas et al 2012).

Parametric analysis of gene set enrichment (PAGE) was performed in JMP Genomics 6.0® (Kim & Volsky, 2005). This analysis is used to determine enriched gene ontologies or gene sets. Parametric analysis of gene set enrichment (PAGE) uses a normal distribution for statistical inference, thereby requiring less computational effort than GSEA. PAGE analysis uses a Z-test of all known gene ontologies and their degree of statistical significance (i.e. a p-value) to identify enriched or over-represented ontologies. Analyzing expression profiles within an ontology (i.e. a group) can provide functional information on how the chemical is affecting the organism. A false discovery

rate for multiple hypothesis testing was set to $p = 0.05$. A false discovery is the proportion of the rejected null hypotheses which were incorrectly rejected (Benjamini & Hochberg, 1995). The negative \log_{10} transformed false discovery rate (FDR) p-value was 3.87. Mean p-values for a gene set (S_m), overall mean of the dataset (μ), standard deviation (δ) and gene set size (m) was calculated. A PAGE Z-score was calculated for each gene set from input p-values of the difference between the control group versus the two groups with the highest doses of dieldrin. The Z score calculation was $Z = (S_m - \mu) * m^{1/2} / \delta$ (Kim & Volsky, 2005). An example of how this analysis is conducted is as follows; the gene ontology term GO:0070469 (respiratory chain) had 24 probes associated with it on the microarray. The mean p-value for all 24 probes in this ontology was 0.26, and the overall mean p-value of all gene ontologies in the entire dataset was 0.47 (standard deviation = 0.03). Thus, this group of probes is lower than the mean. In this case, the calculated Z-score was 3.48, which corresponds to a p-value < 0.001 . Thus, genes in the respiratory chain showed lower p-values as a group than that expected by chance, based on the mean p-value of all gene ontologies. Thus, genes in the respiratory chain were “enriched” in the hypothalamus of zebrafish in the 0.15 $\mu\text{g} / \text{g}$ treatment compared to controls. A gene ontology term would not be enriched if the distribution of p-values for genes within that ontology were randomly distributed across the entire probe dataset.

2.5.5. Sub-network Enrichment Analysis

Sub-network enrichment analysis (SNEA) was performed in Pathway Studio 9.0 (Ariadne, Rockville, MD, USA) using the ResNet 9.0, a mammalian database curated by

Ariadne (Nikitin et al 2003). SNEA identifies gene networks related to cellular processes or diseases that change with a treatment or disease. A total number of 20,902 gene probes for zebrafish were successfully mapped to mammalian homologs using the official gene Name + Alias function. SNEA was performed to identify gene networks that were significantly affected by the dieldrin treatments. Both cell process and disease sub-networks were queried and there were 500 permutations of the data to generate the distributions. Briefly, SNEA uses known relationships among genes (e.g. relationships based on co-expression patterns, binding, or involvement in common pathways) to build networks focused around gene hubs. These interaction maps are generated using information from the ResNet 9 database. The database contains over 20 million PubMed abstracts and approximately 2.4 million full-text articles (September 22, 2014). Thus, these are pre-defined molecular networks based on literature (i.e. it is the background or reference group). A gene list is then imported into the program along with fold changes and p-values for each probe, and the signed fold change (relative to a control group) for each probe is mapped onto the networks. The algorithm also considers the probe p-value. A statistical comparison between the experimental sub-networks mapped to known networks and the entire background of known networks (reference group) is conducted using a Mann-Whitney U-Test and a p-value is generated that indicates the statistical significance of difference between two distributions (additional details on the method can be found in the technical bulletin pg. 717 from Pathway Studios 7.0). If there are many genes involved in a defined network that all show low p-values, more so than by random chance, then this network is an enriched network that is affected by dieldrin. A median fold change for the entire network can

then be obtained as the fold change for individual genes in the network are known (if present on the microarray). A network that has an equal number of genes with both “low” and “high” p-values (indicating a random distribution in the network) would not be preferentially affected by dieldrin. The enrichment p-value for a gene set was set at $p < 0.05$.

It is pointed out here that this thesis does not present all data from the microarray or pathway analysis but rather presents a selection of relevant processes related to the known mode of action of dieldrin and those processes most prevalent (such as in the case of pathways related to the immune system). However, all data generated during this research will be submitted as an Appendix in any subsequent publications.

3.0. Results

3.1. Body burden of dieldrin in zebrafish whole bodies

The mean (\pm SEM) concentrations of dieldrin in zebrafish was 0.006 (\pm 0.006) (detection limit), 0.011 (\pm 0.005), 0.058 (\pm 0.016) and 0.473 (\pm 0.097) μg dieldrin /g d.w for individuals fed the control, low, medium, and high dose feed, respectively (Figure 2B). The body burden level of dieldrin in individuals the fed low-feed treatment was not significantly different from control animals but was above reporting limits of the analytical assay. Both the medium and high dose treatments were different than control fish ($p < 0.05$) and there was a significant increase in dieldrin concentration in the zebrafish with dose ($F_{(3,8)} = 20.65$; $p < 0.001$). Mean surrogate recovery, MSs and CRMs were 80 % (\pm 6.5, $n = 13$), 99% and 78%, respectively.

3.2. Behavioural responses

Over the course of the 180 second novel tank test was there any significant changes in latency to first exit bottom, time spent in bottom and time spent in middle either between treatments or sexes. Latency to first exit from the bottom of the novel tank was not different across sexes ($F_{(1,55)} = 2.55$; $p = 0.12$) or treatments ($F_{(3,55)} = 0.58$; $p = 0.63$) (Figure 3A), with no trends in the data observed. The fish spent the majority of time in the bottom of tank but there was no significant differences between sexes ($F_{(1,62)} = 3.32$; $p = 0.07$) or treatment ($F_{(3,62)} = 0.71$; $p = 0.86$) (Figure 3B). Time spent in middle was not significantly different between treatments ($F_{(3,62)} = 0.73$; $p = 0.54$) and sexes ($F_{(1,62)} = 2.48$; $p = 0.12$) (Figure 3C). The latency to first entering the top portion of the tank had a significant interaction effect between sex and treatment ($F_{(3,36)} = 3.15$; $p = 0.04$) with a significant increase in latency to first entering into the top portion of the tank in males fed the low dose of dieldrin (d.f.= 36; $t = 2.52$; $p = 0.02$) compared to males fed control pellets (Figure 3D). However, no behavioural endpoint was significant after a correction for multiple comparisons using a Bonferroni test.

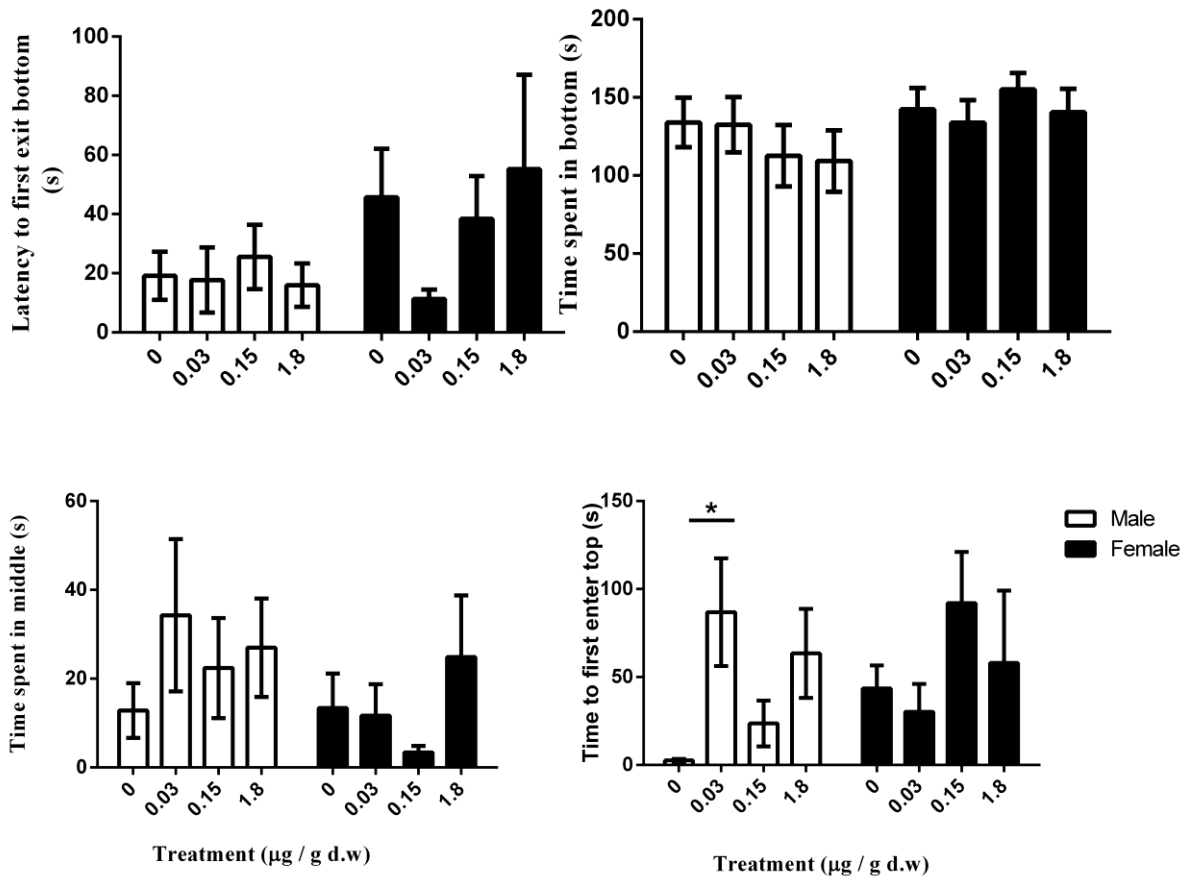


Figure 3: The behaviour of zebrafish did not significantly differ between treatments or sexes for latency to first exit bottom, time spent in bottom, and time spent in middle, (in seconds) (A-C). The latency to first entering the top portion of the tank had a significant interaction effect between sex and treatment ($F_{3,36} = 3.15$; $p = 0.04$) with a significant increase in latency to first entering into the top portion of the tank in males fed the low dose of dieldrin ($d.f. = 36$; $t = 2.52$; $p = 0.02$) compared to males fed control pellets (D).

3.3. Microarray analysis and differentially expressed probes in the hypothalamus

Prior to an FDR correction, there were 2470 probes (2453 unique genes) in the hypothalamus that were differentially expressed compared to the control group in the 0.15 µg/g treatment (p-value < 0.05). A total of 1240 probes were down-regulated and 1230 probes were up regulated. Some examples of genes that were differentially expressed included *solute carrier family 30 (zinc transporter), member 1b (slc30a1b)*, FC= -2.74, t= -4.39, p< 0.001), and *solute carrier family 4, anion exchanger, member 1a (slc4a1)*, FC= 2.80, t = 3.74, p= 0.002). Transcript *gamma-glutamylamine cyclotransferase, tandem duplicate 2 (ggact)* was significantly down-regulated in the 0.15 µg / g treatment (FC = -11.08; t = -2.79, p = 0.015) as well as in the 1.8 µg treatment (FC = -6.71; t = -2.31; p = 0.038). Microarray analysis identified 1822 probes (1814 unique genes) in the hypothalamus that were differentially expressed from controls in the 1.8 µg/g treatment (p-value < 0.05).

In the 1.8 µg/g treatment, a total of 1027 probes were down-regulated and 795 probes were up regulated. Some examples of genes that were differentially expressed (fold change from controls = FC) included *solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10 (slc25a10)*, FC= 2.46; t= 2.47; p=0.028) and *solute carrier family 6 (neurotransmitter transporter, glycine), member 5 (slc6a5)*, FC= -2.55, t=-4.08, p=0.001). In both the 0.15 and 1.8 µg/g d.w. treatments, there were 454 unique probes in common (10.6 %, p-value < 0.05) (Figure 5). Differentially expressed genes in common to both doses included *solute carrier family 20, member 1a (slc20a1)*; FC= -3.51; t= -2.25, p= 0.042) and *glutathione S-transferase, C-terminal domain containing (gstcd)*, FC= -2.61, t= -2.26, p= 0.041). There were no significant changes in any transcript following an FDR post-hoc correction. When clustering

the probes ($p < 0.05$), each of the three treatment groups separated out into individual clades in the hierarchical cluster (Figure 4). This suggests that overall there was an effect of dose on the hypothalamic transcriptome.

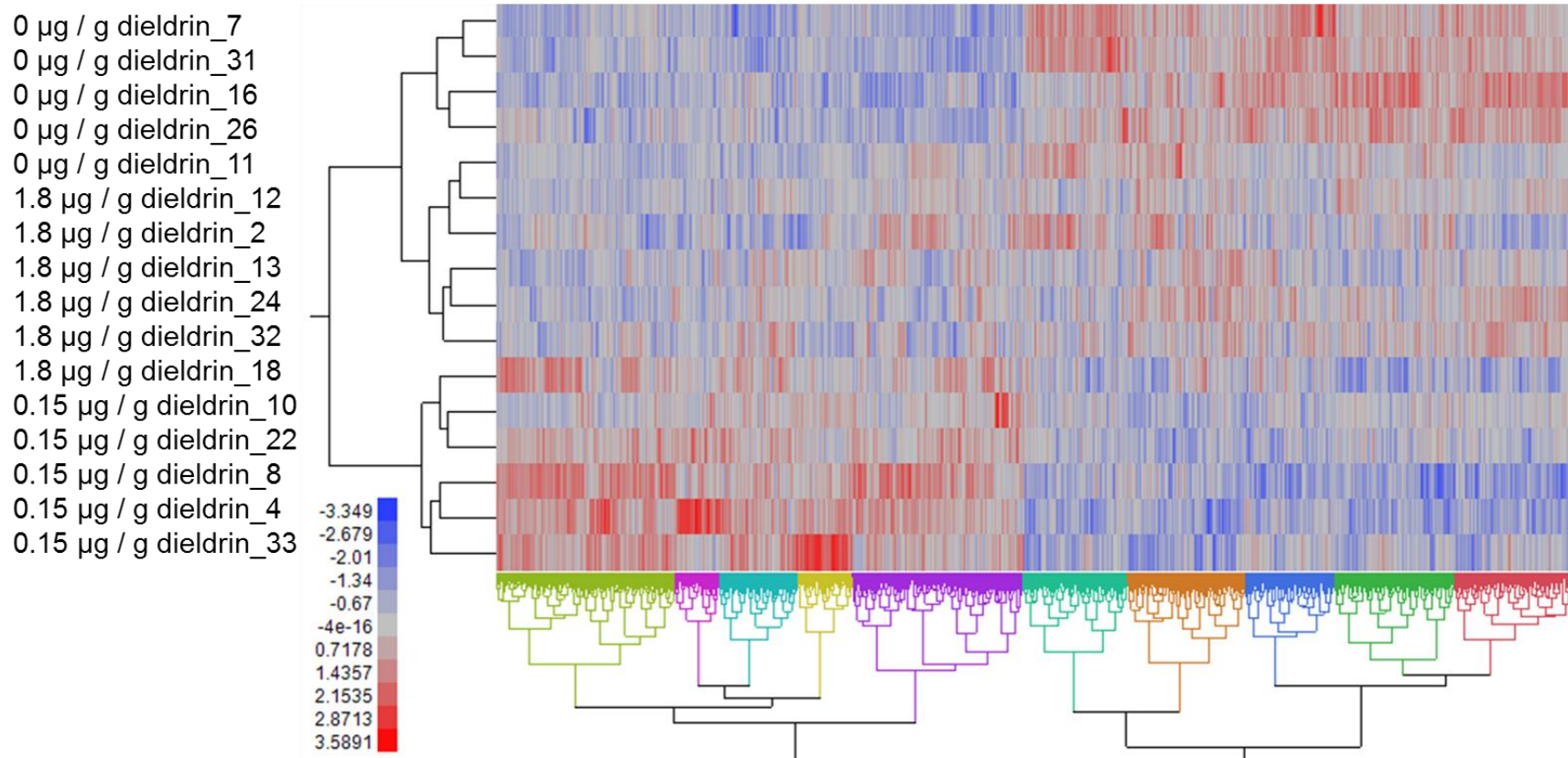


Figure 4: Hierarchical cluster analysis of differentially expressed transcripts showed separate clades for each treatment group. The first clade to separate was 0.15 µg/g d.w. treatments from 1.8 µg/g d.w. and 0 µg/g d.w. treatments. Those two treatments separated in the next clade. The blue to red scale bar is the z-score transformed intensity with dark blue being the most negative (lowest intensity) and dark red being the most positive (highest intensity). The x-axis depicts all unadjusted significant p-values ($p < 0.05$) transcripts measured on the microarray, they grouped into 10 clades. The y-axis depicts the 16 samples grouped into the three treatment groups.

3.3.1. Parametric analysis of gene set enrichment analysis

Parametric analysis of gene set enrichment analysis (PAGE) analysis was used to determine if there were any gene ontology terms (e.g. biological processes, molecular function, and cellular components) that were preferentially expressed in the hypothalamus following dieldrin exposure. In both treatments, there were 14 gene ontologies that were differentially expressed after FDR adjustment and 565 out of 2841 gene sets were differentially expressed before FDR adjustment. In the 0.15 $\mu\text{g/g}$ treatment, a total of 6 gene sets were over-represented including hemoglobin complex ($z = -5.25$, FDR $p = 2.21e^{-4}$), oxygen binding ($z = -5.05$, FDR $p = 5.04e^{-4}$); oxygen transport ($z = -4.93$, FDR $p = 7.74e^{-4}$), oxygen transporter activity ($z = -4.7$, FDR $p = 1.85e^{-3}$), mitochondrial membrane ($z = -4.23$, FDR $p = 0.01$), and intermediate filament ($z = -4.17$, FDR $p = 0.01$). In the 1.8 $\mu\text{g/g}$ treatment, a total of 8 gene sets were over-represented in the hypothalamus and these included lipid transporter activity ($z = -7.09$, FDR $p = 7.8e^{-9}$), lipid transport ($z = -5.93$, FDR $p = 8.69e^{-6}$), DNA photolyase activity ($z = -5.78$, FDR $p = 1.48e^{-5}$), response to estrogen stimulus ($z = -4.7$, FDR $p = 1.85e^{-3}$), DNA-dependent negative regulation of transcription ($z = -4.22$, FDR $p = 0.01$), signal complex assembly ($z = -4.05$, FDR $p = 0.01$), mitochondrial membrane ($z = -4.05$, FDR $p = 0.02$), and translation elongation factor activity ($z = -4.0$, $p = 0.02$).

Mitochondrial dysfunction was a major theme affected at the transcript level, based on the PAGE analysis, as a number of gene ontologies related to this process were over-represented in both treatments. Three over-represented gene ontologies that were related to the mitochondria and were affected in both treatments included mitochondrial

membrane (GO: 0031966), mitochondrial matrix (GO: 0005759), and adenosine triphosphate (ATP) synthesis coupled electron transport (GO: 0042773). Mitochondrial-related gene ontologies that were identified in the 0.15 µg/g treatment group included the respiratory chain (GO: 0070469), electron transport chain (GO: 0022900), hydrogen ion transporting ATP synthase activity (GO: 0046933), nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) activity (GO: 0008137), mitochondrial electron transport, NADH to ubiquinone (GO: 0006120), proton-transporting ATP synthase complex, catalytic core F (GO: 0045261), mitochondrial envelope (GO: 0005740), proton-transporting v-type ATPase, v0 domain (GO: 0033179), mitochondrial iron ion transport (GO: 0048250), and mitochondrial respiratory chain complex IV (GO: 0005751). Unique gene ontologies identified in the 1.8 µg/g treatment were proton-transporting ATP synthase complex, coupling factor F(o) (GO: 0045263), mitochondrial transport (GO: 0006839), mitochondrial part (GO: 0044429), mitochondrial electron transport, ubiquinol to cytochrome c (GO: 0006122), NADH dehydrogenase activity (GO: 0003954), ATP synthesis coupled proton transport (GO: 0015986), and mitochondrion (GO: 0005739) ($p < 0.05$), suggesting that genes related to different regions of the mitochondria may be affected by dieldrin. Between the two treatments, a total of 565 (268 in 0.15 µg/g and 297 in 1.8 µg/g) gene sets were differentially expressed (unadjusted p -value < 0.05) from a total of 2841 gene sets. There were 41 gene sets that were identified as differentially affected in both doses ($p < 0.05$) (Figure 5).

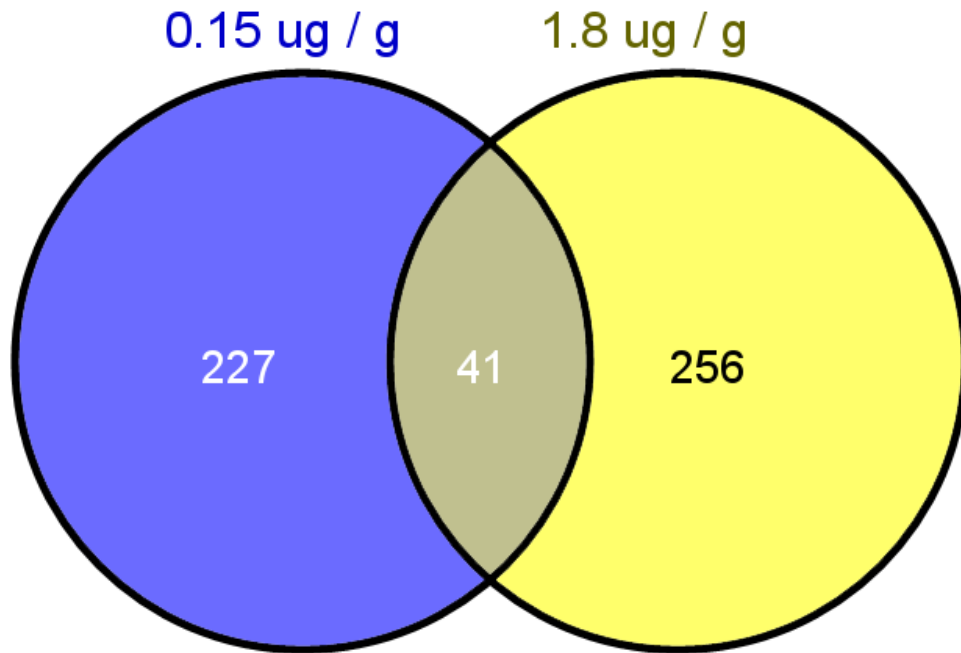


Figure 5: Venn diagram representing the relationship of differentially expressed transcripts after 21 day exposure of zebrafish to 0.15 µg dieldrin/g d.w. and 1.8 µg dieldrin/g d.w.

3.3.2. Cell signaling pathways identified by Gene Set Enrichment Analysis

Gene set enrichment analysis (Pathway Studio) revealed that T-cell receptor signaling pathways were preferentially down-regulated in both treatments. Other pathways down-regulated included activator protein 1 (AP-1), activating transcription factors (ATF) / cAMP response element (CREB), CREB-binding protein (CREBBP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), nuclear factor of activated T-cells (NFATC), and signal transducers and activator of transcription (STAT) (Table 1). The signaling pathway showing the highest degree of down-regulation was STAT signaling, with a median fold change of -1.23 ($p = 0.012$) and -1.33 ($p < 0.001$) in the 0.15 $\mu\text{g/g}$ and 1.8 $\mu\text{g/g}$ treatments, respectively. The median fold change of the “T Cell activation” pathway was - 1.07 ($p = 0.012$) and - 1.06 ($p < 0.001$) in the 0.15 $\mu\text{g/g}$ and 1.8 $\mu\text{g/g}$ treatments, respectively.

Interleukin signaling pathways were down-regulated in both treatments, and there were 4 signaling pathways down-regulated in the 0.15 $\mu\text{g/g}$ d.w. treatment and 11 signaling pathways down-regulated in the 1.8 $\mu\text{g/g}$ d.w. treatment (Table 2). NF – κ B / NFATC mediated by IL15 R was down-regulated in the 0.15 $\mu\text{g/g}$ d.w. treatment (FC = -1.14, $p = 0.041$) and in the 1.8 $\mu\text{g/g}$ d.w. (FC = -1.11, $p = 0.008$) treatment. STAT signaling mediated by the IL21R was down-regulated in both treatments with a FC = - 1.30 ($p = 0.013$, 0.15 $\mu\text{g/g}$ treatment) and FC = -1.32 ($p = 0.002$, 1.8 $\mu\text{g/g}$ d.w. treatment). The STAT signaling pathway was also down-regulated by IL4R in both treatments with a FC = -1.19 ($p = 0.023$, 0.15 $\mu\text{g/g}$ treatment) and FC = -1.30 ($p = 0.008$, 1.8 $\mu\text{g/g}$ treatment) (Figure 6). The FOXO / NF – κ B signaling pathway was also down-

regulated in both treatments with a FC = -1.18 ($p = 0.019$, 0.15 $\mu\text{g/g}$ treatment) and FC = -1.12 ($p = 0.020$, 1.8 $\mu\text{g/g}$ treatment). Entities related to neuromodulators were significantly down-regulated in the 0.15 $\mu\text{g/g}$ (FC = -1.11, $p < 0.001$) and 1.8 $\mu\text{g/g}$ (FC = -1.04, $p = 0.002$) treatments. The group of neurotransmitter uptake entities were significantly down-regulated in the 0.15 $\mu\text{g/g}$ (FC = -1.13, $p = 0.002$) and 1.8 $\mu\text{g/g}$ (FC = -1.09, $p = 0.036$) treatments. The molecular entities involved in estrogen and androgen metabolism were, as a group, significantly down-regulated in the 0.15 $\mu\text{g/g}$ (FC = -1.04, $p = 0.032$) and in the 1.8 $\mu\text{g/g}$ (FC = -1.03, $p = 0.033$) treatments. More than 100 molecular groups or signaling pathways were determined to be differentially affected at the gene level by one or more of the treatments.

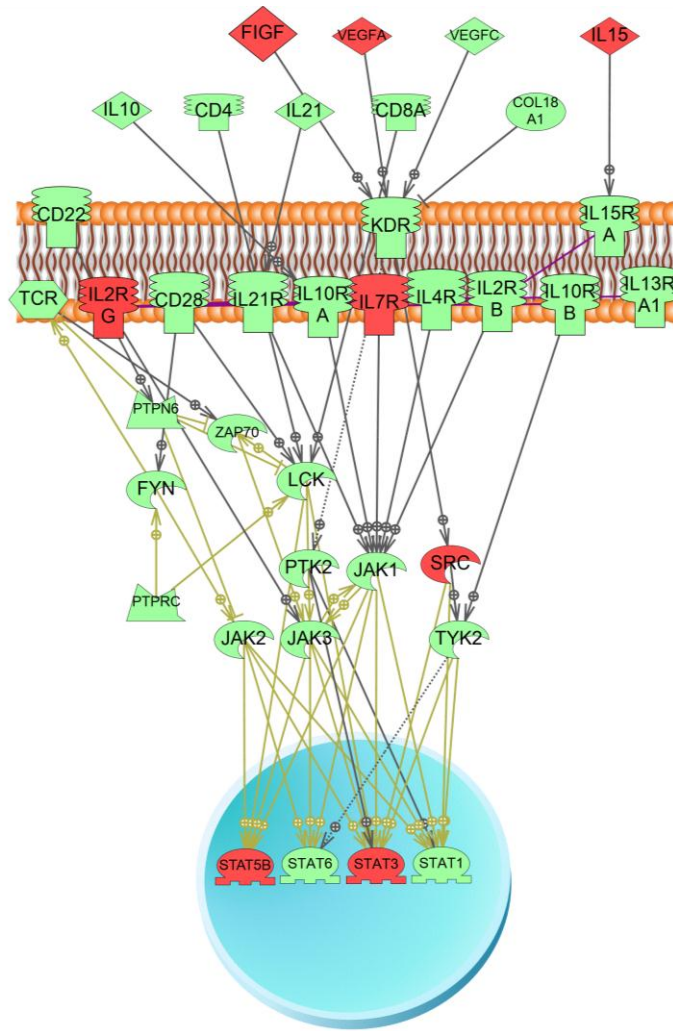


Figure 6: Immune system dysregulation in zebrafish hypothalamus after exposure to 0.15 μg dieldrin/g d.w. and 1.8 μg dieldrin/g d.w.

Table 1: T-cell receptor pathways in zebrafish hypothalamus that showed dysregulation after exposure to 0.15 μg dieldrin/g d.w. and 1.8 μg dieldrin/g d.w. Seven signaling pathways regulated by the T-cell receptor were down-regulated. The total entities are the total number of transcripts in the pathway according to Pathway Studio and measured entities are the number of transcripts measured on the microarray.

Name	Total Entities	Measured Entities	1.8 μg / g treatment		0.15 μg / g treatment	
			Median change	p-value	Median change	p-value
AP-1 signaling	180	48	-1.17	0.021	-1.13	0.01
ATF/CREB signaling	195	59	-1.13	0.005	-1.14	0.004
CREBBP signaling	176	38	-1.21	0.016	-1.19	0.001
NFATC signaling	172	39	-1.21	0.019	-1.16	0.002
NF-kB signaling	176	45	-1.17	0.001	-1.19	<0.001
STAT signaling	142	17	-1.33	<0.001	-1.23	0.012
T Cell Activation	957	553	-1.06	<0.001	-1.07	0.012

Table 2: Hypothalamic signaling pathways in the zebrafish affected at the gene level following exposure to 0.15 µg dieldrin/g d.w. and 1.8 µg dieldrin/g d.w. Interleukin signaling pathways were down regulated in both treatments. There were 4 signaling pathways that were down-regulated in the 0.15 µg/g treatment and 11 that were down-regulated in the 1.8 µg/g treatment. There were 4 signaling pathways down-regulated in both treatments, and these are italicized and underlined in the table. The total entities are the total number of transcripts in the pathway according to Pathway Studio and measured entities are the number of transcripts measured on the microarray. NS = non-significant.

Name	Total Entities	Measured Entities	0.15 µg/g		1.8 µg/g	
			Median change	p-value	Median change	p-value
IL10R -> STAT signaling	8	7	NS	NS	-1.33	0.009
IL13R -> STAT6 signaling	7	6	NS	NS	-1.14	0.015
<u><i>IL15R -> NF-kB/NFATC signaling</i></u>	35	30	-1.14	0.041	-1.11	0.008
IL15R -> STAT signaling	11	10	NS	NS	-1.12	0.001
<u><i>IL21R -> STAT signaling</i></u>	8	7	-1.30	0.013	-1.32	0.002
IL2R -> ELK-SRF/MYC signaling	58	50	NS	NS	-1.06	0.040
IL2R -> STAT signaling	10	7	NS	NS	-1.12	0.003
<u><i>IL4R -> STAT signaling</i></u>	7	5	-1.19	0.023	-1.30	0.008
<u><i>IL7R -> FOXO/NF-kB signaling</i></u>	36	31	-1.18	0.019	-1.12	0.020
IL7R -> STAT signaling	9	7	NS	NS	1.10	0.022
IL9R -> STAT signaling	8	5	NS	NS	-1.12	0.011

3.3.3. Cell processes identified by sub-network enrichment analysis

The immune system was identified by the SNEA analysis (Pathway Studio) as a theme significantly affected at the gene level. Similar to the GSEA analysis for the 0.15 µg/g treatment group, a total of 8 cell processes related to T-cells were down-regulated including lymphocyte proliferation and development. In the 1.8 µg/g treatment, a total of 9 cell processes related to T-cells were down-regulated including T-cell suppression and function. The T-cell processes that were down-regulated in both treatments were T-cell suppression, T lymphocyte proliferation, T-cell response, T-helper lymphocyte response and γ - δ -T-cell proliferation ($p < 0.05$) (Table 3). In addition to these processes related to the immune system, those related to the adaptive immune response, cellular immune response, immunity, and inflammatory response were also all down-regulated at the transcript level in both treatments. All data from the sub-network enrichment analysis for cellular processes are provided upon submission for publication, and all supplemental data will be available online. In summary, genes involved in immune system networks were preferentially decreased in both treatment groups.

Table 3: Isolated hypothalamus from zebrafish treated with 0.15 $\mu\text{g/g}$ d.w. or 1.8 $\mu\text{g/g}$ d.w deregulated cell processes in T-cells. In the 0.15 $\mu\text{g/g}$ treatment, 8 cell processes related to T-cells were down-regulated including lymphocyte proliferation and development. A total of 9 cell processes related to T-cells were down-regulated in the 1.8 $\mu\text{g/g}$ treatment. The total entities are the total number of transcripts in the pathway according to Pathway Studio and measured entities are the number of transcripts measured on the microarray. NS = non-significant.

Name	Total Entities	Measured Entities	0.15 $\mu\text{g/g}$ treatment		1.8 $\mu\text{g/g}$ treatment	
			Median change	p-value	Median change	p-value
T cell aggregation	17	7	-1.35	0.009	NS	NS
T cell suppression	68	45	-1.1	0.014	-1.09	0.006
T lymphocyte proliferation	604	391	-1.09	0.001	-1.09	<0.001
T-cell function	428	275	NS	NS	-1.04	<0.001
T-cell homeostasis	100	66	NS	NS	-1.07	0.002
T-cell response	567	326	-1.07	0.004	-1.06	0.005
T-cell selection	27	16	NS	NS	-1.12	0.012
T-helper lymphocyte response	130	80	-1.07	0.004	-1.07	0.016
Thymic T-cell selection	64	35	NS	NS	-1.17	0.005
γ - δ -T-cell proliferation	10	8	-1.27	0.009	-1.24	0.006
T-cell development	542	375	-1.07	0.009	NS	NS
T-helper 2 cell differentiation	60	40	-1.14	0.005	NS	NS

3.3.4. Diseases identified by Sub-network Enrichment Analysis

Transcript profiling revealed that there were 183 sub-networks related to diseases that were preferentially affected by dieldrin. A total of 67 disease outcomes were in common between the two treatments. Three immune diseases were identified by sub-network enrichment analysis including T-helper lymphocyte activity, autoimmune diseases, and T-cell dysfunction. Transcripts related to CNS depression were overall up-regulated in the 0.15 $\mu\text{g/g}$ treatment (FC = 1.14, $p = 0.049$) but not in the 1.8 $\mu\text{g/g}$ treatment. A down-regulation of immune complex disease transcripts was observed in the 1.8 $\mu\text{g/g}$ treatment (FC = -1.26, $p = 0.024$). The transcript pathway for T-cell count was down-regulated (FC = -1.30, $p = 0.011$) in 1.8 $\mu\text{g/g}$ treatment. Transcripts of diseases related to the CNS were also regulated in both treatments. Down-regulation of CNS neoplasm transcripts was observed in 1.8 $\mu\text{g/g}$ treatment (FC = -1.09, $p = 0.010$). It should be pointed out that the analysis does not indicate that dieldrin is the cause of these diseases but rather, that transcripts related to these diseases are influenced by dieldrin.

4. Discussion

4.1. Quality Control and Assurance

Prior to microarray analysis, it is necessary to determine the quality and integrity of RNA samples. The RIN number reports this on a scale ranging from 1 to 10, with a value of 1 indicating degraded RNA and a value of 10 indicating intact RNA. These are calculated using a number of different metrics, including the ratio area under the curve for the 18s and 28s bands (the most abundant RNA in the cell) as well as the breakdown products in the 5S region of the electropherograms. In comparison with 28S to 18S ratio, the RIN is a strong predictor of microarray quality according to Kiewe et al. (2009) based on a meta-analysis on 484 microarrays. For microarray studies, it is recommended that the RNA quality must be 7.15 or higher (Kiewe et al. 2009). For this study, the mean (\pm SD) RNA RIN was 9.0 (\pm 0.7) and RNA integrity was therefore sufficient for a reliable and robust experiment.

A CRM allows the researcher to analyze dieldrin concentrations in fish tissue, taking into account the complexity of the matrix created by the fish proteins and cellular structure. The CRM, surrogate (PCB209), and method spike recovery of dieldrin ranged from 78.0% to 125.0%, 66.8% to 124.4% and 76.0% to 99.5%, respectively. These numbers were within the allowable limits for qualifications of dieldrin (A. Mercer, personal communication). These high percentages are not entirely unexpected, since the extraction of dieldrin from tissue involves multiple steps. This can increase the variability in the measurements for dieldrin in the fish tissues. The mean percentage

recovery for the internal standards for the assessment of dieldrin and other organochlorine pesticides was 80% for PCB30 (Kidd et al. 2001). In a simplified method using GC-MS, the average recovery ranged from 78.7 % to 113.7 % for 19 pesticides, and dieldrin showed more than 90 % recovery (Chen et al. 2009). The recoveries of OCPs in certified reference material (CRM 430) ranged from 79 to 101 % and dieldrin was recovered at 88 % (Manirakiza et al. 2002). Thus, the recovery of dieldrin in this experiment was comparable to other studies and extraction protocols.

False Discovery Rate is a term referring to the frequency of type I error which is acceptance of the null hypothesis when an effect does not exist. This is different from type II error which is failure to observe an effect when one exists. One of the main issues with FDRs is inadequate samples sizes (Ioannidis et al. 2001, 2003), and this problem exists when the number of variables greatly exceeds the number of samples. This is a significant limitation for microarray studies. In this study, we applied a 5 % FDR, however others in the field of environmental toxicology such as the United State Environmental Protection Agency uses 15-25 % FDR (Villeneuve et al. 2009).

4.2. Accumulation and Biomagnification of Dieldrin

This study reports a significant accumulation of dieldrin in the whole body of zebrafish over a relatively short feeding duration. Dieldrin has been shown to bioaccumulate in the tissue of fish in both laboratory and field studies. In laboratory experiments, LMB fed approximately 3.0 µg dieldrin/g feed for 2 months showed a measured level of 0.036 µg dieldrin/g wet weight in muscle (1-2% of the total feed) with no dieldrin in controls (Martyniuk et al. 2010a). In another laboratory study, LMB

accumulated ~40 % dieldrin of the total dieldrin fed to the fish after either 30 or 50 days of exposure (Muller et al. 2004). Fish collected in the natural environment also accumulate significant levels of dieldrin in tissues. Common carp, grass carp, bighead carp, and yellow catfish sampled from Baiyangdian Lake in China exhibited high levels of dieldrin in the muscle that ranged from 0.098 to 0.432 $\mu\text{g/g}$ lipid weight (Hu et al 2009). In herring (*Clupea harengus*) sampled in the northern part of the Baltic Sea, concentrations of dieldrin were 0.120 $\mu\text{g/g}$ lipid (Strandberg et al 1998). In Bow Lake (Banff National Park, Canada), Mountain whitefish (*Prosopium williamsoni*) and Lake trout (*Salvelinus namaycush*) had mean concentrations of 0.0007 and 0.0023 $\mu\text{g/g}$ wet weight, respectively (Campbell et al 2000). In addition, Chinook salmon (*Oncorhynchus tshawytscha*) returning to the Fraser River from the Thompson River in Canada during the 2007 spawning migration contained dieldrin at levels of ~ 0.001 $\mu\text{g/g}$ lipid wet weight (Kelly et al. 2011a). Farmed and wild salmon in British Columbia, Canada, had dieldrin levels about 0.0001 $\mu\text{g/g}$ wet weight (Kelly et al. 2011b). In the present study, the level of dieldrin in zebrafish was consistent with what has been observed in fish from the Baltic Sea and Baiyangdian Lake, but was higher than that reported for fish collected from Bow Lake in Canada. Thus, our target body concentrations for dieldrin were within the range of environmentally- relevant levels in fish. Although dieldrin is still measured in 100s ng/g level, these levels were still causing immune disruption in the zebrafish hypothalamus. Despite the fact that dieldrin was banned from most developed countries in the 1970s, this pesticide is still detectable in fish tissues collected from the environment.

4.3. Behavioural Changes in Zebrafish Exposed Neuroactive Chemicals

This study found that zebrafish fed a diet with 0.03 µg dieldrin/g d.w. over 21 days showed a significant increase in the time it took to first enter the top portion of a novel tank and this is interpreted as a delay in exploratory behaviour and anxiety.

Behaviour is an important phenotype for studying neuroactive chemicals and screening of psychoactive drugs. For example, zebrafish exposed to 250 µg/L lysergic acid diethylamide (LSD) for as little as 30 minutes significantly increased the time spent in the upper portion of the tank compared to control fish, and decreased their average velocity and freezing time in a novel tank test (Grossman et al. 2010). LSD also affected the duration spent in “white light” and “average entry duration into white light” during a 6 minute light-dark test (Grossman et al. 2010). These findings demonstrate that the psychoactive chemical LSD changes the behaviour of the zebrafish from inhabiting the “safer” (darker or deeper environment) environment to using the entire environment.

Zebrafish exposed to the pharmaceutical chlordiazepoxide showed increased sedation while diazepam and buspirone reduced the amount of time the zebrafish spent at the bottom of the novel tank (Bencan et al. 2009). For GABA_A receptor antagonists, much of the focus on behaviour has been on the larval stages. For example, fipronil was one of the replacement pesticides for dieldrin and a study showed that larvae had decreased motor function as well as notochord degeneration and abnormal axial muscle morphology (Stehr et al. 2006). Although in this study only dieldrin in the lowest dose (0.03 µg dieldrin/g) reduced the time spent in the tank bottom, it is clear that other chemicals, such as the psychoactive pharmaceutical diazepam and LSD, can alter zebrafish exploration behaviour in a novel tank and that it is a meaningful apical endpoint for ecotoxicology

studies. Additional studies are needed to associate behavioural changes to global molecular responses.

4.4. The effects of dieldrin on mitochondrial function

Transcriptomics data suggested that there was a significant effect on the expression of genes related to mitochondrial function and oxidative respiration in the hypothalamus. Transcripts such as ND4, ND1, sdhaf2, cox16, and cox7a1 and gene ontologies such as mitochondrial membrane mitochondrial matrix and ATP synthesis coupled electron transport were differentially expressed after dieldrin treatment, suggestive of mitochondrial disruption. These data are consistent with the mode of action of dieldrin, which acts to inhibit complex III of the mitochondrial electron transport system (Bergen, 1971). Our data in zebrafish are also consistent with controlled feeding studies in LMB, in which proteins related to mitochondria and mitochondrial intermembrane protein transporter complex were significantly affected in the hypothalamus after dieldrin treatments (Martyniuk et al. 2010a). The researchers observed a significant increase in ATP synthase mitochondrial complexes in the LMB hypothalamus following dietary ingestion of dieldrin. In the mitochondria of the mosquitofish (*Gambusia affinis*), dieldrin decreased the activity of succinic dehydrogenase (Moffett & Yarbrough 1972), also known as the respiratory Complex II enzyme involved in cellular respiration. Additional in vitro studies support the hypothesis that dieldrin results in mitochondrial dysfunction in the CNS. In rat dopaminergic neural (N27) cells treated with dieldrin, there was a rapid increase in ROS

followed by a release of cytochrome c into the cytosol (Kanthasamy et al. 2008). Dieldrin may impair mitochondrial function irreversibly by arresting the flow of electrons at or near cytochrome b via NADH dehydrogenase and succinate dehydrogenase, thus lowering cellular ATP production (Bergen, 1971). The changes in transcripts involved in the electron transport chain and oxidative phosphorylation support that idea that dieldrin disrupts the mitochondrial membrane function. A concern for human health is that many neuroactive pesticides target the mitochondria and electron transport system in the brain, thus mitochondria dysfunction is one mechanism by which neuroactive pesticides exacerbated human disease etiologies.

4.5. Dieldrin and other mitochondria disruptors are associated with Parkinson-like symptoms

Dieldrin and other mitochondria disruptors (including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP), paraquat, and rotenone have been associated with Parkinson-like symptoms (Langston & Ballard, 1984; Rathinam et al. 2013). Interestingly in the mouse MPTP, a model for PD, showed increased T-cell infiltration into the substantia nigra which correlated with increasing amounts of neuronal damage (Cztonkowska et al. 2002). It should be pointed out that in zebrafish, these model compounds do not always result in conserved molecular and organismal responses compared to their mammalian counterparts due to different anatomical organization. For example, studies with larval zebrafish and MPTP have revealed that different populations of DA neurons are affected compared to mammals, and this may underlie locomotor behavioural changes observed in other studies (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005; Thirumalai

and Chine, 2008; Wen et al., 2008; Sallinen et al., 2009a,b). A decrease in dopamine, noradrenaline and serotonin levels in larval zebrafish were observed after exposure to MPTP (Sallinen et al., 2009b). Paraquat and rotenone did not decrease locomotion 7 day post-fertilization (dpf) or affect dopaminergic cell staining in larval zebrafish (Breteau et al., 2004). Dieldrin may also be associated to other neurodegenerative diseases such as Alzheimers disease (Martyniuk et al. 2010a). Interestingly, in our study, there was no difference between dieldrin-treated and control fish with respect to genes related to Parkinson's. Individual transcripts related to Parkinson's were not significantly affected by dieldrin. Pathway analysis results from feeding zebrafish dieldrin did not show any indication of malfunction of pathways involved in neurodegenerative diseases, this could have been from the lower exposure time as previous studies with dieldrin usually have a 60 day exposure.

4.6. Dieldrin affects T-cell signaling cascades at the transcript level in the hypothalamus

The T-cell receptor and interleukin receptor signaling pathways were significantly down-regulated in 0.15 and 1.8 $\mu\text{g} / \text{g}$ treatments suggesting that the immune system may be a target for dieldrin neurotoxicity. These cellular processes included T-cell response, T lymphocyte proliferation and T-helper lymphocyte response, and each was down-regulated with both doses of dieldrin examined. Transcriptome profiling in the hypothalamus revealed that dieldrin also affected transcripts involved in T-cell immune function and the immune system. Both epidemiological and ecotoxicological studies provide evidence for compromised immune systems following dieldrin treatments and

organochlorine pesticides (OCPs) appear to act as immuno-suppressers in different taxa. For example, higher concentrations of dieldrin in breast milk fat in Inuit infants lead to an increased incidence of acute otitis media, which is a middle ear infection (Dewailly et al. 2000). After a 10 week exposure to 1 or 5 µg dieldrin/g, BALB/c (Bagg Albino) mice showed increased mortality when a challenged with malaria or *Leishmania* compared to control (Loose, 1982). Dieldrin also induced immunosuppression of primary IgM response to thymo-dependent and T cell-independent antigens in primary sheep blood culture (Bernier et al. 1987). Martyniuk et al. (2010b) demonstrated that feeding dieldrin to LMB induced transcripts such as *coagulation factor XI (f11)*, *toll-interleukin-1 receptor interacting protein II (tlr2)*, *alpha-2-macroglobulin (a2m)*, and *small inducible cytokine subfamily E member 1 (scye1)* in the hypothalamus. This is the first evidence of dieldrin causing immunosuppression in zebrafish, and increases understanding of the mechanisms underlying pesticide-induced immunosuppression.

4.7. Contribution to research and future direction:

To the best of my knowledge, this thesis presents the first study on dieldrin and its effects on behavior in a teleost fish, finding that dieldrin did affect behavior in novel environment exploration as zebrafish fed 0.03 µg/g feed over 21 days showed an increase in time it took to first enter the top portion of a novel tank. There were no other effects observed on the behaviour of the zebrafish exposed to the three doses of dieldrin.

The molecular data here support studies showing that dieldrin affects mitochondrial function. Dieldrin may result in mitochondrial dysfunction in the zebrafish hypothalamus by affecting the mitochondria membrane and complexes I and III. To test

this hypothesis, future studies should expose isolated zebrafish mitochondria to different doses of dieldrin, followed by analyzing the effects of each dose on the electron transport complex and overall ATP production; secondly, one could use isolated tissue or primary neuronal cultures and expose them to different doses of dieldrin while assessing the electron transport chain and ATP production.

Dieldrin has been associated with neurodegenerative diseases and has also been linked to immunosuppression. The data presented in this thesis suggest that one potential mechanism underlying this association is immune system dysfunction. To address the hypothesis that the immune system is affected by dieldrin, an experiment on primary glial cells (immune cell of the CNS) could be performed with dieldrin to determine if there are adverse effects on immune cell responses (e.g. gene expression and protein expression).

Dieldrin has been associated to neurodegeneration through mechanisms of GABA dysregulation and in epidemiological studies. Dysregulation of GABA is shown to affect the viability of neuron and lead to cell death or apoptotic events, the question is whether GABA dysregulation that is mediated by dieldrin antagonism leads to neurodegeneration, or if dysregulation of GABA results in immune function impairment, resulting in neurodegeneration. Another probable cause of Parkinson's disease is dysfunction of the mitochondria, allowing for the cell to create insufficient ATP (energy) thereby causing apoptosis and cell death. Cztonkowska et al. (2002) proposed that PD could be caused by immune system dysregulation, and data collected from our study would demonstrate that dieldrin first causes immune system distress before the damaging the GABA system and causing neuron cell death. To address this question, an *in vitro* model for zebrafish glial

cells could be used as these are the native immune cells of the brain, to determine if dieldrin was in fact changing the regulation of immune function. In addition, an *in vitro* model of zebrafish neurons could be used to determine if dieldrin does not affect the zebrafish GABAergic system as it does in other animals. A third question that would need to address is whether or not the non-neuronal immune system in fact migrating into the brain region. Zebrafish provide a powerful model for this as they can be imaged real time and have transgenic lines with fluorescent tagged immune proteins that would allow for visualization of the immune cells in the brain. In summary, these data offer new research directions for pesticide induced neurodegeneration and suggests that studies focus on mitochondria function an the immune system.

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Appendix Title

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Glossary

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Curriculum Vitae

Candidate's full name: Andrew M. Cowie

Universities attended (with dates and degrees obtained):

2008-2012 University of New Brunswick B.Sc. (Hons) Biology-Psychology

2012-2015 University of New Brunswick M.Sc. Biology

Publications:

Cowie, AM., Wood, RK., Chishti, Y., Feswick, A., Loughery, JR., Martyniuk, CJ. Individual transcript variability and physiological correlates in the fathead minnow ovary: Implications for sample size and experimental power. *Comparative Biochemistry and Physiology: Part B*. Submitted.

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Li, H., **Cowie, AM.**, Johnson, JA., Webster, D., Martyniuk, CJ., Gray, CA. 2014. Can microarray decipher the anti-mycobacterial mode of action of C17 diynes? Maritime Natural Product Conference, Charlottetown, Aug 18-21. Prince Edward Island, Canada (Oral, regional, MSc work).

Martyniuk, CJ., **Cowie, AM.**, Wood, R., Chishti, Y., Mudge J., Loughery, JR., Houlahan, J. 2014. Considerations in variability for molecular endpoints in ecotoxicology. SETAC North America 35th Annual Meeting, November 9-13, Vancouver, BC, Canada.

Martyniuk, CJ., **Cowie, AM.**, Wood, R., Chishti, Y., Mudge J., Loughery, JR., Houlahan, J. 2014. Variability in transcriptomics: Considerations for environmental applications. Aquatic Toxicity Workshop, September 28-October 1, Ottawa, Ont. Canada.

Cowie AM., Loughery JR., Wood RK., Chishti Y., Martyniuk CJ. 2013. Individual gene variability and correlates in the fathead minnow ovary: Implications for critical effect size and experimental power. Oct 18-20. 6th Annual Atlantic Regional Comparative Physiology Workshop. Saint Andrew's, New Brunswick.

Cowie AM., Riazanov A., Baker CJO., Martyniuk CJ. 2013. Meta-analysis of toxicants with Gamma-aminobutyric acid (GABA)_A receptor non-competitive antagonist mode of action and linking them to neurodegeneration. March 26th. UNB Graduate Research Conference, Fredericton, New Brunswick.

Cowie AM., Hindle M, Riazanov A, Goudreau S, Ornostay A, Baker CJO, Martyniuk CJ. 2012. Using bioinformatics to enhance biological information in fish physiology and toxicology. Oct 12-14. 5th Annual Atlantic Regional Comparative Physiology Workshop. Saint Andrew's, New Brunswick.

Martyniuk CJ, **Cowie AM**, Hindle M, Riazanov A, Goudreau S, Ornostay A, Baker CJO. 2012. Novel bioinformatics methods to exploit omics data in fish. Sept 30- Oct 3rd. 39th Aquatic Toxicology Workshop, Sun Peaks, BC.

Martyniuk CJ, Ornostay A, Alvarez S, **Cowie A**, Hindle M, Baker C, Hogan NS, Lo B, Kennedy CJ, Elphick J, Marlatt VL. 2012. Towards better understanding of the mechanisms of action of the ureic based herbicide linuron. SETAC North America 33rd Annual Meeting November 11-15, Long Beach, CA, USA

Cowie AM., Ornostay A., Martyniuk, CJ. 2012. Sub-Network Enrichment Analysis identifies MAP3K1 signalling as a target of the herbicide LIN in *Pimephales promelas* ovary. Mar 2-4. Science Atlantic Undergraduate Biology Conference, Sackville, NB, Canada

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Martyniuk CJ, LougheryJR, Wood RK, Chishti Y, Feswick A, **Cowie AM**. 2015. Transcript variability for genes in the fathead minnow ovary: Implications for molecular reproductive studies. North American Society of Comparative Endocrinology, June 21-26, Ottawa, CAN.

Slade L, Cowie A, Trivedia P, **Martyniuk CJ**, Kienesberger P , Pulinilkunnil T. 2014. Dieldrin augments mTOR signaling and inhibits autophagy in the adult zebrafish heart (*Danio rerio*). The 6th Annual New Brunswick Health Research Conference. Nov 13-14, Moncton, NB, CAN.

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Cowie AM., Riazanov A., Goudreau ES., Hindle MM., Baker CJO., Martyniuk CJ. 2012. Exploitation of OMICS data using Semantic Automated Discovery and Integration (SADI) Web Services for Fish Toxicology. Sept 28-29th. Canadian Rivers Institute Symposia, Quebec City, Quebec.

Cowie AM., Ornostay A., Hindle H., Baker C., Martyniuk CJ. 2012. Support Vector Machine Classification of Linuron as an Androgen or Anti-androgen. Canadian Society of Zoologists, CSZ 2012 Section Symposia. Mount Allison University, Sackville, NB

Cowie AM., Ornostay A., Hindle H., Baker C., Martyniuk CJ. 2012. Support Vector Machine Classification of Linuron's Mode of Action as an Androgen or Anti-androgen. Presented as a partial requirement of Honors thesis according to UNBSJ requirements.