PERSISTENT LOCOMOTOR AND BEHAVIORAL EFFECTS RESULTING FROM

DEVELOPMENTAL EXPOSURE TO PYRETHROID PESTICIDE DELTAMETHRIN

ARE MEDIATED BY DOPAMINERGIC AND SEROTONERGIC SYSTEM

DYSFUNCTION

by

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ABSTRACT OF THE DISSERTATION

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Pyrethroid pesticides are generally considered to be a safer alternative to other classes of
insecticides. However, there is increasing concern that children are more susceptible to
the adverse effects of pesticides. The hypothesis tested in this thesis is that exposure to
pyrethroid pesticide deltamethrin, at concentrations below the LOAEL, during the critical
developmental period would result in persistent behavioral deficits, which are due, in
part, to changes in dopamine and serotonin system gene expression and neurochemistry.
Zebrafish embryos were treated with deltamethrin (0.25 μg/L - 0.5 μg/L) during the
embryonic period (3-72hpf), and then reared in treatment free water until the larval (2-
week) and adult stages. Deltamethrin exposure during development resulted in increased
locomotor activity, decreased *drd1* and *drd2a* transcripts, and increased levels of
dopamine (DA) metabolite, homovanillic acid, at the larval stage. Manipulating the DA
system by concomitant knockdown of the dopamine transporter (DAT) during exposure
rescued deltamethrin induced locomotor activity. Acute methylphenidate (DAT inhibitor)

exposure increased locomotor activity in control larvae but reduced locomotor activity in larvae previously exposed to deltamethrin. These studies indicate that dopaminergic dysfunction mediates the behavioral effects observed in larval zebrafish following deltamethrin exposure during development. Behavioral characterization of adult zebrafish revealed a sex specific response in fish that had been exposed to deltamethrin during development, including increased distance travelled, velocity, bouts of high mobility, and aggression in males, and increased swim velocity, thigmotaxis, and altered rates of habituation in females. In adult males, transcript levels of *serta*, *sertb*, and *drd2a*, positively correlated with the magnitude of aggression. However, this correlation was lost in the population of male fish that had been developmentally exposed to deltamethrin. Fluoxetine exposure attenuated aggression exhibited by dominant male zebrafish demonstrating the involvement of the serotonin system in mediating aggressive behavior. In conclusion, this research demonstrates that environmental influences during critical neurodevelopmental stages results in persistent neurobehavioral deficits. Also, we add to zebrafish ethology using the experimental behavioral paradigms adapted for our laboratory. Finally, we provide a mode of action for deltamethrin induced behavioral deficits which can assist in the cumulative risk assessment of this class of compounds.

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Table of Contents

Chapter 2: Developmental deltamethrin exposure causes persistent changes in dopaminergic gene expression, neurochemistry, and locomotor activity in zebrafish

Chapter 3: The persistent behavioral deficits in adult zebrafish caused by developmental exposure are gender dimorphic

Chapter 4: The effects of developmental deltamethrin exposure on the 5-HT system and social aggression in the adult zebrafish

Chapter 5: General Discussion and Conclusions

List of Figures

List of Tables

Abbreviations

3-PBA	3-phenoxybenzoic acid
5-HIAA	5- 5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTR	serotonin receptor (subtypes 1a, 1b, 2a, 2c)
AAAD	L-amino acid decarboxylase
CNS	central nervous system
CO-MO	control morpholino
COMT	catechol-o-methyltransferase
cAMP	cyclic AMP
DA	dopamine
DAT	dopamine transporter (slc6a3)
DLM	deltamethrin
DMF	N,N-dimethylformamide
DOHaD	developmental origins of health and disease
DOPAC	3,4-Dihydroxyphenylacetic acid
dpf	days post fertilization
DR	dopamine receptor (subtype d1, d2, d3, d4, or d5)
EC50	effective concentration 50
FQPA	Food Quality Protection Act
GD	gestational day
HPLC-ECD	High-performance liquid chromatography-electro chemical detection
HVA	homovanillic acid
kg	kilogram

KO	knockout
LOAEL	lowest observable adverse effect level
MAO	monoamine oxidase
mg	milligram
ml	milliliter
MPD	methylphenidate
MO	morpholino
mRNA	messenger ribonucleic acid
PND	postnatal day
qPCR	quantitative polymerase chain reaction
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
USEPA	United States Environmental Protection Agency
VMAT	vesicular monoamine transporter
VSSC	voltage sensitive sodium channel
VTA	ventral tegmental area
wpf	weeks post fertilization
μM	micromolar

Chapter 1 Introduction

1.1 General Introduction

This dissertation examined the effects of developmental exposure to low doses of the pyrethroid pesticide deltamethrin on larval and adult zebrafish behavior. In addition, the role of the dopamine and serotonin neurotransmission pathways in mediating these resulting behavioral deficits was also characterized. In Chapter 2, the effects of developmental exposure to deltamethrin on larval swim activity, dopamine (DA) gene expression, and DA neurochemistry were investigated. The dopamine system was then manipulated by dopamine transporter (DAT) knockdown and pharmacological inhibition to demonstrate that the DA system is involved in producing altered locomotor effects. In Chapter 3, the long term sex-specific behavioral effects resulting from developmental exposures to deltamethrin were characterized in adult zebrafish using the open field test and mirror induced aggression assay. Finally, in Chapter 4, we demonstrate that dopaminergic and serotonergic dysfunction, through changes in gene expression, neurochemistry, and pharmacological response, is likely responsible for the long-term behavioral deficits caused by developmental exposure to deltamethrin. Based on these studies, it can be concluded that exposure pyrethroid pesticide deltamethrin, at concentrations below the LOAEL, during critical neurodevelopmental periods causes permanent long-term behavioral consequences. This work further confirms the unique sensitivity of developing organisms to the adverse effects of pesticides and demonstrates that deltamethrin is a potential developmental neurotoxicant. Establishing dopaminergic and serotonergic dysfunction as a possible mode of action underlying the behavioral

deficits caused by developmental deltamethrin exposure could contribute to the ongoing EPA reregistration review process for pyrethroid pesticides.

1.2 Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that a multitude of adult disorders are predisposed by stress occurring during the prenatal and developmental stages. This hypothesis suggests that perturbation during critical windows of development results in enduring changes in organ structure and physiology. DOHaD is the current iteration of the original "Barker hypothesis" which stemmed from geographical epidemiological studies that found a positive correlation between low birth weight and coronary heart disease, indicated by mortality from cardiovascular disease, elevated systolic blood pressure (Barker et al. 1989; Osmond et al. 1993), increased serum cholesterol (Barker et al. 1993), and incidence of diabetes mellitus (Barker et al. 1993; Hales et al. 1991). It is thought that the ensuing hormonal, metabolic, structural, and physiological changes that take place to overcome this nutritional deficit and deprived fetal environment results in increased risk for disease later in life (Barker 1990). The "Barker hypothesis" has since expanded into the DOHaD paradigm, as mounting evidence suggests that an adverse intrauterine milieu has lasting impacts on not only adult cardiovascular and metabolic disorders, but encompasses most chronic non-communicable diseases and disorders including neurological, reproductive, and inflammatory disorders.

Because brain development is a highly dynamic and plastic process and is itself reliant on adaptation to internal and external cues (de Kloet et al. 2005), it is not

surprising that any additional or inappropriate stressors would have permanent and long lasting effects on brain function and behavior (Swanson and Wadhwa 2008; Van den Bergh 2011; Wadhwa et al. 2009). As such, there is increasing attention being paid to the persisting neurological and behavioral effects of low dose neurotoxicant exposure during development (Fox et al. 2012; Grandjean and Landrigan 2006). In 1993, the National Academy of Sciences report released by the National Research Council documented that children are uniquely susceptible to pesticides (National Research Council (U.S.). Committee on Pesticides in the Diets of Infants and Children. 1993) and ushered in a new era of risk assessment focused on protecting the health of infants and children (Landrigan 2001). In response, the Food Quality Protection Act (FQPA) was passed in 1996 and incorporated into law stricter safety standards, specifically with special considerations for infants and children. In addition, it required the US Environmental Protection Agency (USEPA) to re-evaluate the toxicity and tolerance levels of all pesticides. One of the most tangible outcomes of FQPA implementation was the USEPA mandated phase out of 2 organophosphate pesticides for residential use (USEPA 2000, 2001). This was spurred by *in vivo* and epidemiological studies establishing a correlation between developmental low dose organophosphate pesticide exposure and affected neurodevelopment in rodent models and children, reviewed in (Eskenazi et al. 1999). As a result of this phase out, the pyrethroid class of pesticides has largely been used as a replacement for residential pest control. Following FQPA requirements, in 2010, the USEPA began the registration review of all pyrethroid pesticides registered after November 1984 (USEPA 2013). To date, the process is still ongoing with initial decisions expected in 2015.

1.3 Pyrethroid Pesticides

1.3.1 Pyrethroid structure and toxicity

Pyrethroids are synthetic derivatives of the natural compounds pyrethrins that are designed to have increased insecticidal activity, photostability, and environmental persistence (Soderlund et al. 2002). The three basic structural components common to most pyrethroids include a 1) chrysanthemic acid or chrysanthemic acid derivative linked to an 2) aromatic alcohol moiety by a 3) central ester bond. Notable structural enhancements include the use of a phenoxybenzyl alcohol and/or halogenated moieties which greatly contribute to chemical stability and the addition of an α-cyano group which significantly enhanced insecticidal activity (Elliott 1971). In addition, most pyrethroids also contain 2 or 3 chiral centers and can exist as mixtures of as many as 8 stereoisomers. Electrophysiological studies demonstrated that only certain stereoisomers are biologically active, suggesting that the overall three-dimensional shape of pyrethroids determines toxicity, reviewed in (Kaneko 2010; Soderlund et al. 2002).

Classically, pyrethroids are divided into two subclasses, type I and type II, based on chemical structural (absence or presence of the α-cyano group on the alcohol moiety respectively) and characteristic symptoms of acute poisoning. Symptoms of type I pyrethroid poisoning, termed the "T-syndrome," begins with hyperaggression, hyperexcitation, and progresses from fine tremors to whole body tremors while prostrate. Acute poisoning with type II pyrethroids elicit symptoms classified as "CS-syndrome" and consists of abnormal locomotion followed by choreoathetosis and salivation (Barnes and Verschoyle 1974; Verschoyle and Barnes 1972; Verschoyle and Aldridge 1980). The primary mode of action of all pyrethroids in vertebrates and invertebrates is modification

of voltage sensitive sodium channels (VSSCs). While pyrethroids can slow the activation gate of the action potential, they greatly delay the rate of sodium current inactivation. Prolonging the open time of VSSCs increases the permeability of the neuronal membrane to sodium ions, creating a more positive membrane potential, and rendering the neuron hyperexcitable (Narahashi 1986, 1992; Soderlund and Bloomquist 1989). The distinct structure-activity relationships associated with type I and type II pyrethroid exposure is likely due to kinetic differences in VSSC modification; type II pyrethroids hold open VSSCs approximately 10x longer than type I pyrethroids. As a result, type I pyrethroids open VSSCs long enough to cause repetitive neuronal discharge, whereas type II pyrethroids prolongs VSSC inactivation for so long that it causes persistent depolarization to the point of conduction failure (Lund and Narahashi 1981, 1982; Ray 2001).

1.3.2 Pyrethroid usage and human exposure

Pyrethroid pesticides are generally considered to be a safer alternative to many insecticides, because they are rapidly metabolized in mammals and are easily photo- and biodegraded in the environment (Demoute 1989). Consequently, pyrethroids are used in a wide range of pest control applications including in agricultural and residential settings, treatment for infestations of human and animal hosts, and proactively applied to textiles and used in spray programs to control vector borne diseases (Spurlock and Lee 2008). Multiple sources indicate that their use is on the rise (Grube 2011; Power and Sudakin 2007) especially in the residential setting where previously popular organophosphate insecticides chlorpyrifos and diazanon have been phased out (Bekarian et al. 2006; Trunnelle et al. 2014). Human exposure is well documented (Becker et al. 2006; Heudorf

and Angerer 2001; Heudorf et al. 2004). Consumption of pyrethroid residues in food has been determined to be the primary route of exposure (Lu et al. 2010; Schettgen et al. 2002) and exposure from contact with a treated environment is another significant contributing source (Bradman et al. 2007; Lu et al. 2006; Morgan et al. 2007).

In the US, 70% of urine samples collected from the 1999-2002 NHANES cohort had detectable levels (0.292 ng/ml - 0.318 ng/ml) of the pyrethroid metabolite, 3 phenoxybenoic acid (3PBA) (Barr et al. 2010). A troubling statistic highlighted by this survey was that 3PBA levels were significantly higher in samples from children (6-11 year old) than from adolescents or adults. Other epidemiological studies have also reported finding pyrethroid metabolites in the urine of pregnant women (Berkowitz et al. 2003; Qi et al. 2012; Whyatt et al. 2002) as well as in children and adolescents (Babina et al. 2012; Becker et al. 2006; Bradman et al. 2007; Couture et al. 2009; Fortin et al. 2008; Heudorf et al. 2004; Morgan et al. 2007; Morgan 2012). Given that children are more susceptible to the toxic effects of pesticides, the extent of pyrethroid exposure is children is concerning.

1.3.3 Sensitivity of developing organisms to pyrethroids

Several lines of study indicate that developing organisms may be more sensitive to the adverse effects of pyrethroids. Both *in vitro* and *in vivo* studies have pointed to age related toxicokinetic differences as reasons for heightened sensitivity. For instance, studies using rats found that neonates are 16x more sensitive to acute lethal doses of deltamethrin (Sheets et al. 1994; Sheets 2000), and 6x and 17x more sensitive to permethrin and cypermethrin, respectively (Cantalamessa 1993). Since pretreatment with tri-o-tolyl phosphate, an esterase inhibitor, did not potentiate the lethality of permethrin

or cypermethrin in neonatal rats, but did increase pyrethroid induced lethality in adult rats (Cantalamessa 1993), it was concluded that the increased toxicity is most likely the result of overwhelming immature detoxification mechanisms. This conclusion is further substantiated by work done *in vitro*. Adult liver microsomes display an increased capacity for deltamethrin metabolism than that of neonatal liver microsomes, which correlated *in vivo* with lower blood deltamethrin levels and decreased acute toxicities in adults (Anand et al. 2006). Sheets et al. 1994 also determined that brain concentrations of deltamethrin were higher in younger pups than adults. Even though pups were administered a dose that was 7-fold lower than that given to adults, the deltamethrin concentration in the brains of pups and adults were comparable, suggesting that both pup and adults received equivalent toxic doses despite differences in administered dose. This notion is supported *in silico* as PBPK modeling of brain dosimetry of deltamethrin predicted that juvenile rats are likely to have 3-fold more deltamethrin in the brain than in adult rats (USEPA 2010).

In addition, toxicodynamic differences between juveniles and adults are thought to be another contributing factor to the increased sensitivity of developing organisms to pyrethroid toxicity. Meacham et al. 2008 report that developmentally expressed sodium channel variants are more susceptible to modification by pyrethroids than sodium channel variants expressed during adulthood (Meacham et al. 2008). Taken together, with the increased use of pyrethroids and increased susceptibility of juveniles to pyrethroid toxicity, understanding the consequences of pyrethroid exposure during development is an important human health question.

1.3.4 Low dose pyrethroid exposure and behavioral effects

Given the increased susceptibility of neonates to pesticides, characterization the long lasting effects of pesticide exposure during critical neurodevelopmental periods would be instrumental for cumulative risk assessment purposes. Currently, there are several reports in rodents that suggest that developmental exposure to pyrethroids results in persistent changes in neurochemical, behavioral, and cognitive endpoints and is reviewed extensively by (Shafer et al. 2005). These studies exposed developing rodents to low, non-lethal and non-toxic doses of pyrethroids and monitored the behavioral consequences at later time points.

The work examining the long-term consequences of developmental pyrethroid exposure has been investigated primarily using deltamethrin, a type II pyrethroid, which is considered one of the most potent and toxic registered pyrethroids (Pham, 1984). Several studies observed consistent increases in spontaneous locomotor activity and lack of habituation out to 5 months postnatal in mice exposed developmentally (PND10-16) to 0.7 mg/kg deltamethrin. (Ahlbom et al. 1994; Eriksson and Fredriksson 1991; Talts et al. 1998) In rat models, gestational exposure (GD5-21) to 0.5-1 mg/kg (Johri et al. 2006) and 7 mg/kg (Husain et al. 1992) deltamethrin resulted in decreased locomotor activity at 3 and 6 weeks postnatal. However, exposure at later postnatal timepoints (PND22-37) resulted in increased locomotor activity at these timepoints (Husain et al. 1994). Similarly, exposure to deltamethrin (0.07 mg/kg) at earlier postnatal days (PND0-7) resulted in reduced locomotion at approximately 13 weeks postnatal. But exposure at later developmental timepoints (PND9-13) resulted in increased locomotion and lack of habituation at 3 weeks and decreased locomotion at 12 weeks (Patro et al. 2009).

Together, these studies demonstrate the importance of the time of exposure, as effects seem to depend on the neurodevelopmental period when exposure occurred. In addition to changes in spontaneous locomotion, learning deficits were also present in adult rats at 6 and 12 weeks following prenatal exposure (GD14-20) to 1 mg/kg deltamethrin (Aziz et al. 2001). Differences in forced swim behavior and changes in spontaneous locomotion immediately following the swim test were also reported in adult male rats following prenatal (GD6-15) low dose deltamethrin exposure (0.08mg/kg) (Lazarini et al. 2001).

In studies utilizing other type II pyrethroids, including cyhalothrin (Gomes Mda et al. 1991; Moniz et al. 1990) and fenvalerate (Husain et al. 1992; Moniz et al. 1999), changes in spontaneous locomotor activity were not observed after developmental exposures in rats. In a more recent study examining the effects of neonatal cypermethrin exposure, Nasuti et al. also did not observe changes in locomotor activity at PND21, similar to that reported in (Husain et al. 1992). However, increased spontaneous locomotion and rearing frequencies appeared by PND35 (Nasuti et al. 2007), suggesting that time of assay is another important factor when assessing developmental neurotoxicity. In terms of other behavioral endpoints, developmental cyhalothrin exposure was associated with decreased motivational response (Gomes Mda et al. 1991) and disrupted inhibitory avoidance tasks (Moniz et al. 1990). Fenvalerate exposure was associated with decreased grip strength (Husain et al. 1992).

Allethrin and permethrin are the only type I pyrethroids to be studied under this paradigm thus far. Similar to the findings with deltamethrin, increased spontaneous locomotor activity and lack of habituation was also observed in mice dosed developmentally (PND10-16) to 0.7 mg/kg bioallethrin (Ahlbom et al. 1994; Eriksson

and Fredriksson 1991; Talts et al. 1998). The only other study using an allethrin consisted of exposing PND 10-16 mice to d-allethrin via an inhalation exposure. However, changes in locomotor activity or water maze behavior were not observed (Tsuji et al. 2002) and can likely be attributed to differences in the routes of exposure. In the case of permethrin, Nasuti et al. 2007 found that the effects of neonatal exposure to non-toxic doses of permethrin was similar to that of cypermethrin; changes in locomoter activity appeared at PND35, but was not present when assayed earlier (PND21) (Nasuti et al. 2007).

In humans, data associating pyrethroid exposure and neurobehavioral deficits are just beginning to emerge. To date only 2 epidemiological studies address this premise and are very preliminary. In the first study, prenatal exposure to piperonyl butoxide, a commonly used pyrethroid synergist and indicator of pyrethroid use, correlated with lower scores on the mental development index of the Bayley Scales of Infant Development (BSID-II) at 36 months, suggestive of decreased cognitive development (Horton et al. 2011). However, permethrin was the only pyrethroid measured and a correlation between plasma permethrin levels and 36-month BSID-II scores was not found. The second study found a correlation between urinary cis-DCCA levels, a specific metabolite of permethrin, cypermethrin and cyfluthrin, and parent reported behavioral problems (Oulhote and Bouchard 2013). Among other things, both studies suffer from the fact that pyrethroids are rapidly metabolized in the body and therefore single spot sampling may not accurately reflect the extent of exposure. However, recognizing the limitations of these studies, they provide preliminary evidence that adverse behavioral outcomes from developmental pyrethroid exposure may exist in human populations.

Clearly, a multitude of experimental factors including time of exposure, time of assay, animal model, and route of exposure, play an important role in the manifestation of behavioral toxicity. In addition, other factors such as the use of commercial formulations (Aziz et al. 2001; Husain et al. 1992, 1994), where the presence of synergists may affect the toxicokinetics of deltamethrin, could alter exposure. Since these formulations are proprietary, it is also possible that unknown ingredients are directly responsible for the effects seen. Despite the inconsistencies with experimental design and the variability of the results, taken together these studies still demonstrate the potential for behavioral modification by developmental pyrethroid exposure. However, to date, there are no studies definitively linking pyrethroid induced molecular events (ie. VSSC modification, receptor activation, neurotransmitter release) to the observed physiological and behavioral endpoints. The paucity of data concerning the modes of action underlying pyrethroid toxicity to the developing nervous system requires additional studies to fill these mechanistic gaps. A better understanding of the mode of action concerning the developing nervous system would be valuable in conducting cumulative risk assessment for the reregistration of pyrethroid pesticides.

1.4 Dopaminergic System

1.4.1 Overview

The mechanisms of pyrethroid induced behavioral and cognitive deficits are unknown. Because the blood plasma half-life of most pyrethroids extends for only several hours, it is unlikely that pyrethroids remain in the system at these later life stages to modify the sodium channels (Kaneko 2010). However, given that the dopamine (DA) system plays an essential role in mediating many of the previously described behavioral

lesions, especially locomotor activity, it is hypothesized that disruption of dopamine biochemistry during development could have permanent neurological effects.

Anatomically, mammalian CNS dopaminergic neurons are primarily localized to the mesencephalon, with smaller populations found in the diencephalon and the olfactory bulb. The mesencephalic dopamine circuitry is further categorized into four anatomically and functionally distinct output pathways which include the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular systems, reviewed in (Bjorklund and Dunnett 2007). Nigrostriatal neurons project from the pars compacta of the substantia nigra to the dorsal striatum (caudate-putamen) and this system is largely responsible for voluntary motor control. Both mesolimibic and mesocortical neurons originate from the ventral tegmental area (VTA) and extend to limbic areas and the prefrontal cortex of the brain, respectively. Limbic structures innervated by mesolimbic dopamine projections such as the nucleus accumbens, septal nuclei, amygdala, and olfactory tubercle mediate emotion-driven behaviors such as motivation and reward. Mesocortical DA input to the prefrontal cortex maintains working memory and is essential for higher cognition (Seamans and Yang 2004).

DA synthesis in the CNS begins with the hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA) via tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis. Subsequently, the decarboxylation of L-DOPA to DA is catalyzed by the enzyme aromatic L-amino acid decarboxylase (AAAD). After synthesis, the majority of DA is transported into synaptic vesicles by vesicular monoamine transporter (VMAT) and stored until its release. Metabolism of DA contributes significantly to the termination of DA neurotransmission and occurs in a multistep

process to yield the final deaminated and O-methylated major metabolite homovanillic acid (HVA). The primary enzymes responsible for DA metabolism, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), can act on DA and its intermediary metabolites in variable order, but it would appear that the initial deamination of DA and formation of 3,4-Dihydroxyphenylacetic acid (DOPAC) is the preferred route.

Dopamine signaling is initiated at the axon terminal as well as dendritic cell bodies (Ludwig and Pittman 2003) by Ca^{2+} dependent release of DA from synaptic vesicles into the extracellular space. Upon release, DA binds to and activates both pre- and post-synaptic DA receptors, which belong to the seven transmembrane domain G-protein coupled receptor family that modulates adenylyl cyclase activity. DA receptors are divided into 2 subtypes, the D1 or D2-class receptors, according to sequence, pharmacological, and biochemical characteristics. The D1-class DA receptors (D1 and D5) are exclusively localized on postsynaptic neurons and associate with stimulatory $G\alpha_{s/olf}$ G proteins that activate cAMP signal transduction. D2-class DA receptors (D2, D3, and D4) are known to associate with inhibitory $G\alpha_{i/o}$ G proteins and inactivate cAMP signal transduction. The D2 and D3 DA receptors are unique in that expression is found pre- and post-synaptic neurons. Pre-synaptic DRD2 and DRD3 function as autoreceptors that provide negative feedback signals to temper DA neurotransmitter signal transduction. Detailed reviews characterizing the various dopamine receptors can be found (Beaulieu and Gainetdinov 2011; Missale et al. 1998).

1.4.2 Behavioral disorders associated with dopamine system dysfunction

Due to the fact that the DA system extensively activates the motor circuit and is involved in pathologies of kinetic disorders such as Parkinson's disease, one of the most

studied behavioral endpoints when examining the dopamine system is locomotor activity (Beninger 1983; Herlenius and Lagercrantz 2001). Neurotoxicants that selectively destroy dopamine neurons, such as 6-hydroxydopamine (6-OHDA) (Beninger 1983) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), produce locomotor deficits (Ferro et al. 2005). In addition, genetic and pharmacological manipulation of the DA system also produces such effects; a brief summary of the effects in animal models can be found in (Table 1.1).

There is ongoing interest in determining the effects of illicit drug use during pregnancy, especially concerning the use of cocaine and amphetamine, which target the DA system. Thus far, the results are far from conclusive; however, minor associations between prenatal drug exposure and mild motor, cognitive, and behavioral impairments have been found in children (Buckingham-Howes et al. 2013; Lambert and Bauer 2012; Thompson et al. 2009). Animal models have for the most part supported this association between prenatal cocaine exposure and behavioral impairments (Chae and Covington 2009; Harvey 2004; Middaugh 1989). In addition, multiple studies in the rabbit model have also demonstrated that prenatal cocaine exposure has long lasting effects on the DA system including, the uncoupling of the D1 receptor from its G-protein (Friedman et al. 1996; Jones et al. 1998; Wang et al. 1995), reduction of dopamine release (Wang et al. 1995) and an altered anatomy of dopamine-rich regions (Stanwood et al. 2001).

Given the rise in the use of pharmaceutical psychostimulants, evidence is also beginning to emerge concerning their effects on neurodevelopment. In rat models, exposure to high doses of methylphenidate, a DAT inhibitor, during development has been linked to persistent behavioral and neurochemical changes in adults, reviewed in

(Marco et al. 2011). For instance, adults exhibited differences in anxiety-like behaviors (Bolanos et al. 2003; Carlezon et al. 2003; McFadyen-Leussis et al. 2004), altered sensitivity to aversive effects of dopaminergic drugs (Andersen et al. 2002; Carlezon et al. 2003), decreased impulsivity (Adriani et al. 2007) and decreased locomotor activity (Bolanos et al. 2003; Ruocco et al. 2010). In addition, developmental methylphenidate exposure was associated with long term decreases in DA and its metabolites in various brain regions (Ruocco et al. 2010), produces long lasting decreases in striatal DAT in rats (Moll et al. 2001), and changes basal firing activity of DA neurons (Brandon et al. 2003).

The studies cited above the long-term behavioral consequences of early DA system perturbation. Given this association, it is possible that other neurotoxicants that modify the DA system during development could give rise to similar behavioral outcomes.

1.4.3 Effects of pyrethroids on DA system

Pyrethroid exposure alters the kinetics of DA transmission by altering DA uptake (Elwan et al. 2006; Karen et al. 2001; Kirby et al. 1999), promoting DA release (Eells and Dubocovich 1988) or both (Bloomquist et al. 2002; Hossain et al. 2006). Because DA re-uptake and clearance from the synapse is mediated by the dopamine transporter (DAT), several studies also measured levels of DAT following pyrethroid exposure. Based on the findings, the differences in DA uptake could be due to the dysregulation of DAT expression (Elwan et al. 2006; Kirby et al. 1999; Pittman et al. 2003) and that altered DAT expression may persist over time (Gillette and Bloomquist 2003). In addition to altered dopamine transmission kinetics, studies in the striatum have shown that pyrethroid exposure changed the concentrations of DA and its metabolites 3,4-

Dihydroxyphenylacetic acid (DOPAC) and Homovanillic acid (HVA). Treatment with toxic but sublethal levels of cypermethrin, fenvalerate, and permethrin increased levels of striatal DOPAC (Doherty et al. 1988; Hudson et al. 1986). Similarly, adult animals dosed with nontoxic doses of deltamethrin show decreased levels of DA in the striatum, while levels of DOPAC and HVA were increased, suggesting that there is increased DA turnover. In addition to increased DA metabolism, the decreased levels of DA may also be attributed to decreased DA biosynthesis as levels of tyrosine hyroxylase mRNA, protein and activity were also found to be decreased in deltamethrin treated animals (Liu and Shi 2006). This finding was recapitulated *in vitro* using PC12 cells (Liu et al. 2006).

To date only a handful of studies establish a correlation between permanent behavioral changes and changes in dopamine neurochemistry following developmental pyrethroid exposure. For instance, rats exposed prenatally to nontoxic doses of a deltamethrin (0.08 mg/kg) formulation exhibited behavioral deficits that appeared during adulthood, including altered swim behavior, decreased locomotion and increased immobility. These animals were also found to have increased levels of striatal DOPAC and an increased DOPAC/DA ratio. These phenomena were only observed in male rats (Lazarini et al. 2001). In another study utilizing only male rats, neonatal exposure to permethrin or cypermethrin resulted in increased locomotor activity, rearing and anxiolytic behavior almost 3 weeks after exposure, but not at an earlier time point. This was complemented by decreased striatal DA levels and increased HVA levels, indicating increased DA turnover (Nasuti et al. 2007). Together, these studies demonstrate the potential for pyrethroid pesticides to modify the dopaminergic system. If DA system

modification by pyrethroids occurs during the plastic and dynamic developmental period, this could give rise to long term behavioral consequences.

1.5 Serotonin system

1.5.1 Overview

Currently, an association between long term behavioral deficits and changes in 5-HT neurotransmission following developmental pyrethroid exposure has not been investigated. However, acute exposure to pyrethroid pesticides has been associated with rapid onset behaviors such as anxiety (De Souza Spinosa et al. 1999; Meng et al. 2011; Ricci et al. 2013; Righi and Palermo-Neto 2003) and aggression (Hossain et al. 2013; McDaniel and Moser 1993; Meng et al. 2011). Since the serotonin system is thought to play a role in anxiety-like behaviors (Handley and McBlane 1993) and aggression (Lesch and Merschdorf 2000; Lesch et al. 2012; Olivier and van Oorschot 2005) it is plausible that perturbation of the 5-HT system by pyrethroids during development can contribute to these phenotypes.

In mammals, CNS 5-HT neurons are relatively confined in the brain, with cell body clusters (raphe nuclei) primarily located in upper brain stem region. However, 5-HT projections reach almost every portion of the brain. Generally speaking, two major groups of raphe nuclei exist and both contain a number of different raphe populations. Neurons in the rostral raphe, localized to the mesencephalon and rostral pons, primarily send projections rostrally to the limbic and sensory areas of the forebrain. Neurons in the caudal raphe, located in the medulla oblongata and caudal pons, sends projection backwards into the caudal brainstem and spinal cord and are positioned to contribute to motor and sympathetic activity, reviewed (Hornung 2003).

Synthesis of 5-HT, similar to the synthesis of catecholamines, begins with the hydroxylation of an amino acid precursor. In this case, L-tryptophan is converted into 5-hydroxytryoptophan via tryptophan hydroxylase. Subsequent action by AAAD, also involved in catecholamine biosynthesis, catalyzes the decarboxylation of 5-hydroxytrptophan to 5-HT. 5-HT is also stored in synaptic vesicles until its release and transport is also mediated by VMAT. The reuptake and degradation of 5-HT is critical for terminating signal transduction and modulating serotonergic tone. The serotonin reuptake transporter (SERT) a 12 transmembrane NA^+/CL^- dependent transporter is responsible for the reuptake of serotonin from the synapse.   5-HT is also chiefly metabolized by MAO and ALDH to form the primary metabolite 5-hydroxy-3-indolacetic acid (5-HIAA).

Similar to catecholamine signal transduction, 5-HT signaling is initiated at the axon terminus by Ca^{2+} dependent release of 5-HT from synaptic vesicles into the extracellular space. 5-HT binds to and activates both pre- and post-synaptic 5-HT receptors, which, all except for one, belong to the seven transmembrane domain G-protein coupled receptor family that modulates adenylyl cyclase activity. 5-HT receptors are divided into 7 families, and within these families a number of subtypes exist based on structural, biochemical, and pharmacological criteria. Currently, at least 14 distinct murine 5-HT receptors exist and detailed reviews characterizing the central 5-HT receptors can be found (Barnes and Sharp 1999; Hannon and Hoyer 2008; Hoyer et al. 2002).

1.5.2 Behavioral disorders associated with serotonin system dysfunction

The serotonin system plays an important role in modulating behaviors such as anxiety and aggression, reviewed in (Hendricks et al. 2003; Lesch and Merschdorf 2000;

Quadros et al. 2010). In humans, selective serotonin reuptake inhibitors (SSRIs) and various 5-HT receptor agonists are commonly used to treat these disorders. In addition, lower levels of 5-HIAA are present in the brain and CSF of subjects with impulsive aggression (REF). Animal models have also contributed significantly to implicating the 5-HT system in mediating these behaviors. Tables 1.2 and 1.3 briefly summarize the effects of genetic and pharmacological manipulation of the 5-HT system on anxiety-like behaviors and aggression, respectively in animal models.

Along the lines of the DOHaD hypothesis, the serotonin system is thought to be the mediator of gene x environment interactions that shape adult behaviors including altered locomotion, aggression, and anxiety, reviewed in (Homberg and van den Hove 2012; Huizink et al. 2004; Lesch et al. 2012). For instance, in animal models, prenatal stress produces anxiety-like behaviors in offspring (Van den Hove et al. 2005; Van den Hove et al. 2014) and is associated with neurochemical changes in the serotonin system including altered 5-HT and TPH2 levels (Van den Hove et al. 2014), decreased 5-HTa1 receptor binding in male rats (Peters 1990; Van den Hove et al. 2005), and increased 5-HT turnover (Hayashi et al. 1998; Peters 1990). In addition, treatment with fluoxetine (an SSRI) ameliorated the anxiety like behaviors induced by maternal stress during gestation (Rayen et al. 2011). Directly targeting the 5-HT system using fluoxetine during neonatal life also produces anxiety-like behaviors in mice offspring (Ansorge et al. 2008; Karpova et al. 2009). Conditional expression of 5-HT1a specifically during development (and not adults) in 5-HT1a knockout mice rescues the anxiolytic effects of the knockout (Gross et al. 2002). This suggests that the anxiety-like behavior observed in adult 5-HT1a mice is likely an effect established during development.

Similar findings have also been reported in terms of aggression. Rats undergoing stress during the peripubescent period exhibit hyperagression as adults and was found to have increased MAOA expression and histone H3 acetylation at the MAOA promoter (Marquez et al. 2013). Accordingly, prenatal exposure to paroxetine, an SSRI, resulted in increased aggression in male mice (Coleman et al. 1999). Early postnatal exposure to partial 5-HT1A receptor agonist buspirone augmented aggression in an aggressive mouse strain and further attenuated aggression in a less aggressive mouse strain (Markina et al. 2006). In a chicken model, exposure to 5-HT during development, via direct injection into the egg, was associated with decreased aggression in adult hens and a sustained increase in 5-HT levels (Dennis et al. 2013). The use of this ex-utero exposure model system directly implicates the serotonin system in mediating these latent behavioral effects since confounders from maternal influence are eliminated.

These studies suggest that behavioral disorders, such anxiety and aggression, are programmable behaviors that may be influenced by the environment during development, specifically an environment that modifies the serotonin system. Therefore, it is plausible that developmental exposure to toxicants that affect the serotonin system could produce such anxiety-like and aggressive behaviors later in life.

1.5.3 Effects of pyrethroids on 5-HT system

The effects of pyrethroid exposure on the 5-HT system are less studied than those on the DA system. Deltamethrin has been shown to increase serotonin release from synaptosomes; though the EC50 for 5-HT release is 2.4 times greater than the EC50 for DA release (Kirby et al. 1999). Treatment of ex-vivo cortical synaptosomes with

permethrin resulted in a dose dependent increase in 5-HT reuptake; however, this effect was 30-fold less sensitive than that of DA reuptake (Bloomquist et al. 2002). These two studies suggest that while pyrethroids can alter the kinetics of 5-HT neurotransmission, the 5-HT system *in vitro* is apparently less sensitive to effects of pyrethroid exposure.

In vivo, microdialysis analysis of extracellular striatal 5-HT levels found that exposure to acute sublethal nontoxic doses of allethrin reduced extracellular 5-HT levels, whereas exposure to sublethal toxic doses increased extracellular 5-HT levels. Deltamethrin exposure decreased extracellular 5-HT levels and cyhalothrin exposure increased extracellular 5-HT levels. In addition, local infusion with tetrodotoxin, a VSSC blocker, rescued the effects of pyrethoids on 5-HT release (Hossain et al. 2013). These data demonstrate that acute high-dose pyrethroid exposure can modulate 5-HT signal transduction *in vivo* and that NA^+ signal transduction is required for pyrethroid induced neurotransmitter release. Since, Na^+ dependent DA release was also found to be altered to the same degree by allethrin, cyhalothrin, and deltamethrin at equivalent doses (Hossain et al. 2006), it is likely that mechanisms independent of Na+ current stimulation are responsible for the heightened sensitivity of the DA transmission system to pyrethroid modification as previously described in (Bloomquist et al. 2002; Kirby et al. 1999).

In terms of the effects of pyrethroids exposure on striatal neurotransmitter levels, rats injected with sublethal but toxic doses of deltamethrin, cyhalothrin, cyfluthrin had decreased levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Martinez-Larranaga et al. 2003). Conversely, deltamethrin exposure via oral gavage in rats was found to increase striatal 5-HT and 5-HIAA levels (Ricci et al. 2013). The discrepancies between these findings could be due to the differences in the route of

exposure as well as the dose used. Ricci et al. 2013 used lower doses that did not cause toxicity. Nevertheless, taken together, all of these studies demonstrate that pyrethroid exposure can modify the 5-HT system.

1.6 Zebrafish Model

1.6.1 Zebrafish (*Danio rerio*) as a model for neurobehavioral toxicology

The zebrafish (*Danio rerio*) is has gained recognition for its utility as a model organism and has played a large role in advancing the fields of developmental biology and toxicology. Zebrafish are an attractive model because of their fecundity, egg transparency, and the availability of a completely sequenced and annotated genome, among other beneficial traits. The zebrafish share a 70-80% genetic homology to humans (Howe et al. 2013), which facilitates the translation of genetic findings to human health. Since developmental processes are highly conserved among vertebrates, the zebrafish model has proven useful in elucidating the temporal and spatial molecular events of development. Zebrafish are actively being used as a platform for mid-high throughput screening to identify developmental neurotoxicants, as well as a model to elucidate the mechanisms of neurobehavioral toxicology (Linney et al. 2004). A wide array of tools and protocols has been developed for behavioral assessment such that even complex behaviors including anxiety and aggression can be quantified (Bailey et al. 2013; de Esch et al. 2012).

1.6.2 Comparative DA system

There is a high degree of conservation of monoaminergic systems in all vertebrates which allows for the use of lower vertebrate models to study these systems (Panula et al. 2010). However, homologues to mammalian midbrain structures such as

the substantia nigra and VTA are not apparent in the zebrafish brain; the zebrafish do not have mesenchaphalic DA neurons (Tay et al. 2011). Instead, dopaminergic populations in the caudal tuberculum (diencephalon) region are considered to be the most similar to the dopamine population of the substantia nigra based on retrograde labeling experiments (Rink and Wullimann 2001) and the sensitivity of these neurons to nigral DA toxicant MPTP (McKinley et al. 2005; Sallinen et al. 2009).

Tyrosine hydroxylase orthologous have been identified in the zebrafish. However, due to the teleost specific genome duplication event, zebrafish express 2 isoforms of th (th1 and th2). Similarly, D1, D2, D3, and D4 receptor orthologous have also characterized and multiple isoforms for drd2 (drd2a, drd2b, drd2c) and drd4 (drd4a and drd4b) exist (Boehmler et al. 2004; Boehmler et al. 2007). Regardless, pharmacological characterization of these receptors indicates that their contribution to locomotor activity is similar to their mammalian orthologues (Irons et al. 2013). The zebrafish DAT (slc6a3) has also been characterized (Holzschuh et al. 2001) and was found to be targeted by mammalian DAT inhibitors (Lange et al. 2012; McKinley et al. 2005). Levin et al. 2011 demonstrated that embryonic methylphenidate (DAT inhibitor) exposure caused behavioral deficits that persist into adulthood (Levin et al. 2011), and confirmed that the zebrafish is an appropriate model for testing the DOHaD hypothesis. A comparison of locomotor responses to pharmacological manipulation of the DA system between mammals and zebrafish larvae can be found in (table 1.4). In general, DR agonist exposure increased locomotor activity and DR antagonists decreased locomotor activity in both mammalian and zebrafish larval models. Together, these data suggest that DA system function is conserved between mammals and the zebrafish, and that the zebrafish

would be an appropriate model to study the effects of dopaminergic neurotoxicants, particularly under the DOHaD paradigm.

1.6.3 Comparative 5-HT system

The serotonergic system is also well conserved between the zebrafish and mammal models (Panula et al. 2006). The raphe nuclei present in the zebrafish hindbrain follow the typical mammalian ascending and descending mammalian trajectories and axonal projections are broadly distributed and reach most brain areas (Lillesaar et al. 2009). However several caveats exist. For instance, additional serotonergic populations are also prominent in pretectal and hypothalamic areas, as well as in the vagal lobes and medulla oblongata; these nuclei are not present in higher vertebrates (Kaslin and Panula 2001). In addition, compared to mammals, there are relatively few raphe nuclei axonal projections to the medial dorsal telencephalon (Lillesaar et al. 2009), a structure that is homologous to the mammalian pallial amygdala (Vargas et al. 2009), which is highly innervated by the raphe nuclei and is involved in anxiety behaviors (Davis 1992). It is likely that in lower vertebrates, the other serotonergic populations are responsible for innervation of these areas.

In contrast, the serotonin receptors in zebrafish are poorly understood and, as it stands, only four serotonin receptors gene have been cloned and characterized, htr1aa, htr1ab, htr1d (Norton et al. 2008) and htr2c (Schneider et al. 2012). A brief search of the zfin database (8/2014) revealed that at least 16 htr genes have been annotated in the zebrafish genome, which is close in number to the 14 identified in mice. The zebrafish also express 2 isoforms of sert (serta and sertb) and 3 isoforms of tph (tph1a, tph1b and tph2). However, despite the limited knowledge regarding the 5-ht receptors, several

studies demonstrate that the pharmacological profiles of the 5-HT system are similar to that of mammals. For example, chronic fluoxetine (SSRi) exposure produces anxiolytic effects under multiple behavioral paradigms (Egan et al. 2009; Maximino et al. 2011) and buspirone, a partial ht1a receptor agonist also has anxiolytic properties in the zebrafish (Bencan et al. 2009; Gebauer et al. 2011; Maximino et al. 2011). The zebrafish sert demonstrates binding to citalopram (SSRi), although to a lesser degree than that of the mammalian sert (Sackerman et al. 2010).

While characterization of the the zebrafish 5-HT system is not as extensive at the DA system, pharmacological evidence suggests that 5-HT function is similar between mammals and the zebrafish. Because the 5-HT system demonstrates high plasticity and is involved in many biological processes, the 5-HT system is a likely mediator of the DOHaD.

1.7 Research Objectives and Hypothesis

The overall goal of this dissertation is to demonstrate that dopaminergic and serotonergic dysfunction is the underlying mode of action by which developmental low dose exposure to pyrethroid pesticide deltamethrin causes long term (larval and adult) behavioral effects using the zebrafish as a model system. We hypothesize that developmental exposure to deltamethrin, at concentrations below the LOAEL, results in persistent behavioral deficits, which is due to changes in dopamine and serotonin system gene expression and neurochemistry. An illustration of the specific experimental paradigm can be found in figure 1.1.

The specific aims were to:

1) To characterize the role of the dopaminergic system in mediating locomotor changes in larval zebrafish resulting from developmental low-dose deltamethrin exposure.

2) To characterize the persistent effects in locomotion and behavior resulting from developmental low-dose deltamethrin exposure in adult zebrafish.

3) To characterize the role of the serotonin and dopaminergic systems in mediating behavioral changes in adult zebrafish resulting from developmental low-dose deltamethrin exposure.

Gene	Agonism	Antagonism	Knockout	Other
Drd1	Increased [a]	Decreased [a,b]	Increased [a, c] Decreased [a, c]	
Drd2	Presynaptic: Decreased [a] Postsynaptic: Increased [a] Decreased [b]	Decreased [d,e]	drd2L KO – decreased [a] drd2 pan KO – decreased [a,b] drd2 knockdown = decreased [f]	
Drd3	Decreased	Increased [g,h]	Increased [a,c] Decreased [a]	
Drd4	No change [b]	No change [b]	Decreased [a,b,c]	
Drd5	N/A	N/A	No change [a] Increased [c]	
Dat	N/A	Increased [i]	Increased [c]	Overexpression: No change[j] or Decreased[k]

Table 1.1 The effects of genetic and pharmacological manipulation of the dopamine system on locomotor activity in rodents. [a]reviewed in (Holmes et al. 2004), [b]reviewed in (Waddington et al. 2001), [c]reviewed in (Glickstein and Schmauss 2001), [d](Fujiwara 1992), [e](Kelly et al. 1998), [f](Zhou et al. 1994), [g](Sautel et al. 1995), [h](Gyertyan and Saghy 2004), [i](Kuczenski and Segal 2001), [j](Salahpour et al. 2008), [k](Donovan et al. 1999).

	Agonism	Antagonism	Knockout[#]
5-HTR$_{1A}$	Decreased [a]	N/A	Increased
5-HTR$_{1B}$	N/A	N/A	Conflicting
5-HTR$_{2A}$	N/A	N/A	Decreased
5-HTR$_{2C}$	N/A	N/A	N/A
5-HTR$_3$	Increased [b]	Decreased [c]	Decreased
5-HTR$_4$	N/A	N/A	Decreased
5-HTR$_{5A}$	N/A	N/A	Increased
5-HTR$_6$	N/A	N/A	No change
5-HTR$_7$	N/A	N/A	Increased
SERT	N/A	N/A	Decreased
TPH2	N/A	N/A	Increased

Table 1.2 The effects of genetic and pharmacological manipulation of the serotonin system on anxiety in rodents. #Adapted from (O'Leary and Cryan 2010), [a]reviewed in (Griebel 1997), [b](Kennett et al. 1989), [c]reviewed in (Eison and Eison 1994).

	Agonism	Antagonism	KO Mice
5-HTR[1A]	Decreased [a, b]	No effect [c, d, e]	Decreased [f]
5-HTR[1B]	Decreased [a, b]	N/A	Increased [f]
5-HTR[2A]	Decreased [a]	Decreased [b]	N/A
5-HTR[2C]	Decreased [a, b]	N/A	N/A
5-HTR[3]	No change [b] Decreased [b]	No change [b] Decreased [b]	N/A
5-HTR[4]	N/A	N/A	N/A
5-HTR[5A]	N/A	N/A	N/A
5-HTR[6]	N/A	N/A	N/A
5-HTR[7]	N/A	N/A	N/A
SERT	N/A	Decreased [g]	Decreased [h]
TPH2	N/A	Increased [i, j]	Increased [k] Decreased [l]

Table 1.3. The effects of genetic and pharmacological manipulation of the serotonin system on aggression in rodents. [a]reviewed in (Olivier and Mos 1992), [b]reviewed in (Bortolato et al. 2013), [c](Mendoza et al. 1999), [d](Miczek et al. 2002), [e](de Boer and Koolhaas 2005), [f]reviewed in (Zhuang et al. 1999), [g](Carrillo et al. 2009), [h]reviewed in (Holmes et al. 2002), [i](Keele 2001), [j](Vergnes et al. 1986), [k](Beaulieu et al. 2008), [l](Osipova et al. 2009).

Drug	Pharmacology	Rodents	Zebrafish
Apomorphine	Non-selective agonist	Increased or U-shape[a]	U-shape[a]
SKF-38393	D1-like agonist	Increased[a]	Increased[a]
Quinpirole	D2-like agonist	Increased or U-shape[a]	Variable[a]
Butaclamol	Non-selective antagonist	Decreased[a]	Inverted U-shape[a]
Chlorpromazine	Non-selective antagonist	Decreased[b]	Decreased[c]
SCH-23390	D1-like antagonist	Decreased[a]	Decreased[a]
Haloperidol	D2-like antagonist	Decreased[a]	Decreased[a]
Fluphenazine	D2-like antagonist	Decreased[d,e]	Decreased[f]
Raclopride	D2/D3-like antagonist	Decreased[b]	No change[g]
L-745,870	D4-like antagonist	Decreased[h]	Decreased[g]
Methylphenidate	DAT inhibitor	Increased[i,j]	Increased[k]
d-amphetamine	Increases activity of monoamines	Inverted-U shape[l]	Inverted-U shape[m]
Bupropion	DAT inhibitor	Increased[n]	Decreased[o]
Cocaine	DAT inhibitor	Increased[l]	Decreased[m]

Table 1.4. Comparison of locomotor responses to DA drug exposure between rodents and larval zebrafish (7 days post fertilization). [a]Adapted from (Irons et al. 2013), [b](Simon et al. 2000), [c](Farrell et al. 2011), [d](Taber et al. 1968), [e](Babbini et al. 1975), [f](Giacomini et al. 2006), [g](Lambert et al. 2012), [h](Bristow et al. 1997), [i](McNamara et al. 1993), [j](Solanto 1998), [k](Lange et al. 2012), [l](Antoniou et al. 1998), [m](Irons et al. 2010), [n](Redolat et al. 2005), [o](Winter et al. 2008).
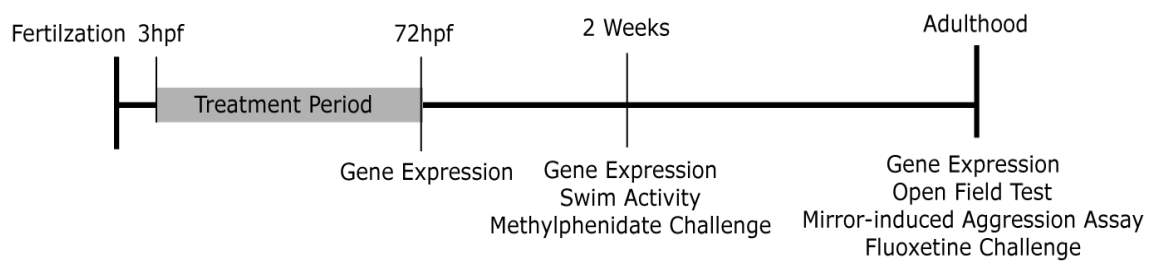
Figure 1.1. Experimental Paradigm

Chapter 2

Developmental deltamethrin exposure causes persistent changes in dopaminergic gene expression, neurochemistry, and locomotor activity in zebrafish

***This is being prepared for publication:**

Kung TS, Richardson JR, Cooper, KR, White LA, Developmental deltamethrin exposure causes persistent changes in dopaminergic gene expression, neurochemistry, and locomotor activity in zebrafish. (*in preparation*).

2.1 Introduction:

Pyrethroids are generally considered to be a safer alternative to other insecticides because they exhibit low mammalian toxicity and low environmental persistence (Demoute 1989). Consequently, their use and popularity is on the rise (Grube 2011). Since it is widely recognized that children are more sensitive to the adverse effects of pesticides (USEPA 2002), a troubling statistic highlighted by the 1999-2002 National Health and Nutrition Examination Survey was the fact that levels of pyrethroid metabolite, 3-phenoxybenzoic acid, were significantly higher in children than adolescents or adults (Barr et al. 2010). Other surveys reported finding pyrethroid metabolites in the urine of pregnant women (Berkowitz et al. 2003; Qi et al. 2012; Whyatt et al. 2002) and children (Babina et al. 2012; Bradman et al. 2007; Couture et al. 2009; Fortin et al. 2008; Heudorf et al. 2004; Morgan et al. 2007) Thus, it is important to understand the consequences of pyrethroid exposure during development.

Recent studies point to toxicant exposure during critical developmental periods as a significant contributing factor to adult disease, including neurological disorders (Fox et al. 2012). Reports in rodents demonstrate that developmental exposure to pyrethroids results in persistent changes in neurochemical, behavioral, and cognitive endpoints (Shafer et al. 2005). Increased locomotor activity was observed in adult mice exposed developmentally to non-toxic doses of deltamethrin (Eriksson and Fredriksson 1991). In rats, gestational exposure to deltamethrin resulted in decreased locomotor activity at 3 and 6 weeks (Husain et al. 1992). The "developmental origins of health and disease" (DOHaD) paradigm provides an explanation for some of the latent effects seen following developmental pyrethroid exposure.

Since the dopamine (DA) system plays an essential role in mediating many of the observed behavioral and locomotor effects, it is possible that disruption of DA neurotransmission during a phase of developmental plasticity is a mode of action for pyrethroid induced locomotor changes. Acutely, pyrethroids can influence the kinetics of DA transmission by altering DA uptake (Elwan et al. 2006; Karen et al. 2001; Kirby et al. 1999), promoting DA release (Eells and Dubocovich 1988) or both (Bloomquist et al. 2002; Hossain et al. 2006). Pyrethroid induced changes in DA uptake has been linked to immediate (Bloomquist et al. 2002; Elwan et al. 2006; Kirby et al. 1999; Pittman et al. 2003) and persistent (Gillette and Bloomquist 2003) changes in dopamine transporter (DAT) expression and activity. In addition, pyrethroid exposure was associated with changes in the concentrations of striatal DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Doherty et al. 1988; Hudson et al. 1986). Thus, if these disturbances occur during a critical period, such

as neurodevelopment, this could reprogram physiological setpoints and result in permanently altered baseline DA neurotransmission.

The vertebrate zebrafish model was utilized to recapitulate the persistent motor deficits caused by embryonic exposure to the pyrethroid pesticide deltamethrin, and to test the hypothesis that dopaminergic dysfunction is responsible for mediating these effects. Developmental exposure (3-72 hpf) to deltamethrin at concentrations below the LOAEL (0.33-0.50 μg/L), lead to increases in locomotor activity following a transition into darkness at the larval stage. Decreased dopamine receptor transcript levels and increased HVA levels are associated with these locomotor effects. Using methylphenidate, a DAT inhibitor, and a DAT morpholino, to knockdown DAT expression, it was shown that DA dysfunction is a possible mechanism for the long term locomotor deficits caused by developmental deltamethrin exposure.

2.2 Methods:

2.2.1 Animal Handling

The AB strain of zebrafish (Zebrafish International Resource Center) was used for all experiments and maintained as previously described (Hillegass et al. 2007). All experiments were conducted in accordance with the zebrafish husbandry and embryonic exposure protocol (#08-025) approved by the Rutgers University Animal Care and Facilities Committee.

2.2.2 Chemicals

Deltamethrin [purity 99.5%] (CAS#52918-63-5) was obtained from ChemService (West Chester, PA). N,N-dimethylformamide (DMF) and methylphenidate HCl were obtained from Sigma-Aldrich (St. Louis, MO).

2.2.3 Pesticide Exposures

A deltamethrin stock solution (2mg/ml) was prepared fresh by dissolving deltamethrin into DMF and subsequently diluted with DMF to make working solutions. 1:10,000 dilutions of the working solutions were performed into aerated egg water (60 μg/ml Instant Ocean in DI water) to obtain final nominal concentrations of 0.25, 0.33 and 0.50 μg/L deltamethrin (0.01% DMF). Embryos exposed to 0.01% DMF were vehicle controls.

Fertilized embryos were staged (Kimmel et al. 1995) and exposure began at 3hpf (512-cell stage). 10 ml static non-renewal bath exposures were performed in 60x15mm glass petri dishes, 1 dish/replicate. Embryos were incubated at 25-26°C in darkness and observed daily using a dissecting microscope for mortality and developmental abnormalities. For larval studies, sac fry larvae (72hpf) were removed from treatment and reared in treatment free water until the larval stage (2-weeks post fertilization (wpf)); each replicate was housed separately.

2.2.4 Reverse-transcriptase quantitative PCR

Embryos were exposed to 0.25, 0.33 and 0.50 μg/L deltamethrin and reared as previously described (section 2.3). At 72hpf or 2wpf, 15-20 embryos were pooled and

snap frozen in liquid nitrogen. (N = 4 pooled replicates/concentration). RNA isolation and RT-qPCR were performed as previously described (Hillegass et al. 2007). Primer sequences are listed in Table 2.1. The experiment was repeated a minimum of three times.

2.2.5 High Performance Liquid Chromatography–Electrochemical Detection (HPLC-ECD)

Embryos were exposed to 0, 0.33, or 0.50 μg/L deltamethrin and reared as previously described (section 2.3). At 2wpf, larvae were snapfrozen in liquid nitrogen (N=6 pooled replicates/treatment, 50 embryos per replicate). 50 μL of cold 0.1N PCA solution (with 300 μM EDTA and 300 μM sodium metabisulfite) was added to each sample and homogenized 2x on ice using a motorized pestle. Samples were spun at 16,000xg for 10 minutes at 4°C and the supernatant was filtered through a 0.22 μM spin filter (EMD Millipore, Billerica, MA). The remaining pellet was dried and dissolved in 200 μl of 0.5N NaOH for protein concentration determination using the Modified Lowry Protein Assay (Thermo Scientific, Waltham, MA). Filtered supernatant was diluted 1:10 in PCA solution and 5 μl was injected onto a Waters Alliance HPLC equipped with a Waters 2465 ECD (Waters Corporation, Milford, MA). Separation and quantitation of DA, DOPAC, and HVA was performed as previously described (Schuh et al. 2009; Sheleg et al. 2013). The experiment was repeated twice.

2.2.6 Activity monitoring of 2 week old larvae

The testing apparatus consisted of 4 IR sensitive Ikegami ICD-49 CCD Cameras with Computar IR manual vari-focal lenses (1/2") and IR filters mounted to the ceiling directly over 4 light boxes. Each light box consisted of a semi-transparent platform on

which the plates were placed and transilluminated by an IR-Room IR illuminator and/or 3 LED lights directly underneath. The camera, lens, filters, and IR illuminators were purchased from Noldus Information Technology, (Leesburg, VA).

Embryos were exposed to 0.25, 0.33 and 0.50 μg/L deltamethrin and reared as previously described (section 2.3). At 2wpf, larvae were placed randomly into white-walled, clear-bottom 24-well plates containing 1.5 mls of room temperature system water. Larvae were allowed to acclimate on the testing apparatus for one hour in light (400 lux). After one hour, lights were turned off to stimulate activity and video was recorded using the Noldus MPEG Recorder 2.1 (Noldus Information Technology, Leesburg, VA) for 30 minutes. Testing occurred between 13:00 and 16:30 h, reducing the time of day parameter (MacPhail et al. 2009).

EthoVision XT 8 (Noldus Information Technology, Leesburg, VA) was used to track the videos and quantify distance travelled. A differencing background subtraction method was applied to detect objects that were darker than the background at a sample rate of 29.97 samples/s. Outliers were considered to be 1) larvae that did not move (<100 mm total distance travelled in 25min) or 2) larvae where at least 3 timebin values exceeded 2 standard deviations from the mean. Studies were repeated three times (from three different breeding sets) and data collapsed such that each larvae was considered an individual and the distance travelled/time bin was an average of all individuals for that treatment group.

2.2.7 DAT Morpholino Injections

An antisense morpolino was designed to target the I2E3 splice junction of *slc6a3* (*dat*) (ENSDART00000129303, Zv7) pre-mRNA, resulting in the deletion of exon 3 in

the mature transcript. The I2E3 splice junction is conserved between the 2 splice variants of *slc6a3*; therefore, the morpholino would target both splice variants. The DAT morpholino 5'-CCCACGGCTGATAAAACAACACACAC-3' was 3' fluorescein tagged and synthesized by GeneTools, LLC (Philomath, OR). A standard control morpholino 5'-CTCTTACCTCAGTTACAATTTATA-3' was used as an injection control. Embryos were injected with 50 μM of the DAT morpholino or control morpholino as previously described (Hillegass et al. 2007). The total injection volume was 8 nl. Embryos exhibiting even distribution of fluorescence 3 hours after injections were used. The injected embryos were treated with 0.33 μg/L deltamethrin or vehicle and raise to 2wpf as described above (section 2.3). At 2wpf, activity monitoring was performed as described above (section 2.6).

2.2.8 Methylphenidate Challenge

Methylphenidate HCL was dissolved in water to obtain a stock concentration of 1mg/ml. Embryos were exposed to 0 or 0.33 μg/L deltamethrin during development and reared until 2wpf as described above (section 2.3). The larvae were subjected to activity monitoring as described above (section 2.6) except that 20 minutes prior to video recording, 1.5 μl of the methylphenidate stock (or water) was added to the appropriate wells to obtain a final concentration of 1 mg/L.

2.2.9 Statistical analyses

Data were analyzed using SigmaPlot v.11 (Systat Software Inc., San Jose, CA). The probability level for statistical significance was ($p \leq 0.05$) for all studies.

2.3 Results:

2.3.1 Developmental deltamethrin exposure increases larval swim activity

Swim activity of 2-week larval fish was measured to determine the long-term effects of developmental low-dose deltamethrin exposure (figure 1). The concentrations tested (0, 0.25, 0.33, or 0.50 µg/L) were below the established LOAEL of 1 µg/L for deltamethrin in zebrafish (DeMicco et al. 2010). Deltamethrin exposure did not cause morphological lesions or acute toxicity (data not shown). There was a significant effect of developmental deltamethrin exposure on swim activity (distance travelled) at 2wpf ($F(3,1103)=8.807$, $p=0.001$). Swim activity across all time bins was significantly increased in larvae that had been developmentally exposed to 0.33 or 0.50 µg/L deltamethrin compared to vehicle control. There was no significant difference between the activities of larvae developmentally exposed to 0.25 µg/L deltamethrin or control. There was not a significant effect of time on swim activity, nor was there a significant interaction between time and deltamethrin treatment. Developmental exposure to 0.33 and 0.50 µg/L deltamethrin resulted in increased swim activity across all time bins following a transition to darkness at the larval stage.

2.3.2 Developmental deltamethrin exposure alters dopaminergic gene expression

Transcript levels of several dopaminergic genes were measured at 72hpf and 2wpf to characterize the immediate and persistent effects of deltamethrin exposure on the dopaminergic system. At 72hpf (figure 2a), there was a significant effect of developmental deltamethrin exposure on *drd1* ($F(3,13)=8.017$, $p=0.005$) and *th* ($F(3,14)=7.828$, $p=0.004$) transcript levels. Developmental exposure to 0.33 and 0.50

µg/L deltamethrin resulted in a significant 1.28 and 1.32–fold decrease in *drd1* transcript levels respectively. Developmental exposure to 0.25 µg/L deltamethrin significantly increased *th* transcripts 1.90-fold. Developmental deltamethrin exposure did not significantly alter *dat*, *drd2a*, or *drd3* transcript levels.

At the larval stage (figure 2b), there was a significant effect of developmental deltamethrin exposure on *drd1* ($\chi^2(3)$=8.747, *p* =0.033) and *drd2a* (*F*(3,15)=5.513, *p* =0.013) transcript levels. Developmental exposure to 0.50 µg/L deltamethrin decreased *drd1* transcript levels by 1.90-fold and *drd2a* transcript levels by 2.72-fold. The transcript levels of *dat*, *drd3*, or *th* at the larval stage were not significantly changed by developmental deltamethrin exposure. The immediate effects of developmental deltamethrin exposure included decreased *drd1* transcript levels and increased *th* transcript levels (figure 2a). *drd1* downregulation persisted to the larval stage and was accompanied by a decrease in *drd2a* transcripts (figure 2b). *th* transcript levels returned to baseline at the 2-week time point (figure 2b).

2.3.3 The effect of developmental deltamethrin exposure on whole body dopaminergic neurotransmitter levels

To determine if developmental deltamethrin exposure results in persistent changes in DA neurochemistry, the levels of DA and its metabolites, HVA and DOPAC, were measured at 2wpf (figure 3a), a time point where deltamethrin induced hyperactivity was observed (figure 1). At the larval stage, there was a significant effect of deltamethrin exposure on HVA levels ($\chi^2(2)$=6.251, *p* =0.034). Developmental exposure to 0.33 µg/L deltamethrin resulted in a 2.14-fold increase in HVA levels. There were no significant

differences in the levels of DA or DOPAC between deltamethrin treated and control embryos.

Metabolite/DA ratios were calculated to examine changes in the rate of DA metabolism (figure 3b). There were no significant differences in DOPAC/DA, HVA/DA, or HVA/DOPAC ratios between control and deltamethrin treated embryos.

2.3.4 Effect of methylphenidate exposure on swim activity in larvae developmentally exposed to deltamethrin

Since DA clearance from the synapse is primarily mediated by its reuptake via the DAT, 2-week old larvae were treated with methylphenidate, a DAT inhibitor, to increase DA availability in the synapse (figure 5). It was hypothesized that saturation of the downregulated dopamine receptors (figure 2b) would rescue deltamethrin induced swim activity (figure 1). There was a significant effect of developmental deltamethrin exposure (F(1,1754)=5.833, p =0.016) and methylphenidate exposure (F(1,1754)=5.586, p =0.018) on larval swim activity. Compared to vehicle treated embryos, developmental exposure to 0.33 µg/L deltamethrin resulted in a significant increase in swim activity across all time bins within the non-methylphenidate treated group, similar to the results in figure 1. There was also a significant interaction between deltamethrin exposure and methylphenidate treatment (F(1,1754)=28.334, p=0.001). In larvae developmentally exposed to vehicle control, subsequent methylphenidate exposure resulted in a significant increase in swim activity across all time bins. Whereas, in larvae developmentally exposed to deltamethrin, subsequent methylphenidate exposure resulted in a significant decrease in swim activity across all time bins, to levels below that of control larvae. No

other significant interactions were detected (deltamethrin x time, methylphenidate x time, deltamethrin x methylphenidate x time). The effect of methylphenidate on swim activity depended on whether the larvae had previously been exposed to deltamethrin during development. Methylphenidate treatment at 2wpf resulted in a significant reduction in swim activity in deltamethrin treated larvae instead of the expected increase in swim activity observed in vehicle control larvae.

2.3.5 DAT morpholino attenuation of deltamethrin induced swim activity

An antisense morpholino (MO) was used to specifically reduce DAT expression (figure 4) in an attempt to saturate the dopamine receptors and attenuate deltamethrin induced hyperactivity. Transient DAT knockdown during development, via DAT-MO (50 µM), did not result in morpholino toxicity (data not shown) or alter larval swim activity ($F(1,1044)=0.448$ $p=0.504$). There was a significant effect of deltamethrin exposure on swim activity ($F(1,1044)=4.066$, $p=0.044$). Within the CO-MO group, treatment with 0.33 µg/L deltamethrin during development resulted in increased swim activity across all time bins compared to control animals, similar to figure 1. However, within the DAT-MO group, there was no significant difference in activity levels of deltamethrin treated and control larvae. DAT-MO knockdown returned deltamethrin induced increases in swim activity to control levels.

2.4 Discussion:

Developmental exposure to non-toxic doses of deltamethrin results in persistent changes in larval swim activity, dopaminergic gene expression and neurochemistry. Our

studies indicate that dopaminergic dysfunction is a likely mode of action for deltamethrin induced increases in locomotion.

The increases in larval swim activity following developmental deltamethrin exposure, even after being reared in treatment free water since 72hpf, are consistent with findings in rodents. Prenatal exposure to non-neurotoxic doses of deltamethrin resulted in increased locomotion in adult mice (Eriksson and Fredriksson 1991) and altered locomotion in adolescent (Husain et al. 1992) and adult rats (Johri et al. 2006; Lazarini et al. 2001). We previously characterized the developmental toxicity associated with an acute exposure to 6 different pyrethroids, and determined that the observed toxicities were consistent with that reported in the mammalian literature (DeMicco et al. 2010). Together, this demonstrates that the zebrafish is an appropriate vertebrate model for studying the long-term behavioral effects of neurotoxicant insult during development.

Given the role of the dopamine system in mediating locomotion and behavior (Levin et al. 2011), the effects of developmental deltamethrin exposure on dopaminergic system was examined at the gene expression level. Exposure to the lowest concentration of deltamethrin resulted in increased *th* expression immediately after exposure; however, this change was transient and returned to basal levels by the 2-week time point. Chronic exposure to deltamethrin has been shown to decrease TH mRNA and protein expression and hydroxylase activity in adult male rats (Liu et al. 2006). The difference in the directionality of *th* expression could be due to inherent differences between the rat and zebrafish models, as well as, to the differential responses of developing and adult organisms to toxicant exposure. However, both studies indicate the potential for deltamethrin to modulate TH expression, thereby altering dopamine biosynthesis.

This study is the first to report deltamethrin induced changes in dopamine receptor transcript levels. Embryos exposed developmentally to deltamethrin exhibited a decrease in *drd1* transcript levels which persisted to the larval stage. Both the dopamine D1 and D2 receptors undergo heterologous and/or homologous desensitization if subject to prolonged activation (Balmforth et al. 1990; Barton and Sibley 1990; Memo et al. 1982). Given that deltamethrin exposure can potentiate DA release (Bloomquist et al. 2002; Eells and Dubocovich 1988; Hossain et al. 2006) or decrease DA uptake (Elwan et al. 2006), these actions may result in increased levels of DA in the synapse and promote DA receptor activation. Accordingly, prolonged exposure to dopamine downregulated *DRD1* expression in SK-N-MC neuroblastoma cells (Sidhu et al. 1999), suggesting that transcriptional regulation may be a mechanism of DA receptor desensitization. At the larval stage, *drd1* downregulation was accompanied by a decrease in *drd2a* transcript levels. Despite the fact that the D1 receptor and D2 autoreceptor have opposing actions on cAMP signal transduction, they act in a synergistic manner to modulate behavior (Robertson 1992). Altering D1 receptor signal transduction often results in corresponding changes in D2 receptor mediated responses; D1 regulates D2 sensitivity (Braun et al. 1997; Hasbi et al. 2011; Hu et al. 1992; Walters et al. 1987). Therefore, it is plausible that a sustained decrease in *drd1* transcript could promote a subsequent downregulation of *drd2a* at the transcript level. Since *Drd1* and *Drd2* knockout mice exhibited altered locomotion (Holmes et al. 2004), together our data demonstrate that reduced D1 and/or D2 levels following developmental deltamethrin exposure likely influences locomotor activity.

Developmental exposure to deltamethrin increased levels of HVA at the larval stage. In adult male rats, developmental exposure to nontoxic doses of deltamethrin resulted in increased levels of striatal DOPAC and DOPAC/DA ratios (Lazarini et al. 2001). Neonatal permethrin or cypermethrin exposure resulted in decreased DA and increased HVA levels in the striatum of male rats 3 weeks after exposure (Nasuti et al. 2007). In both these studies, the affected animals had indications of increased DA metabolism, which was associated with locomotor deficits. In our studies, there were no significant differences in levels of DA, DOPAC, or the metabolite/DA ratios between control and deltamethrin treated embryos at the larval stage. This could be due to the fact that our data represent neurotransmitter levels from whole body preparations as opposed to very specific brain regions, such as the striatum. Therefore, the assay was in all likelihood not sensitive enough to detect differences over the background contributions of DA or DOPAC from other brain regions or from peripheral sources. Additionally, since little is known about DA metabolism in the zebrafish, more research would be necessary to determine the preferred pathway of DA metabolism. However, since CSF, plasma, and/or urinary HVA levels is often used as a biomarker of CNS dopaminergic dysfunction in humans, the increased HVA levels in deltamethrin treated fish is likely indicative of an altered dopamine state.

Since developmental deltamethrin exposure results in decreased transcript levels of *drd1, drd2a* and increased swim activity during the larval stage, we hypothesized that stimulating the dopamine receptors would attenuate this behavioral phenotype. Methylphenidate, an indirect dopamine agonist, was used to increase extracellular levels of dopamine in the synapse in an attempt to saturate the dopamine receptors.

Methylphenidate was chosen because it rescues hyperactive phenotypes in zebrafish (Lange et al. 2012) and rodents (Sagvolden et al. 2005). The present study demonstrates that developmental exposure to deltamethrin modifies the susceptibility of the dopaminergic system in larval animals. In control animals, methylphenidate exposure increased activity. Methylphenidate, a psychostimulant, is known to increase locomotor activity in rodents (McNamara et al. 1993; Solanto 1998) and zebrafish (Lange et al. 2012). However, in larvae that had been developmentally exposed to deltamethrin, methylphenidate reduced swim activity to below the basal levels of control animals. This opposite response is similar to the reported paradoxical effects of methylphenidate on locomotion and behavior in rodents exhibiting altered dopamine states (Del'Guidice et al. 2014; Shaywitz et al. 1978; Tilley and Gu 2008). Taken together, developmental deltamethrin exposure alters the sensitivity of larval fish to methylphenidate and potentially other pharmaceuticals that target the dopaminergic system.

Because methylphenidate also functions as a norepinephrine reuptake inhibitor, a splice morpholino specifically targeting the dopamine transporter was employed to knockdown DAT expression. Since morpholinos are penetrant through 72hpf (Nasevicius and Ekker 2000), DAT knockdown would coincide exactly within the deltamethrin exposure window. In control animals, disruption of dopamine transmission during development by the DAT morpholino did not phenocopy the effects of developmental deltamethrin exposure in terms of persistent activity changes. This could indicate that the effects of deltamethrin neurotoxicity are broad and not limited to dopamine receptor activation Because pyrethroids exert their toxicity primarily through interaction with sodium channels (DeMicco et al. 2010; Hossain and Richardson 2011; Soderlund and

Bloomquist 1989; Soderlund et al. 2002), exposure likely influences multiple components of the dopamine system and other neurotransmitter systems as well. The lack of effect of DAT morpholino knockdown could also be due to the ability of zebrafish embryos to overcome the transitory effects elicited by the concentration of DAT morpholino used. Despite this, in deltamethrin exposed animals, simultaneous DAT morpholino knockdown was sufficient to attenuate deltamethrin induced increases in swim activity at the larval stage.

In conclusion, developmental exposure to deltamethrin resulted in increased swim activity following a transition to darkness in larval zebrafish. This was associated with changes in dopamine neurochemistry and gene expression as evidenced by increased levels of HVA, and decreased levels of the *drd1* and *drd2a* transcripts. *dat* knockdown during development attenuated deltamethrin induced hyperactivity and methylphenidate exposure resulted in a paradoxical reduction of activity in deltamethrin treated fish. Together, these data indicate that dopaminergic dysfunction is a likely mode of action for the persistent hyperactivity observed in the zebrafish exposed developmentally to deltamethrin. These results highlight the risk of low dose (below the NOAEL) neurotoxicant exposure during the critical developmental period on more subtle endpoints such as locomotor activity and drug response.
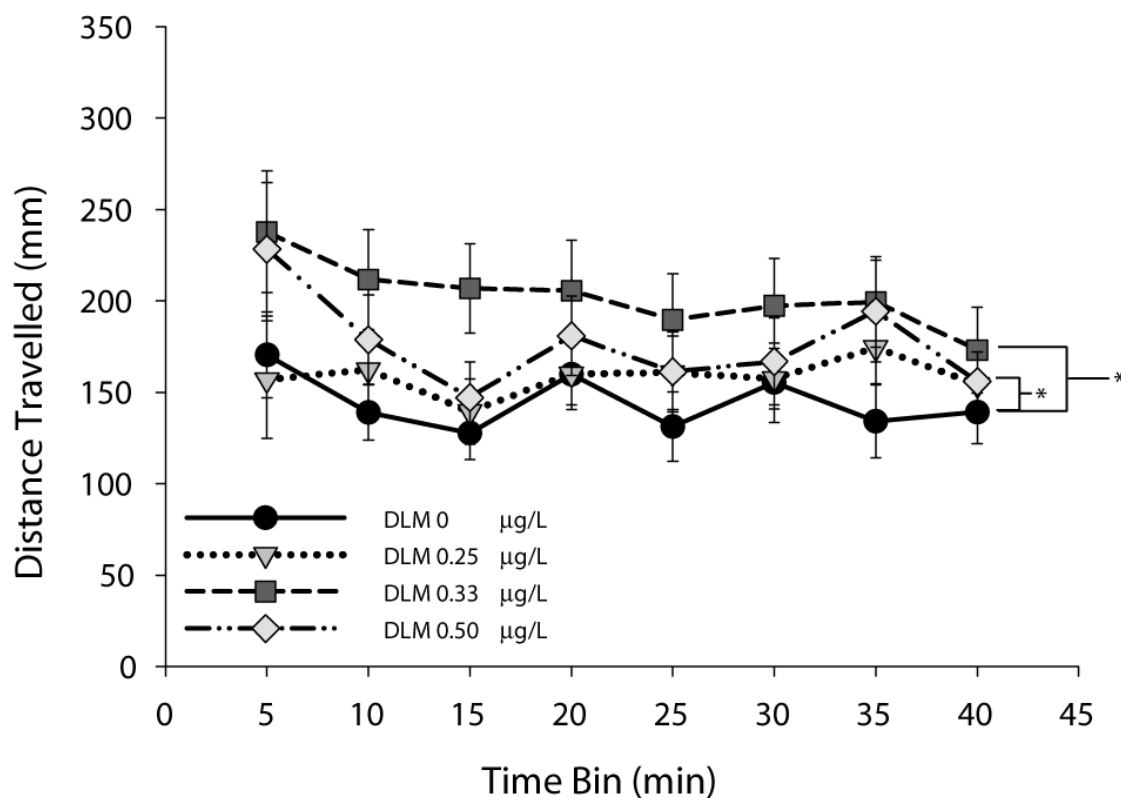
Figure 2.1: Swim activity following a transition into darkness of 2-week old larval zebrafish developmentally exposed to deltamethrin (DLM). Data points are presented as mean distance traveled (mm) ± S.E.M. in 5-min time bins during 40-min sessions (n=31-38). A two-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure across time on larval swim activity. (*) indicates that distance travelled across time in animals developmentally exposed to 0.333 µg/L and 0.5 µg/L deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
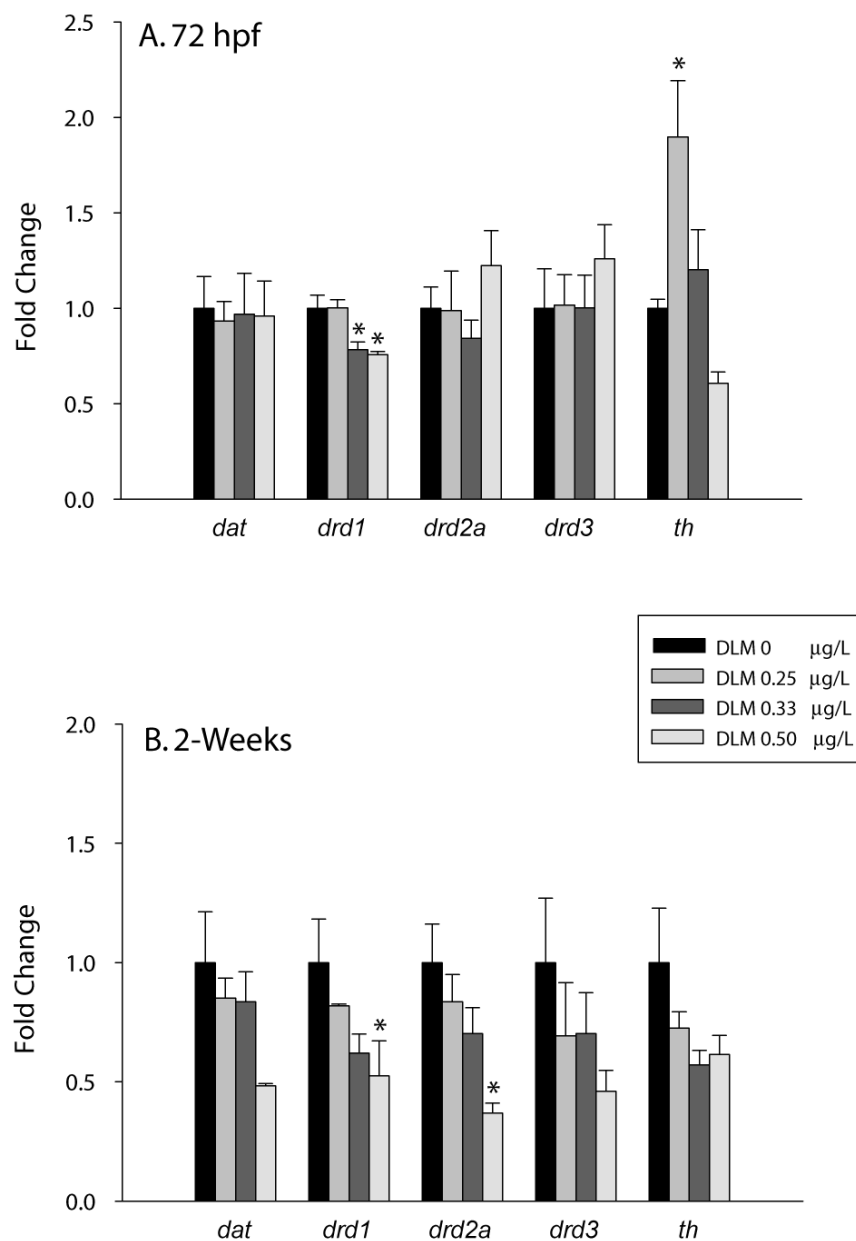
Figure 2.2: RNA transcript levels of genes related to dopamine transport (*dat*), reception (*drd1, drd2a, drd3*), and synthesis (*th*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at A) 72hpf and B) 2-weeks post fertilization. The graphs represents the mean fold change in transcript copy number ± S.E.M of one representative experimental replicate (n=4). A one-way ANOVA or a Kruskal-Wallis ANOVA on Ranks was used to determine if there was a significant effect of deltamethrin exposure on transcript levels where appropriate. (*) indicate a significant difference versus control as determined by Holm-Sidak or Dunn's post hoc analyses where appropriate ($p \leq 0.05$).
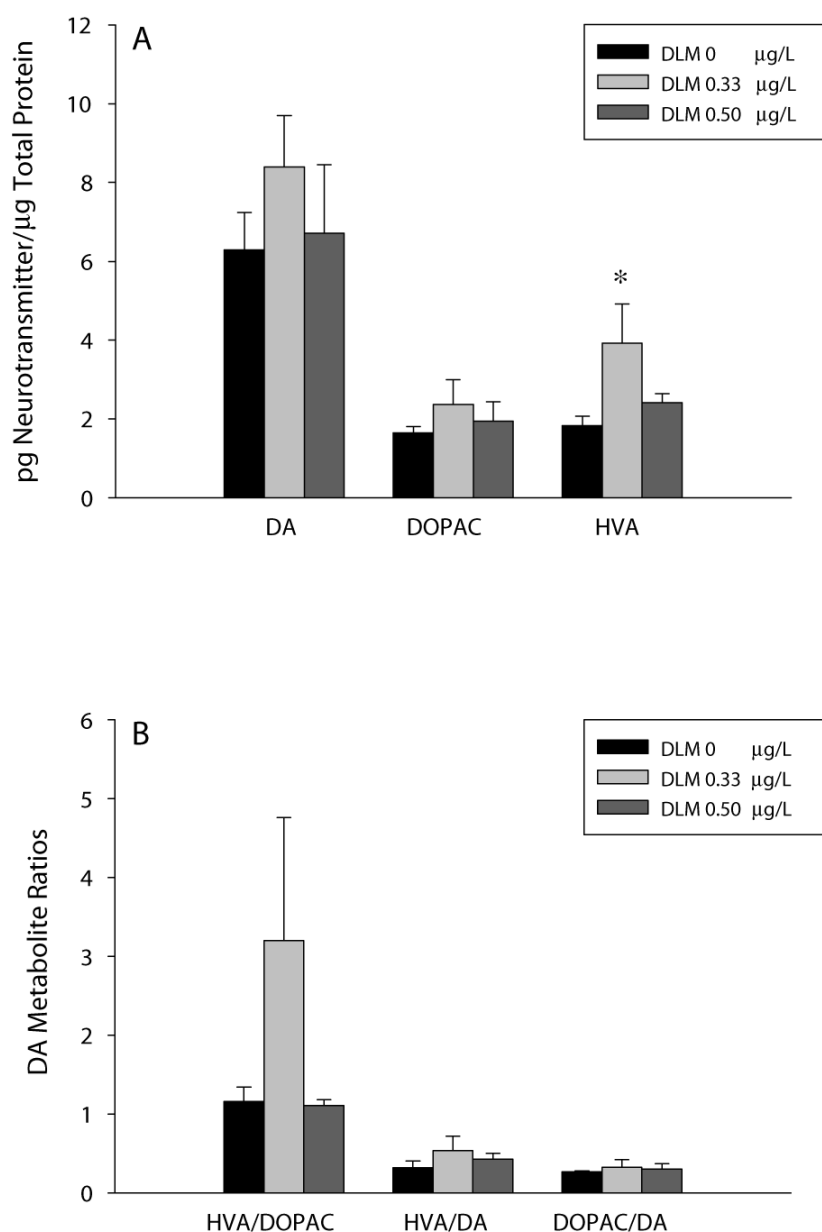
Figure 2.3: The effects of developmental deltamethrin (DLM) exposure on dopamine neurotransmitter levels at 2-weeks of age. A) Whole body concentrations of dopamine (DA), metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and B) metabolite ratios. Values represent the mean ± SEM (n=5) of one representative experimental replicate. A one-way ANOVA was performed to determine if there was a significant effect of developmental deltamethrin exposure. (*) indicates a significant difference versus control animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
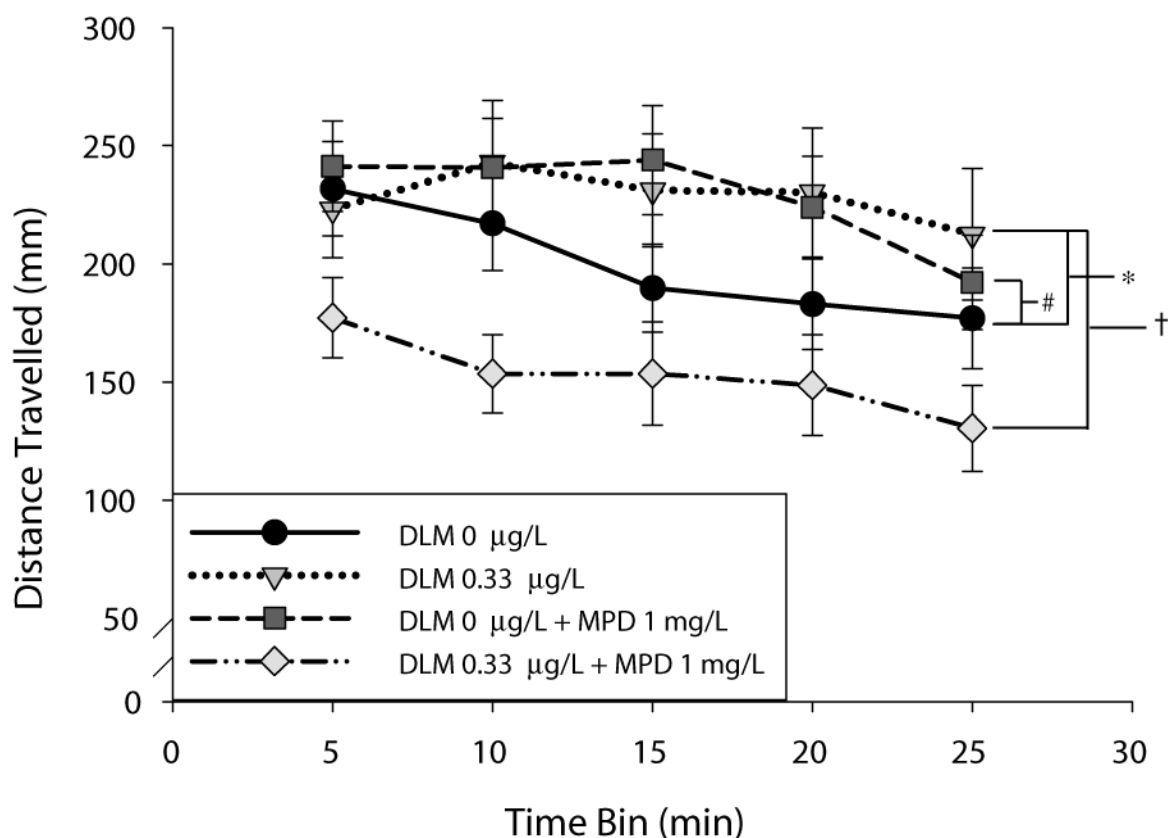
Figure 2.4: Effects of methylphenidate (MPD) on larval swim activity following a transition into darkness in zebrafish developmentally exposed to deltamethrin (DLM). Data points are presented as mean distance travelled (mm) ± S.E.M. in 5-min time bins during 25-min sessions (n=76-97). A three-way ANOVA was used to determine if there was a significant effect of deltamethrin, methylphenidate, and time on larval swim activity as well as an interaction (deltamethrin x DAT morpholino x time). As determined by Holm-sidak multiple comparisons, (*) indicates that distance travelled across time in deltamethrin exposed animals are significantly different from vehicle control exposed animals ($p \leq 0.05$). (#) indicates that there is a significant effect of methylphenidate on the distance travelled across time within vehicle control exposed animals ($p \leq 0.05$). (†) indicates that there is a significant effect of methylphenidate on the distance travelled across time within deltamethrin exposed animals ($p \leq 0.05$).
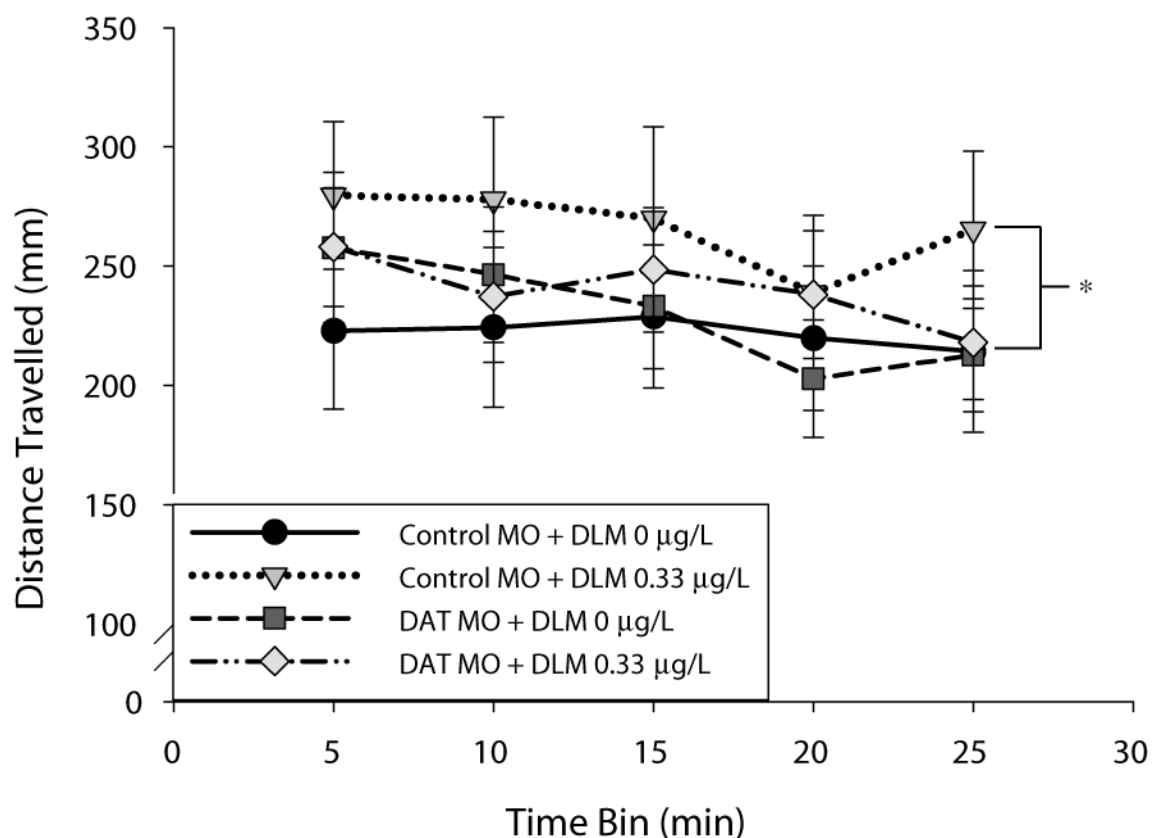
Figure 2.5: Effects of developmental dopamine transporter (DAT) knockdown and deltamethrin (DLM) exposure on larval swim activity following a transition into darkness. Data points are presented as mean distance travelled (mm) ± S.E.M. in 5-min time bins during a 25-min session (n=40-62). A three-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure, DAT morpholino (MO), and time on larval swim activity as well as an interaction (deltamethrin x DAT morpholino x time). As determined by Holm-Sidak multiple comparisons, (*) indicates that total distance travelled in deltamethrin exposed animals is significantly different from vehicle control exposed animals within the control morpholino group ($p \leq 0.05$). There is no significant difference in total distance travelled between deltamethrin and vehicle control exposed animals within the DAT morpholino group ($p \geq 0.05$).

Chapter 3

The persistent behavioral deficits in adult zebrafish caused by developmental deltamethrin exposure are sex specific

*This work is being prepared for submission

Kung TS, Richardson JR, Cooper, KR, White LA, The persistent effects of developmental deltamethrin exposure on adult zebrafish behavior and serotonergic and dopaminergic gene expression are sex-specific. (*in preparation*).

3.1 Introduction

Pyrethroid pesticides are one of the most commonly used insecticides and account for approximately 23% of the world's insecticide market (Casida and Quistad 1998). Recent sources indicate that pyrethroid use is increasing (Grube 2011; Power and Sudakin 2007), a trend most prevalent in the residential setting in the United States due to the phase out of two previously popular organophosphate insecticides (Bekarian et al. 2006; Trunnelle et al. 2014). The popularity of pyrethroids is attributed to their low mammalian toxicity and rapid degradation; thus, pyrethroids are considered a safer alternative to other classes of insecticides (Demoute 1989). Therefore, it is not surprising that pyrethroid metabolites are commonly found in humans during biomonitoring surveys (Barr et al. 2010; Becker et al. 2006; Heudorf and Angerer 2001; Heudorf et al. 2004). While the acute toxic effects of pyrethroid exposure are well characterized, less is known about the effects of low dose exposures on neurobehavioral endpoints, especially in vulnerable populations such as children.

In 1993, the National Research Council report "Pesticides in the Diets of Infants and Children," established that children are more susceptible to the adverse effects of pesticides (National Research Council (U.S.). Committee on Pesticides in the Diets of Infants and Children. 1993). Furthermore, it would appear that many adult diseases are predisposed by adverse environments experienced during development, likely due to altered physiological programming brought about by these environmental stressors (Gluckman et al. 2007). The "developmental origins of health and disease" (DOHaD) hypothesis has been found to apply to many diseases of the brain including neurodegenerative (Modgil et al. 2014), neurobehavioral (Schlotz and Phillips 2009; Van den Bergh 2011) and cognitive (Schlotz and Phillips 2009) disorders. In addition, neurotoxicant exposure is considered a prenatal factor associated with mental health disorders later in life (Fox et al. 2012). Because pyrethroid pesticides are known neurotoxicants (Soderlund and Bloomquist 1989) and can be found in the urine of pregnant women (Berkowitz et al. 2003; Qi et al. 2012; Whyatt et al. 2002) as well as in children and adolescents (Babina et al. 2012; Barr et al. 2010; Becker et al. 2006; Bradman et al. 2007; Couture et al. 2009; Fortin et al. 2008; Heudorf et al. 2004; Morgan et al. 2007; Morgan 2012), it is possible that developmental pyrethroid exposure could pose a long-term human health risk.

Recent animal studies have highlighted the potential for developmental pyrethroid exposure, using nontoxic doses, cause persistant behavioral effects that last into adulthood, long after cessation of exposure, reviewed in (Shafer et al. 2005). For instance, mice exposed to deltamethrin (0.7 mg/kg) between PND10-16 exhibited increased locomotor activity and lack of habituation as adults (4 months) (Ahlbom et al.

1994; Eriksson and Fredriksson 1991; Talts et al. 1998). Similarly in rats, exposure to deltamethrin (0.07 mg/kg) at early developmental time points (PND0-7) resulted in reduced locomotor activity as adults (13 weeks); whereas, exposure during later developmental time points (PND 9-13) resulted in increased locomotor activity during adulthood (Patro et al. 2009). In addition to changes in locomotor activity, adults rats (12 weeks) that had been exposed prenatally (GD14-20) to deltamethrin (1 mg/kg) exhibited learning deficits (Aziz et al. 2001). Pprenatal (GD6-15) exposure to deltamethrin (0.08mg/kg) was also associated with differences in forced swim behavior and spontaneous locomotion 15 minutes after the swim test in adult male rats (Lazarini et al. 2001). Together, these results demonstrate that exposure to deltamethrin during different developmental periods causes locomotor and behavioral deficits that persist into adulthood.

Zebrafish provide a rapid, cost effective, and eukaryotic model for developmental neurotoxicity testing (Gerlai 2012). Assessment of locomotor activity in both larval (Selderslaghs et al. 2010) and adult (Stewart et al. 2014) zebrafish is commonly used to determine the effects of neurotoxicant exposure. In addition, adult zebrafish engage in a wide repertoire of behaviors including, but not limited to, social behaviors, such as aggression (Filby et al. 2010) and shoaling (Gerlai 2014), anxiety/fear-related behaviors (Guo et al. 2012; Jesuthasan 2012), and cognitive behaviors, such as learning (Gerlai 2011) and attention (Echevarria et al. 2011). To measure these complex behavioral endpoints, a number of rodent behavioral assays have been adapted for use with adult zebrafish, such as the open field tests, light/dark boxes, mazes and etc (Champagne et al.

2010; Norton and Bally-Cuif 2010). Because of this, zebrafish are beginning to emerge as a viable organism to model complex human brain disorders (Kalueff et al. 2014).

We previously demonstrated that embryonic exposure to low doses of deltamethrin results in increased locomotor activity in larval zebrafish (2-weeks) (figure 2.1). Given the prevalence of deltamethrin-induced locomotor effects persisting into adulthood in rodent models, we hypothesize that developmental exposure to low doses of deltamethrin would also cause locomotor and behavioral deficits that persists into adulthood in the zebrafish model. Zebrafish embryos were exposed to deltamethrin (0.25 and 0.5 µg/L) at concentrations below the LOAEL, but previously shown to cause increased locomotor activity at the larval stage, during the embryonic period (3-72hpf) (figure 2.1). After which, larvae was removed from treatment and reared to adulthood (6 months – 1 year) in treatment free water. Using the open field test/novel tank test and mirror-induced aggression assay, developmental deltamethrin exposure was associated with gender specific changes in locomotion (distance travelled and velocity), intersession locomotion, thigmotaxis (tendency to stay by edges), and aggression in adult zebrafish.

3.2 Materials and Methods

3.2.1 Zebrafish Husbandry

The AB strain zebrafish (Zebrafish International Resource Center, Eugene, OR) were used for all experiments. Breeding stocks were bred and housed in Aquatic Habitats (Apopka, FL) recirculating systems under a 14:10 hour light:dark cycle. System water was obtained by carbon/sand filtration of municipal tap water and water quality was maintained at <0.05 ppm nitrite, <0.2 ppm ammonia, pH between 7.2 and 7.7, and

between 26 and 28°C. All experiments were conducted in accordance with the zebrafish husbandry protocol and embryonic exposure protocol (#08-025) approved by the Rutgers University Animal Care and Facilities Committee.

3.2.2 Chemicals

Deltamethrin [purity 99.5 %] (CAS# 52918-63-5) was purchased from ChemService (West Chester, PA). N,N-dimethylformamide (DMF) was purchased from Sigma-Aldrich (St. Louis, MO).

3.2.3 Pesticide Exposures

Deltamethrin stock solutions (2 mg/ml) were prepared fresh the day of treatment by dissolving deltamethrin into DMF. Working solutions were made by diluting the stock solution into DMF. Treatments were prepared by performing 1:10,000 dilutions of the working solution into aerated egg water (60 µg/ml Instant Ocean in DI water) to obtain final nominal concentrations of 0.25, 0.33 and 0.50 µg/L deltamethrin (0.01% DMF). Embryos exposed to 0.01% DMF served as vehicle controls.

Fertilized embryos were staged (Kimmel et al. 1995) and exposure began approximately between the 512-cell to oblong stages. Exposures were performed in 60x15mm glass petri dishes using a static non-renewal bath exposure in a total volume of 10mls (30 embryos/dish). Embryos were incubated in the dark at 25-26 °C and were observed daily using a dissecting microscope for the presence of mortality and developmental abnormalities. At 72hpf, sac fry larvae were removed from treatment and reared in treatment free water until assessment. At maturation, adults of the same

treatment group were group housed in 3-liter tanks at a stocking density of 20-25 fish/tank.

3.2.4 Zebrafish horizontal open field test

Embryos were exposed to 0, 0.25, or 0.50 μg/L deltamethrin and reared to adulthood (6 months – 1.5 years) as described above (section 2.3).

The open field apparatus, modified from (Champagne et al. 2010), consisted of a 3L aquatic habitats tank filled with 1.5L clean system water to minimize vertical swimming and covered with white paper to minimize external visual stimulus. The length of the tank was then shortened to 16.5 cm on the vertical side using a plastic backing. 4 Ikegami ICD-49 CCD cameras with Computar Infra-Red manual vari-focal lenses (1/2") (Noldus Information Technology, Leesburg, VA) were mounted to the ceiling directly above the each OFT tank. Each camera was able to capture 2 OFT tanks in one frame. Illumination via fluorescent room lights was consistent with housing conditions (300-400 lux).

Adult zebrafish were singly placed into a novel empty tank using a net. Video recording using the Noldus MPEG Recorder 2.1 (Noldus Information Technology, Leesburg, VA) began immediately after transfer. The total trial length was 30 min, after which the fish were removed and were individually housed for the remainder of the experiment. The OFT tanks were rinsed and water was renewed in between trials to remove waterborne pheromones. Testing occurred between 12:00 and 16:00 hours each day. Each fish was subject to the same procedure once a day, every day, for 5 days to assess intersession habituation.

Distance travelled, velocity (a measure of distance travelled per unit time), and mobility (a spatially independent measure of body movement), are endpoints commonly used to measure motor aspects of zebrafish swimming (Kalueff and Cachat 2011). EthoVision XT 8 (Noldus Information Technology, Leesburg, VA) was used to analyze the obtained videos offline and quantify horizontal locomotor activity, including distance travelled (cm), average velocity (cm/s), duration spent in a defined middle zone (thigmotaxis) (s), and frequency and duration (s) of high mobility and immobility (thresholds of >60% and <20% total body displacement/sample, respectively). A dynamic background subtraction method was applied to detect objects that were darker than the background and the sample rate was set to sample 29.97 samples/s. Following tracking, fish that remained immobile for 50% of the total 30 minute trial were excluded from data analysis. For distance travelled and velocity analysis, fish were determined to be outliers if at least 3 timebin values exceeded 2 standard deviations from the mean. For thigmotaxis and frequency of high mobility analysis, a grubbs outlier test was used to determine outliers within each treatment group.

3.2.5 Zebrafish Aggression: mirror-induced stimulation

Deltamethrin treated and vehicle treated adult zebrafish used in the open field test (section 2.4) were subject to mirror-induced stimulation immediately following the last OFT trial (day 5). The assay was adapted from (Norton et al. 2011). A mirror (7.5 x 7.5 cm) was slotted into trapezoidal end of the tank following the angle of the trapezoid and video was recorded for 10 minutes. Videos were viewed by 2 independent blind reviewers and the number of bites and pushes against the mirror were counted. A pearson's correlation test was used to confirm the consistency between the 2 reviewers.

3.2.6 Statistical analyses

All data were analyzed using the SigmaPlot version 11 computer software package (Systat Software Inc., San Jose, CA). Details of the tests used can be found in the figure legends. The probability level for statistical significance was $p \leq 0.05$ for all studies.

3.3 Results:

3.3.1 Effects of developmental deltamethrin exposure on adult swim activity

The locomotor activity of adult fish was measured using an open field test/novel tank test paradigm, to assess the long-term behavioral effects of developmental deltamethrin exposure (figure 3.1, 3.2, and 3.3). The concentrations tested (0.25 and 0.5 µg/L) represent concentrations that previously did not and did cause increased larval swim activity, respectively (figure 2.1). In male zebrafish, there was a significant effect of deltamethrin exposure during development on adult swim activity (distance travelled) $(F(2,134)=5.157, p=0.007)$. The distance travelled across all timebins was significantly increased in adult male zebrafish that had been developmentally exposed to 0.5 µg/L deltamethrin (figure 3.1a). Developmental exposure to 0.25 µg/L deltamethrin did not significantly change the distance travelled in adult male zebrafish (figure 3.1a). In terms of swim velocity, there was also a significant effect of developmental deltamethrin exposure on swim velocities in male adults $(F(2,139)=4.140, p=0.018)$. The swim velocity across all timebins was significantly increased in adult male zebrafish exposed to developmentally to 0.5 µg/L deltamethrin (figure 3.2a). There was no significant effect of developmental exposure to 0.25 µg/L deltamethrin on the swim velocities of adult male

zebrafish (figure 3.2a). Finally, in male zebrafish there was a significant effect of developmental deltamethrin exposure on the frequency of high mobility swimming ($F_{(2,30)}=4.257$, p=0.024). Male zebrafish that had been developmentally exposed to 0.5 µg/L of deltamethrin exhibited more bouts of high mobility swimming (figure 3.3a).

In female zebrafish, developmental exposure to 0.25 µg/L or 0.5 µg/L deltamethrin did not have any significant effects on adult swim activity (figure 3.1b) or the frequency of high mobility swimming (figure 3.3b). However, developmental deltamethrin exposure had a significant effect on adult swim velocity in female zebrafish ($F_{(2,185)}=3.438$, p=0.034). Adult females exhibited increased swim velocities across all timebins if they had been exposed to 0.25 µg/L deltamethrin during development (figure 3.2b). Developmental exposure to the higher concentration of deltamethrin (0.5 µg/L) was not associated with any significant changes in swim velocities in adult female zebrafish (figure 3.2b). For either sex, there was not a significant effect of time on swim activity, nor was there a significant interaction between time and deltamethrin treatment. In conclusion, under the open field test paradigm, developmental exposure to the higher dose of deltamethrin (0.5 µg/L) resulted in increased distance travelled, swim velocity, and frequency of high mobility swimming in adult male zebrafish; whereas, exposure to the lower dose of deltamethrin (0.25 µg/L) was associated with increased swim velocities in adult female zebrafish.

3.3.2 Effects of developmental deltamethrin exposure on intersession distance travelled in the open field test

To determine whether developmental deltamethrin exposure alters more complex behavioral phenotypes, the rate of intersession habituation during the open field test was characterized in adult zebrafish (figure 3.4). The rate of intersession habituation was calculated by dividing the total distance travelled in the first 5 minute time bin on day 1 by the total distance travelled in the first 5 minute time bin on day 5 for each individual fish. The baseline values of habituation for control animals indicate that there is no difference in the total distance travelled between day 1 and day 5. The ratios of habituation were 1.0 and 0.9 for male and female zebrafish respectively. In male zebrafish, developmental deltamethrin exposure did not affect the rate of intersession habituation (figure 3.4a). However, in female zebrafish, developmental deltamethrin exposure was associated with a significant change in habituation ($F(2,32)=3.522$, $p=0.042$). Developmental exposure to 0.25 µg/L deltamethrin resulted in a 1.5 fold higher rate of intersession habituation in adult female zebrafish (figure 3.4b).

3.3.3 Effects of developmental deltamethrin exposure on adult thigmotaxis

The thigmotactic tendencies of adult zebrafish were measured to determine whether developmental deltamethrin exposure altered this anxiety-like response (figure 3.5). In male zebrafish, developmental exposure to deltamethrin (0.25 or 0.5 µg/L) was not associated with any significant changes in thigmotaxis during the open field test (figure 3.5a). Across all treatment groups, male zebrafish spent an average of 31% of the time in the center of the open field. In female zebrafish, developmental deltamethrin

exposure was found to have a significant effect on the duration of time spent in along the edges of the open field ($F(2,36)=5.230$, $p=0.010$). Developmental exposure 0.5 µg/L deltamethrin was associated with a 1.5-fold increase in thigmotactic tendencies in the adult female zebrafish under the open field test paradigm (figure 3.5b). Control fish spent approximately 45% of the time in the center of the open field. Whereas, fish that had been developmentally exposed to 0.5 µg/L deltamethrin spent 29% of the time in the center of the open field, indicating that more time was spent in the periphery

3.3.4 Effects of developmental deltamethrin exposure on adult aggression

In male zebrafish, data indicate that there is a statistically significant interaction between deltamethrin treatment and social status (dominant/subordinate) ($F(1,43)=16.887$, $p\leq0.001$). In males, within the dominant group, developmental deltamethrin exposure resulted in a significant increase in the number of aggressive attacks performed (figure 3.6a). There were no significant differences in the magnitude of aggression between control and deltamethrin treated subordinate male zebrafish (figure 3.6a). In addition, there were no significant effects of deltamethrin treatment on the magnitude of aggression in female zebrafish, nor was there a significant interaction between developmental deltamethrin exposure and social status (figure 3.6b).

3.4 Discussion

Results of this study demonstrate that developmental exposure to non-toxic doses of deltamethrin was associated with locomotor and behavioral changes that persisted into adulthood. In addition, we found gender differences in adult open field behaviors and aggression between control and deltamethrin treated animals.

Distance travelled, velocity (a measure of distance travelled per unit time), and mobility (a spatially independent measure of body movement), are endpoints commonly used to measure motor aspects of zebrafish swimming (Kalueff and Cachat 2011). In adult male zebrafish, developmental exposure to 0.5 µg/L deltamethrin, but not 0.25 µg/L deltamethrin, resulted in increased locomotor activity (distance travelled, velocity, and bouts of high mobility). Similarly, in terms of distance travelled, we previously demonstrated the same dose-response trend in 2-week old larval zebrafish (chapter 2). The deltamethrin-induced increases in locomotor activity exhibited by male zebrafish are consistent with findings in rodents, as increases in locomotor activity were also found in adult male mice (Eriksson and Fredriksson 1991) following prenatal exposure to deltamethrin. Together, this demonstrate that developmental exposure to 0.5 µg/L deltamethrin causes increases in swim activity (distance travelled) which is present at the larval stage and persists into adulthood in male zebrafish.

On the other hand, adult female zebrafish did not generally exhibit significant differences in locomotor activity. The only observed effect was an increase in swim velocity in the group exposed to 0.25 µg/L deltamethrin during development. Since most preclinical studies are performed using only male animals, relatively less information is known about the behavior of females. Of the studies cited in Shafer et. al 2005, reviewing the long-term behavioral deficits associated with developmental pyrethroid exposure, the majority only tested behavioral outcomes in male animals (Ahlbom et al. 1994; Eriksson and Fredriksson 1991; Husain et al. 1994; Johri et al. 2006; Moniz et al. 1990; Moniz et al. 1999; Nasuti et al. 2007; Talts et al. 1998). Others did not specify the sex or the sex ratio used for studies (Husain et al. 1992; Patro et al. 2009) or used both sexes for testing

but did not distinguish between the sexes when reporting data (Aziz et al. 2001; Gomes Mda et al. 1991). Only 2 studies specifically characterized sex differences in adult behavior following prenatal pyrethroid exposure (Lazarini et al. 2001; Tsuji et al. 2002). Tsuji et al. 2002 did not find any differences in locomotor activity in either male or female animals after prenatal exposure to d-allethrin. However, Lazarini et al. 2001 report a male specific deficit in swimming behavior, locomotor activity after swimming and alterations in DA neurochemistry associated with prenatal deltamethrin exposure. Although testing different endpoints, these studies, along with our data, demonstrate a sex-specific behavioral response following developmental deltamethrin exposure.

When examining endpoints such as thigmotaxis and inter-session locomotor activity we found that developmental deltamethrin exposure did not affect these behaviors in adult male zebrafish; whereas, developmental exposure to deltamethrin increased thigmotaxis and altered intersession locomotor activities in adult female zebrafish. Thigmotaxis, a component of exploratory behavior, is the tendency to move along walls and is a behavior commonly observed in rodents (Lamprea et al. 2008) and has been reported to occur in zebrafish as well. In zebrafish, baseline thigmotaxis activity is variable across the literature with reports of occurrence ranging from 50-90% of the total time tested (Champagne et al. 2010; Grossman et al. 2010; Maximino et al. 2010b). The variability in findings is likely due to different definitions of center (proportion of center zone to total area of tank), type of open field used (size, color, and shape of tank), and strain differences (high-anxiety strain vs low-anxiety strain). However, all studies do indicate that zebrafish engage in thigmotaxis and consistently spend more time along the periphery of the field when subject to a novel environment. Similar to the horizontal open

field test paradigm used in Grossman et al. 2010, we also found that control adult zebrafish spent approximately 40% of the time in the center zone and thus 60% of the time in the periphery. Thigmotactic behavior is also often used as an indicator of anxiety evoked by stress such as novelty in rodents (Simon et al. 1994) and has been thought to elicit the same reaction in zebrafish (Blaser et al. 2010; Champagne et al. 2010). Since we found that developmental deltamethrin treatment increased thigmotaxis in adult female zebrafish, it possible that exposed adult female zebrafish are more anxious and sensitive to stress.

Intersession habituation to novelty is generally thought to involve long-term spatial memory and, in mice, is generally associated with a reduction in locomotor activity across sessions (Bolivar 2009). If we adapt the same intersession habituation parameters from mice to the zebrafish model, control fish did not habituate to repeated daily exposure to the same novel open field environment; the total distance travelled on day 1 of testing was the same as the total distance travelled on day 5 of testing (figure 3.1). It is possible that this inconsistency is due to species differences. In zebrafish, little is known about the manner of intersession habituation in novelty-based paradigms. Wong et al. 2010 described intersession habituation to repeated exposure to a novel environment as an increase in the number of times the zebrafish swam to the top of a tank, an increase in time spent in the top, and a reduction in freezing bouts. Because different testing paradigms were used between (Wong et al. 2010) and our studies (the novel tank diving test measures vertical movement whereas our open field test measures horizontal movement) it is difficult to determine if our findings are directly comparable. When measuring thigmotaxis as an endpoint, intersession exposure to a new environment

did not elicit a consistent habituation response in the zebrafish (Buske and Gerlai 2012; Maximino et al. 2010a). Therefore, it is also likely that when measuring only horizontal distance travelled, adult zebrafish do not appear to habituate. Furthermore, in mice, the overall trends of intersession habituation are strain dependent (Bolivar et al. 2000; Bolivar 2009). The highly inbred AB strain of zebrafish used in our studies generally exhibit higher basal locomotor activity (distance travelled, velocity, and frequency of high mobility) relative to the other strains (Lange et al. 2013), highlighting strain differences in behavior of the Danio species. Wong et al. 2010 used an outbred strain purchased from local distributors and (Buske and Gerlai 2012) and (Maximino et al. 2010a) used the AB zebrafish strain, also used in our studies. Therefore, owing to differences in endpoint analysis and strain selection, the discrepancies in intersession habituation is not surprising. However, under our open field paradigm, using the AB strain of zebrafish, adult female zebrafish exposed to 0.25 µg/L deltamethrin during development exhibited an increase in the rate of intersession habituation (figure 3.4b). When examining the total distance travelled (inset of figure 3.4b), it is apparent that female adults developmentally exposed to 0.25 µg/L deltamethrin develop an exaggerated response to novelty. They exhibit hyperactivity on day 1 of testing and hypoactivity on day 5 of testing (figure 3.4c). The theory behind the open field test is that anxiety and stress is likely the result of 1) social isolation and 2) exposure to a novel, brightly lit arena (Prut and Belzung 2003). This suggests that deltamethrin treated female zebrafish are reacting and adapting inappropriately to a stressor such as the novel environment.

Developmental deltamethrin exposure also heightened aggression in dominant male zebrafish, a condition not present in treated females. Dominant-subordinate social hierarchies are well characterized in adult zebrafish (Dahlbom et al. 2012; Larson et al. 2006; Paull et al. 2010; Spence et al. 2008). Plotting the number of aggressive attacks of each fish using a scatterplot allows for visualization of such dominant and subordinate relationships in zebrafish (figure 3.6). In our studies, control male and female zebrafish do not exhibit significant differences in aggression, which is consistent with several studies in the literature (Dahlbom et al. 2012; Moretz et al. 2007). However, as demonstrated by Moretz et al. 2007, basal aggression levels are highly dependent on strain. As such, other studies have demonstrated that dominant males are more aggressive than dominant females (Filby et al. 2010; Paull et al. 2010). Therefore, it is also possible that the lack of response in the female zebrafish to developmental deltamethrin exposure could be due to the fact that basal aggression levels for females were already heightened due to strain selection.

There is growing evidence demonstrating sex-specific responses, in terms of behavior, to stressors during development. For instance, the incidence of affective disorders is higher in females, whereas, the incidence of autism spectrum disorder is higher in males and it is thought that sex-specific responses to prenatal adversity underlie these findings (Davis and Pfaff 2014). Table 3.1 provides a summary of the gender specific behavioral outcomes of developmental deltamethrin exposure. Generally, the behavioral outcomes of developmental deltamethrin exposure exhibited by adult male zebrafish are related to motor function and aggression; while, the behavioral outcomes of developmental deltamethrin exposure are possibly anxiety-like (affective) disorders. As

previously mentioned, open field parameters such as distance travelled, velocity, and mobility reflect the motor aspects of zebrafish swimming (Kalueff and Cachat 2011). Whereas, thigmotaxis, increased velocity (an indirect measure of erratic movement), and rate of habituation are common endpoints used to assess anxiety-like behaviors in the zebrafish (Champagne et al. 2010). While not a comprehensive battery, developmental deltamethrin exposure is associated with significant changes in all of these parameters in adult female zebrafish; therefore it is likely that exposure effects the stress-response of these fish. Additional testing using assays such as the novel tank test or scototaxis paradigms would help confirm this finding.

In conclusion, developmental exposure to deltamethrin resulted in increased locomotor activity and aggression in adult male zebrafish, as well as, increased swim velocity, thigmotaxis, and altered habituation in adult female zebrafish. With the recent NIH initiative to balance sex representation in preclinical studies (Clayton and Collins 2014), our findings are aligned with the number of studies that fueled this directive. In addition, our data also lend support to the DOHaD hypothesis as we highlight the risk of neurotoxicant exposure during the critical developmental period in mediating longer-term behavioral disorders.
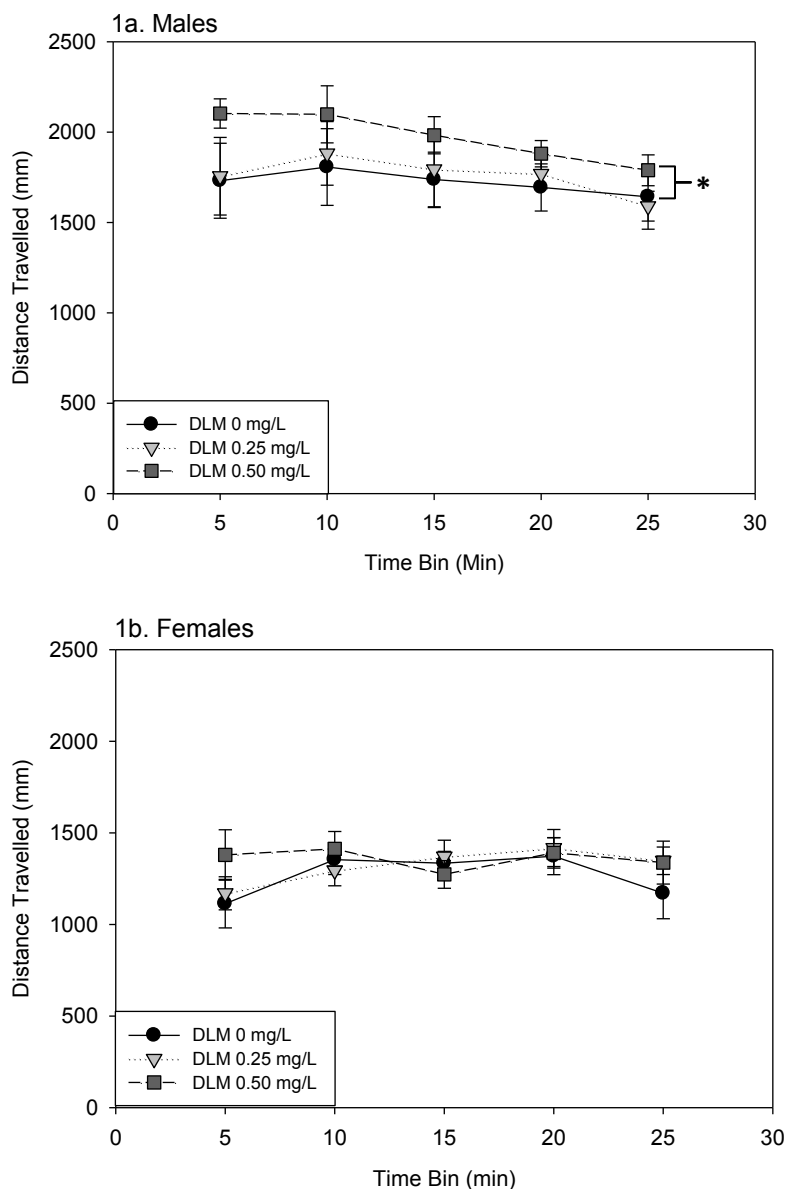
Figure 3.1: Distance travelled in the open field test of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Data points are presented as mean distance traveled (mm) ± S.E.M. in 5-min time bins during 30-min sessions (n=6-13). A two-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure across time on distance travelled. (*) indicate that distance travelled across time in animals developmentally exposed to 0.5 μg/L deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
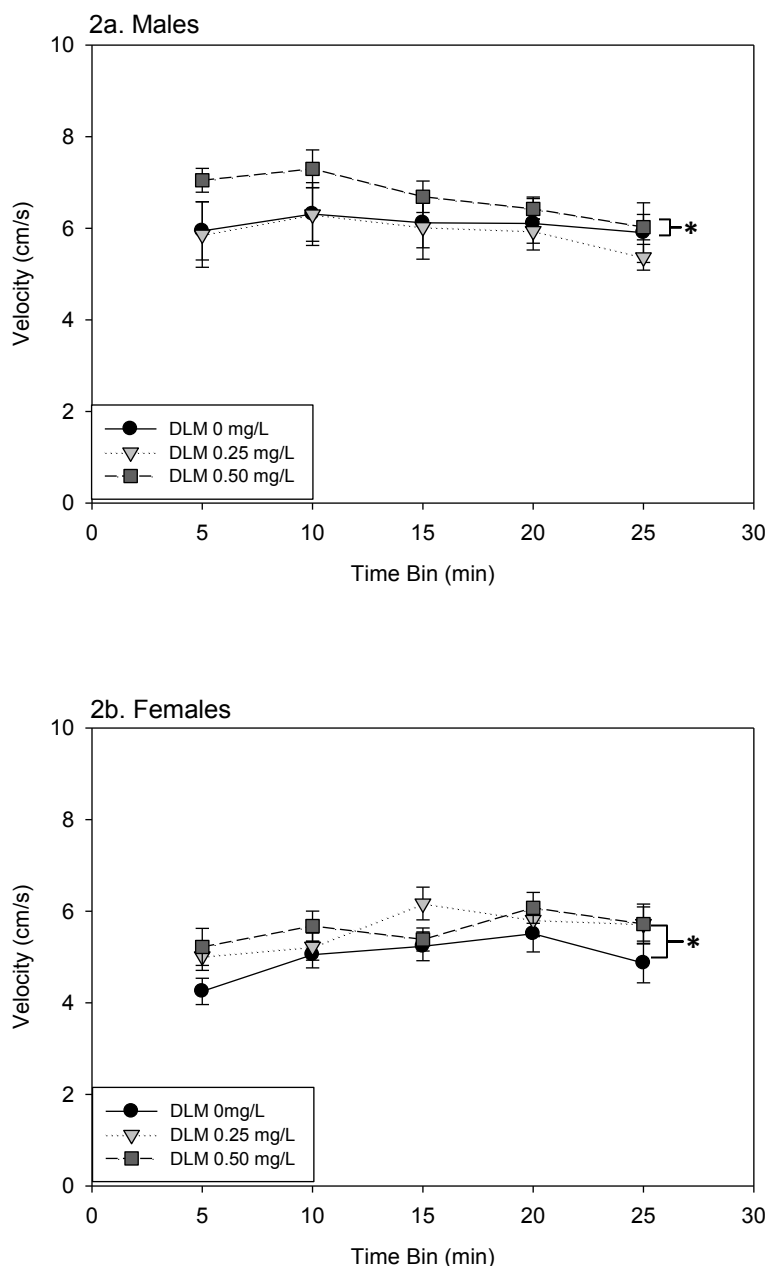
Figure 3.2: Average swim velocity in the open field test of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Data points are presented as average swim velocity (cm/s) ± S.E.M. in 5-min time bins during 30-min sessions (n=6-13). A two-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure across time on adult swim velocity. (*) indicate that average swim velocity across time in animals developmentally exposed to deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
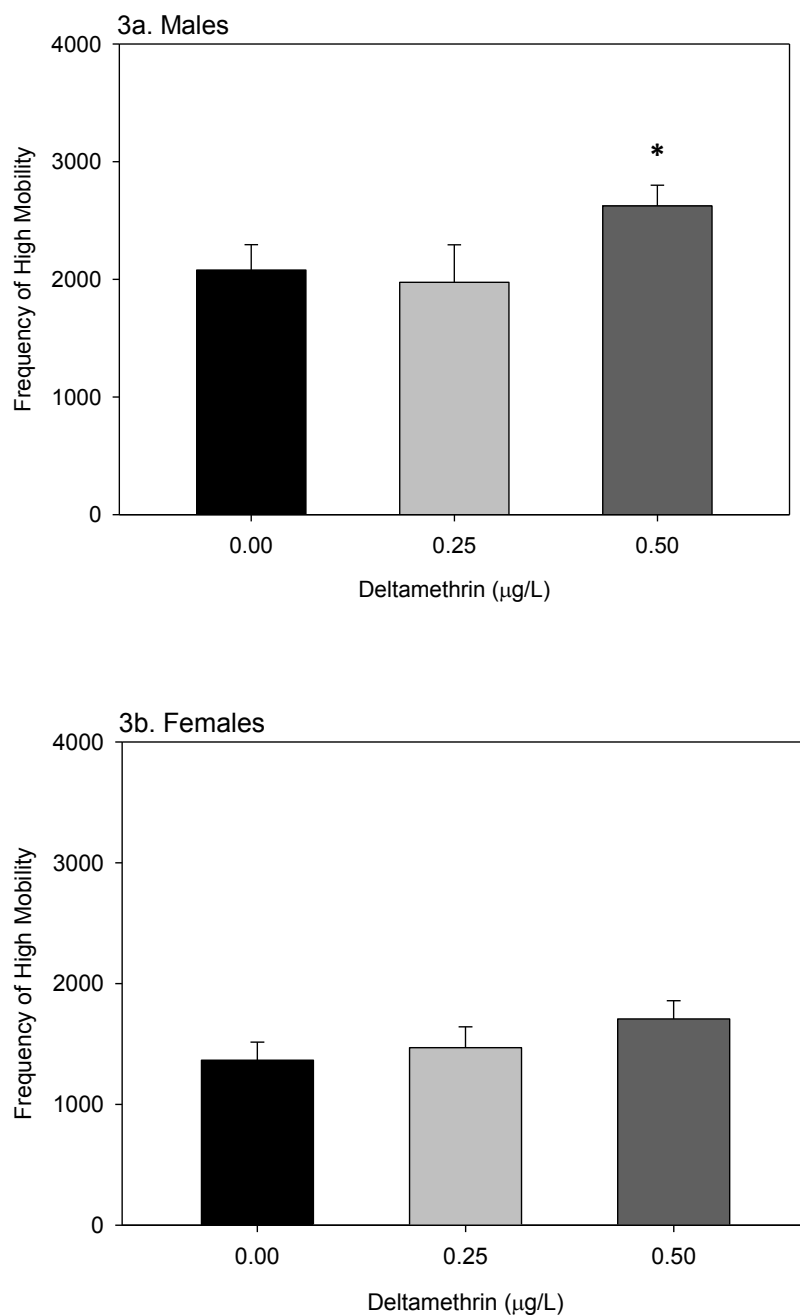
Figure 3.3: Frequency of high mobility swimming in the open field test of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Data are presented as mean frequency of high mobility swimming ± S.E.M. during the entire 30 minute session (n=6-13). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on high mobility swimming bouts. (*) indicate that the frequency of high mobility swimming in animals developmentally exposed to deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq$ 0.05).
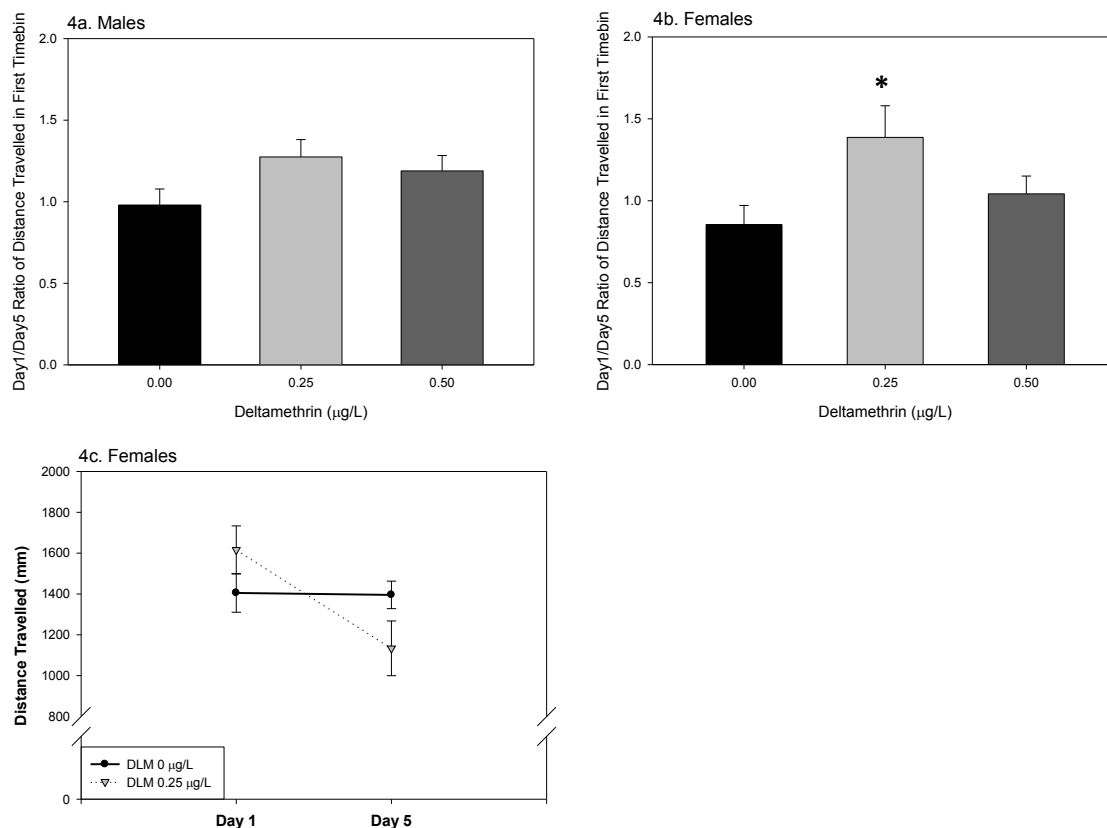
Figure 3.4: Rate of intersession habituation in the open field test of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Bars are presented as the mean ratio of total distance travelled in the first 5 minute timebin between day 1 and day 5 of testing ± S.E.M. (n=6-14). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on the rate of intersession habituation. (*) indicate that the frequency of high mobility swimming in animals developmentally exposed to deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$). Panel (c) represents the mean distance travelled ± SEM in the first 5 minute time bin on day 1 and day 5 testing of female control fish and fish developmentally exposed to 0.25 ug/L deltamethrin.

5a. Males: Thigmotaxis

5b. Females: Thigmotaxis
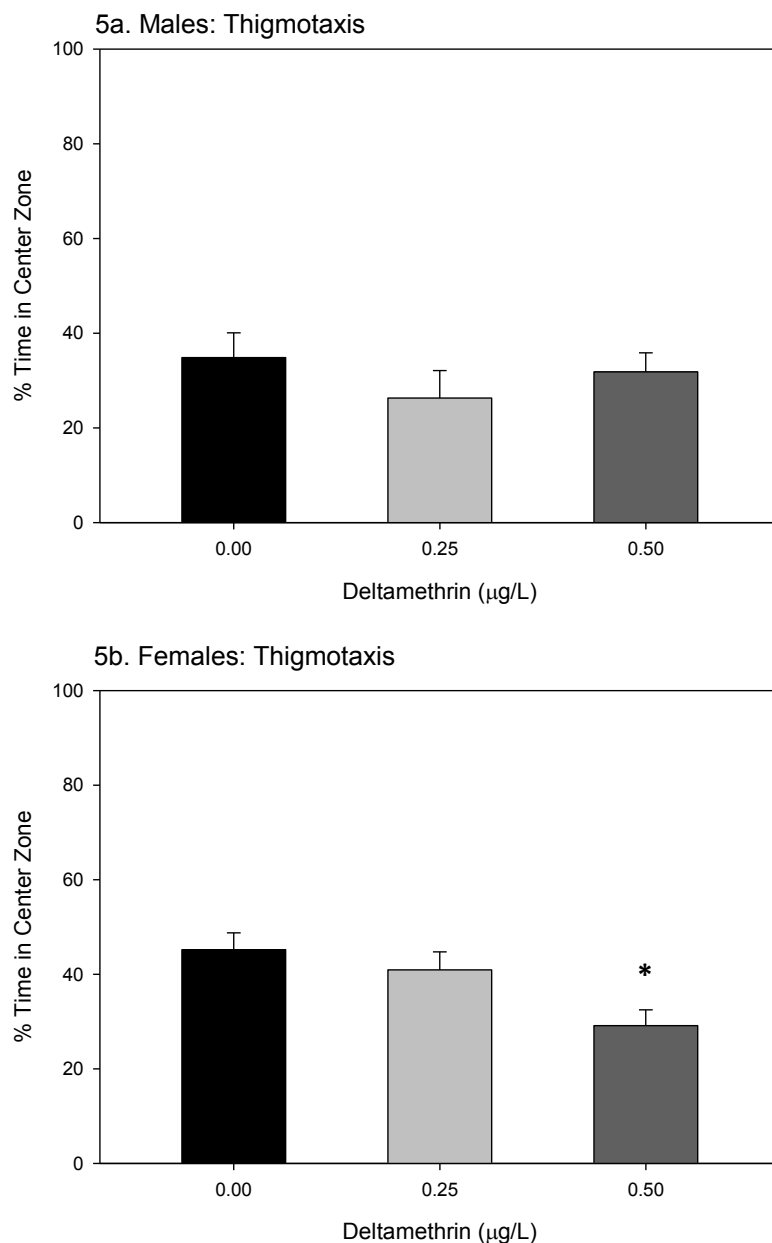
Figure 3.5: Thigmotaxis in the open field test of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Data are presented as mean % time spent in the center zone ± S.E.M. during the entire 30 minute session (n=6-13). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on the amount (%) of time spent in the center zone. (*) indicate that % time spent in the center zone in animals developmentally exposed to deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
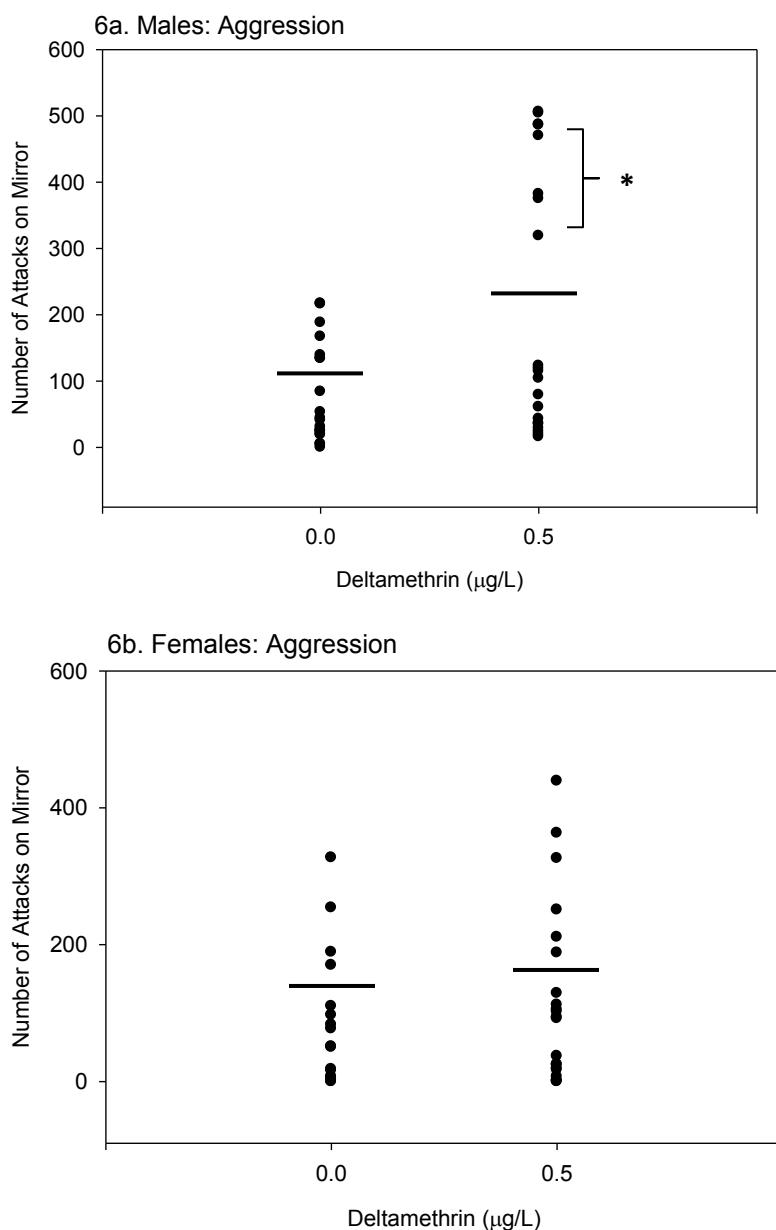
Figure 3.6: Number of aggressive attacks using the mirror induced aggression assay of adult male (a) and female (b) zebrafish developmentally exposed to deltamethrin (DLM). Each data point represents the number of aggressive attacks performed by one individual fish during a 9 minute trial. (n=19-23). Lines present in the graphs indicate where the dominant/subordinate cutoffs were made. A two-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on the number of aggressive attacks in dominant and subordinate fish. (*) indicate that fish developmentally exposed to deltamethrin were significantly different from fish exposed to vehicle control within the dominant group by Holm-Sidak multiple comparisons ($p \leq 0.05$).

	Endpoint	Males	Females
Motor Function	Distance Travelled	Increased	No change
	Average Swim Velocity	Increased	Increased
	Frequency of High Mobility	Increased	No change
Affective disorders	Thigmotaxis	No change	Increased
	Intersession habituation	No change	Altered
	Aggression	Increased	No change

Table 3.1 Summary of behavioral effects found in adult male and female zebrafish following developmental exposure to deltamethrin

Chapter 4

The effects of developmental deltamethrin exposure on the 5-HT system and social aggression in the adult zebrafish

4.1 Introduction

The role of serotonin (5-HT) in behavioral control, systems physiology, and disease far exceeds that of any other neurotransmitter system (Azmitia 2001). The widespread distribution of 5-HT projections throughout the brain and body alludes to the diverse functions of the serotonergic system. Important in regulating behaviors such as depression, affective disorders, and aggression(Hendricks et al. 2003; Lesch and Merschdorf 2000; Quadros et al. 2010), the 5-HT system is a common pharmaceutical target for these diseases. In addition, the 5-HT system is found to be a key modulator of adaption to positive and negative life experiences, reviewed by (Oberlander 2012). Studies have shown that behaviors such as aggression and anxiety are programmable behaviors and can be influence by environmental factors experienced during development (Marquez et al. 2013; Van den Hove et al. 2014). 5-HT is thought to be a "homeostatic regulator which integrates mind and body with the outside world" (Azmitia 2001). As such, the plastic and dynamic nature of the serotonin system, the ability for this system to be influenced by external factors, and the involvement of 5-HT in a variety of biological processes, makes the 5-HT system a candidate mediator of gene x environment interactions in the "developmental origins of health and disease" (DOHaD) paradigm, reviewed in (Homberg and van den Hove 2012; Huizink et al. 2004; Lesch et al. 2012).

The DOHaD hypothesis suggests that most disease states experienced later in life are the consequence of an adverse conditions experienced during critical developmental

periods. When exposed to adversity, developing organism must reprogram physiological endpoints to adapt to these prenatal stressors which have long lasting effects, reviewed in (Gluckman and Hanson 2004; Gluckman et al. 2007). In animal models, disorders such as anxiety and aggression have been found to have fetal links and the serotonin system is largely implicated for mediating these effects. For instance, prenatal stress (daily repeated restraint in bright light) was associated with anxiety-like behaviors in offspring (Van den Hove et al. 2005; Van den Hove et al. 2014) which was reduced following administration of a selective serotonin reuptake inhibitor (SSRI) (Rayen et al. 2011). In addition, molecular events such as altered gene expression of the serotonin transporter (SERT) and 5-tryptophan hydroxylase (TPH2) (Van den Hove et al. 2014), decreased 5-HT receptor binding (Peters 1990; Van den Hove et al. 2005), and increased 5-HT metabolism (Hayashi 1998; peters 1990) are also associated with prenatal stress. Direct manipulation of the 5-HT system using fluoxetine during development also produced anxiety-like behaviors in mice (Ansorge et al. 2008; Karpova et al. 2009). Finally, conditional developmental expression of 5-HT1a in 5-HT1a knockout mice prevents the occurrence of anxiety-like behaviors inherent in this knockout model, suggesting that this disorder is set during development (Gross et al. 2002).

Similarly, studies have demonstrated that aggression disorders have developmental origins and likely involve serotonergic dysfunction. Peripubescent stress was associated with increased aggression and MAOA expression in adult rats (Marquez et al. 2013). Directly targeting the 5-HT system with SSRIs was associated with increased aggression in male mice (Coleman et al. 1999). Postnatal exposure to 5-HT1A receptor agonist buspirone heightened aggression in an aggressive mouse strain and

further ameliorated aggression in a less aggressive mouse strain (Markina et al. 2006). Finally, direct exposure to 5-HT during development, was associated with decreased aggression in adult hens (Dennis et al. 2013). Taken together, these studies show that the 5-HT system can be influenced during early life, which can predispose an individual to anxiety-like and aggression disorders later in life.

Previously we found that developmental exposure to deltamethrin, using concentrations below the LOAEL, results in persistent locomotor and behavioral impairment (hyperactivity, anxiety-like behaviors, and aggression) in larval and adult zebrafish respectively. In addition, dopaminergic dysfunction was shown to be a possible mode of action for pyrethroid induced neurobehavioral toxicity. However, pyrethroids pesticides have also been shown to alter serotonin neurotransmission (Bloomquist et al. 2002; Hossain et al. 2013; Kirby et al. 1999; Martinez-Larranaga et al. 2003; Ricci et al. 2013). Therefore, because pyrethroids can modify the highly plastic 5-HT system and because the 5-HT is involved in regulating behaviors such as anxiety and aggression, (exhibited by adult zebrafish previously exposed to deltamethrin), we hypothesized that the behavioral deficits caused by developmental deltamethrin exposure has a serotonin component. Our findings demonstrate that developmental deltamethrin exposure is associated with an early increase in serotonin transporter transcript levels. In addition, we find a strong positive correlation between the magnitude of aggression exhibited by adult male zebrafish and transcript levels of *drd2a*, *serta*, and *sertb,* a trend which is lost in the population of adult males that had been developmentally exposed to deltamethrin. Using fluoxetine, a serotonin transporter inhibitor, to rescue the hyperaggressive phenotype

exhibited by adult male zebrafish previously exposed to deltamethrin, it was shown that developmental deltamethrin exposure has lasting effects on the monoamine systems.

4.2 Materials and Methods

4.2.1 Zebrafish Husbandry

The AB strain zebrafish, used in all experiments, was originally obtained from the Zebrafish International Resource Center (Eugene, OR). Adult fish were bred and housed in Aquatic Habitats (Apopka, FL) recirculating systems supplied with system water which consisted of carbon and sand filtered municipal water. Water quality was maintained at <0.05 ppm nitrite, <0.2 ppm ammonia, pH between 7.2 and 7.7, and between 26 and 28°C. Fish were maintained under a 14:10 hour light:dark cycle. Fish were fed twice a day, once in the morning with freshly hatched artemia (Aquatic Habitats, Apopka, FL) and once at night with aquatox/tetramin flake food (Aquatic Habitats, Apopka, FL). All experiments were conducted in accordance with the zebrafish husbandry protocol and embryonic exposure protocol (#08-025) approved by the Rutgers University Animal Care and Facilities Committee.

4.2.2 Chemicals

Deltamethrin [purity 99.5 %] (CAS# 52918-63-5) was purchased from ChemService (West Chester, PA). N,N-dimethylformamide (DMF) and fluoxetine hydrochloride (CAS# 56296-78-7) were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.3 Pesticide Exposures

Deltamethrin solutions and exposures were performed as previously described (3.2.3). Briefly, zebrafish embryos were treated between 3-72hpf to 0, 0.25, 0.33, and 0.5 µg/L deltamethrin (nominal concentrations). The final DMF concentration was 0.01% for

all exposures. Exposures were performed in 60x15mm glass petri dishes using 10 ml of treatment solutions. 30 embryos per dish were exposed in a static non-renewal bath and incubated at 25-26 °C in the dark. Embryos were observed daily to monitor proper development and to ensure that symptoms of toxicity described in (DeMicco et al. 2010) were not present. At 72hpf, sac fry larvae were removed from treatment and transferred to 600 ml beakers containing fresh system water and reared in an incubator maintained at 26°C under a 14:10 light:dark cycle until 1 month of age (1 treatment dish/beaker). After which they were transferred to the main housing facility (described in 4.2.1). Adults of the same treatment group were group housed in 3L aquatic habitat tanks at a stocking density of 20-25 fish/tank, with both sexes co-mingled at a approximately a 1:1 ratio.

4.2.4 Analysis of whole body neurotransmitter levels by HPLC-ECD

Zebrafish embryos were exposed to 0, 0.333, or 0.5 µg/L deltamethrin and raised to the 2-week time point as previously described (section 4.2.3). (N=6 pooled replicates/concentration, 50 embryos per replicate). At 2-weeks, larvae were snapfrozen. 50 µL of cold 0.1N PCA solution (supplemented with 300 µM EDTA and 300 µM sodium metabisulfite) was added to each sample and homogenized 2x on ice using a motorized pestle. Samples were spun at 16xg for 10 minutes at 4°C and the supernatant was filtered through a 0.22µM spin column (EMD Millipore, Billerica, MA). The remaining pellet was dried in a 37°C incubator and dissolved in 200 µl of 0.5N NaOH overnight. The protein concentration of each sample was determined using the Modified Lowry Protein Assay (Thermo Fisher Scientific, Waltham. MA). HPLC-EDC quantitation of DA, DOPAC, and HVA was performed as (Schuh et al., 2009; Sheleg et

al., 2013). Briefly, the filtered supernatant was diluted 1:10 in PCA solution and 5 ul was injected directly onto a Waters Alliance high performance liquid chromatograph equipped with a Waters 2465 electrochemical detector (Waters Corporation, Milford, MA). DA, DOPAC, and HVA were separated on a Microdialysis MD-150 x 3.2 column (Thermo Scientific, Waltham, MA) using isocratic MDTM Mobile Phase (Thermo Scientific, Waltham, MA) with NaCl (2 mM) at a flow rate of 0.5ml/min. The compounds identified by electrochemical detection were quantified by area under the curve with known standards. The experiment, from dosing to analysis, was repeated twice.

4.2.5. Analysis of mRNA transcript levels by real-time quantitative PCR

Embryos were exposed to 0.25, 0.333, or 0.5 μg/L deltamethrin as described above (section 4.2.3). At 72hpf or 2wpf, approximately 15-20 embryos were pooled and snap frozen in liquid nitrogen (N = 4 pooled replicates/concentration). The entire experiment, from dosing to analysis, was repeated a minimum of three times.

Following adult aggression analysis (described in 4.2.6), fish were anesthetized with MS222 (Aquatic Habitats, Apopka, FL) and decapitated. Brains were dissected out (Gupta, Jove video) on ice and immediately snap frozen in liquid nitrogen.

RNA isolation, reverse transcription, and real-time qPCR were performed as previously described in (Hillegass et al. 2007). Briefly, mRNA was isolated using RNAzol® Reagent (Sigma-Aldrich, St. Louis, MO), and contaminating DNA was removed with the DNA-*free*™ kit (Life Technologies). Reverse transcription was performed with the iScript™ cDNA synthesis kit (Bio-Rad) and real-time qPCR was

performed using iQ™ SYBR® Green Supermix (Bio-Rad). Primer sets (listed in table 2.1.1) were optimized for the following qPCR protocol: 35 cycles of: 95°C for 15 seconds and 60°C for 1 minute. mRNA was quantified using standard curves and normalized to the housekeeping gene, 28s ribosomal RNA (embryos and larvae) or B-actin (adults).

4.2.6 Chronic Fluoxetine Exposure and Mirror Induced Aggression assay.

Adult zebrafish (~1 year) that had been previously exposed to deltamethrin (0.5 µg/L or vehicle control) during development were exposed to fluoxetine (100 µg/L) in 3L aquatic habitat tanks containing 2L system water for 2 weeks. A 50% water change was performed and treatments renewed every 2 days. Respective controls did not receive drug treatment and were housed and maintained under identical conditions. 10 fish of the same group (vehicle control or deltamethrin treated) were treated at a time, each tank containing a 1:1 male/female ratio. The concentration chosen and duration of exposure was based off the studies published by Egan et al. 2009, which were found to elicit an anxiolytic effect.

After 2 weeks of chronic fluoxetine exposure, the adult zebrafish were subject to the same open field conditions as previously described (3.2.4) for 30 minutes, every 24 hours, for 5 days, to allow for habituation to handling and exposure to the novel field. In between sessions, fish were individually housed and fluoxetine exposure was maintained to ensure that there were no effects of withdrawal. On the 5th day, immediately after the last trial session, a mirror (7.5x7.5 cm) was slotted into the trapezoidal end of the tank following the slant of the tank and video was recorded for 10 minutes. Videos were

viewed by 2 independent blind reviewers and the number of aggressive events (bites and pushes against the mirror) was counted.

4.2.7. Statistical analyses

All data were analyzed using the SigmaPlot version 11 computer software package (Systat Software Inc., San Jose, CA). The exact statistical test ran for each condition can be found in the corresponding figure legend. The probability level for statistical significance was $p \leq 0.05$ for all studies.

4.3 Results:

4.3.1 The effects of developmental deltamethrin exposure on whole body neurotransmitter levels in larval zebrafish

To determine if developmental deltamethrin exposure results in persistent changes in 5-HT neurochemistry, the levels of 5-HT and its metabolite 5-HIAA were measured at 2 weeks (figure 4.1); a timepoint associated with deltamethrin induced increased locomotor activity (figure 2.1). There were no significant effects of deltamethrin exposure on 5-HT or 5-HIAA levels. In addition, 5-HT/5-HIAA ratios were calculated to determine if developmental deltamethrin exposure was associated with changes in 5-HT metabolism. At the 2-week larval stage (figure 4.1), there was a significant effect of developmental deltamethrin exposure on the 5-HT/5-HIAA Ratio ($F(2,12)=24.181$, $p =0.002$) transcript levels. Developmental exposure to 0.5 µg/L deltamethrin resulted in a significant 2.7–fold increase in the 5-HT/5-HIAA ratio.

4.3.2 The effects of developmental deltamethrin exposure on serotonergic gene expression

The transcript levels of several genes involved in the serotonin system were measured immediately after exposure (72hpf) and at the larval stage (2-weeks) to characterize the immediate and persistent effects of deltamethrin exposure. At 72hpf (figure 4.2a), there was a significant effect of developmental deltamethrin exposure on *serta* ($F(2,8)=23.761$, $p =0.001$) transcript levels. Developmental exposure to 0.25 µg/L deltamethrin resulted in a significant 2–fold increase in *serta* transcript levels. Developmental deltamethrin exposure did not significantly alter the expression of *sertb*, *htr1aa*, *htr1b*, or *tph1b* at 72hpf.

At the larval stage (figure 4.2b), developmental deltamethrin exposure did not significantly alter the gene expression levels of any gene tested: *serta*, *sertb*, *htr1aa*, *htr1b*, and *tph1b*. The immediate effects of developmental deltamethrin exposure included increased *serta* transcript levels (figure 4.2a); however, these values returned to baseline levels at the 2-week time point (figure 4.2b).

4.3.3 Correlation between transcript levels of key serotonin and dopaminergic genes and magnitude of aggression

To determine if the gene expression levels of *dat*, *drd1*, *drd2*, *drd3*, *th*, *serta*, and *sertb* was correlated to the magnitude of aggression exhibited by adult zebrafish, a Pearson's correlation was performed. These genes were selected because developmental deltamethrin exposure was found to affect their expression previously, with the exception of *dat* and *drd3*. In male control zebrafish, a significant and strong positive correlation

was found between the expression levels of *drd2a* (r=0.810, p=0.015), *serta* (r=0.948, p=0.001) or *sertb* (r=0.772, p=0.042) and the magnitude of aggression. Thus indicating that in control animals, increased expression of *drd2a*, *serta*, or *sertb* was correlated to increased aggression. In adult male zebrafish that had been developmentally exposed to 0.5 µg/L deltametrhin, these correlations are weak and insignificant: *drd2a* (r=-0.104, p=0.701), *serta* (r=0.014, p=0.961) and *sertb* (r=0.186, p=0.489). Thus indicating in the deltamethrin treated population, there is no correlation between the gene expression levels of *drd2a*, *serta*, or *sertb* with aggression.

In female control animals, there is no significant correlation between the levels of *dat*, *drd1*, *drd2*, *drd3*, *th*, *serta*, or *sertb* with the magnitude of aggression. However, in female adult zebrafish that had been developmentally exposed to deltamethrin, a correlation between the gene expression level of *serta* and the magnitude of aggression appears (r=0.653, p=0.015).

4.3.4 The effects of fluoxetine treatment on aggression

Adult zebrafish were exposed to fluoxetine (100 µg/L) for 2-weeks prior to mirror induce aggression testing in an attempt to rescue the hyperaggressive phenotype exhibited by dominant male zebrafish that been exposed to deltamethrin during development. Statistical analysis could not be run to test for a developmental deltamethrin exposure x dominant x fluoxetine treatment interaction due to the fact that fluoxetine treatment ablated aggression in adult zebrafish such that dominant/subordinate social status could not be determined. However, since this occurred in both control and

deltamethrin exposed zebrafish, this result supports the fact that aggression and hyperaggression in both these populations involve the 5-HT system.

4.4 Discussion

Developmental exposure to non-toxic doses of deltamethrin results in early increases in *serta* gene expression which returned to basal levels by the 2-week time point. In addition at the larval stage, changes in 5-HT or 5-HIAA levels were not present; however, the ratio of 5-HT to 5-HIAA was found to be increased. Furthermore, the transcript levels of genes associated with the 5-HT and DA systems that were previously found to be altered by developmental deltamethrin exposure returned to control levels in adult male and female zebrafish. In adult male zebrafish, fluoxetine exposure reduced aggression in both control fish and fish that had been exposed to deltamethrin during development.

Since we previously observed increased aggression and anxiety-like behaviors in adult zebrafish that had been exposed to deltamethrin during development (chapter 3), and because the 5-HT system plays an important role in mediating these behaviors (Hendricks et al. 2003; Lesch et al. 2012; Quadros et al. 2010), the effects of deltamethrin exposure on the 5-HT system was examined. First, 5-HT and its metabolite 5-HIAA levels were measured at the larval stage, a time point associated with deltamethrin induced increases in locomotor activity (figure 2.1). Our studies find that there are no differences in larval 5-HT or 5-HIAA levels following developmental deltamethrin exposure. In the rodent literature, exposure to high doses (20 mg/kg) of deltamethrin, which were sublethal but toxic, decreased striatal 5-HT and 5-HIAA (Martinez-Larranaga

et al. 2003); whereas, exposure to lower non-toxic doses of deltamethrin (10 mg/kg) increased striatal 5-HT and 5-HIAA levels (Ricci et al. 2013). The apparent lack of effect of deltamethrin exposure on 5-HT levels observed in our studies could be because our assays measure whole body 5-HT levels. Whereas in the rodent studies, investigators measured 5-HT levels in the striatum, an area of the brain that where there is a high concentration of 5-HT neuron terminals. Since ~95% of the body's serotonin is located in the GI (Gershon and Tack 2007), these peripheral sources of 5-HT could significantly dilute any changes that may occur in the CNS. However, our data demonstrate an increase in the ratio of 5-HT to 5-HIAA in deltamethrin treated larvae, suggesting that these animals exhibit decreased conversion of 5-HT to 5-HIAA. In rats, pre-gestational stress was associated with lower 5-HIAA/5-HT ratios in the fetuses (Huang et al. 2012).

Gene expression analysis of several genes involved in 5-HT reuptake, synthesis, and transmission determined that exposure to 0.25 µg/L was associated with increased levels of *serta* transcript immediately after exposure (72 hpf). However, this change returned to control levels by the 2-week timepoint (figure 4.2b) and was not present in at the adult stage in neither male (figure 4.3a) or female (figure 4.3b) zebrafish. This experiment is the first to report changes in serotonin transporter transcript levels following low does (below LOAEL) deltamethrin exposure. Because there is much interindividual variability in the expression levels of the analyzed genes, when looking for the effects of deltamethrin treatment between the two populations as a whole (control vs deltamethrin treated) differences cannot be detected (figure 4.3).

Since many factors can predispose an individual to different serotonergic states, such as social status and propensity for aggression, a correlation between the magnitude

of aggression and transcript levels of various serotonergic and dopaminergic genes was examined. There is a strong positive correlation between the magnitude of aggression and serta, sertb, and drd2a transcript levels in adult male zebrafish, indicating that zebrafish that are more aggressive have higher levels of *serta*, *sertb*, and *drd2a* transcript. Increased levels of sert would result in the increased reuptake of 5-HT from the synapse, producing a hyposerotonergic state and reduced 5-HT activity, which according the "serotonin deficiency hypothesis" is associated with a propensity of aggression (Quadros et al. 2010). Evidence in support of this hypothesis comes mostly from pharmacological studies which show that increased 5-HT activity via inhibition of serotonin reuptake (increasing 5-HT availability) or 5-HT1 receptor agonism decreases aggression in various animal models. In addition, selective serotonin transporter inhibitors have demonstrated efficacy in treating violent and excessively aggressive patients, reviewed in (Miczek et al. 2007).

Studies also show that intact DA activity is required for the initiation and execution of aggressive behavior (Miczek et al. 2007; Miczek and Haney 1994; Miczek et al. 2002). In addition pharmacological manipulation of the DA system using amphetamines demonstrate that the DA stimulation can enhance and prologue aggressive tendencies, reviewed in (Miczek et al. 2007). Because DA receptors mediate neurotransmission, increased levels of *drd2a* could result in increased DA activity and therefore enhance aggression, supporting the positive correlation found between drd2a levels and aggression.

In the population of adult male zebrafish that had been developmentally exposed to deltamethrin, the correlation between the magnitude of aggression and *serta*, *sertb*, and

drd2a transcript levels is lost. Since, the total gene expression levels of serta (figure 4.2) and drd2a (figure 2.2) are affected by developmental deltamethrin exposure it makes sense that these genes do not follow normal patterns of expression in deltamethrin treated fish.

Fluoxetine treatment was hypothesized to attenuate the hyperaggression exhibited by dominant male zebrafish that had been developmentally exposed to deltamethrin (figure 3.6a) due involvement of the 5-HT system in mediating aggression (table 4.1) and due to the increases in *serta* expression resulting from deltamethrin exposure (figure 4.2). Fluoxetine treatment ablated aggression associated with social status in both control and deltamethrin treated male fish indicating that the 5-HT plays a role in dominant/subordinate aggression in the zebrafish. Since we previously implicate DA system dysfunction as a likely mode of action in deltamethrin induced locomotor deficits, it is possible that DA system dysfunction underlies the specific hyperaggressive phenotype exhibited by male fish since, as stated previously, DA system activity enhances and prolongs aggressive tendencies (Miczek et al. 2007). Therefore, taken together, it is possible that developmental deltamethrin exposure causes a hyperdopaminergic state. However, this would need to be investigated via pharmacological intervention.

In female, zebrafish, correlations between the magnitude of aggression and the transcript levels of the genes analyzed does not exist (table 4.1). In addition, fluoxetine exposure did not mitigate aggression (figure 4.4b). This suggests that aggression related to social status in females is likely mediated by different mechanisms all together. Given

the behavioral gender dimorphisms present in many behaviors (Bekker and van Mens-Verhulst 2007), including aggression this finding is not unanticipated.

In conclusion, developmental deltamethrin exposure caused early increases in *serta* transcript levels, which returned to baseline levels at the larval and adult stages. Whole body 5-HT and 5-HIAA levels were not affected by developmental deltamethrin exposure. In adult male zebrafish, a strong positive correlation between the magnitude of aggression and *serta*, *sertb*, and *drd2a* transcript levels exists; this correlation is lost in zebrafish that had been developmentally exposed to deltamethrin. Fluoxetine exposure in control and deltamethrin treated male zebrafish reduced aggression. Together, our studies demonstrate that deltamethrin also modulates the serotonin system, which likely contributes to the behavioral deficits observed.
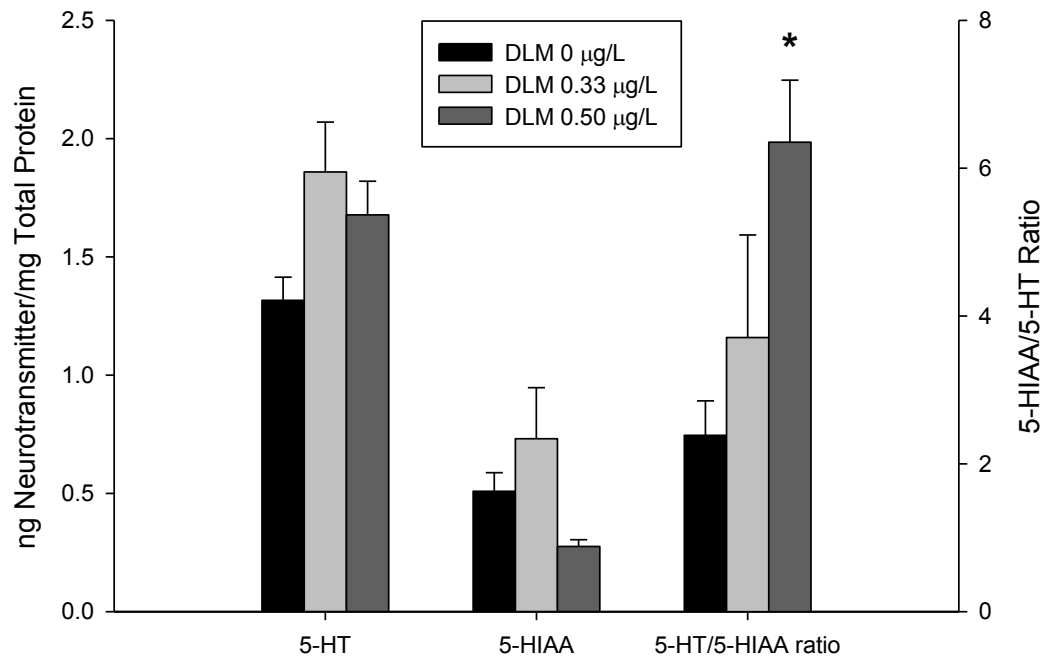
Figure 4.1: The effects of developmental deltamethrin (DLM) exposure on serotonin (5-HT) levels, serotoin metabolite levels, 5-Hydroxyindoleacetic acid (5-HIAA) and 5-HT/5-HIAA ratios at 2-weeks of age. Values represent the mean ± SEM (n=6). A one-way ANOVA was performed to determine if there was a significant effect of developmental deltamethrin exposure. (*) indicate a significant difference versus control as determined by Holm-Sidak post hoc analyses where appropriate ($p \leq 0.05$).
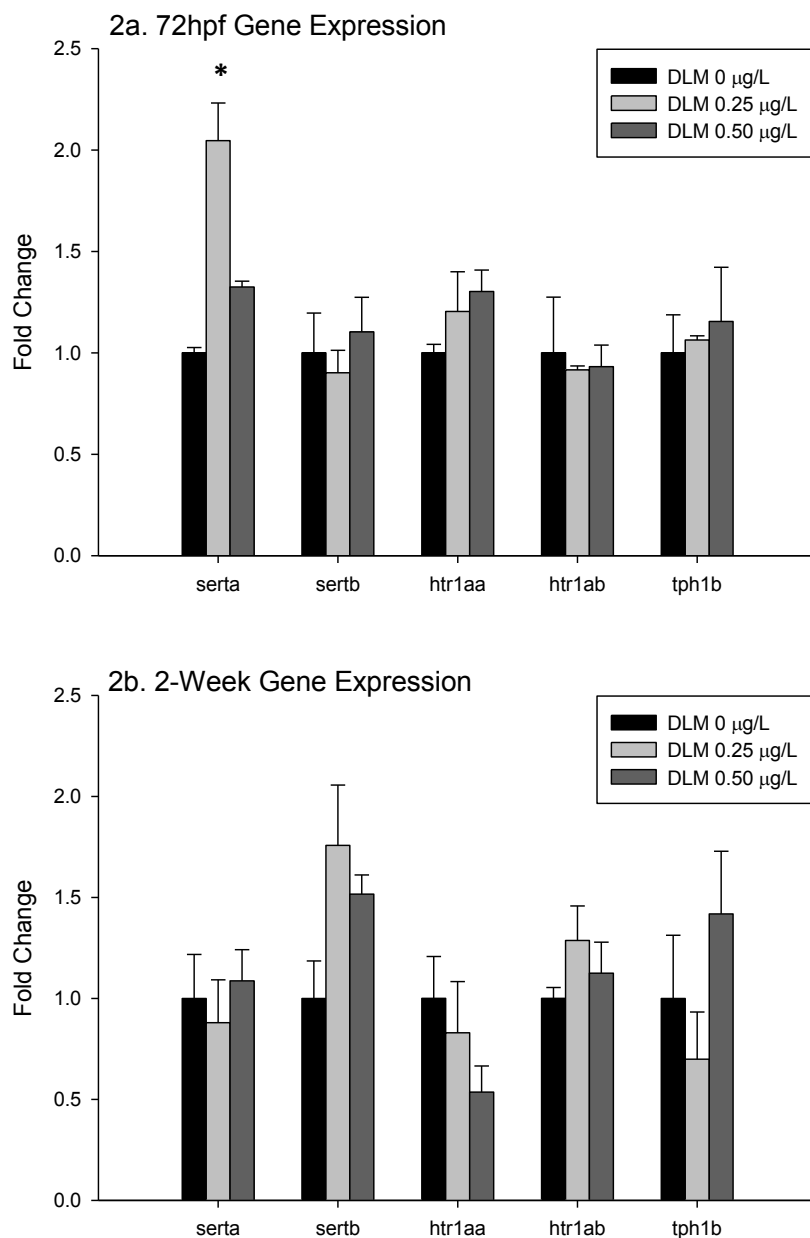
Figure 4.2: RNA transcript levels of genes related to serotonin transport (*serta, sertb*), reception (*htr1aa, htr1ab*), and synthesis (*tph1b*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at a) 72hpf and b) 2-weeks post fertilization. The graph represents the mean fold change in transcript copy number (n=4). The error bars represent standard error of the mean.). A one-way ANOVA was performed to determine if there was a significant effect of developmental deltamethrin exposure. (*) indicate a significant difference versus control as determined by Holm-Sidak or Dunn's post hoc analyses where appropriate ($p \leq 0.05$).
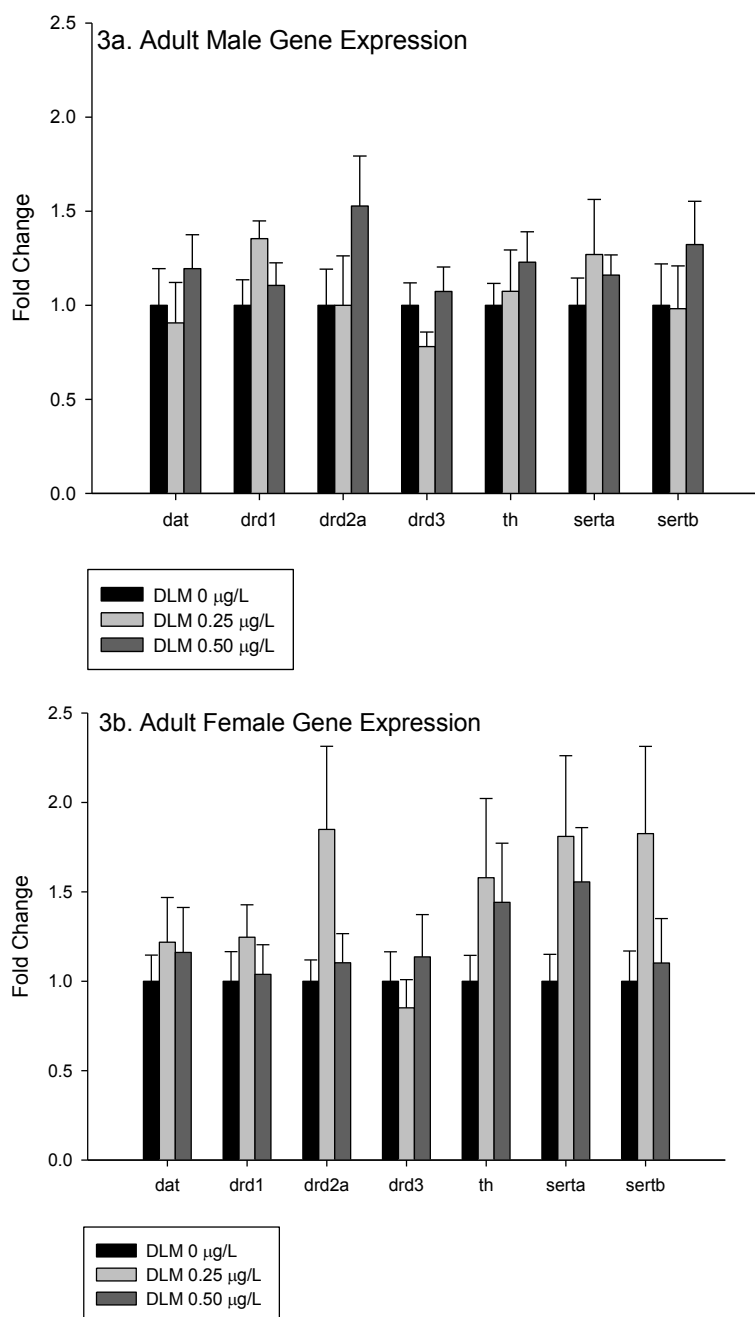
Figure 4.3: RNA transcript levels of genes related to serotonin transport (*serta*, *sertb*), reception (*htr1aa, htr1ab*), and synthesis (*tph1b*) in zebrafish exposed to deltamethrin (DLM) during development for a) adult males (n = 7-15) and b) adult females (n = 12-14). The bars represents the mean fold change in transcript copy number. The error bars represent standard error of the mean. A one-way ANOVA. There was no significant effect of developmental deltamethrin exposure on 5-HT gene expression of the gene analyzed.

Males	Pearson's statistic	dat	drd1	drd2a	drd3	th	serta	sertb
DLM 0µg/L	r	0.617	0.590	0.810*	0.062	0.459	0.948*	0.772*
	p	0.103	0.124	0.015*	0.884	0.253	0.001*	0.042*
DLM 0.5µg/L	r	0.188	0.326	-0.104	-0.456	-0.227	0.014	0.186
	p	0.502	0.235	0.701	0.076	0.416	0.961	0.489

Females	Pearson's statistic	dat	drd1	drd2a	drd3	th	serta	sertb
DLM 0ug/L	r	-0.125	-0.171	-0.152	-0.029	-0.097	0.128	-0.181
	p	0.699	0.596	0.636	0.930	0.765	0.691	0.573
DLM 0.5µg/L	r	-0.163	-0.048	0.048	-0.330	-0.343	0.653*	-0.171
	p	0.613	0.876	0.883	0.295	0.275	0.015*	0.595

Table 4.1: Pearson's correlation coefficients and p-values comparing transcript number of the indicated gene and number of aggressive attacks performed in the mirror induced aggression assay in adult male and female zebrafish. These fish had been developmentally exposed to 0 or 0.5 µg/L deltamethrin as indicated. *$p \leq 0.05$.
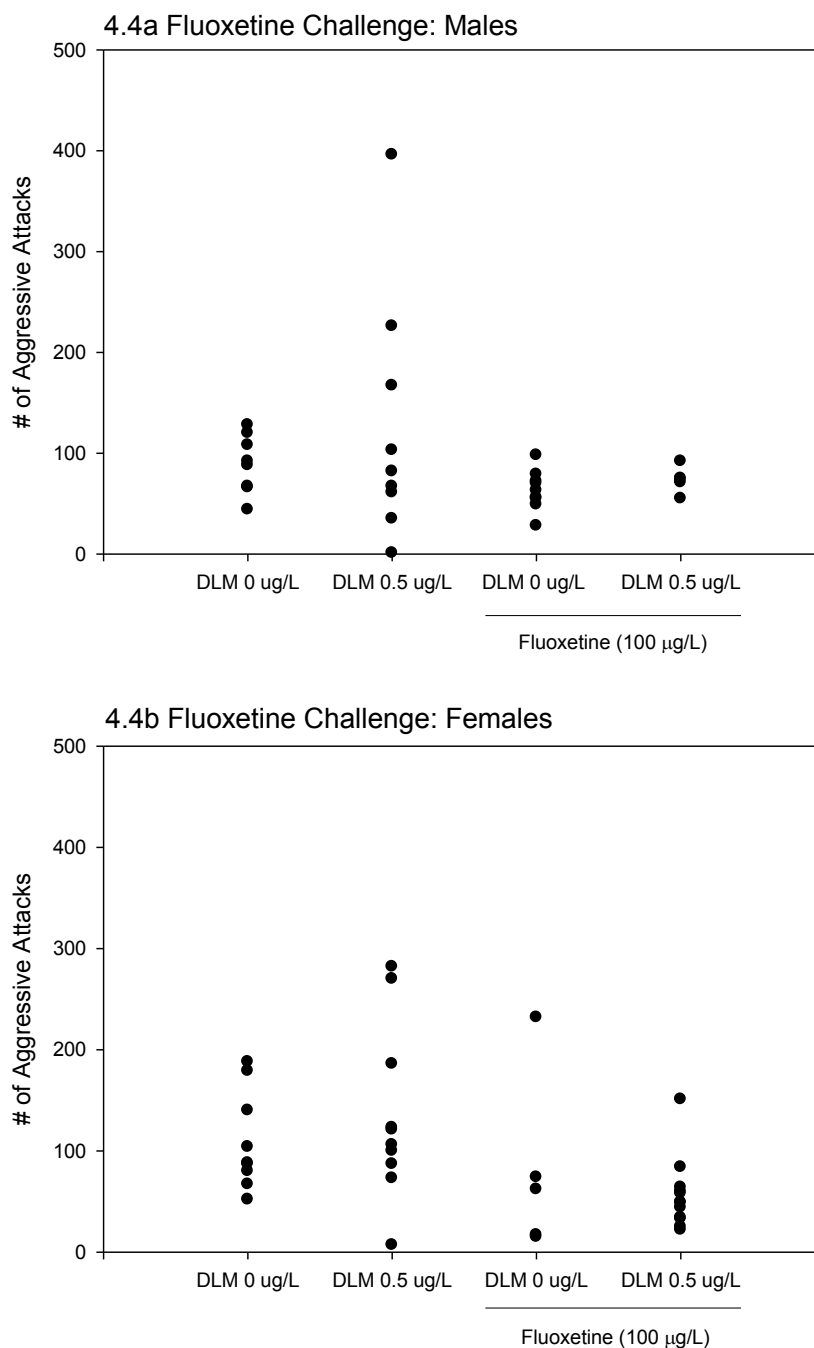
Figure 4.4: Fluoxetine challenge. Number of aggressive attacks using the mirror induced aggression assay of adult (a) male (b) and female zebrafish developmentally exposed to deltamethrin (DLM). Each data point represents the number of aggressive attacks performed by one individual fish during a 9 minute trial. (n=6-12). Dominant/subordinate social status cannot be distinguished in male zebrafish treated with fluoxetine.

Chapter 5

General Discussion and Conclusions

5.1 Major Findings

The results presented in this thesis support the hypothesis that developmental exposure to concentrations of deltamethrin below the LOAEL results in persistent behavioral deficits which are due, in part, to changes in dopamine and serotonin system gene expression and neurochemistry. Developmental deltamethrin exposure resulted in increased larval swim activity, decreased expression of genes involved in DA and 5-HT neurotransmission, as well as increased levels of DA metabolite HVA. Manipulating the DA system by knocking down DAT simultaneously during deltamethrin exposure rescued the deltamethrin induced locomotor effects. Acute methylphenidate (DAT inhibitor) exposure was found to increase locomotor activity in control larvae while reducing locomotor activity in larvae previously exposed to deltamethrin. Both of these studies strongly suggest that dopaminergic dysfunction mediates the behavioral effects of developmental deltamethrin exposure. The deltamethrin induced locomotor activity observed in larval zebrafish was found to persist into adulthood, and further behavioral characterization of adult male and female zebrafish revealed a gender dimorphic response to developmental deltamethrin exposure. In adult males, transcript levels of *serta*, *sertb*, and *drd2a*, positively correlated with the level of aggression. However, this correlation was lost in the population of male fish that had been developmentally exposed to deltamethrin. Furthermore, fluoxetine exposure attenuated aggression exhibited by dominant male zebrafish demonstrating the involvement of the serotonin system in mediating aggressive social behavior. In conclusion, this research demonstrates that

deltamethrin is a developmental neurotoxicant that causes persistent locomotor and behavioral effects. Also, we add to the ethology of zebrafish, by incorporating the experimental behavioral paradigms adapted in our lab. Finally, we provide a mode of action for deltamethrin induced behavioral toxicity which can assist in the cumulative risk assessment of these compounds.

5.2 Developmental Origins of Health and Disease

Developmental deltamethrin exposure caused locomotor and behavioral deficits in larval and adult zebrafish, effects seen long after cessation of treatment. These findings support the DOHaD hypothesis that an adverse developmental environment results in enduring effects. Exposure to deltamethrin during the embryonic period increased locomotor activity in larval zebrafish (figure 2.1) which persisted into adulthood in male zebrafish (figure 3.1a). In addition male zebrafish exhibited increased swim velocity (figure 3.2a), increased bouts of high mobility (figure 3.3a) and increased aggression (figure 3.6a). Female zebrafish that had been developmentally exposed to deltamethrin exhibited increased swim velocity (figure 3.2b), increased thigmotaxis (figure 3.5b) and altered habituation rates (figure 3.4b). Similarly, latent and sex-specific behavioral effects following developmental deltamethrin exposure have been observed in rats. (Lazarini et al. 2001).

Additional support for the DOHaD hypothesis comes from our DAT morpholino knockdown and inhibition studies. Since morpholinos are typically penetrant through 72hpf, DAT knockdown occurred simultaneously with deltamethrin exposure. This early intervention was sufficient to rescue the deltamethrin induced locomotor activity at 2-weeks of age. One would expect methylphenidate exposure at the larval stage to also

rescue the deltamethrin induced locomotor activity due to the fact that both conditions would effectively elicit the same effect: lower DAT activity. However, this was not the case, as methylphenidate exposure produced a substantial reduction in locomotor activity in larvae that had previously been exposed to deltamethrin. In this scenario, the DA system had been already adapted to effects of developmental deltamethrin exposure. These larvae were likely functioning at a new baseline level, rendering them susceptible to subsequent modification. Our studies are theoretically similar to those performed in 5-HTR1a KO mice. In 5-HTR1a KO mice, which exhibit anxiety-like behaviors as adults, conditional re-expression of 5-HTR1a only during the early postnantal period was able to prevent the occurrence of anxiety-like behaviors in adults. However, conditional expression of 5-HTR1a during adulthood did not ameliorate the anxiety-like behaviors exhibited by these KO mice (Gross et al. 2002). This study indicates that the anxiety-like behaviors exhibited by adult 5-HTR1a KO mice are the result of 5-HTR1a deficiency during development and not adulthood. The lack of effect of 5-HTR1a re-expression during adulthood suggests that modifying the serotonin system during development (by 5-HTR1a knockout) had already predisposed the animal to anxiety-like behaviors. Together, our studies show that early intervention, during the critical developmental window, was capable of preventing long-term changes in behavior caused by early perturbations to the 5-HT and DA systems. These findings reinforce the idea that the 5-HT and DA monoamine systems are highly plastic systems that can be permanently modified during development and support the DOHaD hypothesis.

5.3 Cross talk of the dopamine and serotonin systems

Our studies show that developmental deltamethrin exposure transiently alters transcript levels in both the 5-HT and DA systems (figure 2.2 and 4.2a). In addition, genes from both the 5-HT (*serta*, *sertb*) and DA (*drd2a*) system were found to positively correlate with the degree of aggression in the adult male zebrafish, a correlation lost in fish that had been developmentally exposure to deltamethrin (table 4.1a). These findings are not surprising since locomotor activity (figure 1.1, 3.1a, 3.2a, 3.3a), affective disorders (figure 3.4b,c, 3.5b), and aggressive behaviors (figure 3.6a) are complex endpoints and are influenced by a number of different neural pathways. The dual role of the DA and 5-HT systems in mediating behaviors such as aggression and anxiety are well characterized. For instance, hyposerotonergic (Coccaro et al. 1989; Virkkunen and Linnoila 1993) and hyperdopaminergic (Netter and Rammsayer 1991; Seo et al. 2008; Yu et al. 2014) states are associated with aggression in animal and humans studies. Blockade of either the DAT or SERT during critical windows of development permanently altered aggressive behaviors in adults (Yu et al. 2014). Furthermore, fluoxetine was found to ameliorate the behavioral effects caused by prentatal methylphenidate exposure (Bolanos et al. 2008).

There is also substantial evidence demonstrating cross talk between the DA and 5-HT neurotransmitter systems. The serotonin system directly interacts and influences dopamine neurotransmission (Kapur and Remington 1996) . 5-HT projections from the dorsal raphe extend into midbrain structures populated by DA neurons (substantia nigra, ventral tegmental area) as well as areas where DA projections terminate (amygdala, striatum, prefrontal cortex) (Broderick and Phelix 1997). In addition, 5-HT1a (Pazos and

Palacios 1985), 5-HT2a (Doherty and Pickel 2000; Nocjar et al. 2002), and 5-HT2c (Di Matteo et al. 2001) receptors are often found on DA neurons in the VTA. Furthermore, administration of selective 5-HT agonists is found to modulate DA neuron firing and DA release and neurotransmission (Bortolozzi et al. 2005; Di Matteo et al. 2001). Conversely, the nigral DA system also projects into the dorsal raphe area (Peyron et al. 1995) and D2 receptors are also present on 5-HT neurons in the dorsal raphe (Mansour et al. 1990). Similarly, administration of DA agonists is associated with increased 5-HT firing; whereas DA antagonists elicit the opposite effect (Ferre et al. 1994). Methylphenidate exposure is also found to decrease 5-HT levels (Marco et al. 2011). In addition, because DA and 5-HT receptors are primarily G-PCRs, studies have also suggested the possibility of 5-HT and DA receptor heteromerization (Albizu et al. 2011; Gurevich and Gurevich 2008).

The DAT is not selective and displays a low affinity for 5-HT (Eshleman et al. 1999; Giros et al. 1991). Because, VMAT2 is a non-selective monoamine transporter, 5-HT can be transported into DA vesicles and be released together with DA during DA neurotransmission (Zhou et al. 2005). Therefore, under conditions where there is excess 5-HT in the synapse (ie. SSRI treatment), 5-HT can outcompete DA for the DAT in areas rich with both DA and 5-HT neurons and alter DA signaling. Therefore, these studies suggest that perturbation of one pathway will likely affect the other.

5.4 Sex-specific differences in behavior

Characterization of adult behavior in adult male and female zebrafish demonstrate that developmental exposure to deltamethrin was associated with increased locomotor and aggressive behaviors in males; whereas, exposure to deltamethrin resulted in anxiety-

like (affective) behaviors in female. This apparent sex-specific vulnerability to certain types of mental disorders is also found in the human populations. For instance, incidence of attention deficit hyperactivity disorder (Waddell and McCarthy 2012), autism (Auyeung et al. 2009), Parkinson's disease (Baldereschi et al. 2000) are higher in males, whereas, incidence of affective disorders (anxiety, mood disorders) are higher in females (Bekker and van Mens-Verhulst 2007). While the mechanisms behind these sex-specific predispositions to mental disease are unknown, the role of sex steroids in mediating these sex-specific behavioral responses is the most obvious etiology.

Estrogen has been implicated to have a neuroprotective effect and has been found to 1) activate estrogen receptors and initiate the transcription of various genes involved in essential brain function, pro-growth signaling, and apoptotic regulation, 2) directly modulate neurotransmitters, their receptors, and signal transduction, 3) function as an antioxidant, reviewed in (Garcia-Segura et al. 2001). Estradiol enhances DA neurotransmission (Thompson and Moss 1994) by increasing DA synthesis, release, and metabolism, and modulates DA neurons firing rates via estrogen receptor activation (Pasqualini et al. 1995; Thompson and Moss 1994; Xiao and Becker 1994). Estrogen deprivation via ovariectomy, was associated with loss of nigral dopamine cells, which was moderately restored with estradiol therapy in primate models (Leranth et al. 2000) as well as rodent models (Johnson et al. 2010b). In addition, estrogen has been found to affect serotonin neurotransmission by altering uptake (Endersby and Wilson 1973; Wirz-Justice et al. 1974), metabolism (Greengrass and Tonge 1974), and altering serotonin receptor density (Biegon and McEwen 1982). E2 replacement in ovariectomized rats is associated with increased levels of 5-HT and 5-HIAA, increased expression of TPH,

SERT, 5-HT2a receptors, decreased levels of 5-HT1a receptors and increased SERT binding, reviewed in (Amin et al. 2005).

Alternatively, testosterone has been found to modulate the DA and 5-HT systems as well. In rat models, androgen receptor agonism has been found to increase the expression levels of several genes in these systems including *Th*, *Dat*, *Drd1*, *Drd2*, *Vmat*, *Comt*, *Maoa*, and *Maob* (Purves-Tyson et al. 2012; Purves-Tyson et al. 2014), increase the levels of DA and 5-HT in the striatum (de Souza Silva 2009), and increase turnover of both DA and 5-HT (Thiblin et al. 1999). Castration was associated with increased TH immunoreactivity in rodents, which was lowered again with testosterone replacement therapy, suggesting that testosterone may suppress the DA system (Johnson et al. 2010a). Castration is also associated with reduced 5-HT1a binding (Fischette et al. 1983) and 5-HT1a mediated behavioral activity in rats (Gogos and van den Buuse 2003). Thus, the opposite influences of the steroidal hormones on the 5-HT and DA systems provide a plausible mechanism for the observed gender dimorphic behaviors. Along the lines of the DOHad hypothesis, the manner by which the DA and 5-HT systems adapt to deltamethrin insult would be dictated by the available sex hormone, both having different effects on these systems.

In addition, there is also a developing theory that the sex-specific behavioral morbidities (affective disorders in females and austism spectrum in males) are due to gender-specific responses of the placenta, reviewed in (Davis and Pfaff 2014). The sex-specific gene expression, protein expression, immune function, and steroid profile of the placenta may be the reason for the differences in how males and females cope with prenatal stress, reviewed in (Clifton 2010). However, because we demonstrate similar

gender-specific behavioral profiles in adult zebrafish, a species that develops ex-utero and thus not affected by maternal or placental contributions, this hypothesis is likely not a mechanism of deltamethrin induced neurobehavioral toxicity.

Besides the sex-specific differences in behavior, we also find that female zebrafish are more sensitive to the effects of developmental deltamethrin exposure. Females exhibited behavioral deficits after exposure to 0.25 µg/L deltamethrin (figure 3.3b and 3.4b,c), a concentration half of that was required to elicit effects in male zebrafish (0.5 µg/L). This can be explained not only by differences in sex-hormone driven neural modulation but also possibly by sex-differences in ADME during development.

5.5 General Conclusions

In conclusion, the studies discussed in this dissertation contribute to the current state of science in 3 major ways. First, we demonstrate that perturbation of the critical neurodevelopment period, via neurotoxicant exposure, has long lasting effects on adult behavior. In particular, we highlight the dynamic and plastic nature of the dopamine and serotonin systems during development. This finding supports the DOHaD hypothesis that environmental influences during development can have long-lasting effects. Therefore, identifying and mitigating these fetal stressors can decrease the global disease burden.

Second, using the behavioral paradigms adapted for our laboratory, our data contribute to zebrafish ethology, specifically characterizing the AB strain of zebrafish. With the increasing use of this model system and the call for their use in behavioral studies, there is a paucity of data concerning their natural behaviors and responses under

different experimental paradigms. In addition, due to the great variability that exists between *Danio* strains, there is a need to establish baseline values for the more common "wildtype" laboratory strains in order to standardize results. In addition, if the zebrafish is to be used as a model of human behavior there is also the need to standardize and understand how the various assessment methods translate to human behavior.

Finally, these findings establish a mode of action for deltamethrin induced neurobehavioral toxicity, and contributes to the cumulative risk assessment of pyrethroids pesticides. Our studies bring into light the potential for this class of compounds to cause persistent behavioral deficits if exposure occurs during development. However, pyrethroid pesticides are still one of the safer insecticide options on the market, and as with all decision making processes, the benefits must outweigh the risks. If actions are taken to mitigate the risk of exposure during critical developmental periods (ie. increased education about the dangers of exposure, more controlled and timed application), then further action may not be warranted. In addition, for risk assessment purposes, our studies further highlight the utility of the zebrafish model to proactively screen compounds that have a common mode of action.

5.6 Future Studies

Although we propose that dopaminergic and serotonergic dysfunction as likely modes of action for the locomotor and behavioral deficits caused by developmental exposure to deltamethrin, there is still a gap of knowledge behind the mechanisms by which pyrethroids modify the dopamine and serotonin systems. At this time there is no indication that pyrethroids specifically target the DA and 5-HT systems. In fact,

pyrethroids have been shown to alter the other major neurotransmission pathways as well (Singh et al. 2012), such as the glutamate and GABA systems (Bloomquist et al. 2002; Hossain et al. 2008) and the cholinergic systems (Ahlbom et al. 1994; Eriksson and Fredriksson 1991; Talts et al. 1998). Whole transcriptome analysis of the rat frontal cortex following non-toxic deltamethrin (0.3 – 3 mg/kg) and permethrin (1 – 100 mg/lg) exposure identified increased expression of genes commonly associated with increased neuronal firing (Harrill et al. 2008). However, it is not known if sodium channel activation (the most direct effect of pyrethroid exposure) directly impacts these neurotransmitter systems, or if the effects seen in these systems are mediated by intermediary pathways. One possible link between sodium channel activation and DA and 5-HT dysfunction is via neurotrophins such as brain derived neurotrophic factor (BDNF). *Bdnf* gene expression is known to be regulated in an activity-dependent manner, such that Ca^+ influx (membrane depolarization) induces its expression (Morimoto et al. 1998). Once released BDNF acts in a pro-survival manner, binds to its respective downstream receptor (TRKB) and activates signal transduction cascades that promote cellular growth and proliferation. BDNF plays a large role in synaptic plasticity, long-term potentiation, and memory, reviewed in (Yamada and Nabeshima 2003). Therefore it is possible that increased depolarization of sodium channels by pyrethroid exposure mimics neuronal activity, promoting the synthesis and release of BDNF, which in turn encourages the aberrant growth and proliferation of neurons. BDNF has also been shown to be involved in the regulation of drd3 expression(Guillin et al. 2001) and promote the development of 5-HT neurons, reviewed in (Martinowich and Lu 2008). *In vivo*, high concentrations of deltamethrin has been shown to inhibit BDNF expression (Imamura et

al. 2000), but is a potent inducer of BDNF expression at low concentrations (Imamura et al. 2006). Studies utilizing the newly available trkb knockout zebrafish strain or using morpholinos to knockdown BDNF or trkb expression during development could test the involvement of BNDF in mediating the long-term effects of developmental deltamethrin exposure.

Because we found that developmental deltamethrin affected both the dopaminergic and serotonergic systems, examining targets common to both systems would be a viable route to pursue. For instance, MAO and COMT are enzymes shared in the degradation pathways of both 5-HT and DA. In addition, polymorphisms in both these genes have been associated with aggression phenotypes and both COMT and MAO knockout mice exhibit increased aggression exclusively in male mice, reviewed in (Volavka et al. 2004). Therefore, since both of these genes are common to 5-HT and DA metabolism, and because we observed increases in HVA levels, and increased 5-HT/5-HIAA ratios (possibly indicative of altered metabolism), as well as a hyperaggressive phenotype in only male animals following developmental deltamethrin exposure, it is possible that changes in COMT and/or MAO are involved. Further examination of the expression and activity levels of MAO or COMT or utilizing the readily available MAOIs or COMT inhibitors to rescue the behavioral effects would be worth investigating.

Recent studies examining the DOHaD hypothesis has shifted focus to mechanisms involving epigenetic modifications by which fetal programming is thought to occur. Epigenetic modifications are an attractive mechanism to explain the phenomena of DOHaD due to fact that these marks can regulate gene transcription without changing

the DNA sequence. Epigenetic modifications of interest include, but are not limited to, chromatin remodeling events such as DNA methylation and histone modification, reviewed in (Morgan et al. 2005). These epigenetic marks, set during development, are mitotically heritable and allude to the permanent nature of fetal programming, reviewed in (Reik et al. 2001). In addition, epigenetic modifications are found to be influenced by a variety of environmental factors including maternal diet and glucocorticoid exposure (Vickaryous and Whitelaw 2006). Currently, there is a paucity of data concerning the potential epigenetic effects of pyrethroid exposure. Fenvalerate exposure was found to change the methylation status of several genes in sperm DNA (Xia et al. 2013); however, these effects are likely due to the endocrine disrupting properties specific to fenvalerate (WHO 1990). Therefore, this area of research is open for exploration. Focusing on the specific genes found to be altered by deltamethrin from our studies (*drd1*, *drd2*, *sert*) could help direct future studies.

One major assumption of this thesis is that mRNA transcript levels correlates with the amount of corresponding protein present. The use of mRNA transcript levels as a proxy for levels of the corresponding protein is due to the limited availability of adequate antibodies that specifically react with zebrafish proteins. However, recent studies indicate that protein levels are largely regulated by post-transcriptional and post-translational events and that the abundance of the mRNA transcript only accounts for 40% of the variance in levels of the respective protein (Vogel and Marcotte 2012). Additional studies validating antibodies specific for the DA receptors would be required. Following that, these antibodies can then be used to determine the effects of

developmental deltamethrin exposure on the relative protein levels, localization, and density of the DA receptors.

Finally, despite the fact that pyrethroid pesticides are readily degraded in the environment through abiotic and biotic processes (Thatheyus and Selvam 2013), a recent United States Geological Survey (USGS) reports finding pyrethroids in stream bed sediment in several metropolitan areas throughout the United States at concentrations toxic to benthic organisms (Kuivila et al. 2012; Moran et al. 2012). Likewise, pyrethroids have also been detected in streams that support salmon spawning in the Pacific Northwest (Weston et al. 2011) and California (Weston and Lydy 2010; Weston et al. 2013). While environmental concentrations of pyrethroids are generally at levels considered to be sublethal to teleost species (Moore and Waring 2001) lethality is an extreme endpoint. The long-term effects following low dose exposure is unknown. Studies by Moore and Waring, 2001, demonstrated that exposure to environmentally relevant levels of cypermethrin resulted in impairment of response to sex pheromones in male salmon and decreased embryo fertilization. In addition, studies have also found that environmentally relevant concentrations of cypermethrin were acutely toxic to common carp embryos (Aydın et al. 2005). Since we demonstrated that developmental low dose exposure to deltamethrin produces behavioral effects in larval and adult zebrafish, this could potentially affect general survivability of fish populations, since engaging in proper behavior (schooling, territorial/resource aggression, and predator avoidance) is necessary for survival. Taken together, all of these studies suggest that pyrethroid contamination of aquatic ecosystems could ultimately impact wild fish populations and warrants further investigation. From an environmental risk assessment standpoint, the results from our

studies provide mechanistic adverse outcome pathway data that can be integrated with individual based simulation models for population forecasting and ecotoxicology modelling (Kramer et al. 2011).

Appendices

Table A5.1 Primer sets used for RT-qPCR

Transcript	GenBank Accession Number	Primer Pair Sequence
dat (slc6a3)	NM_131755	F: 5'-acaactgctacagagacgccatca-3' R: 5'-ggccacgttgtgtttctgtgacat-3'
drd1b	NM_001135976	F: 5'-tcatgtgttccactgcctccatct-3' R: 5'-atagcgaaacgggcttgaaatggc-3'
drd2a	NM_183068	F: 5'-acgatgctctctgtgtgattgcga-3' R: 5'-caaacgcacatgcccagttacaga-3'
drd3	NM_183067	F: 5'-cttcagaccaccaccaactac-3' R: 5'-gccgaccacttccaaataaac-3'
th	NM_131149	F: 5'-tccaccatcttgaaaccagaccca-3' R: 5'-tcaaagagctgaccagcgtgctaa-3'
serta	NM_001039972	F: 5'-gcctggtgtgtgtctgttat-3' R: 5'-ctgtggcgtactcctcaaatag-3'
sertb	NM_001177459	F: 5'- tgggctctgttctacttctact-3' R: 5'-gtccaggtcacgttgtcttt-3'
htr1aa	NM_001123321	F: 5'-ggttttgaataaatggactttggg-3' R: 5'-gtagtctatgggatcatcggtgatggc-3'
htr1ab	NM_001145766	F: 5'-gtgaagactctgggcatcataa-3' R: 5'-acacaagtcggacagaaagg-3'
tph1b	NM_001001843	F: 5'- gtcacagctcagatcctctttac-3' R: 5'-gagagaagccaaccctaattcc-3'
eaat2	NM_199979	F: 5'-tatctgtccaacgccaccaaacct-3' R: 5'-tccgatcagacccagcacattcat-3'
eaat3	NM_001002666	F: 5'-tgagaatgctaaagatggtcatcc-3' R: 5'-tgacggcaataatggtggtggaga-3'
gat1	NM_001007362	F: 5'-ggaggagatgatcggatataagcc-3' R: 5'-accagcccacgccttgcccccact-3'
gabra1	NM_001077326	F: 5'-tcactatgaccaccctaagtatca-3' R: 5'-tgagagcagagaagacaaaggcgt-3'
gad67	NM_194419	F: 5'-tctgaacatagtcattactcaatc-3' R: 5'-tactgtagtaccagccgtggcatt-3'

avp	NM_178293	F: 5'-gagcatctcaggagaaactcg-3' R: 5'-taggcgatgtgttcagaaagg-3'
nkcc1	NM_001002080.1* NM_001163654.1*	F: 5'-tcaagcacagccaacctcat-3' R: 5'-ataaagggcactggagacgg-3'
kcc2	JN688966.1	F: 5'-gcacagcactgttcgctttc-3' R: 5'-aacggatgtaccagcctcac-3'
bdnf	NM_131595*	F: 5'-aggtccccgtgactaatggt-3' R: 5'-cgcttgtctattcctcggca-3'
28S		F: 5'-cctcacgatccttctggctt-3' R: 5'-aattctgcttcacaatgata-3'
β-actin	NM_131031	F: 5'- cgagcaggagatgggaacc-3' R: 5'-caacggaaacgctcattgc-3'

*Spans all transcript variants
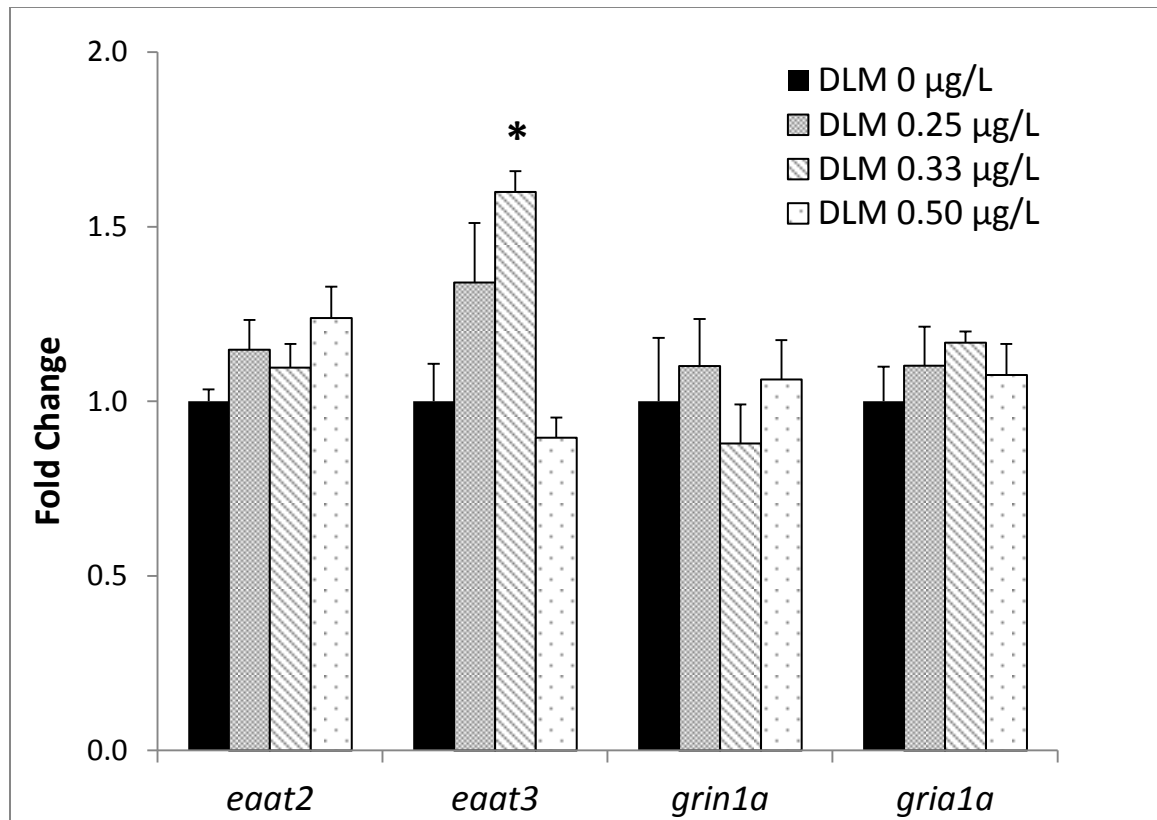
Figure A5.1: RNA transcript levels of genes related to glutamate transport (*eaat2*, eaat3) and reception (*grin1a* and *gria1a*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at 72hpf. The bars represents the mean fold change in transcript copy number ± S.E.M of one representative experimental replicate (n=4). A one-way ANOVA or a Kruskal-Wallis ANOVA on Ranks was used to determine if there was a significant effect of deltamethrin exposure on transcript levels where appropriate. (*) indicate a significant difference versus control as determined by Holm-Sidak or Dunn's post hoc analyses where appropriate ($p \leq 0.05$).
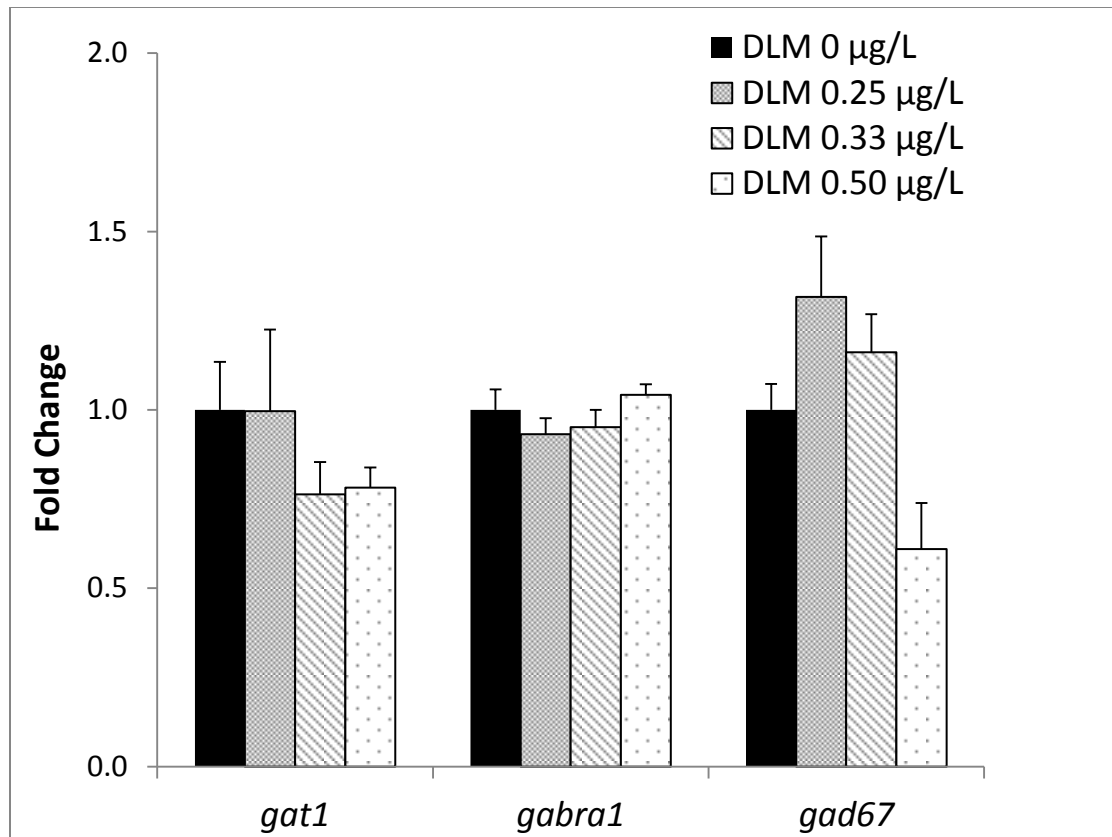
Figure A5.2: RNA transcript levels of genes related to GABA transport (*gat1*), reception (*gabra1*) and synthesis (*gad67*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at 72hpf. The bars represents the mean fold change in transcript copy number ± S.E.M of one representative experimental replicate (n=4). A one-way ANOVA or a Kruskal-Wallis ANOVA on Ranks was used to determine if there was a significant effect of deltamethrin exposure on transcript levels where appropriate. Compared to control animals, there was no significant effect of developmental deltamethrin exposure on *gat1*, *gabra1*, or *gad67* RNA transcript levels.
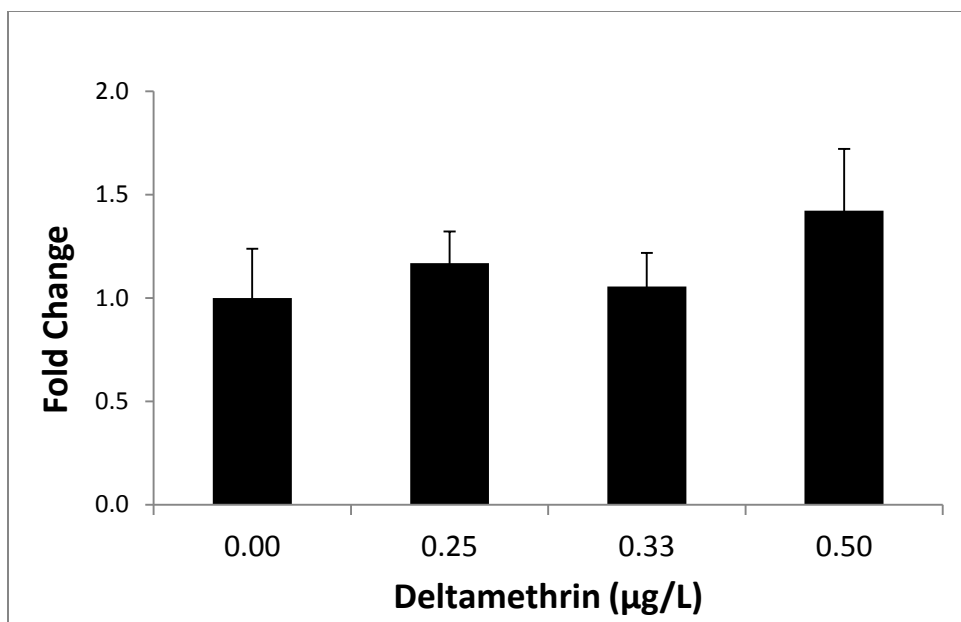
Figure A5.3: RNA transcript levels of arginine vasopressin (*avp*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at 72hpf. The bars represent the mean fold change in transcript copy number ± S.E.M of one representative experimental replicate (n=4). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on transcript levels. Compared to control animals, there was no significant effect of developmental deltamethrin exposure on *avp* expression.
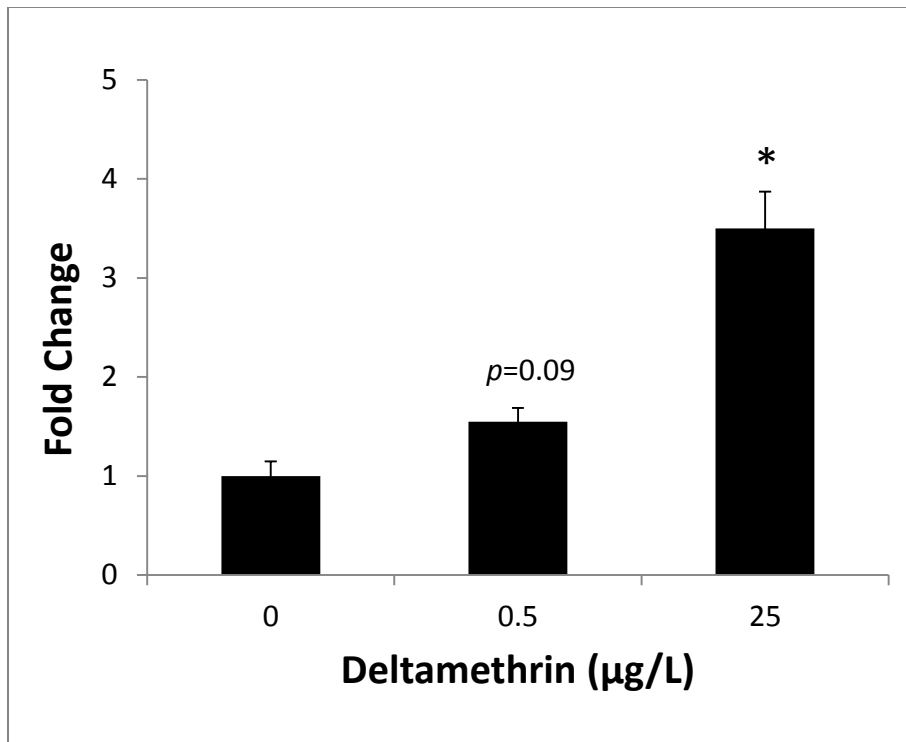
Figure A5.4: RNA transcript levels of brain derived neurotrophic factor (pan-*bdnf*) in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at 72hpf. The bars represent the mean fold change in transcript copy number ± S.E.M of one representative experimental replicate (n=4). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on transcript levels. (*) indicate that distance travelled across time in animals developmentally exposed to 25 µg/L deltamethrin were significantly different from vehicle control exposed animals as determined by Holm-Sidak multiple comparisons ($p \leq 0.05$).
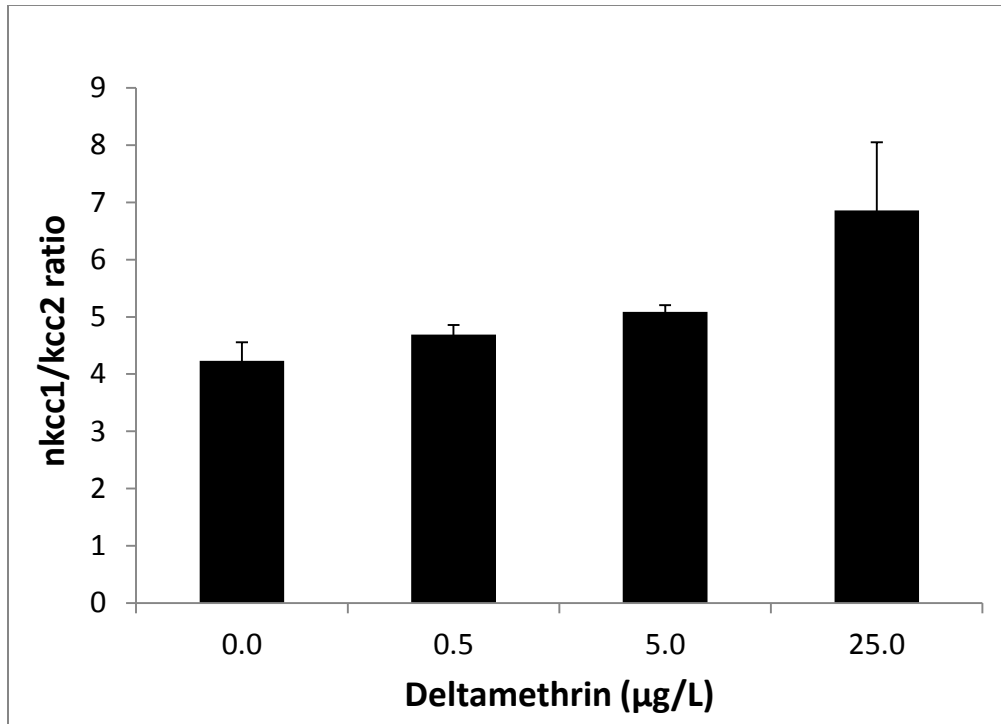
Figure A5.5: Ratio of *nkcc1/kcc2* transcripts in zebrafish exposed to deltamethrin (DLM) during development. Transcript levels were assayed at 72hpf. The bars represent the mean fold change in transcript copy number ratio $\pm$ S.E.M of one representative experimental replicate (n=4). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on transcript levels. Compared to control animals, there was no significant effect of developmental deltamethrin exposure on the ratio of *nkcc1/kcc2* transcript.
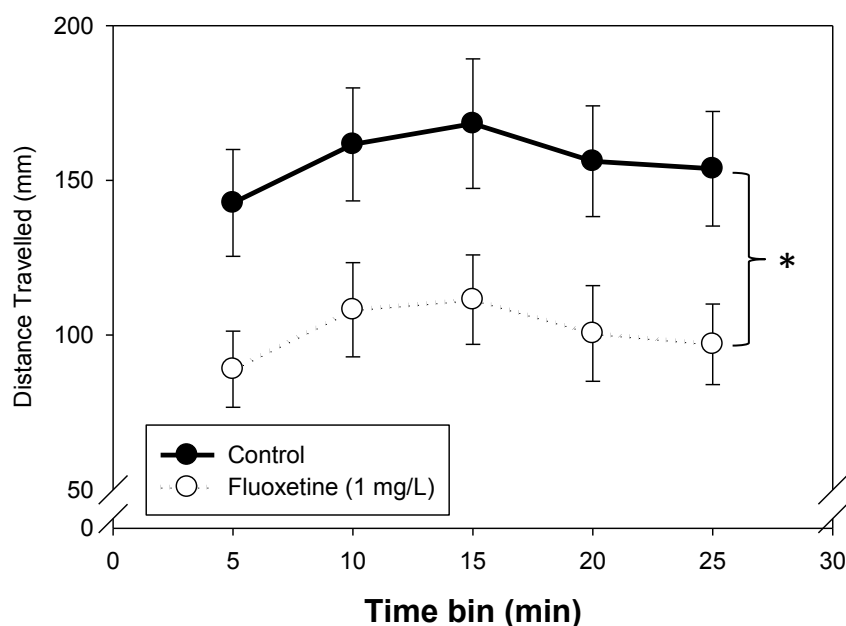
Figure A5.6: Effects of acute fluoxetine exposure on swim activity following a transition into darkness in 2-week old larval zebrafish. Larval zebrafish were acutely exposed to 1 mg/L fluoxetine via bath exposure for 1 hour prior to activity assay. The activity assay was performed as described in chapter 2. Data points are presented as mean distance travelled (mm) ± S.E.M. in 5-min time bins during 25-min sessions (n=42-50). A two-way ANOVA was used to determine if there was a significant effect of fluoxetine exposure and time on larval swim activity. As determined by Holm-sidak multiple comparisons, (*) indicates that distance travelled across time in fluoxetine exposed animals are significantly reduced when compared to vehicle control (water) exposed animals ($p \leq 0.05$).
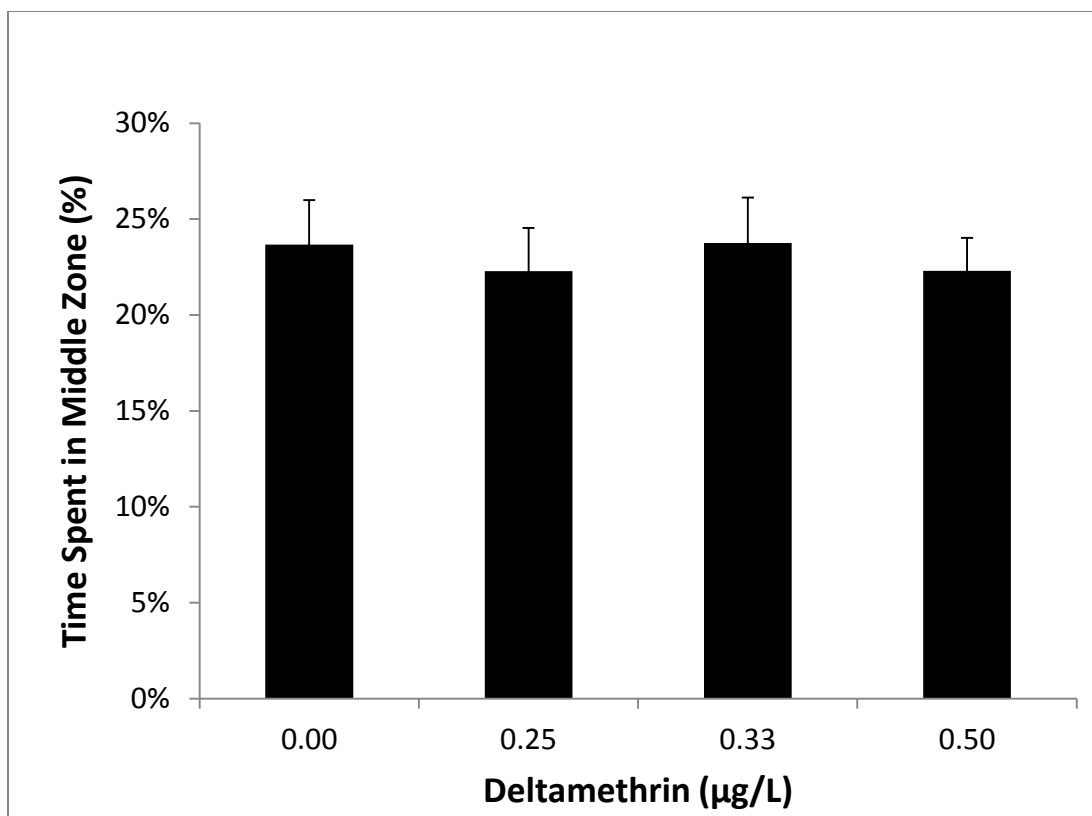
Figure A5.7: Thigmotaxis in 2-week old larval zebrafish developmentally exposed to deltamethrin (DLM). Data are presented as mean % time spent in the center zone ± S.E.M. during the entire 30 minute session (n=28-33). A one-way ANOVA was used to determine if there was a significant effect of deltamethrin exposure on the amount (%) of time spent in the center zone. Compared to control animals, there was no significant effect of developmental deltamethrin exposure on thigmotaxis in larval zebrafish.

	DLM 0 µg/L		DLM 0.5 µg/L	
	%	N	%	N
Females	46.3%	37	54.7%	47
Males	54.8%	43	45.3%	39

Table A5.2: Sex ratio of adult zebrafish developmentally exposed to vehicle control or 0.5 µg/L deltamethrin (DLM). Chi-square analysis was used to determine if developmental deltamethrin exposure alters the sex ratio of zebrafish. There is no significant effect of developmental deltamethrin exposure on the sex ratio of zebrafish.

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