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Effects of Bifenthrin on the Estrogenic and Dopaminergic Signaling Pathways in Fish

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Effects of Bifenthrin on the Estrogenic and Dopaminergic Signaling Pathways in Fish

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Luísa Becker Bertotto

June 2018

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Dedication

This dissertation is dedicated to my husband Colin Lawrence and my lab family, without their support and understanding this would not have been possible.

ABSTRACT OF THE DISSERTATION

Effects of Bifenthrin on the Estrogenic and Dopaminergic
Signaling Pathways in Fish

by

Luísa Becker Bertotto

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, June 2018
Dr. Daniel Schlenk, Chairperson

Bifenthrin (BF) is a pyrethroid insecticide used in urban and agricultural applications. Previous studies have shown that environmentally relevant (ng/L) concentrations of BF increased plasma concentrations of 17β-estradiol (E2) and altered the expression of dopaminergic (DA) pathway components. DA neurons can indirectly regulate E2 biosynthesis, suggesting that BF may disrupt the hypothalamic-pituitary-gonadal (HPG) axis. This study sought to answer the hypothesis that BF affects dopaminergic and E2 signaling pathways through a unique target, causing *in vivo* estrogenic activity in fish. Since embryos do not have a complete HPG axis, the hypothesis that BF impairs DA regulation was tested in embryonic and one-month-old juvenile zebrafish (*Danio rerio*) with exposure to measured concentrations of 0.34 and 3.1 μg/L BF for 96 hours. Significant decreases of TH and DR1 transcripts, and HVA levels, as well as ratios of HVA/DA and HVA+DOPAC/DA in zebrafish embryos were observed after BF treatment. In juveniles, a significant increase in the expression of ERβ1 and the DOPAC/DA ratio was noted. These results show a possible anti-estrogenic effect of BF in embryos, and estrogenicity in juveniles. To examine the role of the estrogen receptor (ER) in BF's toxicity, embryos were exposed for 96 hours to a

mixture of BF and an ER agonist (17α-ethynylestradiol – EE2). Results showed that only exposure to EE2 had a significant effect on gene expression and DA, HVA, and DOPAC levels after co-exposure with BF. Additionally, ERα knockdown did not rescue BF effects in embryos of zebrafish. Results demonstrated that ER might not be the target of BF's toxicity at the zebrafish embryonic stage. Furthermore, the hypothesis that BF impairs sex differentiation was tested in larval and embryonic Japanese Medaka. Fish were exposed to BF, an ER antagonist (ICI 182,780), and an ER agonist (E2). Results showed a trend towards masculinization of Japanese Medaka exposed at the larval stage for 30 days to the lowest concentration of BF. However, genotypic gender ratios were not altered. Exposures at embryonic stages of development also did not alter phenotypic gender. These results show sex differentiation was not significantly altered by embryonic and larval exposure to BF. Overall, these results will be very useful in risk assessment strategies and inform studies of developmental mechanisms of toxicity of bifenthrin.

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Chapter 1: Introduction

Fish Reproduction

Hypothalamus-Pituitary-Gonadal Axis

Hypothalamus-pituitary-gonadal (HPG) axis is a system very important for the control of reproduction in vertebrates. The brain controls the release of hormones, that in turn modulate bodily functions. Studies by Benoit and Assenmacher (1952) and Donovan and Harris (1954) were the first to discover a linkage between the brain and the gonads. It was not until 1966 that the brain-hormone relationship in fish started to be revealed, from studies on pituitary grafts (Olivereau & Ball, 1966), followed by the proof that hypothalamic extracts stimulated gonadotropins (GtH) in carp (Breton *et al.*, 1971; Breton & Weil, 1973). Gonadotropin releasing hormone (GnRH), was discovered by Amoss *et al.* (1971) and Matsuo *et al.* (1971) in 1971.

In fish, as in all vertebrates, the hypothalamus is a part of the brain that synthesizes neurohormones that will further control the activity of the pituitary gland. The hypothalamus connects directly to the pituitary gland via neurosecretory fibers, which are actually axons from neurons in the hypothalamus that send projections to the pituitary, near their target cells. This is one major difference between mammals and fish. Mammals present a hypothalamus-pituitary portal system, located at the external zone of the median eminence. Neurohormones are released by hypophysiotropic neurons close to the median eminence blood vessels, reaching the pituitary gland via blood circulation. In general, most innervation responsible for the control of a certain cell type in fish are localized around the same group of cells (Olivereau & Ball, 1964). For example, GnRH fibers mostly innervate the pituitary proximal pars distalis, where gonadotroph cells are

present (Zohar *et al.*, 2010). Figure 1.1 shows a representation of the morphological differences in the hypothalamus-pituitary system between mammals and fish. Studies in zebrafish show that innervation to the pituitary cells comes from the preoptic area and the medio basal hypothalamus, considered to be the areas responsible for controlling reproduction functions (Johnston & Maler, 1992; Mananos *et al.*, 1999).

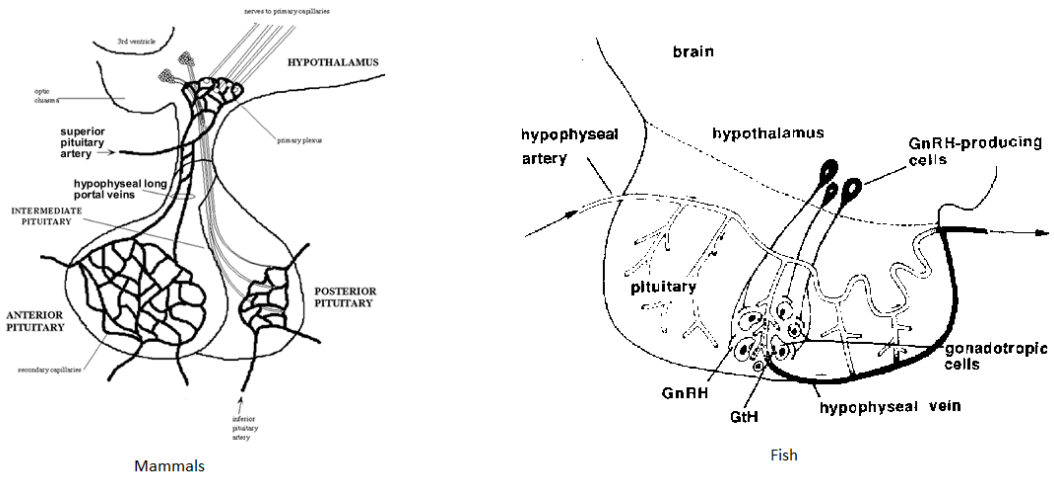

Figure 1.1 – Representation of the hypothalamus-pituitary system in mammals (left) and fish (right). Mammals secret their hypothalamic hormones in a portal system, whereas fish have neuronal axons (represented by GnRH-producing cells) ending directly on endocrine cells in the pituitary. Adapted from TSN (2017) and NZDL (2017).

The pituitary gland, also called the hypophysis, is divided in the adeno and the neurohypophysis. The former is considered the glandular part of the pituitary, where secretion occurs. The neurohypophysis consists of neurosecretory fibers coming from different regions of the brain, innervating the pituitary gland. In fish, the adenohypophysis is divided into a pars distalis, which is equivalent to the anterior lobe of terrestrial vertebrates. The pars distalis is divided into the rostral and proximal pars distalis, as well as the pars intermedia, which is the intermediate lobe of terrestrial vertebrates (Olivereau & Ball, 1964). The rostral pars distalis contains corticotroph,

mammotroph and in some species thyrotroph cells. These cells produce, respectively, adrenocorticotropic (ACTH), prolactin (PRL) and thyroid-stimulating (TSH) hormones. The proximal pars distalis contains somatotroph, gonadotroph and thyrotroph cells. These types of cells produce respectively, growth (GH), follicle-stimulating (FSH) and luteinizing hormones (LH); last two hormones being produced both by the gonadotroph cells (Olivereau & Ball, 1964). In fish, cells of a given subtype, contrary to mammals, tend to group together in certain regions of the pars distalis, forming specialized sectors (Zohar *et al.*, 2010).

One of the most important hypothalamic hormones that controls gonadal steroids is GnRH. There are 24 variants of GnRH found in vertebrates and invertebrates (Morgan & Millar, 2004; Lethimonier *et al.*, 2004; Kah *et al.*, 2007; Okubo & Nagahama, 2008). Teleost fish present the most number of isoforms among vertebrates, having 8 variants (Guilgur *et al.*, 2006). A phylogenetic analysis showed three different branches of GnRH variants (White *et al.*, 1995; Fernald & White, 1999). Hypophysiotropic variants are categorized as GnRH-1. Another branch clusters all GnRH forms consistently expressed in the synencephalon/mesencephalon of vertebrates, from fish to mammals, and they are classified as GnRH-2. A third GnRH branch includes only the fish (salmon) GnRH isoforms, mainly expressed in the rostral forebrain, and are classified as GnRH3. This last branch originated from gene duplication that happened in all teleost fish (Guilgur *et al.*, 2006; Kah *et al.*, 2007; Okubo & Nagahama, 2008).

The involvement of GnRH in regulating the release of the pituitary hormone LH is established throughout teleosts. However, for FSH, except for salmonids, measurements of the hormone peptide have not been possible (Zohar et al., 2010). Nevertheless, the relationship of GnRH and FSH release has been well studied in salmonids (Dickey & Swanson, 1998; Breton *et al.*, 1998; Mananos *et al.*, 1999; Weil *et al*., 1999). GnRH control of GtH varies depending on species and reproductive physiology, with the hormone modulating gene transcription and or hormone

secretion (Yaron *et al.*, 2003). GtHs are heterodimeric glycoproteins that share a common α subunit but unique β subunits. Once released, LH and FSH stimulate the gonads to synthesize gonadal hormones, and both also have different roles depending on gender. In male red seabream (*Pagrus major*), LH and FSH stimulate testicular 11-ketosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) production (Swanson *et al.*, 2003). In females, GtH acts at thecal and granulosa cells and stimulates steroidogenesis. The thecal cell layer then secretes testosterone, that is subsequently transported to the granulosa cell layer to be metabolized to E2 by aromatase. During oogenesis, both granulosa and thecal layers show an increase in GtH receptors (Arcand-Hoy & Benson, 2001).

The constant communication between brain, pituitary and the gonads is very important to control reproductive cycles in all species. The sex steroids produced by the gonads are regulated by the brain but also used as indicators of sexual status. They can affect expression of neurotransmitters, neuropeptides and their corresponding receptors in the brain and pituitary, as well as control sexual behavior and spawning. Regulation is achieved through positive and negative feedback systems, which have been reported for sex steroids on both LH and FSH release in teleost fish (Zohar *et al.* 2010).

Dopamine synthesis and signaling pathway

A critical signaling system in the CNS that regulates the HPG axis is the dopaminergic system. Dopamine (DA) is a neurotransmitter that can exert a wide variety of functions in the vertebrate brain, such as reward-motivated behavior, and motor control (Zohar *et al*., 2010). Dopamine inhibits release of GnRH, LH and FSH in several fish, including goldfish (*Carassius auratus*) and rainbow trout (*O. mykiss*) (Peter & Paulencu, 1980, Chang & Peter, 1983, Vacher *et al.*, 2000,

2002). DA is involved in spermiation and precocious LH release prevention, as well as blockade of puberty (Sloley *et al.*, 1992; Vacher *et al.*, 2000, 2002). A study assessing the relationship between the HPG axis and DA in zebrafish showed that, when sexually regressed female zebrafish were coexposed with a DA DR2 antagonist (domperidone) and a GnRH agonist, an increase in LHβ mRNA transcript levels, gonadosomatic index and ovarian vitellogenesis was observed (Fontaine *et al.* 2013). These results indicate that only removal of the inhibitory effect of DA allows GnRH to exert its stimulatory function, showing that DA can consequently modulate E2 levels in fish.

Dopamine is derived from the amino acid tyrosine, which is converted to L-DOPA by tyrosine hydroxylase (TH) and is considered the rate-limiting step of DA production. L-DOPA is metabolized to DA by the enzyme DOPA decarboxylase (DDC). Dopamine is catabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO), and then to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT), which is excreted (Ashcroft, 1969). Dopamine is stored in synaptic vesicles at the presynaptic neuron and is released by action potentials. Once released in the synaptic cleft, DA can bind to one of its two metabotropic receptor families: dopamine 1-like receptor (DR1) and dopamine 2-like receptor (DR2). D1-like receptors stimulate the G-protein $G_{\alpha s}$ family, which in turn activate adenyl cyclase (AC) increasing cyclic adenosine monophosphate (cAMP) and calcium levels. D2-like receptors couple G-protein $G_{\alpha i/o}$, a family that inhibits AC, decreasing concentrations of cAMP and calcium levels. Once there is an excess of DA in the synaptic cleft, it undergoes reuptake by the dopaminergic pre-synaptic neuron through the DA active transporter (DAT), to be then metabolized by MAO to DOPAC (Beaulieu & Gainetdinov, 2011).

The distribution of DA in the brain of fish has been extensively studied (Nieuwenhuys *et al.*, 1998). Dopaminergic neurons can be categorized into A8–A10 (diencephalon–midbrain), A11–A15 (diencephalon), A16 (olfactory bulb), and A17 (retinal) groups (Smeets & Gonzalez 2000).

These neurons express TH1. TH2-positive dopaminergic neurons are also found in vertebrates, except for mammals (Yamamoto *et al*. 2010). This second TH has been identified in the zebrafish through sequence homology studies. However, recent characterization of this possible homologue suggests that the zebrafish TH2 encodes for a protein with tryptophan, not tyrosine, hydroxylase activity, which is a marker for serotonergic neurons (Ren *et al*., 2013). Teleost dopaminergic neurons in the anteroventral part of the preoptic area regulate the release of LH from the anterior pituitary, similar to mammalian A12 neurons, but with some differences in the pattern of projection (Kah *et al.*, 1984; Busby *et al.*, 2010). In mammals, A12 neurons project to the median eminence, which is not protected by the blood–brain barrier, as previously mentioned. Here, DA is released into portal blood vessels and inhibits LH release from the anterior pituitary (Dailey *et al*. 1978; Leebaw *et al.* 1978). In contrast, teleost DA neurons in the anteroventral part of the preoptic area directly innervate the anterior pituitary (Kah *et al*. 1984).

Estrogen synthesis and signaling pathway

Estrogens are synthesized from cholesterol molecules by several reductases and a number of cytochrome P450 monooxygenase enzymes. Cytochrome p450 monooxygenases are a group of heme-containing enzymes that not only are important for estrogen synthesis but also catalyze the biotransformation of endogenous as well as exogenous chemicals (Klingenberg, 1958; Omiecinski *et al*., 2010). The pathway to synthesize estrogens includes several important intermediary hormones such as aldosterone, cortisol and androgens.

Estrogen synthesis is initiated in the mitochondrial membrane, when cholesterol is transported to the mitochondria inner membrane by the steroidogenic acute regulatory protein (StAR), where the side chain moiety from cholesterol is cleaved, forming pregnenolone (Miller, 2002, 2008).

Pregnenolone can be hydroxylated by cytochrome p450 17a (CYP17a) or converted by 3β-dehydrogenase to form 17-hydroxypregnenolone or progesterone, respectively. Then, 17-hydroxypregnenolone is converted to dehydroepiandrosterone (DHEA) by CYP17a, and further metabolized to androstenedione by an oxidoreductase known as 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone can also undergo hydroxylation by CYP17a into 17-hydroxyprogesterone, which is afterwards converted into androstenedione. Finally, androstenedione can be metabolized to estrone by CYP19 or into testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). Estrone and testosterone are both precursors of E2, with the former hydrolyzed to E2 by 17β -HSD and the latter converted to E2 by CYP19 (Figure 1.2).

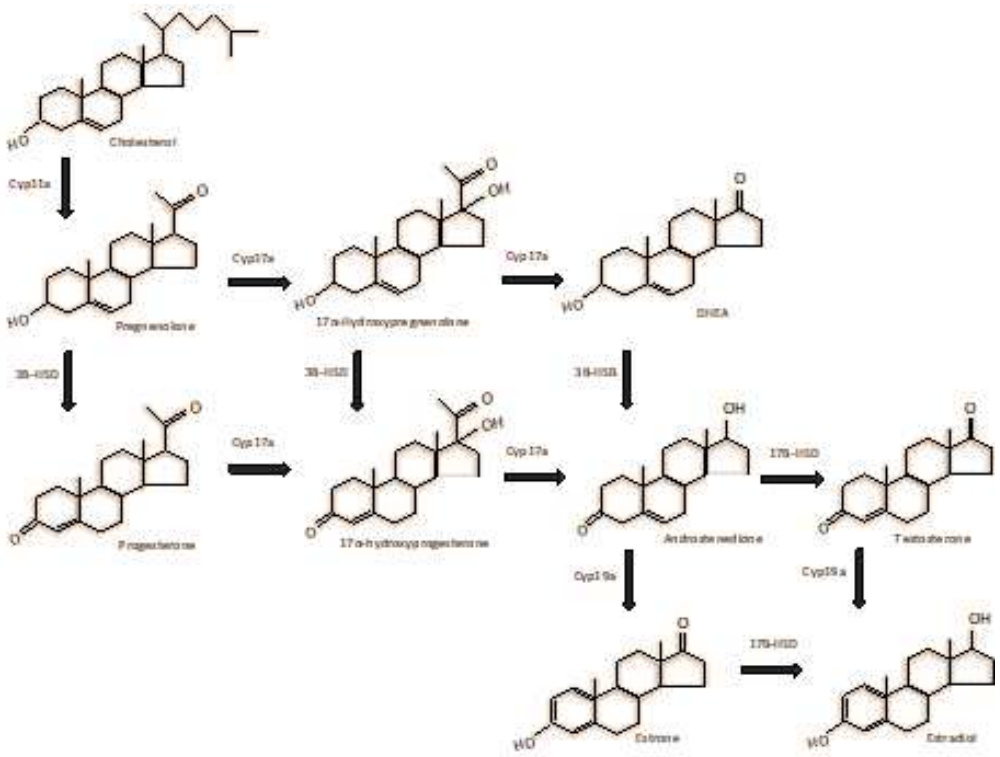

Figure 1.2 - Steroid biosynthetic pathway.

In the gonads, cholesterol is converted to androstenedione and testosterone in either the theca and granulosa cells of the ovaries or in the Leydig cells of the testis. However, conversion of androstenedione to estrone and testosterone to estradiol (E2) occurs only in the granulosa cells (Miller, 2002, 2008 – Figure 1.3). In addition, the teleost fish brain has high activity of aromatase. Teleosts have two separate aromatase genes, *cyp19a1a* and *cyp19a1b*, or aromatase A and B. Aromatase A is exclusively expressed in the gonads, and aromatase B is only expressed in radial glial cells of the brain (Chiang *et al.*, 2001a, b). Therefore, many effects of androgens such as testosterone (T) can be mediated by estrogen receptors (ER) after conversion of T to E2 in brain.

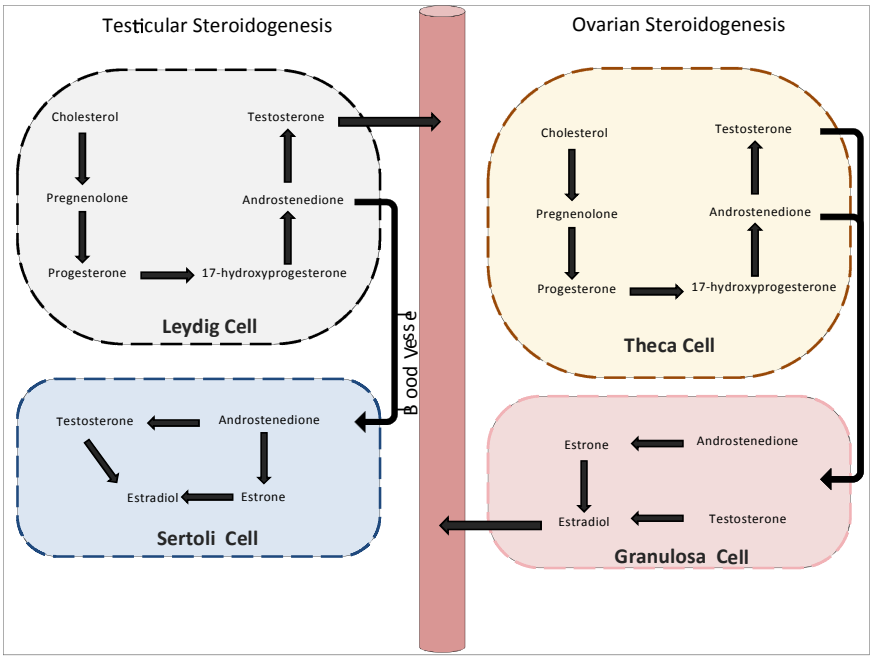

Figure 1.3 - Conversion of cholesterol to sex steroid hormones in the testis and the ovary.

Regulation of StAR alters the production of estrogens through the manipulation of cholesterol transport in the cell (Miller & Strauss, 1999; Miller 2002). Synthesis of E2 and estrone (E3) can also be regulated transcriptionally through the expression of cytochrome P450 enzymes. Recent

evidence has shown that these enzymes can also be epigenetically regulated (Demura and Bulun, 2008; Vanselow *et al.*, 2005).

17β-estradiol is the endogenous agonist with the highest affinity for ERs among all-natural estrogens (E2, E3 and estriol) (Kuiper *et al*., 1997; Heldring *et al*., 2007). The classical genomic pathway of estrogen signaling involves binding of E2 to nuclear ERs. In the absence of a ligand, ERs are inhibited by heat shock proteins. When E2 binds the receptor, it will initiate a conformational change resulting in the release of heat shock proteins. Then, ER dimerizes and migrate to the nucleus. In the nucleus, the dimer binds to estrogen response elements (EREs) in the promoting region of ER-regulated genes. Each element has a specific palindromic DNA sequence (AGGTCAnnnTGACCT). ERs also associate with other transcription factors such as stimulating factor-1 (Sp-1) and activation protein-1 (AP-1) to induce transcription of target genes, which allows E2 to regulate transcription of genes that do not have EREs or have imperfect or half of the ERE sequence in their promoting region (Martin *et al*., 1994; Heldring *et al*., 2007 - Figure 1.4).

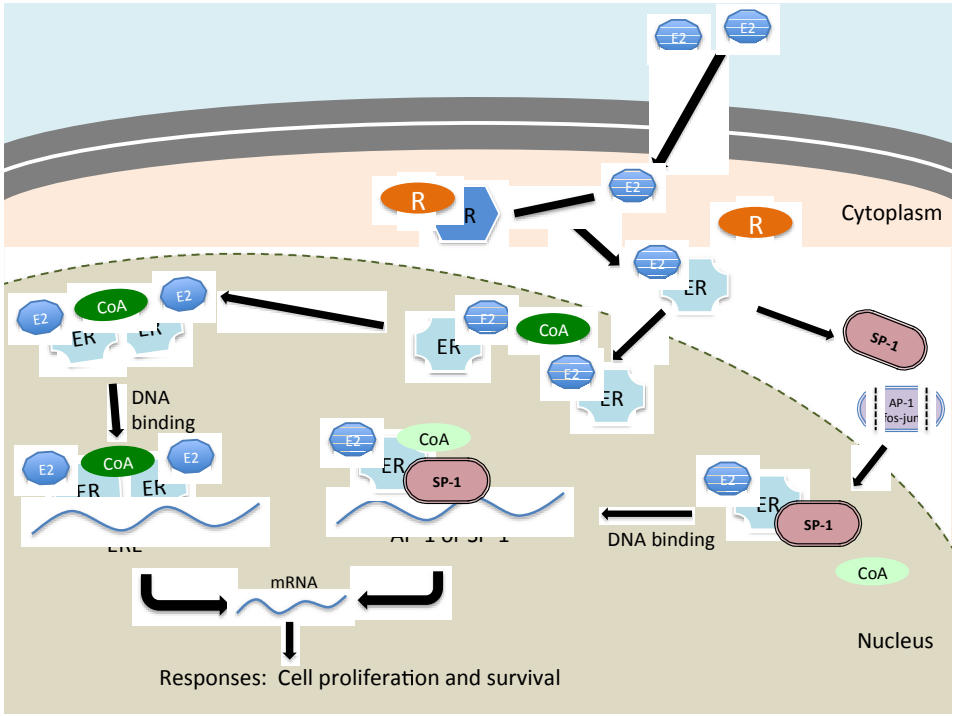

Figure 1.4 - The classical genomic activity of estrogens is mediated through the signaling of nuclear estrogen receptors (ERs). Unbound ER is bound to repressor proteins (R). 17β-estradiol (E2) binding induces formation of ER dimers and causes the movement of the complex into the nucleus. After dimerization, ERs recruits co-activators (CoA) and binds to estrogen response elements (ERE) on DNA. ERs also associate with other transcription factors such as stimulating factor-1 (Sp-1) and activating protein-1 (AP-1) to induce transcription of target genes.

The ER structure has 6 different domains that are labeled A to F. The A/B domain is localized at the N-terminal region and contains a ligand independent transcription activation function site (AF-1). This is the most variable region among species and can be phosphorylated by mitogen activated protein kinase (MAPK) and serine/threonine residues (Kato *et al*., 1995). The C domain comprises the DNA-binding domain, being the most highly conserved region among species (Schwabe *et al*., 1993a, b). The D domain can elicit nuclear localization signals, and studies also suggest this domain can interact with nuclear receptor co-repressors (Aranda & Pascual, 2001). The ligand binding domain is in domain E, where a second transcription activation function site (AF-2) can be found. AF-2 is responsible for activating ligand dependent transcription and is present at the

C-terminal portion of the E domain (Wurtz *et al*., 1998). AF-2 can recruit co-activators such as GRIP1, TIF1 and SRC-1, which in turn can bind to other transcriptional factors such as CREB and p300 proteins, resulting in histone modification and recruitment of the transcription machinery complex (Webb *et al.*, 1998 – Figure 1.5).

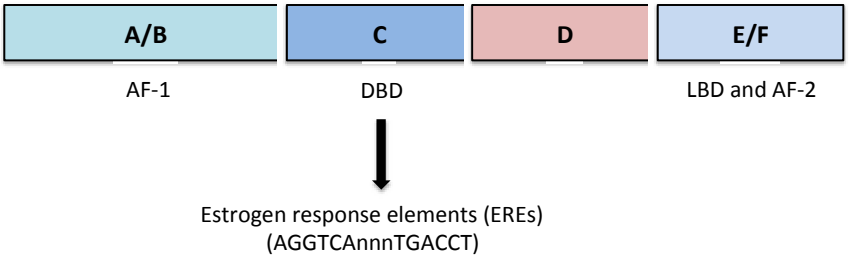

Figure 1.5 - Functional domains of the nuclear estrogen receptors.

The two main forms of nuclear ERs in mammals are ERα and ERβ. However, teleost fish have an additional ERβ subtype, ERβ2, which was first reported by Hawkins *et al.* (2000). ERα is homologous across vertebrates. Fish ERβ2 shares the most identity with mammalian ERβ, while fish ERβ1 likely arose from gene duplication, has several key amino acid changes, and has no mammalian homologue (Hawkins and Thomas, 2004; Hawkins *et al*., 2000).

Estrogen can also exert its effects through a non-classical, non-genomic pathway, via a membrane bound receptor. The most studied membrane receptor and the one identified in fish is the g-protein coupled receptor (GPER), that can be present at the plasma and other organelle membranes, such as the endoplasmic reticulum (Thomas *et al.,* 2005; Funakoshi *et al.* 2006). The signaling pathway activated by GPER leads to alteration in levels of cAMP and calcium (Szego & Davis, 1967; Aronica *et al.* 1994). This response is faster than the classical genomic pathway, leading to rapid responses of the organism to E2. In breast cancer cells, E2 was shown to bind to

GPER activating AC and causing an increase in cAMP levels (Thomas *et al*., 2005). Another study in vascular smooth muscle cells has shown that E2 can promote apoptosis by inhibition of PKA via GPER (Ding *et al*., 2009).

Dopamine control of HPG and Steroidogenesis

DA signaling can also be regulated by estrogenic feedback loop responses. Increased levels of E2 can stimulate DA inhibitory tone in the preoptic area toward final maturation in female rainbow trout (*O. mykiss*) (Linard *et al.*, 1995; Saligaut *et al.*, 1999; Vacher *et al.*, 2002) and male goldfish (*C. auratus*) (Dulka *et al.*, 1992). In catfish (*Heteropneustes fossilis*), E2 might be responsible for regulating the activity of TH (Chaube & Joy, 2002, 2005) and MAO (Senthilkumaran & Joy, 1995). ERs were found to be expressed in the preoptic area of several species of fish, corroborating the hypothesis of direct regulation of DA by E2 (Dufour *et al.*, 2010). Furthermore, Crago & Schlenk (2015) demonstrated a relationship between DA and E2 biosynthesis, by showing a trend towards an increase in plasma E2 and a significant increase in the E2 regulated protein vitellogenin (VTG), as well as decreased DA receptor 2A (DR2A) mRNA expression in rainbow trout (*O. mykiss*) after exposure of 0.15 and 1.5 μg/L bifenthrin for 2 weeks. Figure 1.6 shows a schematic of the HPG axis and its relationship with the dopaminergic pathway.

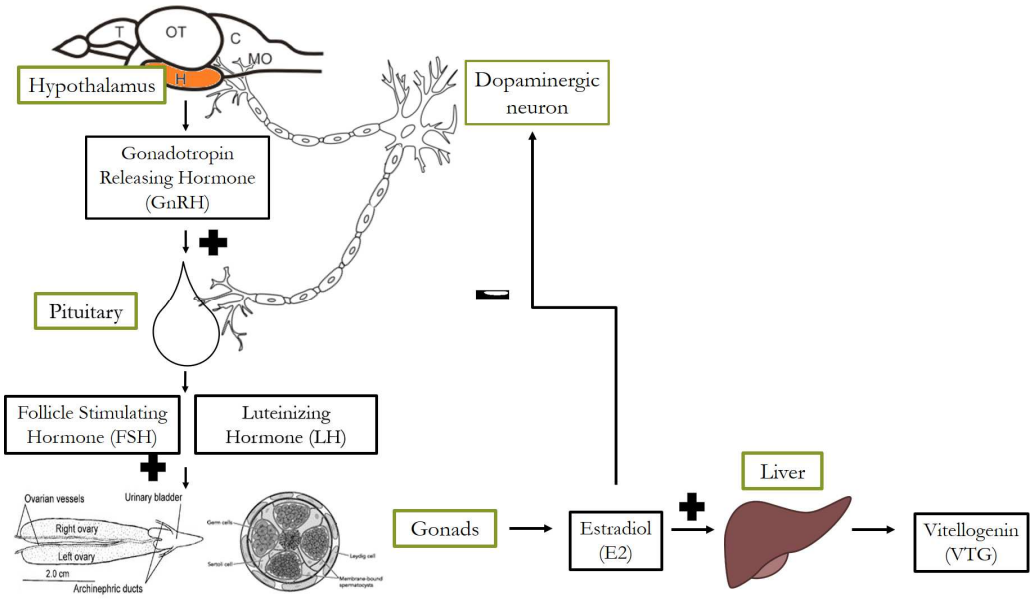

Figure 1.6 – The hypothalamus-pituitary-gonadal (HPG) axis in teleost fish, showing the negative feedback trigger by increase in E2 levels activating the dopaminergic pathway

Gonadal Development

Sexual determination and differentiation

In most fish species, sex determination and differentiation are similar to that of mammals: sex is determined by sex chromosomes after fertilization and differentiated by the presence or absence of a "testis-determining factor" (Jobling, 1995). Germ cell development and positioning are an important marker of sex determination, and in most teleost fish development occurs outside the gonadal region, and then migrates to the gonads. This process varies among species and gender, with gonadal differentiation usually occurring first in females (Satoh and Egami, 1972). However, in contrast to mammals, fish gonads frequently retain the ability to change due to hormone levels, independent of chromosomal gender. For example, immature teleost gonads can be stimulated to differentiate into testis or ovary, turning fish into sequential hermaphrodites (Francis, 1992).

In Japanese medaka (*Oryzias latipes*) sexual differentiation occurs at hatching, where female germ cells are in meiotic prophase in the first 24 hours of hatching, but male germ cells stop proliferating after hatch (Satoh and Egami, 1972). In the male medaka, sexual differences were only identifiable 10 days post fertilization, but the actual testis tubular structure was only visible when fish were 15-20mm, which corresponds to the period when meiosis should happen (Kanamori *et al.* 1985).

Female and male medaka are easily distinguishable by several secondary sex characteristics, including the shape and size of the anal and dorsal fin. Females have a triangular-shaped anal fin and connected dorsal fin rays, while males have a parallelogram-shaped anal fin and a separation on the dorsal fin (Kinoshita, *et al*., 2009). Medaka have an XX-XY sex determination system like mammals, and these chromosomes are morphologically identical. Medaka's sex determining gene is also known. It is called *DMY* (or *dmrt1bY*, that stands for doublesex and mab-3 related transcription factor 1, subtype b, related to the Y chromosome) and has its evolutionary origins in the dmrt1 gene. This gene encodes a putative transcription factor with an intertwined zinc-finger DNA binding domain (Schartl, 2004).

Zebrafish do not have specific sex chromosomes identified. Both XY-like chromosome and heterogametic females were proposed for zebrafish (Uchida *et al.*, 2002; Devlin & Nagahama, 2002). Gonads of all zebrafish larvae start to differentiate into an immature gonad at 10-12 days post hatching. Gonads begin to transform into testis 23-25 days, and the process is complete 40 days of age (Takahashi, 1977).

Testicular Development and Spermatogenesis

The main sperm duct (vas deferens) develops from the elongated paired tubule system attached to the dorsal wall of the testis. The vas deferens leads to an opening called urogenital papilla. This lobular testicular structure is the most commonly found in teleost fish. The lobules are separated by connective tissue, containing primary spermatogonia that undergoes mitosis to form cysts. Sertori cells are the forming unit of cysts, also holding the spermatogonial cells that undergo maturation, a process called spermatogenesis. This process involves the development of primary spermatocytes, which undergo subsequent divisions all the way to spermatozoa, and in turn will undergo spermiogenesis, when nuclear and cytoplasmic organization, as well as the development of a flagellum, occurs. When the entire process of spermatogenesis is complete, the cyst ruptures and the mature sperm enters the testicular lumen to the connecting sperm duct (Nagahama, 1983).

Ovarian Development, Oogenesis and Maturation

The primary function of the teleost ovary is to hold and support developing oocytes, with usually two paired cavities. Fish have several reproductive strategies depending on species, and oogenesis is correspondingly different. There are three categories of reproductive strategies and oocyte development: synchronous, group synchronous, and asynchronous. Synchronous ovaries possess all oocytes in the same stage of development. These fish usually spawn only one time a year, as part of their migration cycle. Asynchronous ovaries are found in fish with oocytes in all developmental stages. Examples would be the zebrafish (*D. rerio*) and medaka (*O. latipes*). They are capable of spawning daily for most months of the year. Group synchronous ovaries have

oocytes grouped in two different stages of development. This represents fish with short annual breeding cycles (Arcand-Hoy & Benson, 2001).

Oogenesis is a process similar to spermatogenesis, where oocytes undergo a sequence of mitotic divisions. A period of growth happens at the first meiotic prophase, when the oocytes arrest (Nagahama, 1983). There is a classification for stages of oogenesis, developed for rainbow trout (*O. mykiss*) by Nagahama (1983). These criteria are based in oocyte size, nuclear and nucleolar appearance, and type and location of cytoplasmic inclusion. Ovarian follicles are formed of two cell layers, thecal and granulosa. As oocyte growth continues, the nucleus increases in size and is surrounded by nucleoli, until a yolk nucleus can be seen. These nuclei contain several cell organelles. This process is followed by the vitellogenic period, which is considered the longer growth term and leads to increase in oocyte size and yolk accumulation (Arcand-Hoy & Benson, 2001).

The next phase of oocyte development is oocyte maturation, consisting of hormonally controlled meiotic divisions. Initially, meiosis starts with the breakdown of the germinal vesicle, chromosome condensation, formation of meiotic spindle and extrusion of first polar body. Meiosis is arrested at the second metaphase, just before ovulation, and these oocytes are ready to be fertilized. The second polar body will appear at fertilization (Nagahama *et al.*, 1994)

Pyrethroids

Pyrethroids are a class of synthetic insecticides structurally similar to the natural insecticide produced by the plant *Chrysanthemum cinerariaefolium* (reviewed in Laskowski, 2002), which is the same genus as common daisies (*Chrysanthemum*) (Sanders & Taff, 1954). Pyrethroids can be used in agriculture, horticulture, forestry, public health and are part of many household products

for insect control (Feo *et al*., 2010). The first synthetic pyrethroids to be developed were allethrin and bioallethrin in 1949, by Milton S. Schechter and colleagues (Sanders & Taff, 1954). In a search for more stable molecules to be used in agriculture, Michael Elliott synthesized permethrin in 1972 and this was the first pyrethroid to be used in field crops (Elliott, 1976).

The use of pyrethroids have other advantages when compared to other older pesticides. Pyrethroids are 20 times more effective in killing insects than dichlorodiphenyltrichloroethane (DDT) (USEPA, 2017). Targeting a wide variety of insects, farmers can apply less insecticide for their crops and still have a satisfactory result (Elliott, 1976). Furthermore, pyrethroids have lower acute toxicity to mammalian species than organophosphates, because mammals have carboxylesterases that can quickly degrade pyrethroids and lower toxicity (Weston *et al,* 2005).

Due to bans and/or limitations on the application of cholinesterase-inhibiting insecticides, such as organophosphates and carbamates (Luo & Zhang, 2011; Feo *et al*., 2010), pyrethroid usage has increased in recent years. Because of lower toxicity, the World Health Organization (WHO) recommends pyrethroids for indoor and bed net spraying against malaria vectors (Walker, 2000; WHOPES, 2005). According to the United States Environmental Protection Agency (USEPA), 1,867,000 lbs. of pyrethroids were used in urban settings in 2011. Around 600,000 lbs. of the total use (32%) was bifenthrin (Myers, 2016)

Environmental Fate and Transport

Abiotic Fate and Transport

Pyrethroids are esters with a hydrophobic acid moiety such as a 3-phenoxybenzyl group substituting a chrysanthemic acid. Other structural features include a less hydrophobic alcohol

moiety, and hydrophobic fluorinated benzyl groups. These features result in a n-octanol/water partition coefficient (log K_{ow}) greater than 6 (reviewed in Laskowski, 2002).

Many factors can affect pyrethroid dissipation and half-life in the field. In a water-sediment system, synthetic pyrethroids are expected to partition into the surface layers of sediment. The depth of the chemical's distribution in the sediment will be dependent on the colloidal matter in pore water, which controls diffusion of hydrophobic compounds (Katagi, 2006). In aquatic environments, most synthetic pyrethroids will be hydrolyzed in alkaline conditions to form acids and alcohols, obeying first order kinetics. Pyrethroids are generally stable at pH 4-7 (Katagi, 2002).

Photolysis can play a role in the degradation of synthetic pyrethroids in the environment, due to the absorbance of ultraviolet (UV) radiation at 300nm. Sunlight hits the ground at a range of 290-400nm of irradiance, exposing pyrethroids to various reactive oxygen species (ROS) and natural sunlight, forming many degradants as a consequence (Katagi, 2004). There can be two types of photochemical reactions occurring with synthetic pyrethroids. Direct photolysis happens by direct absorption of light energy, causing mainly homolityc cleavage to form intermediate radials, and photo-induced ester hydrolysis. Indirect photolysis is defined as reactions of a ground-state molecule with the other excited molecule or photochemically produced reactive species, proceeding via reaction with ROS such as hydroxyl radical ($OH^{\bullet}$), ozone (O_3), and singlet oxygen ($^1O_2$) (Katagi, 2004).

Most pyrethroids used in agriculture have low vapor pressures, around 10^{-8} mmHg at an ambient temperature. Pyrethroids have relatively low Henry's law constants, ($1x10^{-3}$-$1x10^{-7}$ atm m^3 mol^{-1}), which indicates a low tendency for volatilization. Therefore, pyrethroid distribution to the air can be considered limited.

Biotic Fate and Transport

Pyrethroids are degraded via oxidative and hydrolytic cleavage to alcohol and acid functional groups through cleavage of the ester bonds (Glickman *et al.*, 1979; Soderlund & Cassisa *et al.*, 1977; Scollon *et al.*¸ 2009; Demoute, 1989). Furthermore, the type of soil may also affect the metabolism rate of pyrethroids. Texture, bacterial species, organic matter content, presence of oxygen (aerobic or anaerobic conditions), may alter degradation. Mixtures of pyrethroids have first order half-lives varying from 2.9 to >200 days in aerobic conditions and 20 to 200 days in anaerobic conditions. Bacteria living in the water column may not be able to degrade pyrethroids, since the insecticide partitions into the sediments so rapidly (Lee *et al.*, 2004). Additionally, dissolved organic matter concentration can also affect bacterial degradation. A study performed by Yang *et al.* (2007) showed that permethrin toxicity was less affected by dissolved organic carbon (DOM) than cyfluthrin. Therefore, hydrophobicity cannot exclusively explain bioavailability of pyrethroids.

Synthetic pyrethroids can consist of one to three chiral centers, containing between two to eight enantiomers and possibly an assortment of *cis* and *trans* varieties (Tsuji *et al.*, 2011). Each isomer can have different environmental fates due to physicochemical characteristics, which also leads to alterations in toxicity and metabolic processing (Liu *et al.*, 2005). For example, bacteria showed selective degradation of 1S *cis* over 1R *cis* bifenthrin, the latter being shown to be the most toxic of the isomers (Lin *et al.*, 2006).

Bioconcentration and Biotransformation

Bioconcentration of pyrethroids are likely, due to relative high hydrophobicity. However, reduced bioavailability of pyrethroids may also occur due to association with DOM and/or adsorption to suspended and bottom sediments (Katagi, 2010). Studies have shown that pyrethroids can bioconcentrate in fish, with bioconcentration factors (BCF) of several hundred to several thousand. However, metabolism limits the amount of bioaccumulation, and depuration can be rapid (reviewed in Laskowski, 2002).

In mammals, pyrethroids are primarily biotransformed in the plasma by hydrolytic ester cleavage. However, in fish, oxidative biotransformation in the liver is greater than esterase activity (Bradbury & Coats, 1989; Glickman & Lech, 1981; Crow *et al.*, 2007; Godin *et al.*, 2007). Cypermethrin can predominantly be metabolized by ester cleavage, while trans-permethrin can undergo two different pathways: oxidative or hydrolytic processes (Scollon *et al.*¸ 2009). Pyrethroids are classified as type I and type II based on structure and biological activity (see "Mode of Action" below), thus, type I and II pyrethroids undergo different metabolism processes. Type I pyrethroids are esters of primary or secondary alcohols and do not have a cyano group. *Trans* isomers are metabolized faster than the *cis* isomers. Ester cleavage can occur via esterases as cytochrome P450 enzymes. Type II pyrethroids are esters of a secondary alcohol containing a cyano group. They can undergo both oxidative and ester cleavage reactions, but the rate of biotransformation is lower than type I compounds. Therefore, type II pyrethroids can be more acutely toxic than type I pyrethroids. The resulting metabolites can conjugate with glucuronide, glycine, taurine or sulfate groups. This increases solubility and therefore excretion (Scollon *et al.*¸ 2009).

Mode of action

Pyrethroids disrupt the voltage-gated sodium channel (VGSC) in neuronal tissues in biota. In mammals, pyrethroids cause persistent opening of the alpha pore forming unit of the channel, which is considered the binding site (Soderlund *et al,* 2002). This results in altered nerve function, which manifests as short or prolonged bursts, leading to repetitive discharge of nerve signals or stimulus-dependent nerve depolarization (Van den Bercken & Vijverberg, 1980).

Verschoyle and Aldridge (1980) intravenously exposed rats to 36 pyrethroids and classified poisoning by pyrethroids in two types. The T (tremor) syndrome consists of restlessness, hyperexcitation, prostration, aggressive sparring, sensitivity to external stimuli, and most importantly body tremors. This syndrome was shown to be mostly related to compounds with non-cyano groups. On the other hand, the CS (choreoathetosis with salivation) syndrome is characterized by symptoms such as hyperactivity, incoordination, convulsions, pawing and burrowing behavior, coarse tremor, writhing, and choreoathetosis with salivation. In contrast, this syndrome was shown to be mostly related to compounds with an α -cyano groups. Lawrence and Casida (1982) confirmed this classification for 29 pyrethroids in mice via intracerebral injection.

The type I/II nomenclature has been used concomitantly with the T/CS nomenclature, so that type I and type II pyrethroids are generally considered to induce T- or CS-syndrome, respectively (Soderlund *et al.*, 2002). However, the relationship between the two syndromes and types have not been confirmed for all pyrethroids.

Studies on the sensitivity of different VGSC isoforms to pyrethroids were initially performed in nerve tissues of rats (Ginsburg & Narahashi, 1993; Song & Narahashi, 1996; Tabarean & Narahashi, 1998). In mammals, there are nine pore-forming sodium channel α-subunit genes, $Na_v1.1–1.9$, and four sodium channel β subunit genes, β 1-4 that can combine to form various

heteromultimeric channels (Goldin, 2001). Heterologously expressed channels in *Xenopus laevis* oocytes were evaluated using voltage-clamp techniques. Rats $Na_v1.2$, which is highly expressed in the central nervous system (CNS), was only slightly modified by pyrethroids (Smith & Soderlund, 1998). In contrast, $Na_v1.8$, a sodium channel mostly present in the peripheral nervous system (PNS), was highly sensitive to pyrethroid modifications (Choi & Soderlund, 2006). Moreover, in the adult rat brain, $Na_v1.6$, was highly sensitive to pyrethroid modification, especially as a use-dependent modifier of this channel, being considered an important target site that is possibly related to the neurotoxicity of pyrethroids (Tan & Soderlund, 2010; Tan & Soderlund, 2011).

Another target that might play a role in pyrethroid toxicity is the voltage-gated calcium channel. Pyrethroids can modulate these channels at concentrations similar to those capable of modifying VGSC (Clark & Symington, 2007). Moreover, several pyrethroid insecticides modulate different types of calcium channels, such as L and T-type channels (Hildebrand *et al.*, 2004; Xiao *et al.*, 2006). A study with 11 commercial pyrethroids evaluated calcium influx and glutamate release in rat brain synaptosomes (Clark & Symington, 2007; Symington *et al.*, 2008). A concentration-dependent response curve was developed, and results were used in a cluster analysis. Type I pyrethroids did not elicit calcium influx nor glutamate release. In contrast, type II pyrethroids were more potent enhancers of calcium influx and glutamate release under depolarizing conditions which may contribute to differences in neurotoxicity between type I and type II compounds. In primary cultures of mouse neocortical neuron cells, calcium influx was measured using a calcium indicator dye fluo-3 after exposure to 11 different types of pyrethroids (Cao *et al.*, 2010). Nine pyrethroids increased intracellular calcium concentration. Calcium influx was blocked by tetrodotoxin (TTX) under resting conditions suggesting that these pyrethroids stimulated calcium influx after their effects on VGSC under resting conditions, contrasting the above-mentioned results under depolarizing conditions (Cao *et al.*, 2010). Cao *et al* (2014) performed a more recent study

evaluating pyrethroids (more specifically bifenthrin) and calcium influx. Primary mouse cortical neurons were exposed to nanomolar concentrations of bifenthrin, altering synchronous calcium oscillations by amplifying glutamatergic glutamate receptor 5 activity, further altering cAMP response element–binding protein (CREB) signaling independent of detectable perturbation in the VGSC. This study shows a novel effect of pyrethroids in calcium signaling.

Acute Toxicity

The acute oral toxicity of pyrethroids in rat is shown in Table 1.1. The dose to cause 50% mortality (LD_{50}) of most compounds was more than 50mg/kg, indicating pyrethroids are considered moderately toxic. Acute inhalation toxicity is also considered moderate, and acute dermal toxicity is considered very low. Toxicity of compounds containing the α-cyano group (type II) is generally higher than compounds without the moiety (type I).

Table 1.1 – Pyrethroids acute oral toxicity, structure class, and type of clinical sign in rats. Retrieved from Tsuji *et al.*, 2011. [a]Soderlund *et al.*, 2002; [b]McGregor, 2002; [c]EPA, 2009; [d]EPA, 2009; [e]EPA, 1996; [f]Matsuo *et al.*, 2005; [g]IPCS, 1990; [h]IPCS 1990; [i]WHO, 2002; [j]IPCS, 1990; [k]Verschoyle & Aldridge, 1980; [l]Lawrence & Casida, 1982; [m]Weiner *et al.,* 2009; [n]Tsuji, 2011.

Compound	Cyano group in structure	Acute oral toxicity LD_{50} (mg/kg)		Types of clinical signs	Comparative FOB study with low dose	
		Male	Female		LOAEL (mg/kg)	Type of clinical sign
Cypermethrin	+	297[a]	372[a]	CS[k,l]	65[m]	CS
Cyfluthrin	+	155*[a]	160*[a]	–	12.5[m]	CS
λ-Cyhalothrin	+	79[a]	56[a]	–	10[m]	CS
Deltamethrin	+	95[a]	87[a]	CS[k,l]	12.5[m]	CS
Esfenvalerate	+	90[b]	90[b]	CS[k,l]	15[m]	Mixed
Cyphenothrin	+	318[c]	419[c]	CS/T[k], CS[l]	60[n]	Mixed
Fenpropathrin	+	71[a]	67[a]	T[k], CS/T[l]	15[m]	Mixed
D-Allethrin	–	2,150**[d]	900**[d]	–	200[n]	T
S-Bioallethrin	–	370[a]	320[a]	T[l]	150[m]	T
Bifenthrin	–	70[a]	54[a]	–	40[m]	T
Imiprothrin	–	1,800**[e]	900**[e]	–	900[n]	T
Metofluthrin	–	>2,000**[f]	2,000**[f]	–	57[n]	T
Permethrin	–	430[g]	470[g]	T[k,l]	200[m]	T
D-Phenothrin	–	>10,000**[h]	>10,000**[h]	T[l]	>5,000[n]	ND
Prallethrin	–	640[i]	460[i]	–	150[n]	T
Pyrethrin	–	710[a]	320[a]	T[l]	400[m]	T
Resmethrin	–	1,695[a]	1,640[a]	T[k,l]	350[m]	T
Tefluthrin	–	22[a]	35[a]	–	10[m]	T
Tetramethrin	–	>5,000[j]	>5,000[j]	T[l]	>5,000[n]	ND

Pyrethroids are known to have low oral acute toxicity to mammals when compared to other organisms such as insects. There are three reasons hypothesized to explain this phenomenon. First, differences in body temperature between insects and mammals make insects' nervous system more susceptible to pyrethroid intoxication. Second, differences in body size allow mammals to metabolize the compound before it reaches the CNS. And lastly, insects metabolize pyrethroids more slowly than mammals (Narahashi 1992).

Several studies have showed developmental toxicity of pyrethroids, indicating age-related differences in their toxicity (Cantalamessa, 1993; Sheets, 2000), mostly due to immature metabolite activity. When it comes to carcinogenic effects, pyrethroids were shown to cause tumors in rodent models. These results, however, do not seem to be specific of a certain pyrethroid. Therefore, it

appears that pyrethroids as a class do not have a common target, instead being a response specific to each compound within the class of pyrethroid insecticides and to each species (Gold *et al.*, 2001).

Pyrethroids are known to be highly toxic to fish and groups of aquatic invertebrates, such as arthropods. Algae and mollusks are not as sensitive to pyrethroids as other aquatic organisms. Furthermore, toxicity values tend to be at or higher than the average pyrethroid solubility, which ranges from 0.01 to 2 µg/L (Lakowski, 2002). Table 1.2 shows acute toxicity values of pyrethroids to various aquatic species.

Table 1.2. Ranges of acute LC_{50} (µg/L) of synthetic pyrethroids to fish, various groups of aquatic invertebrates, and algae (Giddings, 2006. Compilation and evaluation of toxicity data or synthetic pyrethroids. Unplubished report of Compliance Services International, Rochester)

Pyrethroid	Range of fish LC_{50} values	Range of crustacean $E(L)C_{50}$ values	Range of insect $E(L)C_{50}$ values	Range of mollusk $E(L)C_{50}$ values[a]	Range of algae EC_{50} values[a]
Bifenthrin	0.1–17.8	0.00397–5.7	0.39–9.1	285	–
Cyfluthrin	0.0247–4.05	0.00246–0.344	3.4	3.42–>100,000	>991
Cypermethrin	0.4–6.3	0.0036–1.37	0.0069–9.8	>5 to >2,270	>1,300
Deltamethrin	0.048–5.13	0.0016–0.44	0.02–0.71	8.2–445	>9,100
Esfenvalerate	0.172–5	0.008–53	0.13–80	>12.5 to >10,000	>1,000
λ-Cyhalothrin	0.078–2.3	0.0023–3.3	0.0028–0.13	>590	>1,000
Permethrin	1.5–246	0.018–2.29	0.027–45	14.9–1,740	12.5–1,600

[a]These values are well above water solubilities

Bifenthrin

One of the most widely used pyrethroids is bifenthrin. Bifenthrin is a type I synthetic pyrethroid insecticide widely used in urban and agricultural applications (Spurlock & Lee, 2008).

Bifenthrin has a log K_{ow} of 6 (Fecko, 1999), and is stable to abiotic hydrolysis at pH 5, 7, and 9, at 25°C over a 30-day period (Fecko, 1999). With a carboxylate ester linkage, it should be prone

to hydrolysis. However, the aqueous photolytic half-life is 276 days for C-14-cyclopropyl and 416 days for C-14-phenyl labeled bifenthrin, indicating photo-stability (EFED, 1999).

Bifenthrin K_{oc} values are considered high and can range from 1.31 - 3.02×10^5. The photodegradation half-life on soil exposed to natural sunlight was 106 days for C-14 phenyl labeled bifenthrin and 147 days for C-14 cyclopropyl labeled bifenthrin (Fecko, 1999). Aerobic soil half-lives for C-14 phenyl labeled bifenthrin ranged from 97 to 156 days depending on the type of soil utilized in the tests. C-14 Cyclopropyl-labeled bifenthrin showed aerobic half-lives of 129 to 250 days, again depending on soil type (Fecko, 1999). In flooded soil tests, bifenthrin was shown to be stable to anaerobic soil metabolism (Fecko, 1999). 4'-Hydroxy bifenthrin (primary degradate) accounted for 4.5% of the total application after 30 days under anaerobic conditions (Fecko, 1999). Field dissipation half-life tests have been conducted for bifenthrin in a wide range of soils and conditions. Half-lives ranged from 122 to 345 days (Fecko, 1999). Figure 1.7 shows bifenthrin degradation pathways in soil, water and biota.

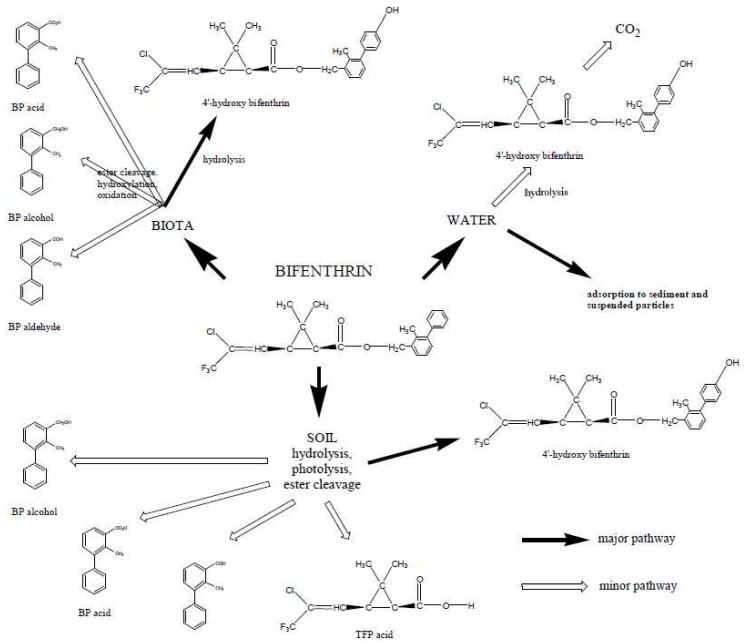

Figure 1.7 – Bifenthrin degradation pathways in soil, water and biota (Fecko, 1999).

Bifenthrin undergoes rapid metabolism and excretion in mammals (USEPA, 1987). Rats treated with 4-5 mg/kg of bifenthrin excreted 70% of the chemical in the urine and 20% in the feces within 7 days. The remaining bifenthrin was found in fat tissues, such as skin and ovaries (USEPA, 1987). Bifenthrin was found to increase the number of tumors in the urinary bladder, lung, liver and caused leukemia (USEPA, 2008; EFSA, 2008). Maternally toxic dose level of bifenthrin did not produce adverse effects on embryonic development in rats (McCarty *et al.*, 2002).

Bifenthrin can be moderately toxic to birds, with a dietary (8 days) LD_{50} of 1,280 ppm for the Mallard duck (*Anas platyrhynchos*) and 1,800 ppm for the Bobwhite quail (*Colinus virginianus*) (Fecko, 1999). However, as most pyrethroid insecticides, bifenthrin can be very toxic to aquatic organisms. The LC_{50} after 96 hours of exposure for the rainbow trout (*Oncorhynchus mykiss*), the bluegill sunfish (*Lepomis macrochirus*) and the *Daphnia magna* is 0.00015, 0.00035 and 0.0016 ppm, respectively (Fecko, 1999).

Bifenthrin as an Endocrine Disrupting Compound

Recent *in vitro* and *in vivo* studies have shown that pyrethroids (including bifenthrin) can disrupt endocrine and/or steroidogenic pathways by mimicking, antagonizing or disrupting the synthesis of endogenous hormones. *In vitro* studies have shown that bifenthrin can have estrogenic and anti-estrogenic effects. Studies by Zhao *et al.* (2010) and Wang *et al.* (2007) showed that bifenthrin increases MCF-7 cells proliferation in the E-SCREEN assay. Furthermore, bifenthrin also increases the mRNA level of ps2, a transcription factor responsive to E2, and that effect can be blocked by ICI 182,780, an ER antagonist (Wang *et al.*, 2007). Bifenthrin can also act as an antiestrogenic compound in the ER CALUX cell line (Brander *et al.* 2012).

Beggel *et al.* (2011) observed reduced levels of VTG and cytochrome p450 3a (CYP3a), an E2-regulated yolk sac protein and a P450 enzyme involved in steroid and pyrethroid metabolism, respectively, in fathead minnow (*Pimephales promelas*) after treatment to 0.7 µg/L of bifenthrin. Furthermore, even lower bifenthrin concentrations of 1 to 100 ng/L can cause altered levels of choriogenin, an estrogen-regulated egg coat protein, as seen in Brander *et al.* (2012). Bifenthrin exposure also affects apical end points in fishes, such as lower gonadosomatic index and increased ovarian follicle size, parallel with increases in plasma E2 levels, when compared to control (Forsgren *et al.*, 2013).

Bifenthrin enantiomers may also play a role in its mode of action. The *1S cis* bifenthrin enantiomer was shown to have greater estrogenicity than *1R cis* in MCF-7 cells and to disrupt LH-induced ovulatory genes in rat ovarian granulosa cells, resulting in a reduction of prostaglandin E2 (Zhao *et al.*, 2007). Zhao *et al.* (2007) also performed docking studies with both bifenthrin enantiomers, demonstrating that *1S cis* bifenthrin can form a hydrogen bond with residue Thr347 in the ERα LBD, whereas *1R cis* bifenthrin cannot. At the ERβ LBD, *1S cis* bifenthrin formed three hydrogen bonds, while *1R cis* bifenthrin formed two. Binding scores for both receptors were higher for *1S cis* bifenthrin.

One common characteristic among studies with bifenthrin, especially in fish, is the biphasic nature of the response elicited by this compound. In the above-mentioned study by Brander et. al. (2012), whole body homogenates of *Menidia beryllina* had higher levels of choriogenin once exposed to the lowest concentration of bifenthrin used, 1 ng/L, in comparison to the other concentrations used. Interestingly, concentrations of choriogenin in fish exposed to 1 ng/L bifenthrin were also higher than the positive control (1ng/L of ethinyl estradiol). Since the best-fit dose response curve developed for bifenthrin was considered biphasic, it was suggested bifenthrin might be causing its effects via different mechanisms depending on its concentration. Another study

by Brander *et al.* (2016b) demonstrated this biphasic response, where 0.5 ng/L bifenthrin inhibited expression of estrogen-responsive genes such as GPER and ERα but higher concentrations (5, 50 ng/L) induced expression of the same genes. In addition, Beggel *et al.* (2011) observed a biphasic response at lower bifenthrin concentrations of 75 to 150 ng/ml, but the response was linear at concentrations higher than 150 ng/ml, in fathead minnow (*P. promelas*). These responses are similar to what is observed with hormones and other EDCs and can be considered a hormetic response. Hormesis is defined by Calabrese and Baldwin (2002) as "an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis.". One possible explanation for this effect is response activation at lower pg to ng/ml concentrations but saturation of the target receptor or enzyme at higher concentrations, eliciting a more general toxic response at higher concentrations (Brander *et al.*, 2016a).

Another concern that bifenthrin raises as an EDC is the evidence that its main metabolite, 4-hydroxy-bifenthrin, might cause even greater endocrine disrupting effects than its parent compound. Studies that used *in vitro* assays to test bifenthrin estrogenicity at environmentally relevant concentrations failed to show significant results (Brander *et al.*, 2012; DeGroot & Brander, 2014; Forsgren *et al.* 2013). However, DeGroot & Brander (2014) also exposed *M. beryllina* to 10 ng/l of bifenthrin, 10 ng/l of 4-hydroxy bifenthrin, and 10 ng/l bifenthrin with 25 µg/l piperonyl butoxide (PBO) – a cytochrome P450 inhibitor. Fish exposed to 4-hydroxy-bifenthrin had higher levels of choriogenin, showing that this bifenthrin metabolite can be more estrogenic than the parent compound.

Bifenthrin was also shown to possibly affect steroidogenesis. Crago & Schlenk (2015) exposed rainbow trout (*O. mykiss*) to 1.5 µg/l bifenthrin for 96 h and 2 weeks, resulting in decreased DR2A

associated with an increase in E2 levels and significant increase in VTG mRNA. Increased TH expression was also observed, indicating over production of DA as a result of bifenthrin exposure. Lastly, GnRH transcript levels were increased after 96 h exposure, but decreased after 2 weeks, consistent with a feedback loop activation of E2. In contrast, in rat granulosa cells, bifenthrin downregulated genes related to steroidogenesis such as StAR, P450scc, progesterone receptor (PR), and CYP19a1 (Liu *et al.*, 2011). Bifenthrin was also shown to modulate the expression of other genes related to steroidogenesis such as GPER, ERα, CYP17 and thyroid receptor alpha (TRα) in MCF-7 cells and *M. beryllina* (Brander *et al.*, 2016b). In the same study, *M. beryllina* exposed to the same concentration of bifenthrin had significantly reduced fertilized eggs output. These findings demonstrated the variety of effects bifenthrin can have on the estrogen signaling pathway.

Hypothesis

Based on previous studies that have shown bifenthrin effects on E2 levels and the dopaminergic pathway, we hypothesize that bifenthrin affects dopaminergic and E2 signaling pathways, causing *in vivo* estrogenic activity in fish. To assess that hypothesis, we have three specific aims. Because bifenthrin has significantly different effects on different developmental periods, we will: 1) examine the effects of bifenthrin in juveniles and embryonic stages of zebrafish, 2) examine the role of ER in bifenthrin's endocrine disruption effects in embryos of zebrafish, and 3) determine whether embryonic exposure can have adverse apical effects on sex reversal. To assess these specific aims we propose to: 1) measure transcripts of the HPG axis and DA pathway as well as DA and E2 levels in juveniles and embryos exposed to environmentally relevant concentrations of bifenthrin, 2) co-expose bifenthrin with E2 receptor agonists, as well as knockdown ERα, to characterize potential effects through this receptor pathway, and 3) evaluate female/male ratios,

secondary sexual characteristics and DMY measurement in the Japanese medaka model after embryonic and larval exposure to bifenthrin.

Our results will construct an adverse outcome pathway for the estrogenic activity of bifenthrin. Novel insights about ER and the DR1 signaling pathways will be assessed and information can be used by molecular and cell biologists in future research to better understand potential reproduction impairment of bifenthrin. Ultimately, results from this research can be extrapolated to population level responses and be used for risk assessments allowing use by government, academia and industrial entities.

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Chapter 2: Effects of Bifenthrin Exposure on the Estrogenic and Dopaminergic Pathways in Zebrafish Embryos and Juveniles

Abstract

Bifenthrin is a pyrethroid insecticide used in urban and agricultural applications. Previous studies have shown that environmentally relevant (ng/L) concentrations of bifenthrin increased plasma concentrations of 17β-estradiol (E2) and altered the expression of dopaminergic pathway components. The dopaminergic neurons can indirectly regulate E2 biosynthesis, suggesting that bifenthrin may disrupt the hypothalamic–pituitary–gonadal (HPG) axis. Because embryos do not have a complete HPG axis, the hypothesis that bifenthrin impairs dopamine regulation was tested in embryonic and 1-mo-old juvenile zebrafish (*Danio rerio*) with exposure to measured concentrations of 0.34 and 3.1μg/L bifenthrin for 96 h. Quantitative reverse transcriptase polymerase chain reaction was used to investigate transcripts of tyrosine hydroxylase (TH), dopamine receptor 1 (DR1) and 2A (DR2A), dopamine active transporter (DAT), estrogen receptor α (ERα), ERβ1, ERβ2, luteinizing hormone β (LHβ), follicle-stimulating hormone β (FSHβ), vitellogenin (VTG), cytochrome P450 cyp19a1a, and cyp19a1b. Levels of E2 were measured by enzyme-linked immunosorbent assay (ELISA). Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations were measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Significant decreases in TH and DR1 transcripts and HVA levels, as well as ratios of HVA/dopamine and HVA+DOPAC/dopamine, in zebrafish embryos were observed after bifenthrin treatment. In juveniles, a significant increase in the expression of ERβ1 and the DOPAC to dopamine ratio was

noted. These results show a possible antiestrogenic effect of bifenthrin in embryos, and estrogenicity in juveniles, indicating life-stage–dependent toxicity in developing fish.

Introduction

Pyrethroids are a class of synthetic insecticides based on the natural insecticide produced by the plant *Chrysanthemum cinerariaefolium* (Laskowski, 2002). Widely used in urban and agricultural applications, pyrethroid use has increased because, relative to organophosphate insecticides, pyrethroids have low acute toxicity to mammalian species and relatively low persistence (Weston *et al.*, 2005; Spurlock & Lee, 2008). Despite high hydrophobicity, which makes pyrethroids prone to bind organic matter of sediments, previous studies have demonstrated the presence of this compound in the water column in concentrations that cause toxic effects in the aquatic environment (Weston *et al.*, 2005) with surface water concentrations varying from 0.005 to 3.79 µg/L (Siepmann & Holm, 2000).

Pyrethroids cause persistent opening of the voltage-gated sodium channel at the alpha pore-forming subunit, which is considered the binding site (Soderlund *et al.*, 2002). Repetitive action potentials from only one depolarization event caused tremors observed during mammalian intoxication (Van den Bercken & Vijverberg, 1980). In addition to acute effects, several studies have shown that pyrethroids such as bifenthrin can act as endocrine-disrupting compounds by mimicking, antagonizing, or disrupting the synthesis of endogenous hormones (reviewed in Brander *et al.* 2016a). Wang *et al.* (2007) showed that bifenthrin has estrogenic activity in the *in vitro* human breast carcinoma MCF-7 cell proliferation assay, and this effect was completely blocked by an estrogen receptor (ER) antagonist (ICI 182,780). In contrast, Brander *et al.* (2012) showed that bifenthrin acted as an antiestrogen following exposure in a CALUX-ER cell system to

concentrations ranging from 1 to 100 ng/L. *In vivo*, bifenthrin induced expression of vitellogenin (VTG) in Japanese medaka (*Oryzias latipes*) (Wang *et al.*, 2007) and the yolk-protein choriogenin in juvenile *Menidia beryllina*. (Brander *et al.* 2012), but also decreased choriogenin, ERα, and reproductive output in adult *M. beryllina* (Brander *et al.* 2016b). Further studies are needed to better understand the mechanism of action of bifenthrin.

Estrogen biosynthesis is regulated through the hypothalamus–pituitary–gonadal (HPG) axis (Zohar *et al.*, 2010). The hypothalamus produces gonadotropin-releasing hormone (GnRH), and its pulsatile release controls biosynthesis of the gonadotropins (GtHs): luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The GtHs are synthesized in the pituitary gland. Furthermore, both LH and FSH will induce egg development, maturation, and release, and they control gonadal hormone synthesis, including 17β-estradiol (E2). Increased levels of E2 will stimulate the liver to express proteins such as VTG and choriogenin. Different feedback responses operate to tune GnRH, LH, and FSH release, including the dopaminergic system. Primarily, dopaminergic neurons will be activated with increased E2 levels, acting on GnRH and GtH neurons (Zohar *et al.*, 2010). Dopamine can block synthesis and release of GnRH (Yu *et al.*, 1991), modulating gonadotropin levels by increasing and decreasing them according to receptor subtype (Chang *et al.*, 1990), which will lead to decreased levels of E2. Furthermore, dopamine can also control E2 levels by regulating brain aromatase, an enzyme that converts androgens to estrogens (Xing *et al.*, 2016). Xing *et al.* (2016) showed that a dopaminergic agonist was able to regulate aromatase B by increasing or decreasing its expression, which is potentially different from the inhibition effect dopamine exerts over GnRH.

Species differences in the HPG axis–dopamine relationship may exist, particularly among synchronous and asynchronous spawners. Synchronous spawners may only breed once in a season or a lifetime, and levels of GtH and gonadal hormones can be well defined depending on oocyte

maturation and spermatogenesis process (Yaron *et al.*, 2003). However, in asynchronous fish such as zebrafish, reproduction cycles are variable (days to weeks), and females can have all stages of oocyte maturation present at the same time (Yaron *et al.*, 2003). This affects the FSH, LH, E2, and dopamine concentration profile, which can be more complex compared with synchronous spawners. A study assessing the relationship between the HPG axis and dopamine in zebrafish was performed by Fontaine *et al.* (2013). That study showed that when sexually regressed female zebrafish were co-exposed with a dopamine DR2 antagonist (domperidone) and a GnRH agonist, there was an increase in LHβ messenger ribonucleic acid (mRNA) transcript levels, gonadosomatic index, and ovarian vitellogenesis. These results indicate that only removal of the inhibitory effect of dopamine allows GnRH to exert its stimulatory function, suggesting that dopamine can consequently modulate E2 levels in zebrafish.

Dopamine is derived from the amino acid tyrosine, which is converted to L-DOPA by the enzyme tyrosine hydroxylase (TH) and is considered the rate-limiting step of dopamine production. Then L-DOPA is metabolized to dopamine by the enzyme DOPA decarboxylase (DDC). Dopamine catabolism occurs through metabolism to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO), and then to homovanillic acid (HVA) by catechol-O-methyltransferase (Ashcroft, 1969). Once released in the synaptic cleft, dopamine can bind to one of its 2 metabotropic receptor families: dopamine receptor 1-like (DR1) and dopamine receptor 2-like (DR2) receptors. The D1-like receptors stimulate the G-protein $G\alpha_s$ family, which in turn activates adenyl cyclase (AC) and production of cyclic adenosine monophosphate (cAMP) and calcium levels. The D2-like receptors couple G-proteinG$\alpha_{i/o}$, a family that inhibits AC, decreasing concentrations of cAMP and calcium levels. Once there is an excess of dopamine in the synaptic cleft, it undergoes reuptake by the dopaminergic presynaptic neuron through the dopamine active transporter (DAT), to be then metabolized by MAO to DOPAC (Beaulieu & Gainetdinov, 2011).

Increases in DAT protein were observed in brain striatal tissues in mice after exposure to another pyrethroid (permethrin) (Gillette & Bloomquist, 2003). Reductions in dopamine receptor 1 (DR1) mRNA, and increased levels of the TH transcript and HVA were observed in larval zebrafish homogenates exposed to deltamethrin (Kung *et al.*, 2015). Furthermore, Crago and Schlenk (2015) demonstrated a relationship between dopamine and E2 biosynthesis, by showing a trend toward an increase in plasma E2 and a significant increase in VTG, as well as decreased DR2A mRNA expression in rainbow trout after exposure of 0.15 and 1.5μg/L bifenthrin for 2wk.

Because there are few studies showing how pyrethroids can affect both dopamine and estrogen signaling pathways in fish, the objective of the present study was to investigate the effect of bifenthrin on both pathways using the zebrafish model. Using a developmental stage that lacks a complete HPG axis (embryo) and comparing this with juveniles that have an intact HPG system, our study assessed the role of HPG in the toxicity of bifenthrin. Because embryonic exposure can be very similar to a cellular *in vitro* assay response, we hypothesized that exposures at the embryonic level would result in an antiestrogenic response. However, based on previous studies on the effects of bifenthrin in juvenile fish (Crago & Schlenk, 2015; Forsgren *et al.*, 2013), we hypothesized that, at the juvenile stage, bifenthrin would have an estrogenic effect.

Materials and Methods

Chemicals

A mix of bifenthrin isomers (98% purity) was purchased from ChemService. Tricaine methanesulfonate (MS-222; 98% purity) was purchased from Sigma-Aldrich. Ethanol (Decon Laboratories) was 200 proof. Astock solution of bifenthrin in 100% ethanol was prepared, and

diluted solutions in aerated freshwater were made at every exposure day. Methanol and acetonitrile, both high-performance liquid chromatography grade, were from Fisher Scientific. Deionized water of 18.2Vwas prepared with an E-Pure model D4641 from the Barnstead/Thermolyne. Formic acid (purity 98%) was obtained from Sigma-Aldrich.

Dopamine hydrochloride (1 mg/mL in methanol), and neat solid standards of DOPAC and HVA were from Sigma-Aldrich. Standards of isotopically labeled compounds (Sigma-Aldrich) used as internal standards were dopamine-1,1,2,2-d4 hydrochloride (DA-d4) and 4-hydroxy-3methoxyphenyl-d3-acetic-d2 acid (HVA-d5; 100µg/mL in methanol). All standards were>98% purity. Stock solutions of DOPAC, HVA, and DA-d4 were prepared at 1 mg/mL in methanol and stored at –20 8C. The concentrations were not corrected for purity or salt content. Solutions of working standards, internal standard, and calibration standards were prepared in 0.1% formic acid in deionized water fresh for each experiment. The internal standard solution contained 5 µg/mL of dopamine-d4 and 10 µg/mL HVA-d5. Spiking solutions, containing 5 µg/mL of dopamine, and 10 µg/mL of DOPAC and HVA (high spike), and 10-fold lower concentrations (low spike) of DA, DOPAC, and HVA were also prepared fresh in 0.1% formic acid in water. All the solutions were kept at –20 8C.

Animal Handling

Adult wild-type (strain 5D) zebrafish were maintained on a 14:10-h light:dark cycle within a 242-tank recirculating system (Aquaneering) containing conditioned reverse osmosis water, and fed ad libitum twice a day with GEMMA Micro 300 diet (Skretting USA). Dissolved oxygen, pH, conductivity, salinity, alkalinity, and temperature within recirculating water were maintained at 4 to 6mg/L, 7.0 to 7.5, 950 to 1000 mS, <1 ppt, 50 to 100mg/L, and 28 8C, respectively; in addition,

levels of ammonia, nitrite, and nitrate within recirculating water were consistently below 0.1, 0.05, and 2mg/L, respectively. Adult females and males were bred directly on-system using in-tank breeding traps suspended within 3-L tanks. For all experiments described in the *Bifenthrin exposures* section below, newly fertilized eggs were staged according to previously described methods (Kimmel *et al.*, 1995) and juveniles were raised in the same recirculating system until time of exposure. All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee protocols at the University of California, Riverside (animal use protocols 20150035 and 20130005).

Bifenthrin Exposures

Embryonic Exposure

Twenty viable embryos (3 h post fertilization [hpf]) were placed in 60- x 15-mm Petri dishes containing 10mL of the working solution. Treatments consisted of vehicle control (0.1% ethanol), 0.15 µg/L bifenthrin, and 1.5 µg/L bifenthrin nominal concentrations based on concentrations observed in the environment (0.15 µg/L) (Weston *et al.*, 2005) and demonstrating endocrine-disrupting effects (Crago & Schlenk, 2015; Forsgren *et al.*, 2013). For quantitative polymerase chain reaction (qPCR) assays, each replicate pool consisted of 20 embryos with 5 to 12 replicate pools/treatment. For the E2 enzyme-linked immunorsorbent assay (ELISA) assay, each replicate pool consisted of 80 embryos (20 embryos/Petri dish) with 6 to 7 replicate pools/treatment. For measurements of dopamine and its metabolites, 200 embryos (20 embryos/Petri dish) were used for each replicate pool, with 3 to 4 replicate pools/treatment group. Measurement of DOPAC and HVA was not possible for one replicate because of poor recovery. Water was sampled before and

after exposure to determine measured values of bifenthrin. Following exposure, fish were euthanized in ice, flash-frozen in liquid nitrogen, and then kept at –80°C until assays were performed.

Juvenile Exposure

Juveniles were transferred to glass beakers at 30 d post fertilization (dpf). Gonadal differentiation and maturation in zebrafish spans from 23 to 40 dpf (Takahashi, 1977), so our exposures occurred during this period. First, juveniles were acclimated for 3 d in 100 mL of freshwater, and then further exposed to vehicle control and 0.15 and 1.5 µg/L bifenthrin nominal concentrations, for 96 h, in 100mL of the working solution. Concentrations and durations of exposure were based on earlier studies in other fish species (Crago & Schlenk, 2015; Forsgren *et al.*, 2013). During the exposure, treatment solution was 50%-exchanged each day, and juveniles were fed GEMMA Micro 300 diet once a day. Five to 8 replicates per treatment were designated for the qPCR assays, and 3 to 6 replicates were designated for the E2 ELISA assay, one juvenile/replicate. For the dopamine and metabolites measurement, 3 juveniles were pooled. This assay resulted in 5 to 6 replicates/treatment group. Measurement of DOPAC and HVA was not possible for 3 replicates because of poor recovery. Water was sampled before and after exposure to determine measured values of bifenthrin. Juveniles were anesthetized in 80 mg/L of MS-222, flash-frozen in liquid nitrogen, and kept at –80°C until assays were performed.

Survival was assessed in embryos and juveniles, and hatch per day in embryos after treatment. No significant difference was identified in hatch per day or survival compared with the control in both zebrafish stages (data not shown). In addition, morphological deformities observed after

exposure to bifenthrin in both developmental stages were not significantly higher than observed in controls (data not shown).

Measurements of Nominal Bifenthrin Concentrations

Measurements of bifenthrin concentration in the working solutions after exposure were performed using liquid–liquid extraction via a modified US Environmental Protection Agency method 3510C. The protocol used was based on previously published work (Jiang *et al.*, 2012). The embryo's solution water was pooled from 10 samples to reach 100 mL for each treatment level. Pooling was not necessary for the juvenile's solution water. Briefly, the 100 mL was placed into a separatory funnel with 10 g of sodium chloride (NaCl) and shaken until the NaCl was completely dissolved. Then 20mL of dichloromethane was added to each funnel. The funnels were shaken for 3 min and allowed to settle for 60 min for complete separation of the water and organic solvent phases. After separation, the dichloromethane portion of the sample was drained through sodium sulfate (Na2SO4) and collected in a round-bottom flask. This process was repeated 3 times. After the third extraction and collection, the dichloromethane was evaporated to approximately 0.5 mL under a gentle vacuum at 30°C on a Büchi RE121 Rotavapor. The final extract was reconstituted in 5.0mL of hexane/acetone (9:1, v/v) and then evaporated under a gentle nitrogen flow at 40°C to near dryness, and the residue was recovered in 1.0mL hexane/acetone (9:1, v/v). The samples were analyzed in an Agilent 6890N Gas Chromatograph equipped with an Agilent 5973 mass selective detector.

RNA Isolation, cDNA Synthesis, and RT qPCR

Expression of genes related to the dopaminergic and estrogenic pathways after exposure to bifenthrin was measured. Total mRNA was isolated from embryos and juveniles using the Lipid Tissue RNeasy kit (Qiagen) following the manufacturer's instructions. The quantity and quality of mRNA were determined using the ND-1000 (NanoDrop). The mRNA (1µg) was converted to cDNA using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Primers were designed using IDT DNA PrimerQuest software, and efficiency was calculated using PCR Miner (Zhao & Fernald, 2005 [Table 2.1]). The exception was the VTG set of primers, which was retrieved from Chen *et al.* (2010). As a housekeeping gene, EF1α was used, because its expression is stable throughout development (McCurley & Callard, 2008). The qPCR was carried out with the iTaq Universal Onestep RT-PCR kit with SYBR Green (Bio-Rad), on a MyiQ5 Thermo cycler (Bio-Rad). The samples were denatured and the polymerase activated at 95°C for 3 min, and then 40 cycles of 10 s at 95°Cand 30 s of 55°C. Samples were subjected to melting curve analysis from 54 to 95°C in 0.5°C increments with continuous fluorescence measurement. The qPCR was analyzed according to Schmittgen and Livak (2008), and fold change was calculated against the vehicle control.

Table 2.1 – Primers and accession numbers used for qRT-PCR

Gene	Accession Number	Forward Primer (5'-3')	Reverse Primer (5'-3')
EF1α	NM_131263.1	ATACATCAAGAAGATCGGCTACAA	CCACAGGTACAGTTCCAATAC
TH1	NM_131149	GCCATACCAAGACCAGACTTAC	GCTTGATGCGGGTCACATA
DR1	NM_001135976	AAGCCCGTTTCGCTATGAG	GCACAGGGATGAAGGAGATAAG
DR2A	NM_183068	TGCGAATCCTGCCTTTGT	TTCCGGAGTACCACGTAGAT
DAT	NM_131755	TCTTCTCCTTCCTGGGATACA	GCAATGGCTTCTGGGTAAATG
CYP19a1a	NM_131154	GGCTGTGGTGGATCTTCTTATC	CAGAGTAGCAGCAGTATT
CYP19a1b	NM_131642	GATGGAGCATGTGGTAAAGGA	GTGCAGCAGAATCAGCATTAAG
ERα	NM_152959	GACTACGCCTCTGGATATCATTAC	TGGTCGCTGGACAAACATAG
ERβ1	NM_174862	CTCTCAGCACCTCTTTCCTTC	CCGCCTCTAGAATACAGCTAAC
ERβ2	NM_180966	GTCCGAGGTCTCAAGAGATAAAG	CTTCCATGATCCGGGAGATTAG
LHβ	NM_205622	CAAGGGATCCCGTTTACAAGAG	AACAGTCGGGCAGGTTAATG
FSHβ	NM_205624	GACTCACCAACATCTCCATCAC	CTCGATCCATTGTCCAGCATAG
VTG	NM_001044897	CTGCAAGAGTGCAACTGATAGTTTC	ACTTGCCAGTGACTTTGTGCTT

ELISA

Homogenates of whole-body embryos and juveniles were used for the measurement of E2 after exposure to bifenthrin in a competitive enzyme immunoassay (EIA) kit for E2 (Cayman Chemical). All assay solutions and buffer were prepared according to kit instructions with deionized water. Absorbance readings were performed on a spectrophotometer (Spectra Max Plus 384, Molecular Devices) at 412 nm. Concentration values were calculated by comparing absorbance of samples with a standard curve, using the analysis tool provided by the kit's website. Samples were analyzed

in duplicate. Embryonic E2 concentrations were normalized against number of embryos, while juvenile concentrations were normalized against individual wet body weight.

Liquid Chromatography – Mass Spectrometry

To measure dopamine and its metabolites, samples were kept on ice during handling and extraction. Sample preparation was based on Tareke *et al.* (2007) with slight modifications. The samples were homogenized using a probe blender prior to extraction. Approximately 100mg of homogenized zebrafish tissues (embryos or juveniles, whole body) were placed into a 2-mL centrifuge tube, and internal standard was added to yield 1 ng DA-d4 and 2 ng of HVA-d5 per 1mg of tissue. Ice-cold 0.1% formic acid in water (0.2 mL) was added, and the extraction was performed using a probe blender (Omni plastic homogenizer probe, Omni International) by vigorous blending for 1 min. The tubes were centrifuged for 5 min at 4000 rpm and 4°C. An aliquot (0.2 mL) of the extract was subjected to solid-phase extraction (SPE) with Strata X polymeric reverse-phase cartridges (33 μm, 60 mg, 3 mL; Phenomenex), as described in Tareke *et al.* (2007). The SPE cartridges were conditioned with 1 mL of 0.1% formic acid in acetonitrile, followed by 1 mL of 0.1% formic acid in methanol, and 1mL of 0.1% formic acid in water. After 0.2 mL of the extracts were added, analytes were eluted with 0.3 mL of 0.1% formic acid in acetonitrile/methanol (1:1, v/v) 4 times. The resulting extracts were evaporated to dryness using Turbovap (Zymark), with nitrogen gas (no heat), and reconstituted in 0.2 mL of 0.1% formic acid in water. After vortexing for 10 s, the extracts were transferred to autosampler vial inserts for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

A Shimadzu Nexera X2 LC system and QTrap 6500 MS (Sciex) were used. The LC column was XBridgeTM C18, 2.5 mm, 2.1x50mm connected to a guard cartridge (Waters). The LC

gradient program ran from 90% A (0.1% formic acid in water), 10% B (0.1% formic acid in acetonitrile/methanol, 1:1, v:v) to 80% A over the course of 3 min, and was then ramped back to 90% A over 1 min, and held at 90% A for 1 min of equilibration, resulting in a 5-min run time. The injection volume was 20 mL.

Dopamine was detected in positive electrospray ionization mode ESI (+), whereas DOPAC and HVA were detected in negative mode ESI (–). The LC gradient allowed sufficient separation of dopamine detected in ESI (+) from DOPAC and HVA detected in ESI (–) with polarity switching at 2 min. In positive mode, the ion spray potential was 5500 V, and in negative mode, the ion spray potential was –4500 V, with the source temperature at 300°C.

Calibration curves were linear from 5 to 2500 ng/100mg for dopamine and from 10 to 5000 ng/100mg for DOPAC and HVA with r^2>0.99 for all compounds. Deuterated dopamine and HVA were used as an internal standard for dopamine and HVA, respectively, and HVA-d5 was also used as an internal standard for DOPAC.

Method detection limits were determined as lowest calibrated levels and were 5 ng and 10 mg/100 mg of fish tissue for dopamine, and DOPAC, HVA, respectively. Recoveries were evaluated at 2 spiking levels: 50 and 500 ng/100 mg for dopamine, and 2 times higher for DOPAC and HVA. Three replicate samples (~100 mg each) of embryos and juveniles were spiked and analyzed for each spiking level. In addition, reagent spikes (100 mg deionized water) were also analyzed in triplicates for both spiking levels. Reagent blanks was run to assure there was no interference from materials and reagents. The internal standard solution was also tested separately with LC–MS/MS to ensure that no peaks of unlabeled compounds occurred.

Because embryos and juveniles contained some amounts of dopamine, HVA, and DOPAC, recoveries were calculated after subtracting measured amounts in matrix blanks from measured amounts in spiked samples. Because DOPAC was the only analyte without an isotopically labeled

matched internal standard, HVA-d5 was used for its quantitation, resulting in DOPAC low recoveries.

Statistical Analysis

Statistical analysis was performed using RStudio (Ver 0.98.1091). Normality and homogeneity of variances were evaluated by Shapiro and Bartlett tests, respectively. All data were tested for extreme outliers using Grubb's test. One-way analysis of variance (ANOVA) followed by Dunn's tests was performed to evaluate statistical differences between control and treatment groups for parametric data. Any non-normal data was log transformed. For data that remained non-normal following log transformation, Kruskal–Wallis followed by Games–Howell testing was performed. The Jonckheere–Terpstra test was used to determine concentration-dependency. Statistical significance was determined at $p \leq 0.05$.

Results

Measured Concentrations of Bifenthrin

Bifenthrin concentrations were measured before and after embryonic and juvenile exposure (Table 2.2). The method detection limit was 0.005 µg/L. The 0.15 µg/L initial solution was 226% higher than the nominal concentration, and the 1.5 µg/L initial solution was 206% higher than the correspondent nominal concentration (0.34 and 3.1 µg/L, respectively). Measured concentrations after 96 h of exposure in embryos were 15 and 2% of the 0.15 and 1.5 µg/L initial concentrations, respectively. Measured concentrations after 96 h of exposure in juveniles were 38 and 14% of the

0.15 and 1.5 µg/L initial concentrations, respectively. Samples showed a range of recovery varying from 77.54 to 101.15%. Reductions in the nominal concentrations are likely because of adsorption to the plastic Petri dish/glass beaker, ingestion/absorption by the juvenile/embryo, loss during exposure, sample handling, or the process of extraction. Throughout the rest of the present study, measured initial concentration will be used to describe the exposure concentrations.

Table 2.2 – Bifenthrin concentrations in water used for embryonic and juvenile exposure[a]

Stage	Nominal conc. (in µg/L)	Measured initial conc. (in µg/L)[b]	Recovery initial conc. (in %)[b]	Measured final conc. (in µg/L)	Recovery final conc. (in %)
Embryos	0.15	0.34 ± 0.06	68.88 ± 4.93	0.05 ± 0.004	89.88 ± 11.9
	1.5	3.1 ± 0.22	70.66 ± 4.9	0.06 ± 0.005	77.54 ± 3.13
Juveniles	0.15	0.34 ± 0.06	68.88 ± 4.93	0.13 ± 0.04	101.15 ± 19.15
	1.5	3.1 ± 0.22	70.66 ± 4.9	0.44 ± 0.06	90.31 ± 26.61

[a]Concentrations of BF in working solution are presented as initial BF concentration at 0 hour of exposure and BF concentration at 96hs post exposure. Recovery is presented as the average of all samples.

[b]Initial concentration and respective recovery were performed in one solution for both embryonic and juvenile exposure

Expression of Genes Within Estrogenic and Dopaminergic Signaling Pathways

Significant differences in gene expression were observed between embryos and juveniles following exposure to bifenthrin. There was a significant 31-fold decrease in TH mRNA and a significant 33-fold decrease in the expression of DR1 mRNA (Figure 2.2) when embryos were exposed to 0.34 µg/L of bifenthrin ($p = 0.007$ and $p = 0.009$, respectively). There was a trend toward decrease in the expression of the majority of the remaining genes, compared with the control

(Figures 2.1–2.3). In contrast, significant 2-fold and 4.5-fold increases in the expression of ERβ1 were observed in juveniles exposed to 0.34 and 3.1 μg/L bifenthrin, respectively (control vs 0.34 μg/L bifenthrin, $p = 0.02$; control vs 3.1 μg/L bifenthrin, $p = 4.22 \times 10^{7}$; Figure 2.4). A trend toward an increase of the majority of the remaining transcripts was observed (Figures 2.4–2.6, especially in the genes related to the HPG axis, Figure 2.6).

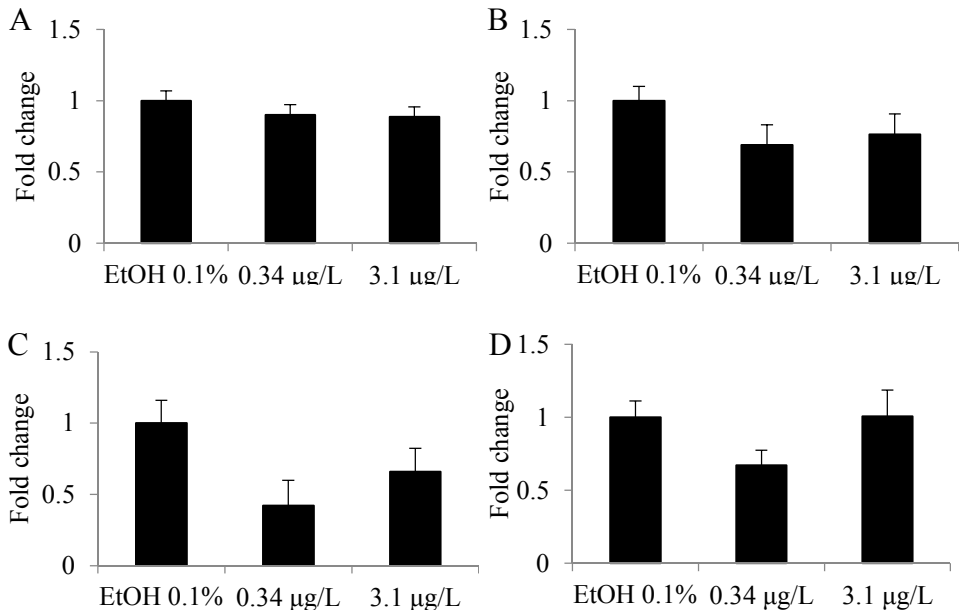

Figure 2.1 - Effects of bifenthrin on transcripts of A) estrogen receptor α, B) estrogen receptor β1, C) estrogen receptor β2 and D) vitellogenin, in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 5-12 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data; Kruskal-Wallis, Games-Howell post hoc for non-parametric data).

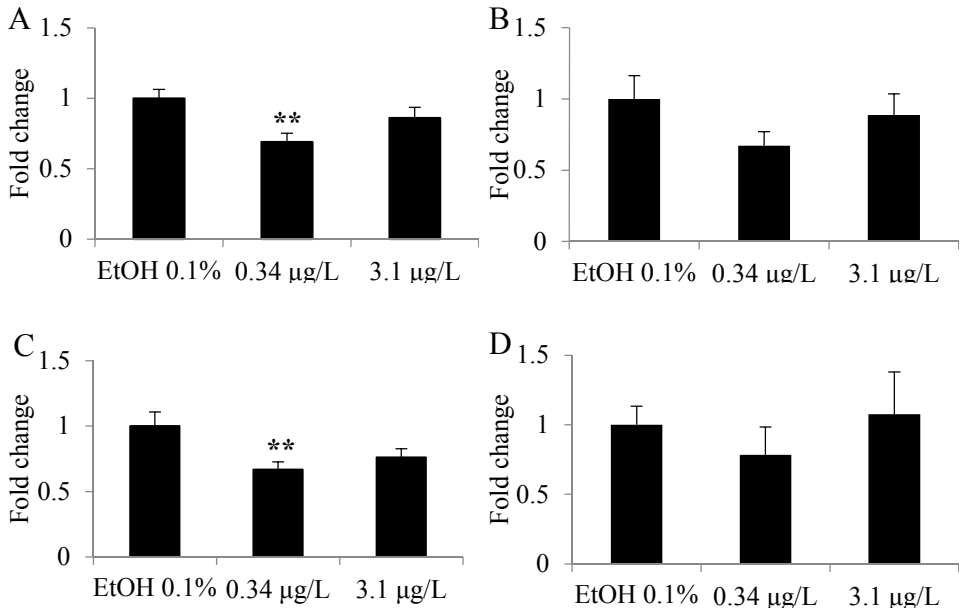

Figure 2.2 - Effects of bifenthrin on transcripts of A) tyrosine hydroxylase 1, B) dopamine active transporter, C) dopamine receptor 1 and D) dopamine receptor 2A, in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 5-12 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data).

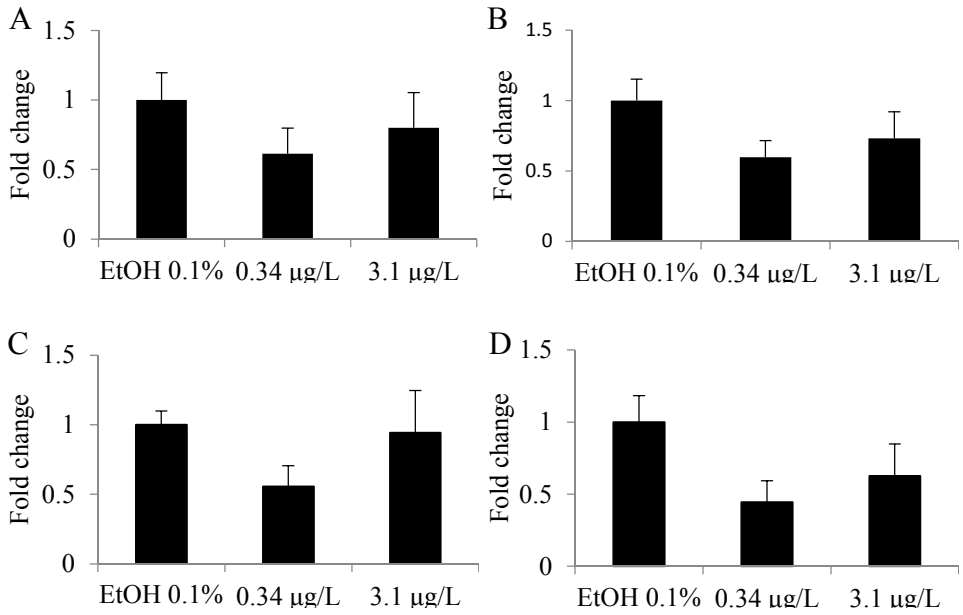

Figure 2.3 - Effects of bifenthrin on transcripts of A) *cyp19a1a*, B) *cyp19a1b*, C) luteinizing hormone β and D) follicle stimulating hormone β, in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 5-8 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data; Kruskal-Wallis, Games-Howell post hoc for non-parametric data).

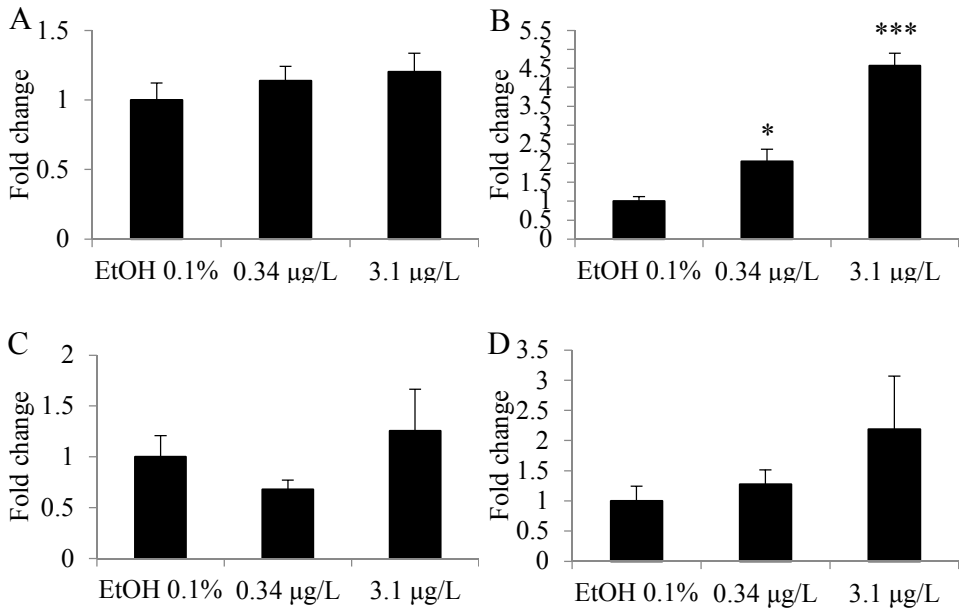

Figure 2.4 - Effects of bifenthrin on transcripts of A) estrogen receptor α, B) estrogen receptor β1, C) estrogen receptor β2 and D) vitellogenin, in zebrafish juveniles after 96 hours of treatment. Each value represents the mean of replicates 5-8 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data.

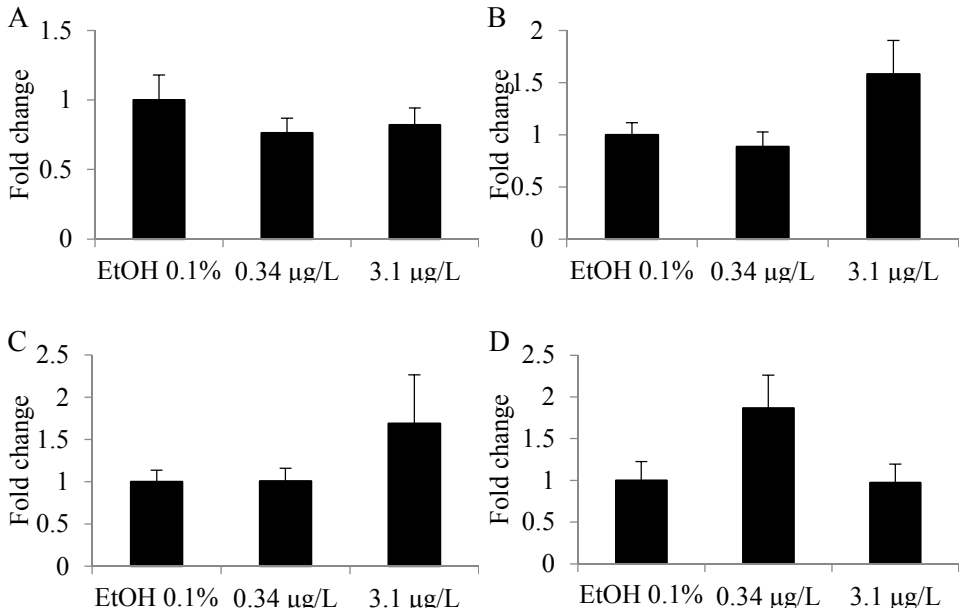

Figure 2.5 - Effects of bifenthrin on transcripts of A) tyrosine hydroxylase 1, B) dopamine active transporter, C) dopamine receptor 1 and D) dopamine receptor 2A, in zebrafish juveniles after 96 hours of treatment. Each value represents the mean of replicates 5-8 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p< 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data).

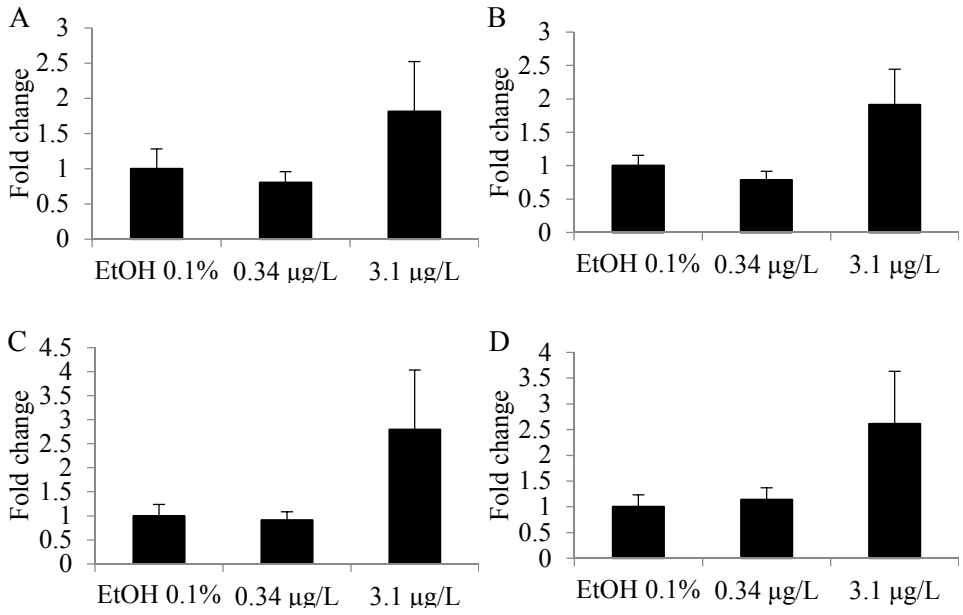

Figure 2.6 - Effects of bifenthrin on transcripts of A) *cyp19a1a*, B) *cyp19a1b*, C) luteinizing hormone β and D) follicle stimulating hormone β, in zebrafish juveniles after 96 hours of treatment. Each value represents the mean of replicates 5-8 ± SE. Treatment groups marked by asterisks have significantly different mRNA levels compared to control * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA, Dunnett's test post-hoc for parametric data.

E2 Concentrations in Tissue

Concentrations of E2 from tissue homogenates showed a trend toward a decrease in embryos following exposure to bifenthrin, compared with the control, but no values were statistically significant ($p > 0.05$; Figure 2.7A). However, there was a significant dose-dependent relationship in the embryonic exposures (Jonckheere–Terpstra test, $p = 0.041$). In juveniles, there was a trend toward an increase in the levels of E2, but no values were statistically significant ($p > 0.05$; Figure 2.7B).

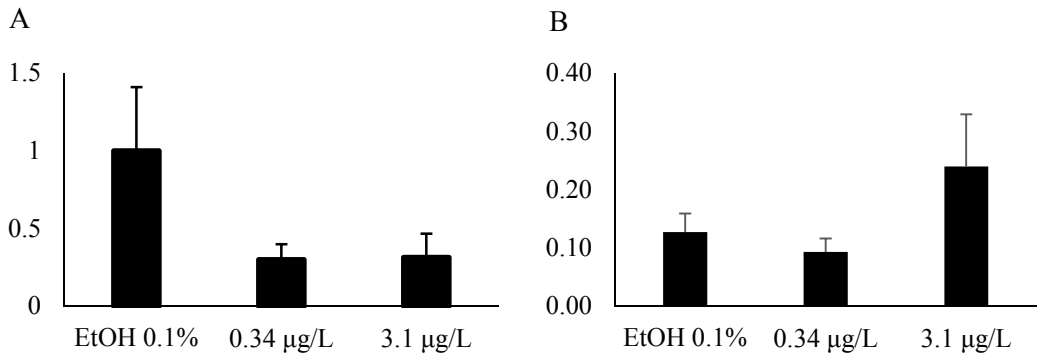

Figure 2.7 – Effects of bifenthrin on 17-β-Estradiol levels in zebrafish embryos (A) and juveniles (B) after 96 hours of treatment. Levels of E2 in embryos are represented as pg/ml per embryo and levels of E2 in juveniles are represented as pg/mg bw. Each value represents the mean of replicates 3-7 ± SE. * $p < 0.05$ (one-way ANOVA, Dunnett's test post-hoc).

Dopamine and Metabolite Concentrations in Tissues

There was a significant 30-fold decrease in homogenate concentrations of HVA in zebrafish embryos exposed to 0.34 µg/L of bifenthrin ($p = 0.038$; Table 2.3). A trend toward decreased HVA levels was observed when embryos were exposed to 3.1 µg/L of bifenthrin ($p = 0.058$; Table 2.3). In addition, there was a significant 33-fold decrease in HVA to dopamine ratios in embryos exposed to the highest concentration of bifenthrin, compared with the control ($p = 0.019$; Figure 2.8A). Furthermore, there was a significant 28-fold decrease in HVA+DOPAC–dopamine ratios in embryos exposed to the highest concentration of bifenthrin, compared with the control ($p = 0.044$; Figure 2.8A). In juveniles, there was a significant increase in the DOPAC to dopamine ratio in animals exposed to the lowest concentration of bifenthrin ($p = 0.024$; Figure 2.8B). It was not possible to measure HVA in the juvenile stage animals because of poor analytical recovery. Reagent blanks did not contain dopamine, HVA, nor DOPAC reagent, matrix spike recoveries ranged from 73 to 119%, and recoveries below 70% were measured for DOPAC (27%) in juvenile spiked samples (Table 2.4).

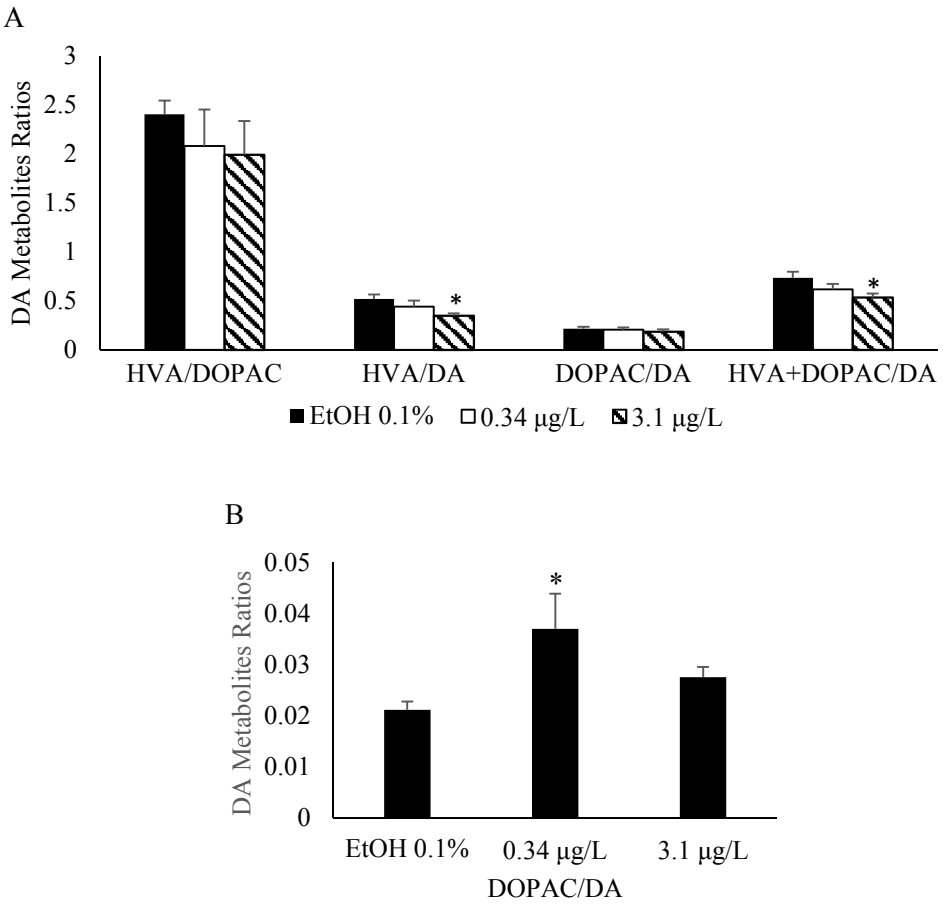

Figure 2.8 – Effects of bifenthrin dopamine (DA), 3,4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) ratios in zebrafish embryos (A) and juveniles (B) after 96 hours of treatment. Each value represents the mean of the ratios of replicates (A) 3-4 ± SE and (B) 5-6 ± SE. * $p < 0.05$ (one-way ANOVA, Dunnett's test post-hoc).

Table 2.3 – Effects of bifenthrin on dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels in zebrafish embryos and juveniles after 96 hours of treatment[a]

Stage	Treatment	Dopamine	DOPAC	HVA
Embryos	EtOH 0.1%	67 ± 11.9	14.2 ± 1	34.2 ± 5.4
	0.34 µg/L BF	58.6 ± 10.7	11.9 ± 2.2	23.7 ± 3.8*
	3.1 µg/L BF	71.3 ± 6.1	13 ± 3	24.8 ± 3.1
Juveniles	EtOH 0.1%	377.6 ± 29.5	7.9 ± 1.6	<LCL
	0.34 µg/L BF	353.5 ± 50.8	12.6 ± 5.3	<LCL
	3.1 µg/L BF	391,.6 ± 69.3	10.5 ± 1	<LCL

[a]Levels of dopamine, DOPAC and HVA are represented as ng/100 mg tissue. Each value represents the mean of replicates embryos 3-4 ± SE and juveniles 5-6 ± SE.
* $p < 0.05$ (one-way ANOVA, Dunnett's test post-hoc).
EtOH = ethanol, BF = bifenthrin, LCL = lowest calibrated level.

Table 2.4 - Recoveries of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)[a]

		Dopamine	**DOPAC**	**HVA**
Reagent	Spiked Low	98 (2)	38 (5)	98 (4)
	Spiked High	90 (1)	57 (7)	94 (2)
Embryos	Spiked Low	109 (24)	34 (32)	77 (22)
	Spiked High	74 (18)	46 (23)	86 (16)
Juveniles	Spiked Low	80 (24)	50 (17)	88 (18)
	Spiked High	77 (6)	73 (12)	86 (6)

[a]Each value represents average recovery (n=3) in percentage (relative standard deviations).

Discussion

Although bifenthrin has low acute toxicity to mammals, it may pose a risk of sublethal toxicity at different stages of development in other species. It can be found in concentrations that are acutely toxic to invertebrates in the water column, and it is the pyrethroid most frequently found in urban wastewater and agricultural discharges in the San Francisco Bay Delta (Weston & Lydy, 2010). There has also been some concern that the concentration may be high enough to elicit endocrine disrupting effects in fish (Brander *et al.*, 2016a).

Bifenthrin showed a possible antiestrogenic effect with a trend toward a decrease in the expression of genes related to the estrogenic pathway and E2 levels in the embryonic stage of zebrafish, with a significant dose-dependent relationship with E2 reduction. In contrast to embryos, bifenthrin caused estrogenic effects in juveniles, demonstrated by the significant increase in the expression of ERβ1 and the trend toward an increase in transcript levels of other HPG and E2 pathway genes, including E2 concentrations in the tissue homogenates. Antiestrogenic results were observed previously *in vitro* with bifenthrin: Brander *et al.* (2012) demonstrated that bifenthrin acted as an antiestrogen following exposure in an ER-CALUX cell system to concentrations ranging from 1 to 100 ng/L of the pyrethroid. Recent studies in female *M. beryllina* have also shown antiestrogenic responses and reductions in fecundity (Brander *et al.* 2016b). However, in contrast, increases in estrogenic biomarkers (such as choriogenin, E2, and VTG) were observed in juvenile and adult male Japanese medaka (*O. latipes*), inland silverside (*M. beryllina*), and *Oncorhynchus mykiss* (Wang *et al.*, 2007; Brander *et al.*, 2016b; Crago & Schlenk, 2015; Forsgren *et al*., 2015).

Differences in estrogenic activity of bifenthrin between the 2 stages may be because of stage-dependent expression of ER or ER activation pathways. Expression and activation of ER genes were clearly observed in zebrafish embryos following treatment with E2, consistent with other

studies (Diamante *et al.*, 2017). After activation, the ER dimerizes, is transported to the nucleus, and binds to estrogen response elements in the promoting region of ER-regulated genes (Martin *et al.*, 1994; Heldring *et al.*, 2007). Bifenthrin has been shown to fit into the ER ligand binding domain pocket forming hydrogen bonds with both ERα and ERβ (Zhao *et al.*, 2014). However, based on *in vitro* studies in cells, the outcome of receptor interaction of bifenthrin with ER appears to be that of antagonism rather than activation. Brander *et al*. (2012) demonstrated an initial concentration-dependent decrease in the E2 competence to activate ER-dependent reporter gene activity in a human ovarian carcinoma cell line (BG-1) exposed to bifenthrin, with concentrations ranging from 1 to 100 ng/L. The gene activity was inhibited by 20% at the 1 ng/L concentration to 60% at the 100 ng/L concentration. Generally, HPG feedback would suggest reductions of ER activation initiating ER biosynthesis. However, E2 concentrations showed a trend toward a decrease, suggesting that other targets upstream of ER binding may be impaired by bifenthrin in embryos. Interestingly, bifenthrin did not significantly affect expression of aromatase (either *cyp19a1a* or *cyp19a1b*) or expression of FSHβ and LHβ, which are subunits of hormones that can regulate aromatase. However, this may be because, as *in vitro* systems, the HPG axis may not be intact within embryos. Alternatively, the biosynthetic enzymes may directly be inhibited by bifenthrin. Consistent with this hypothesis, radial glial cells from goldfish (*Carassius auratus*) indicated regulation of aromatase B (*cyp19a1b*) by E2 via DR1 (Xing *et al*, 2016), which had transcripts significantly altered in our study.

Significant decreases in the expression of DR1 and the rate limiting enzyme for dopamine biosynthesis, TH, was observed in embryos treated with bifenthrin. In addition, significant decreases were noted in embryonic HVA, and the ratios of HVA: dopamine as well as HVA+DOPAC: dopamine. Dopamine and metabolite ratios are used as estimates of dopamine release and turnover. Lower ratios of dopamine and its metabolites might demonstrate reduced

turnover, because of lower levels of E2 as a consequence of bifenthrin exposure. Plasma and urine levels of HVA and DOPAC are used as indicators of dopaminergic signaling dysfunction in humans (Kung *et al.*, 2015). Thus, altered levels of dopamine and its metabolites after exposure to bifenthrin suggest disruption in dopaminergic function, which can further lead to disruption in E2 synthesis and regulation. Diminished dopamine signaling is consistent with reduced E2 concentrations. Generally, increases in E2 concentrations lead to higher levels of dopamine (Zohar *et al.* 2010), which tends to down-regulate biosynthesis of E2 in animals with a complete HPG axis. The corresponding reductions in dopamine with E2 in embryos by bifenthrin suggest a similar interaction. Kung *et al.* (2015) also observed a down-regulation of DR1 in zebrafish embryos by deltamethrin (another pyrethroid insecticide). However, in contrast to our results, that study reported an increase in the levels of TH mRNA and HVA concentrations in embryo homogenates, which did not alter dopamine: metabolites ratios. It should be noted that there might be temporal differences in TH regulation, because the Kung *et al.* (2015) study exposed embryos until the 72-hpf stage, which was 24 h earlier than the exposures in our study. Another possibility may be that downregulation of TH transcript levels might be the result of a negative feedback loop in the dopaminergic system. Because dopamine can regulate TH expression through dopamine receptors, high levels of the catecholamine would trigger a down-regulation of TH mRNA levels, leading to low levels of dopamine and consequently HVA (Kumer & Vrana, 1996). Additional studies are needed to better understand this relationship.

In contrast to embryos, a significant increase in the DOPAC: dopamine ratio was observed in juveniles treated with bifenthrin, which corresponds to estrogenic activity. Other compounds have been shown to increase ratios of DOPAC: dopamine in fish. Aroclor 1254 (a polychlorinated biphenyl mixture) exposure increased the DOPAC: dopamine ratio in the preoptic-anterior hypothalamus and medial and posterior hypothalamus areas in male Atlantic croaker

(*Micropogonias undulates*) (Khan & Thomas, 1996). In contrast to embryos, increases of dopamine metabolite ratios might demonstrate increased turnover because of higher concentrations of E2. Crago *et al.* (2015) exposed juvenile rainbow trout (*O. mykiss*) to 0.15 and 1.5 µg/L bifenthrin for 96 h and 2wk and assessed E2 levels and expression of gonadotropin-releasing hormone (GnRH2), VTG, TH, and DR2A mRNA. That study indicated a significant decrease in DR2A mRNA at 96 h and 2wk and an increase in TH transcript levels at 96 h in the brains of 1.5 µg/L bifenthrin treated rainbow trout. A significant increase in the relative expression of GnRH2 was observed at 96 h, but a significant decrease was noted after 2 wk of exposure, indicating potential feedback loop activation. These results indicate that the estrogenic effects of bifenthrin may result in part from changes in signaling within the dopaminergic pathway, but that other feedback pathways may also be involved. Additional studies explaining the kinetics of E2 biosynthesis would likely help to determine the target of bifenthrin in the HPG axis feedback loop.

In summary, environmentally relevant concentrations of bifenthrin caused an estrogenic response in zebrafish juveniles, but an antiestrogenic response in zebrafish embryos. This demonstration of life stage–dependent toxicity shows the importance of identifying susceptible stages for thresholds in risk assessments. Further studies are needed to consider the role of estrogen and dopamine receptor signaling, to develop an adverse outcome pathway for sublethal bifenthrin toxicity in fish.

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Chapter 3: Evaluation of the Estrogen Receptor as a Possible Target of Bifenthrin Toxicity in the Estrogenic and Dopaminergic Signaling Pathways in Zebrafish Embryos

Abstract

Bifenthrin (BF) is a pyrethroid insecticide widely used in urban and agricultural applications. Previous studies in embryos of zebrafish have shown that BF can affect estradiol biosynthesis and the dopaminergic system. To examine the role of the estrogen receptor (ER) in the endocrine effects of BF, embryos were exposed for 96 hours to a mixture of 0.15 and 1.5 μg/L BF and an ER agonist (17α-ethynylestradiol – EE2) at 0.09 μg/L. Transcripts related to estrogenic (vitellogenin - VTG) and dopaminergic (tyrosine hydroxylase (TH), dopamine receptor 1 (DR1), monoamine oxidase (MAO), and catechol-O-methyltransferase b (COMTb)) signaling pathways were investigated by qRT-PCR. Dopamine (DA) and its metabolites (homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC)) were also measured. There was a significant increase in VTG, DR1, MAO and COMTb mRNA levels and HVA-DA ratios within all zebrafish embryos exposed to EE2, including EE2 alone, 0.15 μg/L BF +EE2 and 1.5 μg/L BF + EE2. A significant decrease in homogenate concentrations of DA was observed within all zebrafish embryos exposed to EE2, which included EE2 alone, 0.15 μg/L BF +EE2 and 1.5 μg/L BF + EE2. Co-exposure of BF with EE2 failed to diminish estrogenic or dopaminergic signaling in embryos. Additionally, embryos with diminished ERα expression by morpholino injection were exposed to 0.15 μg/L BF, 1.5 μg/L BF and 0.09 μg/L EE2, with subsequent gene expression measurements. ERα knockdown did not rescue the effects of BF in embryos of zebrafish, indicating ER may have a limited role in the estrogenic and dopaminergic effects caused by BF in zebrafish embryos.

Introduction

Pyrethroids are a family of synthetic insecticides used in urban and agricultural settings. Relative to other insecticides such as organophosphates, pyrethroids have low acute toxicity to mammals and relatively low persistence in the environment. Consequently, pyrethroid use has increased in recent years (Weston *et al.,* 2005; Spurlock & Lee, 2008). However, previous studies have detected pyrethroids in the water column after storm events in concentrations that cause estrogenic activity in aquatic organisms (Weston *et al.,* 2005). One of the most frequently detected pyrethroids in surface waters is bifenthrin (BF), a type I synthetic pyrethroid, with concentrations varying from 0.005 to 3.79 μg/L (Siepmann & Holm, 2000).

The primary molecular target of BF is the voltage-gated sodium channel (VGSC) (Soderlund *et al.,* 2002). Binding to VGSC leads to depolarization of the neuronal membrane, causing enhanced neurotransmitter release, and potentially to increased mortality from acute neurotoxicity (Van den Bercken & Vijverberg, 1980). BF also affects estrogen signaling pathways, demonstrating both estrogenic and anti-estrogenic activity (reviewed in Brander *et al.,* 2016a). Brander *et al.* (2012) exposed cell-lines containing both estrogen receptor alpha (ERα) and beta (ERβ) to concentrations of BF ranging from 1 to 100 ng/L and observed anti-estrogenic activity. *In vivo*, BF decreased reproductive output, choriogenin, and ERα mRNA levels in adult inland silverside (*Menidia beryllina*) (Brander *et al.,* 2016b). In contrast, BF caused estrogenic activity through induction of vitellogenin (VTG) expression in male Japanese medaka (*Oryzias latipes*) (Wang *et al.,* 2007) and rainbow trout (*Oncorhynchus mykiss*) (Crago & Schlenk, 2015). Induction of the yolk-protein choriogenin was also observed in juvenile inland silverside exposed to concentrations as low as 1 ng/L of BF (*M. beryllina*) (Brander *et al.* 2012).

BF also affects the dopaminergic signaling pathway. Crago & Schlenk (2015) showed decreased levels of DR2A mRNA in rainbow trout (*O. mykiss*) after exposure to 0.15 and 1.5 µg/L of BF for 2 weeks. In adult male Winstar rats, BF treatment for 30 days at 3.5 and 7 mg kg^{1} doses significantly reduced levels of dopamine (DA) and 3,4 dihydroxyphenyl acetic acid (DOPAC), but significantly increased levels of homovanillic acid (HVA) in the frontal cortex, hippocampus and corpus striatum of the rats (Syed *et al.,* 2018). Additionally, after administration of 5 to 20mg/kg BF, mice showed decreased levels of tyrosine hydroxylase (TH) (Han *et al.,* 2017).

Estrogenic and dopaminergic signaling pathways are intimately associated in vertebrates, through the hypothalamus-pituitary-gonadal (HPG) axis (Zohar *et al.,* 2010). Dopaminergic neurons are activated by increases in E2 concentrations, further inhibiting release of gonadotropin releasing hormone (GnRH), and gonadotropin hormones (GtH), both synthesized by the hypothalamus and the pituitary, respectively (Zohar *et al.,* 2010), reducing levels of E2. Fontaine *et al.* (2013) showed that, when sexually regressed female zebrafish were co-exposed with a DA receptor 2 (DR2) antagonist (domperidone) and a GnRH agonist, an increase in LHβ mRNA transcript levels, gonadosomatic index and ovarian vitellogenesis was observed. These results indicate that only removal of the inhibitory effect of dopamine allows GnRH to exert its stimulatory function, showing that DA can consequently modulate E2 levels in zebrafish. Dopamine is synthesized from tyrosine by TH which is the rate-limiting step enzyme in DA biosynthesis. Dopamine is catabolized to DOPAC by monoamino oxidase (MAO), and then to HVA by catechol-O-methyltransferase (COMT), which is targeted for excretion (Ashcroft, 1969). In the brain, DA activates at least two metabotropic receptor families, dopamine receptor 1-like (DR1) and dopamine receptor 2-like (DR2) (Ashcroft, 1969). Estrogenic signaling primarily occurs through the activation of nuclear ERs. In the absence of a ligand, ERs are inhibited by heat shock proteins. When E2 binds its receptor, it will initiate a conformational change resulting in the release of the

heat shock proteins. Then, ER will dimerize and migrate to the nucleus. In the nucleus, the dimer binds to estrogen response elements (EREs) in the promoting region of ER-regulated genes (Martin *et al.,* 1994; Heldring *et al.,* 2007). In contrast to GnRH neurons, the dopaminergic neurons of the preoptic area express ERα in rainbow trout (*O. mykiss*) (Linard *et al.,* 1995) and in other species (Zohar *et al.,* 2010), demonstrating crosstalk between each signaling pathway.

Our previous study showed anti-estrogenic effects of BF in embryos of zebrafish with significant decreases in mRNA levels of TH and DR1, as well as significant decreases in HVA levels. VTG gene expression and E2 levels were also diminished although not significantly (Bertotto *et al.,* 2018). The objective of the present study was to investigate the role of the estrogen receptor in the estrogenic and dopaminergic pathways impacted by BF in zebrafish embryos. To investigate if ER is the target of BF anti-estrogenic effects, we co-exposed BF with a known potent ER agonist, 17α-ethynylestradiol (EE2). We hypothesize that EE2 will reverse BF antiestrogenic and corresponding DA effects in embryos. To further evaluate the role of ER in the endocrine toxicity of BF, we also used a morpholino to knockdown ERα expression, which would also mimic anti-estrogenic responses observed with BF in zebrafish embryos and modulate DA metabolism. Overall, neither experimental system indicated a significant role of ER in the endocrine effects of BF in zebrafish embryos and suggests other targets may be more important in the endocrine effects of BF.

Materials and Methods

Chemicals

A mix of BF isomers (98% purity) was purchased from ChemService, Inc. Ethinylestradiol (EE2) was purchased from Sigma Aldrich. Ethanol (Decon Laboratories Inc.) was 200 proof. A stock solution of BF in 100% ethanol (EtOH) was prepared, and diluted solutions in embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4, in pH 7) were made at every exposure day. Methanol (MeOH) and acetonitrile (MeCN), both OPTIMA™ grade, were from Fisher Scientific. Deionized water of 18.2Ω was prepared with an Milli-Q water purification system (Millipore). Formic acid (FA - OPTIMA™ grade) was obtained from Fisher Scientific. Dopamine hydrochloride (DA) (1 mg/mL in MeOH), and neat solid standards of 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were from Sigma Aldrich. Standard of isotopically labeled compound used as internal standard (IS), dopamine-1,1,2,2-d4 hydrochloride (DA-d4), were from Sigma Aldrich. All standards were >98% purity. Stock solutions of DOPAC, HVA, and DA-d4 were prepared at 1 mg/mL in methanol, and stored at -80°C. The concentrations were not corrected for purity or salt content. Solutions of working standards and internal standard (IS) were prepared in MeOH. Calibration standards were prepared in 0.1% formic acid in DI water. All the solutions were kept in -80°C.

Animal Handling

Adult wildtype (strain 5D) zebrafish were maintained on a 14-h:10-h light:dark cycle within a 242-tank recirculating system (Aquaneering, Inc.) containing conditioned reverse osmosis water,

and fed *ad libitum* twice per day with GEMMA Micro 300 diet (Skretting USA). Dissolved oxygen, pH, conductivity, salinity, alkalinity, and temperature within recirculating water were maintained at 4 to 6 mg/L, 7.0 to 7.5, 950 to 1000 µS, <1 ppt, 50 to100 mg/L, and 28°C, respectively; in addition, levels of ammonia, nitrite, and nitrate within recirculating water were consistently below 0.1 mg/L, 0.05 mg/L, and 2 mg/L, respectively. Adult females and males were bred directly on-system using in-tank breeding traps suspended within 3 L tanks. For all experiments described in the section "Chemical exposures", newly fertilized eggs were staged according to previously described methods (Kimmel *et al.,* 1995). All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee protocols at the University of California, Riverside (animal use protocols #20150035 and #20130005).

Bifenthrin + 17α-ethynylestradiol co-exposure

Twenty viable embryos (3 hours post-fertilization (hpf)) were placed in 60 × 15 mm Petri dishes containing 10 ml of the working solution. Treatments consisted of vehicle control (0.2% EtOH), nominal concentrations of 0.09 µg/L (0.32 nM) EE2, 0.15 µg/L (0.35 nM), 1.5 µg/L (3.5 nM) BF and co-exposures of 0.09 µg/L BF and 0.09 µg/L EE2 and 1.5 µg/L BF and 0.09 µg/L EE2. The EE2 concentration was the maximum tolerated dose on a dose-response curve that did not significantly show mortality nor increased deformities (Figure 3.1). Actual BF concentrations were not measured during exposure. A similar study in our lab by Bertotto *et al.* (2018) using the same 96h treatment regimen of BF at the same nominal concentrations (0.15 µg/L and 1.5 µg/L) found that initial measured concentration of BF were 0.34 µg/L for the 0.15 µg/L and 3.1 µg/L for the 1.5 µg/L dose. For qPCR assays after BF co-exposure with EE2, each replicate pool consisted of 20 embryos with 4 to 7 replicate pools per treatment. For qPCR assays after ERα knockdown, each

replicate pool consisted of 20 embryos with 4 to 7 replicate pools per treatment. For measurements of DA and its metabolites, 200 embryos (20 embryos per petri dish) were used for each replicate pool, with 3 replicates pool per treatment group. Following exposure of 96 h, fish were euthanized in ice and flash frozen in liquid nitrogen, and then kept in -80°C until assays were performed.

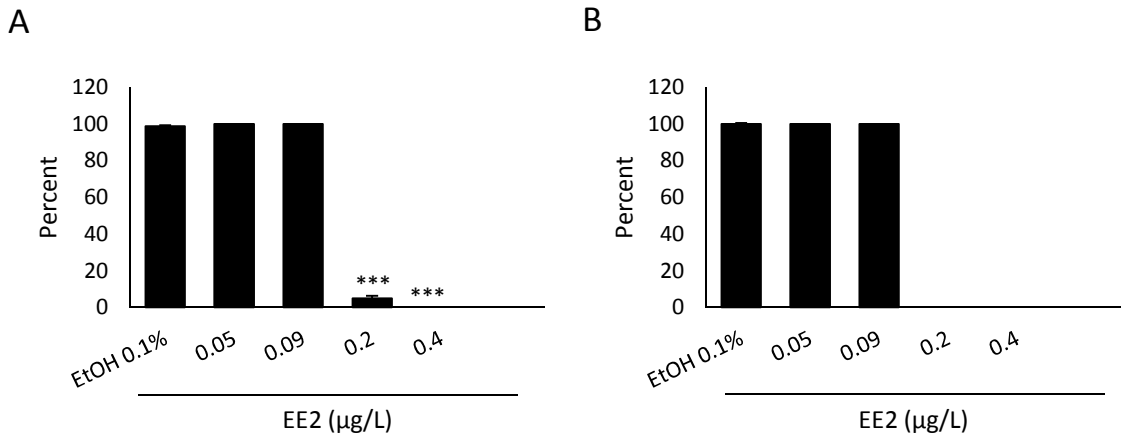

Figure 3.1 Percent survival (A) and percent hatch (B) after embryos of zebrafish were exposed for 96 hours to 17α-ethynylestradiol (EE2). Each value represents the mean of replicates 2-3 ± SE. Treatment groups marked by star have significantly different survival/hatch to the control (generalized linear model).

ERα knockdown experiments

Morpholino (MO) antisense oligos were synthesized and obtained from Gene Tools, Inc. (Philomath, OR). A translational-blocking MO (zfesr1-MO: 5′-ACGAAGGTTCCTCCAGGGCTTCTCT-3′) based on the zebrafish ERα mRNA sequence (GenBank Accession ID: AF349412.1) was used for all experiments described below. Additionally, Gene Tools' standard negative control MO (nc-tMO:5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used to account for potential nontarget MO toxicity and to use as a base for the maximum tolerated concentration of the morpholino. Negative control-tMO were tagged with fluorescein at

the 3′ end by Gene Tools to confirm MO delivery to embryos following injection, and a zebrafish-specific, fluorescein-tagged chordin MO (*chd*-MO: 5′-ATCCACAGCAGCCCCTCCATCATCC-3′) was used as a positive control during the injection and exposure experiments. Molecular-biology grade (MBG) water injections were used for the gene expression experiments. MO injection stocks (1 mM) were prepared by resuspending lyophilized MOs in MBG water, and stocks were stored at room temperature in the dark.

On the same day as injections, working solutions of nc-MOs, *chd*-MOs, and zfesr1-MOs were diluted to 62,5 μM, 125 μM and 62,5 μM in MBG water, respectively. Concentrations of nc-MOs and zfesr1-MOs chosen did not cause significant mortality nor deformities (data not shown). Newly fertilized (one-to-eight-cell stage) wild-type (5D) zebrafish embryos were microinjected with MOs or water (~3 nL per embryo) using a motorized Eppendorf Injectman NI2 and FemtoJet 4× similar to previously described protocols (McGee *et al.,* 2013). At 3 hpf, embryos injected with water or zfesr1-MO were exposed to vehicle control (0.2% EtOH), a positive control of 0.09 μg/L EE2, 0.15 μg/L BF and 1.5 μg/L BF, as described above. Approximately 4 h after injection, embryos were evaluated using a Leica MZ10 F stereomicroscope equipped with a DMC2900 camera and a GFP filter cube to confirm nc-MO and *chd*-MO delivery. Embryos that did not have MO delivery confirmed were removed from exposure. Final pool of zebrafish embryos was ~15 embryos. Following exposure of 96 h, fish were euthanized in ice and flash frozen in liquid nitrogen, and then kept in -80°C until assays were performed.

RNA isolation, cDNA synthesis and RT qPCR

Expression of genes related to the dopaminergic and estrogenic pathways after exposure to BF and EE2, and after ERα knockdown was measured. Total mRNA was isolated from embryos using

the Lipid Tissue RNeasy kit (Qiagen) following the manufacturer's instructions. mRNA quantity and quality were determined using the ND-1000 (NanoDrop). mRNA (1 µg) was converted to cDNA using the Reverse Transcription System (Promega Corporation) according to the manufacturer's instructions. The primers used in this study were already evaluated for efficiency in a previous study done by the same lab (Bertotto *et al.,* 2018). The exception was monoamine oxidase (MAO - forward primer = 5′-GTCTGGCCAACTGTGAACTTA-3′ and reverse primer = 5′-CAGAGAGGCACGATTCACTAAC-3′) and catechol-O-methyltransferase b (COMTb - forward primer = 5′- CCTGGAGTCCCAGACTATCTT-3′ and reverse primer = 5′-CTGCCCTGGTGTACTCTAAATG-3′) set of primers, which were designed using IDT DNA PrimerQuest software and efficiency was calculated using PCR Miner (Zhao & Fernald, 2005). EF1α was used as the housekeeping gene, since its expression is stable throughout development (McCurley & Callard, 2008). qPCR was carried out with the iTaq Universal One-step RT-PCR kit with SYBR Green (Bio-Rad), on a CFX Connect thermos cycler (Biorad). The samples were denatured, and the polymerase activated at 95°C for 5 min, then 40 cycles of 10 s at 95°C and 30 s of 55°C. Samples were subjected to melting curve analysis from 54 to 95°C in 0.5°C increments with continuous fluorescence measurement. qPCR was analyzed according to Schmittgen and Livak (2008) and fold change was calculated against the vehicle control.

Ultra Performance Liquid Chromatography

To measure DA and its metabolites samples were stored in -80°C prior to analysis, and on ice during handling and extraction. Sample preparation was based on Tareke *et al.* (2007) with slight modifications. Approximately 100 mg of homogenized zebrafish tissues (whole body) were placed into a 2 mL centrifuge tube, internal standard (IS) was added to yield 3 µg DA-d4 per ml of final

extract, for recovery measurements. Ice cold 0.1% formic acid in water (270 µL) was added, samples were incubated in ice for 30 minutes, and the extraction was performed using a homogenizer (Fisherbrand) by vigorous blending. The tubes were centrifuged for 5 min at 12000 g at 4°C. This process was repeat twice, however IS was not added to the samples on the second time. An aliquot (600 µL) of the extract was subjected to solid phase extraction (SPE) with Strata X polymeric reverse-phase cartridges (33 µm, 60 mg, 3 mL - Phenomenex), as described in Tareke *et al.* (2007). The SPE cartridges were conditioned with 1 mL of 0.1% formic acid in MeCN, following by 1 mL of 0.1% formic acid in MeOH, and 1 mL of 0.1% formic acid in water. After adding 600 µL of the extracts, analytes were eluted with 3 mL of 0.1% formic acid in MeCN/MeOH (1:1, v/v). The resulting extracts were evaporated to dryness with nitrogen gas (no heat) and reconstituted in 400 µL of 0.1% formic acid in water. Prior to analysis, samples were filtered using a PP 0.22 µm syringe filter (Tisch Scientific). After vortexing for 10 s, the extracts were transferred to autosampler vial inserts for UPLC analysis.

A Waters ACQUITY ultra-performance liquid chromatography (UPLC) combined with a Waters Micromass Triple Quadrupole mass spectrometer (qQq) equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA) was used for analysis. Separation was achieved using an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 µm, Waters.) at 40 °C. A binary gradient system was used to separate analytes comprising of mobile phase A, DI water (18 Ω) acidified using 0.1% FA, and mobile phase B composed of MeOH/MeCN (1:1, v/v) acidified using 0.1% FA. The solvent gradient program, in terms of mobile phase A, was as follows: initial condition began with 95% for 1 min when it was decreased linearly to 40% for 1 min, it was further decreased to 10% for 1.5 min then decreased linearly to 0% for 0.5 min, then increased linear to 10% for 0.5 min, finally it was increased 95% where it was left to equilibrated for 0.5 min for a total run time of 5.50 min. The flow rate was 0.3 mL min^{-1} and the injection volume was set to 5µL.

Mass data was acquired using Intellistart® (Waters) in the multiple reactions monitoring (MRM) mode and product ion scan in the positive ESI mode. The specific instrument settings were as follows: capillary source voltage 3.00 kV, dwell time 0.028 s, source temperature 150 °C, desolvation temperature 600 °C, desolvation gas 1200 L h-1 and cone gas 150 L h^{-1} the collision gas was Argon 99.9% pure. Cone voltage and collision voltage were generated using IntelliStart software (Waters) and were as follows: 50 (V), and 20 (V). Quantification ions were also generated using IntelliStart software and are shown in table 3.1. Individual compound peaks were detected and integrated using TargetLynx XS software.

Table 3.1 Quantification ions

Analyte	Precursor ion (m/z)	Product ion (m/z)
Dopamine	154.0381	90.9673 118.9987
3,4-dihydroxyphenylacetic acid (DOPAC)	169.0319	123.0351
Homovanillic acid (HVA)	183.0957	97.8735 137.0271
Dopamine-1,1,2,2-d_4 hydrochloride (DA-d_4)	158.1043	94.9891

Statistical Analysis

Statistical analysis was performed using RStudio (version 0.98.1091). Normality and homogeneity of variances were evaluated by Shapiro and Bartlett tests, respectively. All data were tested for extreme outliers using the Grubb's test. Two-way ANOVA were performed to evaluate statistical differences between control and treatment groups. Any non-normal data was log

transformed. If interaction was significant after two-way ANOVA, Tukey HSD was performed. Survival and hatching was evaluated by generalized linear model. Pairwise comparison between zebrafish control and exposed to EE2 was performed to evaluate positive control effectiveness. Statistical significance was determined at $p \leq 0.05$.

Results

Expression of genes within estrogenic and dopaminergic signaling pathways

There was a significant increase in VTG, DR1, MAO and COMTb mRNA levels within all zebrafish embryos exposed to EE2, which included exposures to EE2 alone, 0.34 µg/L BF +EE2 and 3.1 µg/L BF + EE2 (1.9-fold, *p*-value = 0.025, figure 3.2; 2.35-fold, *p*-value = 0.013, figure 3.3; 1.48-fold, *p*-value = 0.039, figure 3.4B; and 1.55-fold, *p*-value = 0.029, figure 3.4C respectively) when compared to embryos that were not exposed to EE2 (vehicle control, 0.34 µg/L BF alone and 3.1 µg/L BF alone). There was no significant interaction between BF and EE2, after zebrafish embryos were co-exposed to BF and EE2 (figure 3.2-3.4). There was a trend towards a dose dependent increase in mRNA levels of TH, COMTb and MAO after exposure to both concentrations of BF (figure 3.4). Pairwise comparisons showed significant 4.2 and 3.6-fold increase in VTG and DR1 mRNA levels in embryos exposed to EE2 alone, compared to vehicle control embryos (*p*-value = 0.0008 and *p*-value =0.009) (Figure 3.2 and 3.3).

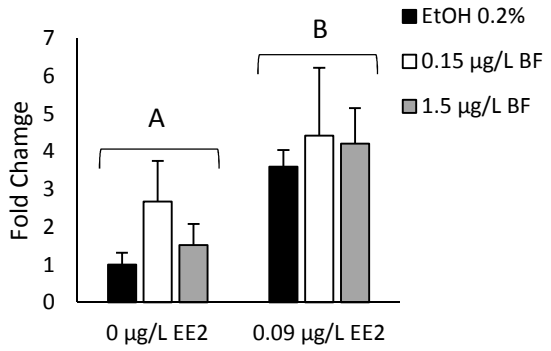

Figure 3.2 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of vitellogenin in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 4-7 ± SE. Treatment groups marked by letter have significantly different mRNA levels compared to other group (two-way ANOVA).

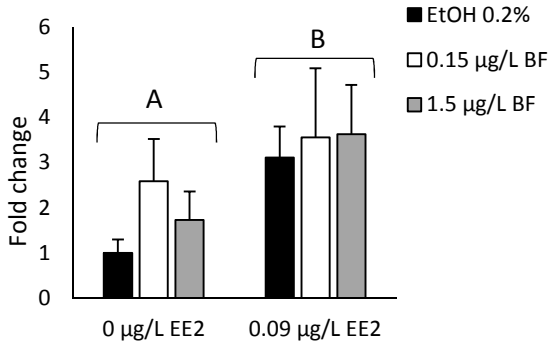

Figure 3.3 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of dopamine receptor 1 in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 4-7 ± SE. Treatment groups marked by letter have significantly different mRNA levels compared to other group (two-way ANOVA).

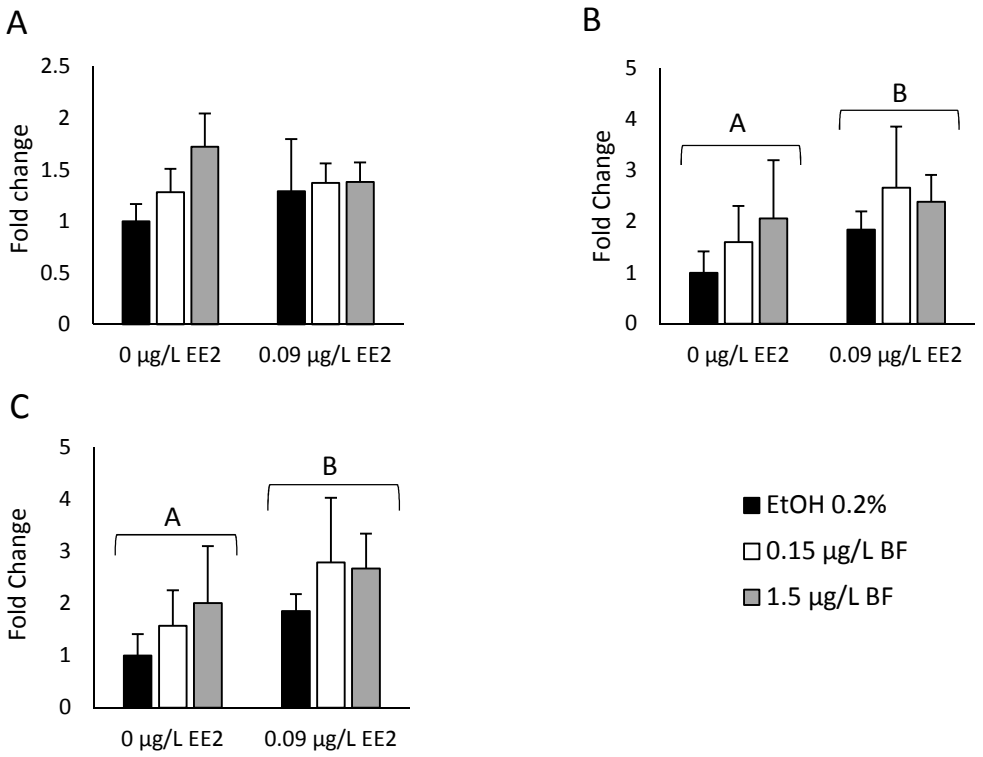

Figure 3.4 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of A) tyrosine hydroxylase, B) monoamino oxidase, and C) catechol-O-amino transferase b in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 4-7 ± SE. Treatment groups marked by letter have significantly different mRNA levels compared to other group (two-way ANOVA).

Dopamine and metabolites concentrations in embryos

A modest but significant 1.73-fold decrease in homogenate concentrations of DA was observed within all zebrafish embryos exposed to EE2, which included exposures to EE2 alone, and in mixtures with BF (*p*-value = 0.005, Figure 3.5A) when compared to embryos that were exposed to BF, but not exposed to EE2. In addition, there was a significant 2.43-fold increase in HVA-DA ratios within all zebrafish embryos exposed to EE2 alone and in mixtures with BF(*p*-value = 0.003, Figure 3.5B). There were no significant effects of BF, EE2 or a combination of both in the

remaining metabolite levels and ratios (Figure 3.6 and 3.7). Recoveries of DA and metabolites ranged from 70-112%.

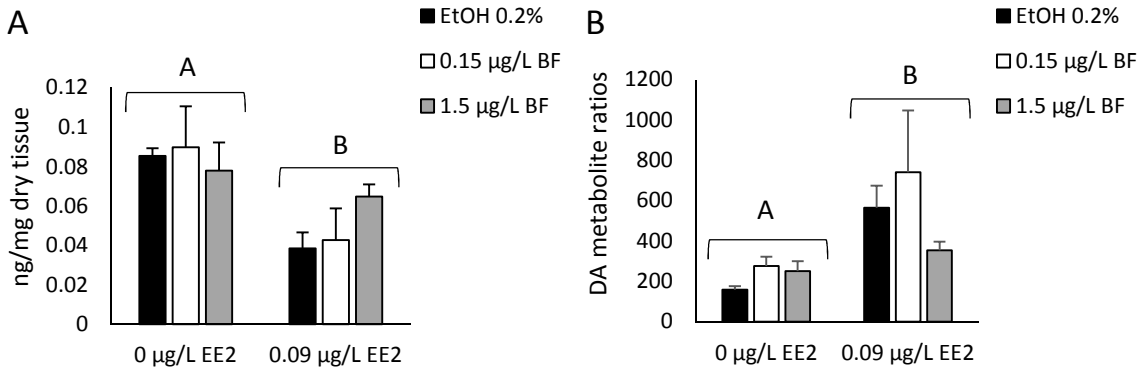

Figure 3.5 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on A) dopamine levels and B) HVA-DA ratios in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 3 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA).

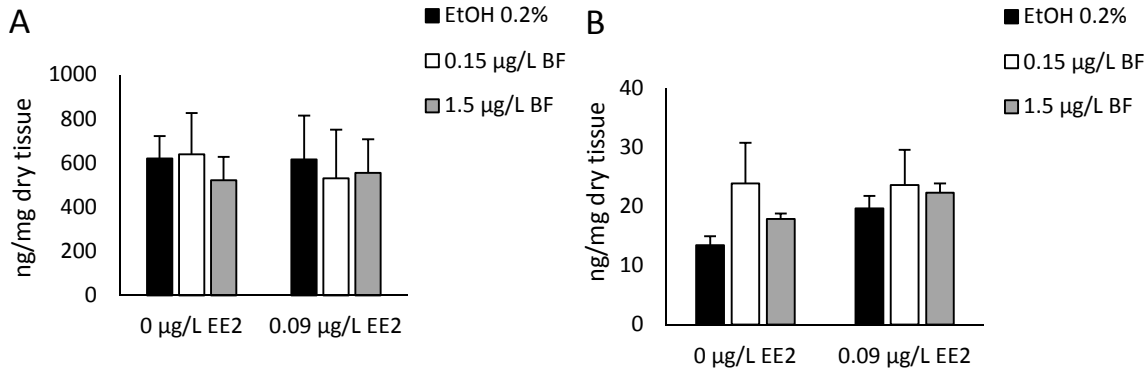

Figure 3.6 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on A) 3,4-dihydroxyphenylacetic acid (DOPAC) and B) and homovanillic acid (HVA) levels in zebrafish embryos after 96 hours of treatment. Each value represents the mean of replicates 3 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA).

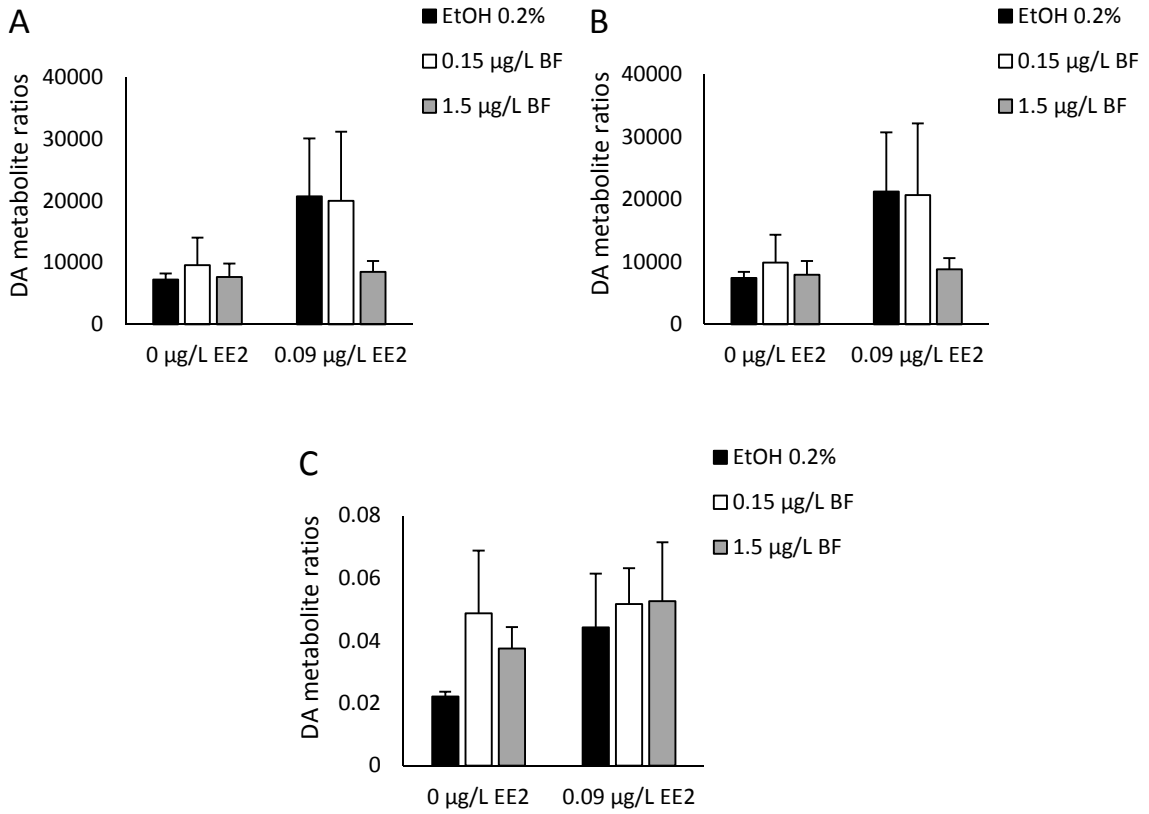

Figure 3.7 Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) ratios in zebrafish embryos after 96 hours of treatment. (A) DOPAC-DA, (B) HVA+DOPAC-DA, and (C) HVA-DOPAC. Each value represents the mean of replicates 3 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA).

ERα knockdown followed by bifenthrin exposure

There was a significant 10.9-fold increase in MAO mRNA levels when injected control embryos were exposed to EE2, compared to the vehicle control ERα knockdown embryos (*p*-value = 0.005). ERα knockdown embryos exposed to EE2 also had a significant 2.64-fold decrease in MAO mRNA levels when compared to the vehicle control injected embryos (*p*-value = 0.04) and a significant 11.7-fold decrease when compared to injected control embryos exposed to EE2 (*p*-value = 0.0006, figure 3.10B). Expression of COMTb mRNA was significantly higher with a 10.4-fold increase in injected control embryos exposed to EE2, when compared to vehicle control ERα

knockdown embryos (*p*-value = 0.007). Expression of COMTb mRNA was also significantly higher with a 10.9-fold increase in injected control embryos exposed to EE2, when compared to ERα knockdown embryos exposed to EE2 (*p*-value = 0.002, figure 3.10C). Injection (control or ERα morpholino) was a significant factor for VTG and DR1 mRNA levels (*p*-value = 0.002, figure 3.8 and *p*-value = 0.01, figure 3.9, respectively). There were no significant differences nor a significant interaction between exposure and injection factors in relation to TH mRNA levels (Figure 3.10A).

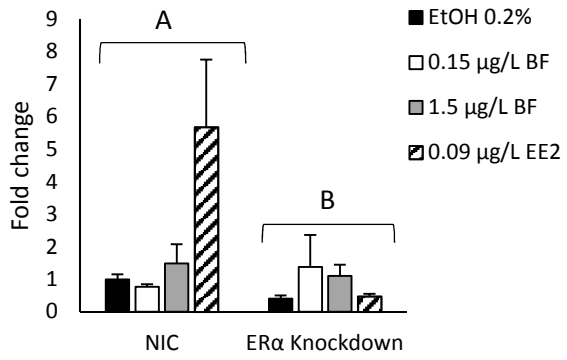

Figure 3.8 Effects of bifenthrin on transcripts of vitellogenin in zebrafish embryos injected with water or an ERα morpholino after 96 hours of treatment. Each value represents the mean of replicates 3-5 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA). NIC = negative injected control.

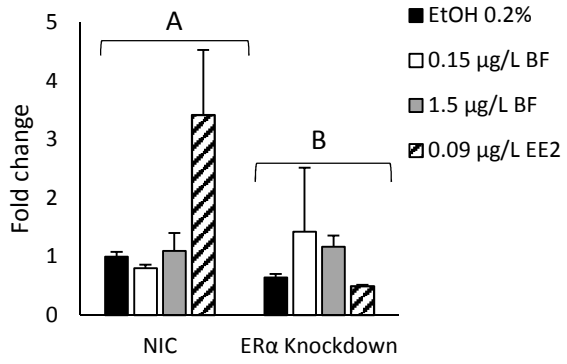

Figure 3.9 Effects of bifenthrin on transcripts of dopamine receptor 1 in zebrafish embryos injected with water or an ERα morpholino after 96 hours of treatment. Each value represents the mean of replicates 3-5 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA). NIC = negative injected control.

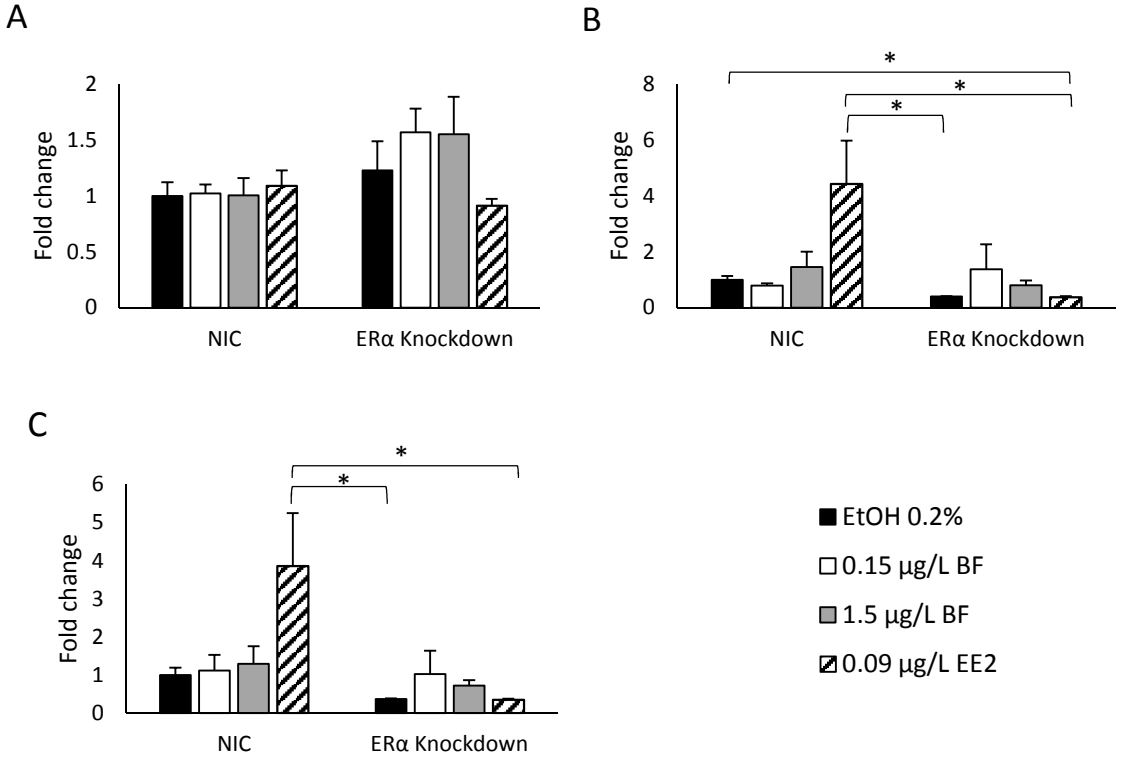

Figure 3.10 Effects of bifenthrin on transcripts of A) tyrosine hydroxylase, B) monoamino oxidase, and C) catechol-O-amino transferase b in zebrafish embryos injected with water or an ERα morpholino after 96 hours of treatment. Each value represents the mean of replicates 3-5 ± SE. Treatment groups marked by letter have significantly different neurotransmitter levels compared to other group (two-way ANOVA, Tukey HSD post-hoc test). NIC = negative injected control.

Discussion

The mechanism of action for endocrine disruption of BF in zebrafish embryos at low (ng/L) concentrations is still uncertain (Crago & Schlenk, 2015; Bertotto *et al.,* 2018). Bifenthrin has an estrogenic response in juveniles of zebrafish and silverside but is an antiestrogenic chemical in *in vitro* assays and embryos of zebrafish (Brander *et al.,* 2012; Bertotto *et al.,* 2018). In this study, the role of ER was evaluated to better understand this mechanism.

Since we demonstrated that BF had antiestrogenic effects at the embryonic stage (exposures starting at 3 hpf) of zebrafish (Bertotto *et al.,* 2018), we sought to co-expose embryos to BF and an ER agonist, EE2, to determine if previously observed anti-estrogenic responses could be reversed. Our results showed that EE2 exposure increased expression of VTG, after zebrafish embryos were exposed to EE2 alone or in mixtures of BF and EE2. EE2 is a model ER agonist (Ankley *et al.,* 2010) used in other studies to antagonize antiestrogenic effects of various chemicals, like tamoxifen, fulvestrant (Hoffmann & Kloas, 2012), methyl-piperidino-pyrazole (MPP) and pyrazolo 1,5-a pyrimidine (PHTPP) (Notch & Mayer, 2011), in concentrations varying from 0.001 to 0.03 µg/L. Since there was a significant difference in VTG mRNA between vehicle control and EE2-exposed zebrafish embryos without BF, EE2 appears to regulate E2-controlled genes in embryos and indicates that the ER signaling pathway is still conserved in this developmental stage and animal model.

As with VTG expression, only when animals were exposed to EE2 with or without BF was a significant change of DR1 mRNA observed. Previously, the endogenous ER ligand, E2, was shown to enhance DR1 activity in primary cultures of anterior pituitary cells from female rats (Maus *et al.,* 1989). A two-fold increase in the DA-DR1 induced stimulation of adenylate cyclase was observed after 24 h exposure to 10^{-9} M E2 (Maus *et al.,* 1989). Lévesque *et al.* (1988) showed that

ovariectomy in female rats decreased striatal DR1 density by 17% compared to intact female rats, demonstrating that DR1 can be controlled by gonadal hormones, including E2, and that this relationship is conserved in zebrafish early developmental stages. Consequently, the lack of change when EE2 was combined with BF indicated ER may not contribute to the effects of BF.

Since a significant decrease in expression of TH and levels of HVA was observed in the previous study (Bertotto *et al.,* 2018), we sought to analyze mRNA levels of MAO and COMTb, two important enzymes in the metabolism of DA. A trend towards dose dependent increase in mRNA levels of TH, MAO and COMTb, and increase in HVA levels after exposure to both concentrations of BF was observed indicating the non-significant increases of MAO and COMTb expression may be responsible for the trend towards increases in HVA levels. BF had significant effects in neurotransmitters and proteins involved in the dopaminergic pathway of rats treated with concentrations ranging from 5-40 mg/kg. HVA was significantly increased in the frontal cortex, hippocampus and corpus striatum of the BF-treated rats (Syed *et al.,* 2018). Additionally, mice showed decreased levels of the tyrosine hydroxylase (TH) protein after administration of 4 doses of BF, ranging from 5 to 20mg/kg (Han *et al,* 2018), in agreement with our previous results in zebrafish embryos (Bertotto *et al.,* 2018). The lack of response observed in the current study may be due to its significantly weaker estrogenic potency at the ER relative to EE2 (Liu *et al.,* 2011).

MAO and COMTb mRNA levels, DA levels and the ratio of DA with HVA were altered in the same pattern when zebrafish exposed to the ER agonist EE2 alone or in combination with BF. These data suggest ER activation had no impacts on the effects of BF on DA metabolism. Physiological (10^{-9}-10^{-7} M) concentrations of estradiol (E2) were shown to down-regulate steady-state 1.3-kb COMT mRNA levels in MCF-7 cells (Xie *et al,* 1999), as well as significantly reduce COMT protein and activity in MCF-7 cells (Jiang *et al.,* 2003). The ER antagonist ICI 182780 blocked these estrogenic effects, and the study concluded that E2 decreased COMT activity through

down-regulation of its mRNA and protein expression via ER interaction. Another study (Kumar *et al.,* 2011) assessed changes in MAO activity in brains of female rats of 3, 12 and 24 months old, and whether these possible changes are affected by administration of E2 (0.1 μg/g body weight for 1 month). E2 significantly decreased MAO activity in all age groups of rats, which contradict our results. Since EE2 exposures significantly increased MAO and COMTb gene expression, as well as decreased levels of DA and increased HVA-DA ratios, ER activation may have increased DA catabolism. However, since HVA and DOPAC levels were not significantly altered by EE2, DA may be converted to other metabolites not targeted for detection in this study. Possibilities include dopamine-3-*O*-sulfate, dopamine-4-*O*-glucuronide or norepinephrine and epinephrine (reviewed by Meiser *et al.,* 2013).

Co-exposure of embryos to EE2 and BF failed to alter expression of VTG, DR1, MAO and COMTb, as well as levels of DA and ratios of DA/HVA. While this suggests ER may not be a significant contributor to the endocrine effects of BF, co-exposure may have had significant effects on the pharmacokinetics of BF. Thus, a second manipulation of the ER pathway was evaluated in our study. ERα knockdown experiments were employed and showed that morpholino injection of ERα was a significant factor in reducing the estrogenic response of VTG mRNA expression in embryos. Knockdown experiments utilizing the morpholino technique are widely used and well established as a mechanistic tool to evaluate the role of a protein in the toxicity of a chemical (McGee *et al.,* 2013; Dasgupta *et al.* 2017). Injected control embryos exposed to EE2 had a 5.7-fold increase in VTG expression, whereas ERα knockdown embryos exposed to EE2 had 2.12-fold decrease in VTG mRNA levels, when compared to vehicle control injected embryos, showing that our knockdown was effective at weakening the estrogenic effect of EE2 in zebrafish embryos. However, it should be noted that ERα knockdown may have had other effects on unknown targets that could also affect VTG expression. However, given the lack of developmental abnormalities

observed in control injections and plethora of studies that have used this model in the same capacity, it is unlikely that non-target effects could explain the down regulation of VTG in morpholino treatments.

ERα morpholino treatment also affected DR1 gene expression. Control injected embryos exposed to EE2 had a 3.4-fold increase in DR1 expression, while ERα knockdown embryos exposed to EE2 had 2-fold decrease in DR1 mRNA levels, when compared to water injection controls. These results are in accordance with previously above-mentioned studies, where E2 increased DR1 activity (Maus *et al.,* 1989) and ovariectomy (which decreases endogenous levels of E2) decreased striatal DR1 density (Lévesque *et al.*, 1988). Again, these results are consistent with an effective ERα knockdown in our study.

Somewhat of a surprise was the effects of ERα knockdown on MAO gene expression. ERα knockdown embryos exposed to EE2 had a significant decrease in MAO gene expression when compared to the vehicle control injected embryos, and when compared to injected control embryos exposed to EE2. Gene expression of COMTb was significantly increased in vehicle control injected embryos exposed to EE2, compared to ERα knockdown embryo exposed to vehicle control and EE2. Although BF activities were not modified by ERα knockdown, MAO and COMTb expression were altered by EE2 through ERα activation. The relationship of ERα and VTG mRNA levels is well established in adult aquatic vertebrates (reviewed in Nagahama, 2002), but not for the expression of MAO and COMTb. The human COMT gene was shown to have a putative ERE (Xie *et al.,* 1999), and it can metabolize E2 as well (Dawling *et al.,* 2001). MAO activity was also shown to be controlled by E2 in rats (Kumar *et al.,* 2011). Therefore, regulation of MAO and COMTb gene expression by an ER agonist seems to be conserved in zebrafish early life stages, where response to EE2 exposure was significantly lost after ERα knockdown.

Contrary to our original hypothesis of ER involvement in the endocrine toxicity of BF, there was no difference in gene expression in ERα knockdown embryos exposed to BF. Several other studies have suggested that the effects of BF as an estrogenic/antiestrogenic compound might be due to its weak activation of the ER (Brander *et al.,* 2012, 2016b; Wang *et al,* 2007; Crago & Schlenk, 2015). Although molecular docking studies indicated interaction between BF enantiomers and human ERα and ERβ, demonstrating that both enantiomers fit in the ligand binding domain for both receptor subtypes, the binding affinities were significantly less than E2 (Liu *et al*., 2011). Zebrafish expresses 3 ER subtypes: a single ERα and two ERβ, 1 and 2. In zebrafish embryos, ERα mRNA levels are higher than the other two ER subtypes, with an increase of 60-fold at 96 hpf in comparison to the <3 hpf stage. Similarly, ERβ1 mRNA expression increases 10-fold after 96 hpf in comparison to the <3 hpf stage, but ERβ2 expression is diminished during development with a small increase in expression at 96 and 120 hpf (Chandrasekar *et al.,* 2010). Consistent with our results, knockdown experiments, with ERα and ERβ1 blocked E2 (0.1 μM) induction of VTG and ERα mRNAs (Griffin *et al.,* 2013). However, knockdown of ERβ2 had no effect on the induction of VTG and ERα mRNAs by E2. Furthermore, Hu *et al.* (2014) performed loss-of-function analysis to identify a subtype of estrogen receptor that affects migration of primordial germ cells (PGC) in zebrafish after EE2 exposure. Only ERβ2 knockdown had an effect in EE2 disruption of PGC migration. Therefore, different ER subtypes could be involved in signaling pathways within this animal model. In reporter studies that expressed the 3 receptors in HeLa-cells, exposures to estrone, estriol and genistein elicited greater activity in the ERα construct compared to the other subtypes (Pinto *et al.* 2014). In contrast, Ferutinin elicited greater activity in the ERβ1 construct and Benzophenone 2 elicited greater activity in the ERβ2 cell-line (Pinto *et al.* 2014). In this study, Pinto *et al.* (2014) also demonstrated EE2 equal agonistic activity on all 3 zebrafish ER subtypes. Consequently, previous studies suggest that while ERα does not appear to play a substantial role in

BF endocrine effects in zebrafish embryos, other ER subtypes may be involved, since exposure to EE2 seems to have different effects with different ER subtypes (Hu *et al.* 2014).

In conclusion, exposure to EE2 significantly altered gene expression in zebrafish embryos, but there was no significant interaction with BF, showing that ER might not be a significant target of BF endocrine activity. ERα knockdown did not alter the effects of BF in embryos of zebrafish but did alter the effects of EE2 on DA metabolism. Further analysis of other differentially expressed genes coupled with endocrine responses are needed to help assess other potential sublethal targets for BF endocrine activation.

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Chapter 4: Effects of Bifenthrin on Sex Differentiation in Japanese Medaka (*Oryzias latipes*)

Abstract

Bifenthrin (BF) is a pyrethroid insecticide used in urban and agricultural applications. In vitro studies in cells have indicated anti-estrogenic activity; however, estrogenic activity has been observed in fish. Japanese Medaka (*Oryzias latipes*) is a unique model that allows the measurement of phenotypic sex reversal, through expression of the *DMY* gene in males. The hypothesis that BF impairs sex differentiation was tested in larval and embryonic Japanese Medaka. Fish were exposed to environmentally relevant concentrations of BF (0.15 µg/L and 1.5 µg/L), a single concentration (0.3 mg/L) of an estrogen receptor (ER) antagonist (ICI 182,780), and an ER agonist (0.1ug/L) (17β-estradiol). Based on earlier studies that identified susceptible windows of sex differentiation in Japanese medaka, 2 separate embryonic exposures were conducted: at 2 hours post fertilization (hpf) for 10 days; and the second in 8 days post hatch (dph) larvae for 30 days. Phenotypic sex, secondary sexual characteristics (SSC) and genotypic sex were investigated at sexual maturity. A trend towards masculinization ($p = 0.06$) based on the presence of papillary processes in anal fin rays of Japanese Medaka was observed in fish exposed at the larval stage for 30 days to the lowest concentration of BF. However, genotypic gender ratios were not altered. Exposures at embryonic stages of development also did not alter phenotypic gender or secondary sexual characteristics. These results show sex differentiation was not significantly altered by embryonic and larval exposure to BF in Japanese medaka.

Introduction

Pyrethroid pesticides have become popular in urban and agricultural applications, largely due to recent reductions in organophosphate pesticide use (Weston & Lydy, 2010). Bifenthrin (BF) is a type I synthetic pyrethroid insecticide heavily used in residential pest control. It is the most frequently detected pyrethroid in the United States (Phillips *et al.,* 2012 Furthermore, concentrations averaging five times the *Hyalella azteca* 96-hr half maximal effective concentration (EC50) have been recorded in tributaries of the San Francisco Bay Delta due to polluted storm water run-off and municipal waste water discharge (Weston & Lydy, 2010, 2012; Forsgren *et al.,* 2013).

The traditional mode of action for BF involves the depolarization of the voltage-gated sodium channels in the nervous system. However, in vitro studies have shown activation and antagonistic activities at the ER by BF using the E-SCREEN assay (Wang *et al.,* 2007) and the CALUX ER assay (Brander *et al.,* 2012), respectively. In contrast, Wang *et al.* (2007) observed in vivo estrogenic activity using Japanese Medaka. Cell proliferation in MCF-7 cells was blocked when BF exposed cells were combined with an antagonist to the ERα, ERβ1 and ERβ2 (Brander *et al.,* 2012). Forsgren *et al.* (2013) reported estrogenic activity in vivo and enhanced 17β-estradiol (E2) plasma concentrations. Crago & Schlenk (2015) investigated the effect of 0.15ug/L and 1.5ug/L BF concentrations on the dopaminergic pathway in rainbow trout (*Oncorhynchus mykiss*) and also saw enhanced E2 plasma concentrations. Bertotto *et al*. (2018) observed estrogenic effects of BF in juvenile zebrafish, with a significant increase in ERβ1 gene expression. In the same study, BF was anti-estrogenic in zebrafish embryos decreasing expression of vitellogenin mRNA and homogenate concentrations of E2.

In 2011, the United States Environmental Protection Agency (USEPA) voiced concern regarding BF and its potential to affect aquatic organisms in a cumulative risk assessment (USEPA, 2011). Exposure to endocrine disrupting compounds (EDC's) at sensitive windows of development can have significant impacts on phenotypic gender (Nimrod & Benson, 1998). In an attempt to evaluate sex differences, a Medaka Reproduction Test (MRT) protocol was developed for the evaluation of developmental impacts of EDC's in biota (USEPA, 2013a; USEPA, 2013b). The MRT was proposed based upon the well described life cycle and rapid sexual development of the Japanese medaka (*Oryzias latipes*) (USEPA, 2013c). Moreover, Japanese medaka have a known sex-determining gene called *DMY* (or *dmrt1bY*, that stands for doublesex and mab-3 related transcription factor 1, subtype b, related to the Y chromosome); therefore, genotypic gender can be easily assessed.

Since BF demonstrates potential endocrine disrupting activities, we hypothesized that BF can affect the differentiation of gender in Japanese medaka. Therefore, the goal of this study was to (1) examine concentration-dependent changes in secondary sexual characteristics in Japanese Medaka exposed at embryonic and larval life stages (Deng *et al.,* 2008); and (2) test whether BF can induce reversal of gender measured by differences in genotypic and phenotypic endpoints. Results from this study will improve our understanding of the potential outcomes of endocrine alterations of BF during development.

Materials and Methods

Chemical reagents

Bifenthrin (≥99% purity), containing both cis-R and cis-S isomers was purchased from ChemService, Inc., and β-estradiol (≥98% purity) was sourced from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Exact concentrations of isomers were uncharacterised. BF stock solutions of 150µg/L and 1500µg/L were dissolved in 200 proof ethanol (Decon Laboratories Inc.). Bifenthrin solutions were prepared biweekly during exposures. ICI 182,780 (>99% purity) was sourced from Tocris Bioscience (Bristol, UK). All other reagents used were analytical grade.

Animal Handling

This study was based on the Medaka Reproduction Test (MRT), a validated Tier 2 Ecotoxicity Test by the USEPA (2013a). Approximately 85 fish were used for embryonic and larval exposures. Embryos were collected from a mature breeding stock of Japanese Medaka (*Oryzias latipes*) cultured at University of California, Riverside in accordance with animal use protocols (AUP # 20140002). Fish were held in 20L tanks maintained at 27°C with a 14:10 light/dark cycle in carbon filtered (Siemens Water Technologies, USA) medium-hard water.

Chemical Exposure

Embryos of Japanese medaka were exposed immediately (1-2 hours post fertilization) in 10ml petri dishes. Larvae for larval-stage exposures were grown out from viable embryos, in freshwater

(<0.1g biomass per liter), until 4 dph where they were acclimated to appropriate sample sizes in 10ml petri dishes for 72 hours prior to exposure. Embryos and larvae were exposed to 0.15 µg/L and 1.5 µg/L nominal concentrations of BF, 0.3 mg/L of ICI 182,780 (ICI) as an anti-estrogenic control and 0.1ug/L of E2 as an estrogenic control for 10 and 30-days, respectively. For the embryonic exposure, 10 days corresponds to the period of their development until hatching. Nimrod & Benson (1998) were able to produce exclusive female populations after exposing larval (5-8 dph) Japanese medaka to E2 at 0.1ug/L for 30 days, so that was the period of exposure chosen for the larval exposure, using E2 as a positive control. ICI was chosen as a negative control in this study due to its estrogen receptor antagonistic properties, and concentration was based on a study by Pinto *et al.* (2014) that demonstrated activity towards the estrogen receptor. Embryos that hatched before the end of the 10-day exposure period were checked and removed daily, to avoid additional exposure.

Embryonic and larval exposures were conducted at 28°C in a 14:10 light/dark cycle, for the duration of the experiment. Upon completion (or hatching of embryos), fish from both the 10-day embryonic exposure and the 30-day larval exposure were removed, washed twice in freshwater (to remove any remaining residues) and moved to freshwater petri dishes/tanks (depending on fish size, not exceeding 0.1g biomass per L) for grow out.

All fish were fed juvenile fish food (GEMMA Micro, Westbrook, ME) *ad libitum* until size and maturity allowed consumption of live brine shrimp. Daily 50% water changes were performed at all stages of acclimation, grow out and exposure. Surplus food was removed during daily water changes. Standard water quality parameters described by U.S. EPA (1991; 1976) were maintained.

Sexually mature fish (16 weeks post hatching) were euthanised using an excess of tricaine methane sulfonate (>0.5g per L). Gender and secondary sex characteristics (SSC) were taken immediately after euthanasia, prior to individual storage (-80°C) for subsequent DNA extraction.

Photos were also taken of fish after SSC measurements. Growth observed in all fish throughout exposure and grow-out stages was similar to Medaka weight gain described in the MMT ISR (USEPA, 2013a). Mortality was recorded and an average of ≥80% survival in negative solvent control treatments was maintained.

Measurement of Nominal Bifenthrin Exposure Concentrations

Water samples were taken before water changes at start, middle (5d and 15d) and when fish reached sexual maturity (16 weeks post hatching) stages of embryonic and larval exposures for BF measurements. Glassware was acid-washed and rinsed twice with methylene chloride (DCM) and hexane prior to use. Extraction based on previously published methods [8] was performed. Briefly, extraction using DCM and sodium chloride was performed in separatory funnels with samples spiked with 1ml 50ng/ml decachlorobiphenyl (PCB 209) as an extraction surrogate. This was followed by solvent evaporation, re-constitution and analysis in an Agilent 6890N Gas Chromatograph equipped with an Agilent 5973 mass selective detector.

Morphometric Measurements

Measurements for four secondary sexual characteristics were recorded following euthanasia of sexually mature fish described by Iwamatsu *et al.* (2003). Measurements taken included the degree of urogenital protuberance (UGP); number of anal fin rays with papillary processes (PP) (Figure 4.1); number of dichotomously branched (DB) anal fin rays; and degree of ventral fin length (VFL). Degree of UGP and ventral fin length was quantified on a scale of 1 to 3, with 1 indicating minimal

growth (or protuberance), and 3 indicating maximal growth (or protuberance resembling a half balloon-like appearance). Degree of ventral fin length was quantified on a scale of 1 to 3:

1. Indicated a short ventral fin not extending to the genital pore region.
2. Indicated a ventral fin that extended to the genital pore region.
3. Indicated a ventral fin extending to or beyond the genital pore.

A gender differentiation score was generated for each fish in control and treatment groups (Iwamatsu *et al.,* 2003). Each score was generated by adding degree of UGP index, number of DB anal fin rays, and ventral fin length index for each fish. A greater gender differentiation score might be attributed to an individual fish exhibiting female morphometric characteristics and a lower score for fish with more male characteristics.

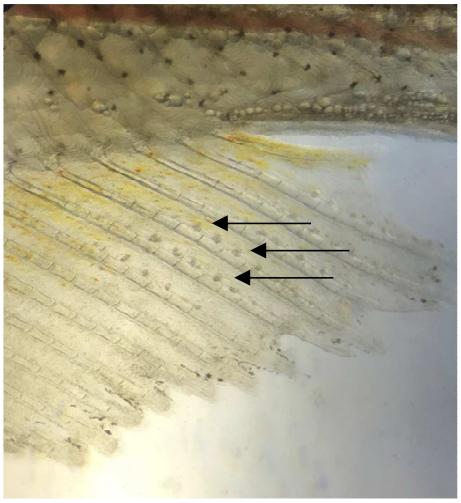

Figure 4.1 Anal fin of a reference Japanese Medaka, arrows pointing to the papillary processes

Genomic DNA sampling protocol

DNA was extracted from caudal fin tissue utilising a proteinase K digestion (U.S. Biological, Salem, MA) and digestion buffer (0.5% SDS; 10mM Tris, pH 8.0; 100mM NaCl; 10mM EDTA,

pH 8.0). The digestion was followed by a phenol:chloroform extraction and ethanol precipitation before drying and resuspension in TE (10mM Tris, pH 8.0; 1mM EDTA, pH 8.0). DNA concentrations were measured using a ND-1000 Nanodrop spectrophotometer (Thermo Fischer Scientific, Wilmington, DE), with acceptable 260/280 and 260/230 ratios of >1.8 and >2.0, respectively. If necessary, samples were cleaned with 2M NaCl and ethanol before resuspension in TE. DNA was stored at 4° C.

DMY assay protocol and PCR conditions

Genetic sex can be observed by the presence of the DMY sex-determining gene (Matsuda *et al.* 2002) for comparison with phenotypic gender (Iwamatsu *et al.,* 2003). If a phenotypic male Japanese medaka does not present the DMY gene, it means it's gender was phenotypically reversed. Primers, (forward = 5' CCG GGT GCC CAA GTG CTC CCG CTG 3'; reverse = 5' GAT CGT CCC TCC ACA GAG AAG AGA 3') designed by Matsuda *et al.* (2002), were purchased from Integrated DNA Technologies (Skokie, IL). A PTC-200 thermocycler (MJ Research Inc., Canada) was used to run the PCR assay, developing a DMY and DMRT1 DNA product of 1ug genomic DNA for gender observations by gel electrophoresis (Figure 4.2). The TaKaRa polymerase kit (Takara Bio Inc., Japan) was used following instructions provided. PCR conditions were as follow: 5 min at 95°C, followed by 30 cycles of 20 sec at 96°C, 30 secs at 55°C, and 30 secs at 72°C, followed by 5 min at 72°C. The length of the DMY PCR product (~1kb) is different from that of DMRT1 (~1.2kb), allowing genetic sex determination using 1% agarose gel electrophoresis (Matsuda *et al.* 2002; Matsuda *et al.,* 2007).

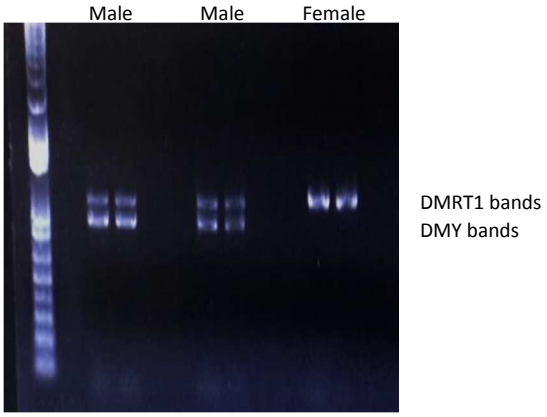

Figure 4.2 DMRT and DMY expression observed by gel electrophoresis

Statistical Analysis

Statistical analysis was performed using RStudio (Version 1.1.383). Normality was evaluated by Jarque-bera. Homogeneity of variances was evaluated by Bartlett tests for parametric data, and by Levenes test for non-parametric data. One-way analysis of variance (ANOVA) followed by Dunn's tests was performed to evaluate statistical differences between control and treatment groups for parametric quantitative data. Kruskal-wallis test was used for nonparametric quantitative data. Chi-square test was used to analyse nominal data, followed by Tukey type pairwise comparison as post-hoc. Poisson regression and hurdle model were used for count data. Statistical significance was determined at $p<0.05$.

Results

Measurement of Nominal Bifenthrin concentrations during exposures

Measured values for nominal 0.15 µg/L and 1.5 µg/L BF concentrations across embryonic exposures averaged 0.5529 µg/L ± 0.009 and 1.5689 µg/L ±0.088, respectively. For larval exposure, average values were 0.3701 µg/L ± 0.0451 and 0.9452 µg/L ± 0.2105, respectively (Table 4.1). The method detection limit was 0.005 µg/L. Average recovery of all measured samples was 96% ± 18.39.

Table 4.1 Bifenthrin concentrations in water used in embryonic and larval exposures

Exposure	Nominal Concentration (µg/L)	Average Measured Concentration (µg/L)
Embryonic	0.15	0.3701 ± 0.0451
	1.5	1.5689 ± 0.088
Larval	0.15	0.5529 ± 0.009
	1.5	0.9452 ± 0.2105

Survival

There was a significant decrease in survival in embryonic and larval exposure to 0.15 µg/L BF ($p = 0.001$ and $p = 0.0002$, respectively), 1.5 µg/L BF ($p = <0.001$ and $p = 0.0001$, respectively) and ICI ($p = 0.019$ and $p = 0.0006$, respectively), when compared to the control.

Morphometric Measurements

A trend towards masculinization ($p = 0.06$) was observed in Japanese Medaka exposed at the larval stage to the lowest concentration of BF, which was demonstrated by the increase in number of anal fin rays with papillary processes (Table 4.3). There was a significant increase ($p = 0.04$) in number of phenotypical females after larval exposure to E2. There was no significance in the UGP gender score among phenotypic females, and gender score among all fish following embryonic and larval exposures.

Table 4.2 Morphological evaluation of reproductive structures and gender after embryonic exposure

	Embryonic				
Parameters	Control	0.15 µg/L BF	1.5 µg/L BF	0.3 mg/L ICI	0.1 µg/L E2
Total fish	41	26	23	18	7
Percent female	53.7	65.4	65.2	50	57.14
Rays with PP (total)	2.68 ± 0.51	2.23 ± 0.64	2.09 ± 0.65	3.39 ± 0.82	2.86 ± 1.35
UGP (total)	1.59 ± 0.12	1.82 ± 0.16	2.04 ± 0.17	1.83 ± 0.22	1.71 ± 0.36
Gender score (Females)	4.13 ± 0.03	4.9 ± 0.6	5.1 ± 0.7	4.7 ± 0.5	3.67 ± 0.7
Gender score (Males)	3.5 ± 0.02	3.6 ± 0.4	3.4 ± 0.5	2.9 ± 0.4	3.0 ± 0.0
Gender score (Total)	3.8 ± 0.02	4.4 ± 0.4	4.5 ± 0.5	3.8 ± 0.4	3.4 ± 0.4
Average survival (%)	80.3 ± 2.6	47.3 ± 4.4**	41.8 ± 5.1***	51.4 ± 2.5*	56 ± 8.6

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way analysis of variance for parametric quantitative data, Dunnett's post hoc test; Kruskal-Wallis for non-parametric quantitative data; chi-square test for nominal data). BF = bifenthrin; ICI = ICI 182, 780; E2 = 17β-Estradiol; PP = papillary processes, UGP = urogenital protuberance.

Table 4.3 Morphological evaluation of reproductive structures and gender after larval exposure

	Larval				
Parameters	Control	0.15 µg/L BF	1.5 µg/L BF	0.3 mg/L ICI	0.1 µg/L E2
Total fish	43	17	22	12	12
Percent female	67.4	41.2	54.5	50.0	83.3*
Rays with PP (total)	1.88 ± 0.43	3.94 ± 0.86	2.82 ± 0.7	3.25 ± 0.99	1.67 ± 0.91
UGP (total)	1.7 ± 0.12	1.82 ± 0.21	2 ± 0.19	1.75 ± 0.25	1.67 ± 0.22
Gender score (Females)	5.5 ± 0.7	5.8 ± 1.2	5.0 ± 0.8	4.8 ± 0.7	3.4 ± 0.4
Gender score (Males)	2.6 ± 0.3	9.4 ± 2.3	6.4 ± 1.2	6.3 ± 1.1	3.67 ± 0.7
Gender score (Total)	4.6 ± 0.3	5.9 ± 1.6	5.0 ± 1.2	5.5 ± 1.2	3.0 ± 0.0
Average survival (%)	86.67 ± 3.8	42.5 ± 7.9***	42 ± 6.3***	43.3 ± 6.1***	83.3 ± 15.7

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way analysis of variance for parametric quantitative data, Dunnett's post hoc test; Kruskal-Wallis for non-parametric quantitative data; chi-square test for nominal data). BF = bifenthrin; ICI = ICI 182, 780; E2 = 17β-Estradiol; PP = papillary processes, UGP = urogenital protuberance.

DMY assay

No difference in the presence or absence of the DMY sex-determining gene in phenotypic males or females of Japanese medaka was observed after exposure to both concentrations of BF, nor single concentrations of ICI nor E2, in either developmental stage (Figure 4.3).

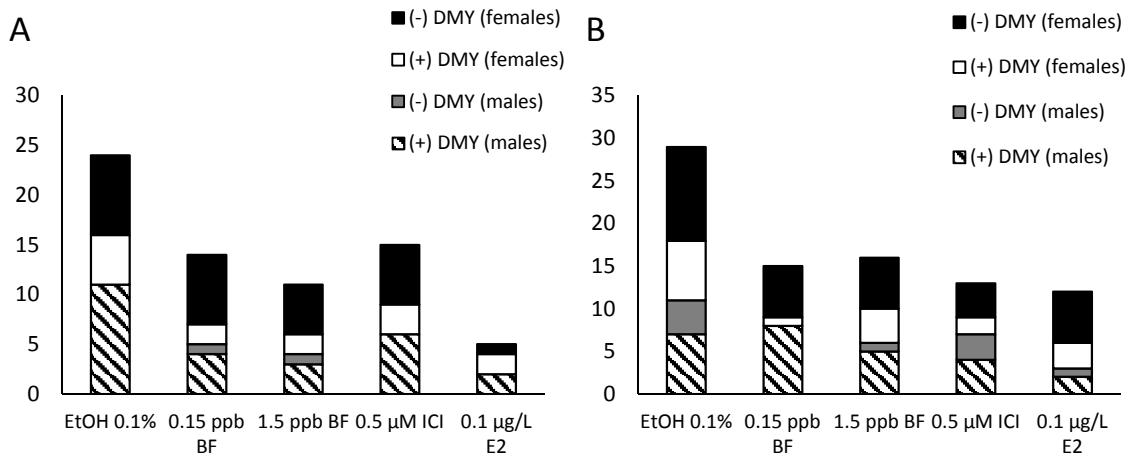

Figure 4.3 Effects of bifenthrin on the presence of the DMY gene after (A) embryonic, and (B) larvae exposure of Japanese medaka after 10 and 30 days of treatment, respectively. Each value represents the presence or absence of the DMY gene in phenotypic males or females. EtOH¼ethanol.

Discussion

Pyrethroids can be found at concentrations in urban wastewater and agricultural discharges in the San Francisco Bay Delta that elicit sublethal and lethal toxicity to aquatic organisms (Weston & Lydy, 2010). Estrogenic and antiestrogenic activity has been previously observed in fish treated with BF (Wang *et al.,* 2007; Brander *et al., 2012;* Crago & Schlenk, 2012; Bertotto *et al.,* 2018; Beggel *et al.,* 2011). To demonstrate the developmental and reproductive impacts as part of an adverse outcome pathway for BF *in vivo*, secondary sexual characteristics and molecular

biomarkers were evaluated after exposure to environmentally relevant concentrations. Weston & Lydy (2012) recorded BF concentrations in the American River averaging 10-30 ng/L; although concentrations above 0.1 µg/L were detected in past rain events. These measurements are comparable to the lower BF concentration used in this study as well as previous studies and were employed to better understand BF impacts on feminisation or masculinisation.

Significant gender skewness towards female characteristics was observed after larval exposure to E2, but gender ratios were unaltered after exposure to BF or ICI. Previous studies suggest phenotypic feminisation can be controlled by exogenous estrogens. Nimrod & Benson (1998) observed 100% sex reversal in Japanese medaka exposed to E2 concentrations as low as 0.1µg/L for 30 days, starting in a period of observed susceptibility (6-10dph). Using a similar technique, larvae were exposed to two BF concentrations (0.15 µg/L and 1.5 µg/L), known to disrupt endocrine function in Rainbow trout (Crago & Schlenk, 2015); but phenotypic changes in sexual sex characteristics were not observed in Japanese medaka. Using a gender score derived by Deng *et al* (2008), no differences were observed after embryonic and larval exposure to both concentrations of BF. However, a trend towards masculinization ($p = 0.06$) in Japanese medaka exposed at the larval stage to the lowest concentration of BF was observed, demonstrated by the increase in number of anal fin rays with papillary processes. The number of papillary processes is a secondary sexual characteristic in Japanese medaka that is used with the MRT protocol (USEPA, 2013a; USEPA, 2013b). Papillary processes are bony structures that can be easily observed under the microscope, and exposure to androgen receptor agonists can induce the development of the characteristic in female Japanese medaka (USEPA, 2013a). A study performed by Seki *et al.* (2006) exposed adult Japanese medaka to 50, 500, and 5,000 ng/L nominal concentrations of the androgen,17β-trenbolone (TB) and observed a significant increase in number of papillary processes in female medaka after exposure to the two highest concentrations, indicating masculinization of

secondary sexual characteristics in females. Although significantly increased mortality was observed after larval and embryonic exposure to both concentrations of BF and ICI 182,780, the trend towards masculinization was not a consequence of increased mortality of one gender after exposure to BF, since we did not see a significant difference in gender ratios. The mechanism of action causing reduced survival is unclear but deserves further study.

Although BF enhanced mortality of larvae, the potential impacts of BF on sex differentiation have not been previously examined. In vitro studies revealed ambiguous results regarding ER agonistic and antagonistic properties of BF, depending on the cell-line used (Wang *et al.,* 2007; Brander *et al.,* 2012). BF, or its metabolites, seem to act as ER agonists *in vivo* (Brander *et al.,* 2012; Crago & Schlenk, 2015) but the same compound, in embryonic stages of development, has been shown to act as an ER antagonist (Bertotto *et al.,* 2018). Endocrine disrupting metabolic by-products from hydroxylation of the phenyl group in BF *in situ* by the MCF-7 cells, via a cytochrome P450 monooxygenase mediated conversion, could explain this discrepancy (DeGroot & Brander, 2014). However, limited biotransformation of BF was noted in earlier studies in fish (Wang *et al.,* 2007). The mechanisms of anti-estrogenicity and estrogenicity is complex and may involve other neurotransmitters such as DA and may not impact ER signalling, previously discussed in chapter 3.

Previous studies have shown that E2 and other potent ER agonist, 17α-Ethynylestradiol (EE2) can successfully reverse Japanese medaka gender. Genotypical males of Japanese medaka exposed to 33.5 and 140.6 ng/L E2 from fertilization until 14 dph presented ova-testis (Hirai *et al.,* 2006). Zhao & Hu (2012) were also able to observe sex-reversal in male Japanese medaka after exposure to 74.8 ng/L EE2 from 1 - 21 dph. However, other weak ER ligands, such as tamoxifen, were not successful in reversing Japanese medaka gender. Consistent with earlier studies, we did not observe significant changes in gender of SSC following ICI exposures. Newly hatched d-rR strain Japanese

medaka fry were exposed to 1000-2000 µg/g of ICI via diet and differences in presence of ova-testis in orange-red males were not observed (Kawahara & Yamashita, 2000). Pinto *et al.* (2014) demonstrated that a concentration of 10^{-6} M of ICI was necessary to suppress activity of zebrafish ERs (zER) in HeLa-based reporter cell line expressing each of the three zERs. However, E2 and EE2, in concentrations that were 100 times lower, elicited 100% activity in the same cell assay. BF was shown to be a weak ER agonist in an E-SCREEN cell assay (Wang *et al.,* 2007) and had a relative proliferative potency (the ratio between E2 and xenoestrogen doses needed to produce maximal cell proliferation) of only 5%. These data are consistent with weak if any estrogenic activity of BF. Whether this is true in other species of fish, or whether other downstream responses from the endocrine effects of BF have significant adverse outcomes deserves further study.

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Chapter 5: Conclusion

Pyrethroids are a class of synthetic insecticides widely used in urban and agricultural applications, and their use has increased because, relative to other insecticides, pyrethroids have low acute toxicity to mammalian species and relatively low persistence (Weston *et al.*, 2005; Spurlock & Lee, 2008). Despite high hydrophobicity, which makes pyrethroids prone to bind organic matter of sediments, previous studies have demonstrated the presence of this compound in the water column in concentrations that cause toxic effects in the aquatic environment (Weston *et al.*, 2005) with surface water concentrations varying from 0.005 to 3.79 μg/L (Siepmann & Holm, 2000).

Pyrethroids cause persistent opening of the voltage-gated sodium channel at the alpha pore-forming subunit, which is considered the binding site (Soderlund *et al.*, 2002). In addition to acute effects, several studies have shown that pyrethroids such as bifenthrin can act as endocrine-disrupting compounds (reviewed in Brander *et al.* 2016). Furthermore, pyrethroids can also affect the dopaminergic pathway. Crago and Schlenk (2015) demonstrated a relationship between dopamine and E2 biosynthesis, by showing a trend toward an increase in plasma E2 and a significant increase in VTG, as well as decreased DR2A mRNA expression in rainbow trout after exposure of 0.15 and 1.5μg/L bifenthrin for 2 weeks.

Estrogen biosynthesis is regulated through the hypothalamus–pituitary–gonadal (HPG) axis (Zohar *et al.*, 2010). The hypothalamus produces gonadotropin-releasing hormone (GnRH), and its pulsatile release controls biosynthesis of the gonadotropins (GtHs): luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The GtHs are synthesized in the pituitary gland. Furthermore, both LH and FSH will induce egg development, maturation, and release, and they control gonadal hormone synthesis, including 17β-estradiol (E2). Increased levels of E2 will stimulate the liver to

express proteins such as VTG and choriogenin. Different feedback responses operate to tune GnRH, LH, and FSH release, including the dopaminergic system. Primarily, dopaminergic neurons will be activated with increased E2 levels, acting on GnRH and GtH neurons (Zohar *et al.*, 2010). Dopamine can block synthesis and release of GnRH (Yu *et al.*, 1991), modulating gonadotropin levels by increasing and decreasing them according to receptor subtype (Chang *et al.*, 1990), which will lead to decreased levels of E2.

Because there are few studies showing how pyrethroids can affect both dopamine and estrogen signaling pathways in fish, the first objective of this study was to investigate the effect of bifenthrin on both pathways using the zebrafish model. Using a developmental stage that lacks a complete HPG axis (embryo) and comparing this with juveniles that have an intact HPG system, our study assessed the role of HPG in the endocrine effects of bifenthrin. Bifenthrin showed a possible antiestrogenic effect with a trend toward a decrease in the expression of genes related to the estrogenic pathway and E2 levels in the embryonic stage of zebrafish, with a significant dose-dependent relationship with E2 reduction. In contrast to embryos, bifenthrin caused estrogenic effects in juveniles, demonstrated by the significant increase in the expression of ERβ1 and the trend toward an increase in transcript levels of other HPG and E2 pathway genes, including E2 concentrations in the tissue homogenates. Antiestrogenic results were observed previously *in vitro* with bifenthrin, as well as estrogenic activity *in vivo*. Differences in estrogenic activity of bifenthrin between the 2 stages may be because of stage-dependent expression of ER or ER activation pathways.

Significant decreases in the expression of DR1 and the rate limiting enzyme for dopamine biosynthesis, TH, was observed in embryos treated with bifenthrin. In addition, significant decreases were noted in embryonic HVA, and the ratios of HVA: dopamine as well as HVA+DOPAC: dopamine. Dopamine and metabolite ratios are used as estimates of dopamine

release and turnover. Lower ratios of dopamine and its metabolites might demonstrate reduced turnover, because of lower levels of E2 as a consequence of bifenthrin exposure. In contrast to embryos, a significant increase in the DOPAC: dopamine ratio was observed in juveniles treated with bifenthrin, which corresponds to estrogenic activity. In contrast to embryos, increases of dopamine metabolite ratios might demonstrate increased turnover because of higher concentrations of E2. These results indicate that the estrogenic effects of bifenthrin may result in part from changes in signaling within the dopaminergic pathway, but that other feedback pathways may also be involved.

To evaluate the role of ER in the endocrine effectsof BF, coexposure to an ER agonist, EE2, was carried out in embryos of zebrafish. Additionally, since a significant decrease in levels of HVA was observed in earlier studies in zebrafish embryos, we then analyzed mRNA levels of MAO and COMTb, two important enzymes in the metabolism of DA. Our results show that the EE2 factor had an effect in the expression of VTG, DR1, MAO and COMTb after two-way ANOVA. However, there was no significant correlation between EE2 and BF exposures. Furthermore, groups exposed to EE2 significantly increased concentrations of DA and decreased HVA-DA ratios after two-way ANOVA, consistent with increases in MAO and COMTb expression.

To further explore the role of ER in BF toxicity, ERα knockdown experiments were conducted and showed only two genes had a significant interaction between injected substance (water or ERα morpholino) and chemical exposure (EtOH, BF or EE2) after a two-way ANOVA: MAO and COMTb. Injection was a significant factor within DR1 and VTG mRNA levels, where injection with water or ERα morpholino made a significant difference in the results, but the injection factor had no effect. There was no difference in gene expression in ERα knockdown embryos, when exposed to BF. Consequently, knocking down ERα translation appear to not have any effect in the

toxicity of BF, demonstrating that ER might not be a significant target of BF at the embryonic stage of zebrafish.

To demonstrate the developmental impacts as part of the adverse outcome pathway for BF *in vivo*, secondary sexual characteristics and molecular biomarkers were evaluated in Japanese medaka after exposures at embryonic and critical developmental stages in larvae. Significant gender skewness towards female characteristics was observed after larval exposure to E2, but gender ratios were unaltered after exposure to BF or the antiestrogen chemical, ICI. A strong trend towards masculinization ($p = 0.06$) in Japanese medaka exposed at the larval stage to the lowest concentration of BF was observed, demonstrated by the increase in number of anal fin rays with papillary processes. However, since no significant ratios were observed between phenotypical and genotypical gender measured by DMRT, BF was considered not to affect Japanese medaka gender.

In summary, environmentally relevant concentrations of bifenthrin caused an estrogenic response in zebrafish juveniles, but an antiestrogenic response in zebrafish embryos at the molecular and cellular level. This demonstration of life stage–dependent activity shows the importance of identifying susceptible stages for thresholds in risk assessments. Furthermore, there was no significant interaction between EE2 and BF, after zebrafish embryos were exposed to these chemicals, showing that ER may not be a significant target for BF endocrine activity. ERα knockdown did not rescue the effects of BF in embryos of zebrafish, corroborating this conclusion. However, BF increased MAO and COMTb gene expression, which was consistent with DOPAC and HVA formation. Further studies are needed to consider the role of estrogen and dopamine receptor signaling, to develop an adverse outcome pathway for sublethal bifenthrin toxicity in fish. Additionally, BF seems not to affect secondary sexual characteristics and gender ratios in Japanese medaka after embryonic and larval exposure, indicating that the endocrine disrupting effect of BF

do not affect apical endpoints of sexual development in fish. Impacts on other developmental processes may be more important and warrant further study.

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