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Impacts of Hypersaline Conditions on the Biotransformation and Toxicity of the Pesticide Bifenthrin in Salmonid Species of the San Francisco Bay Delta

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of the requirements for the degree of

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by

Navneet Riar

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Thesis Committee:
Dr. Daniel Schlenk, Chairperson
Dr. Robert Krieger
Dr. Margarita Curras-Collazo

The Thesis of Navneet Riar is approved:

Committee Chairperson

University of California, Riverside

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This Thesis is dedicated to:

My best friend and husband, Matthew Schworetzky

ABSTRACT OF THE THESIS

Impacts of Hypersaline Conditions on the Biotransformation and Toxicity of the Pesticide Bifenthrin in Salmonid Species of the San Francisco Bay Delta

by

Navneet Riar

Master of Science, Graduate Program in Environmental Toxicology
University of California, Riverside, June 2012
Dr. Daniel Schlenk, Chairperson

Pyrethroids are synthetic derivatives of pyrethrin insecticides whose urban usage and continuous municipal wastewater discharge results in "pseudo-persistent" levels in Northern California waterways. Climate change causes warmer global temperatures which diminishes snowfall and reduces freshwater input into the San Francisco Bay Delta (SFBD) resulting in increased salinity over time. The SFBD and its drainage serve as spawning and rearing habitat for salmonid species and is under threat by multiple stressors including pesticide pollution.

Endocrine disrupting effects of bifenthrin at low-level exposures were investigated in laboratory and field experiments. 96-Hour laboratory exposures to bifenthrin (1 ng/L to 10 µg/L) showed no changes in plasma levels of the estrogenic biomarker, vitellogenin (VTG) protein, nor changes of the sex steroids testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) levels in two salmonid

species: *Oncorhynchus mykiss and Oncorhynchus tshawytscha*. Field exposures using storm water containing bifenthrin, also showed no significant change in VTG or sex steroid levels in both fish species, suggesting short-term low dose exposure to bifenthrin does not result in estrogenic activity in salmonids.

To evaluate the impacts of hypersaline conditions on bifenthrin toxicity, a 14-day laboratory exposure was performed using two populations of *O. mykiss* acclimated to freshwater, 8 g/L, and 17g/L salinity and then exposed to 0, 0.1, and 1.5 µg/L bifenthrin. The first population (Jess Ranch) was previously treated as embryos with high levels of E2 to obtain uni-gender populations, while the second population (Nimbus Hatchery) was cultured without hormone treatment. Only Jess Ranch fish exhibited significant mortality following exposure to 1.5 µg/L bifenthrin in freshwater. No significant difference was observed in VTG levels in either population. Jess Ranch Fish showed no significant difference in T, 11-KT, or E2 levels. Nimbus fish only showed a significant increase in E2 following exposure to 1.5 µg/L bifenthrin in freshwater. *In vitro* biotransformation studies using livers were also performed. Saltwater acclimation significantly reduced conversion of bifenthrin to polar metabolites in Jess Ranch fish. This study suggests that embryonic exposure to estrogens can increase susceptibility to bifenthrin in freshwater fish and saline acclimation protects against acute lethality through an unknown mechanism.

Table of Contents

Chapter 1
Introduction: An Evaluation of the Pyrethroid Bifenthrin and Climate Change in Relation to the San Francisco Bay Delta 1

Part 1: Introduction to Pyrethroids and Bifenthrin 2

Part 2: Introduction to the San Francisco Bay Delta and Salmonids 13

Chapter 2
American River Case Study: The Impact of Urbanization and the Pyrethroid Bifenthrin on Endocrine Disruption in Two Salmonid Species of the San Francisco Bay Delta 24

Abstract 25

Introduction 26

Materials and Methods 29

Results 34

Discussion 43

References 48

Chapter 3
Investigating Endocrine Disruption and Toxicity in *Oncorhynchus mykiss* Acclimated to Salinity and Exposed to the Pyrethroid Bifenthrin 51

Abstract 52

Introduction 54

Materials and Methods 58

Results 64

Discussion 70

References 77

Conclusions 82

List of Figures

Figure 1-1. The chemical structure of bifenthrin enantiomers 1R-cis-bifenthrin and 1S-cis-bifenthrin. 2
Figure 1-2. Proposed biotransformation pathway for bifenthrin in rats. 8
Figure 1-3. Map of the San Francisco Bay Delta Region. 14
Figure 1-4. Salinity in Suisun Bay from 1941-1990. 15
Figure 2-1. Effects of bifenthrin on vitellogenin plasma concentrations in female and male steelhead trout (*O. mykiss*) after 4-day laboratory exposures. 35
Figure 2-2. Effects of bifenthrin on vitellogenin plasma concentrations in Chinook salmon (O. tshawytscha) after 4-day laboratory exposures. 36
Figure 2-3. Effects of bifenthrin on plasma concentrations of sex steroids steelhead trout (*O. mykiss*) after 4-day laboratory exposures. 37
Figure 2-4. Effects of bifenthrin on plasma concentrations of testosterone, 17β-estradiol, and 11-keto-testosterone in juvenile Chinook salmon (*O. tshawytscha*) after 4-day laboratory exposures. 38
Figure 2-5. Vitellogenin plasma concentrations in female and male steelhead trout (*O. mykiss*) from field exposures. 39
Figure 2-6. Pooled vitellogenin protein levels in Chinook blood plasma from field exposures. 40
Figure 2-7. Effects of bifenthrin on plasma concentrations of sex steroids steelhead trout (*O. mykiss*) in field exposures. 41
Figure 2-8. Effects of bifenthrin on plasma concentrations of testosterone, 17β-estradiol, and 11-keto-testosterone in juvenile Chinook salmon (*O. tshawytscha*) after field exposures. 42
Figure 3-1. Survival of Jess Ranch and Nimbus Hatchery *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. 65
Figure 3-2. Vitellogenin plasma concentrations in Jess Ranch and Nimbus Hatchery *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. 66
Figure 3-3. Sex steroid levels of testosterone, 17β-estradiol, and 11-ketotestosterone in Jess Ranch *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. 67
Figure 3-4. Sex Steroid levels of testosterone, 17β-estradiol, and 11-ketotestosterone in Nimbus Hatchery *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. 68
Figure 3-5. Percent conversion of bifenthrin in microsomal incubations containing NADPH compared to boiled protein controls. 69
Figure 3-7. A diagram showing the function of a chloride cell 75

List of Tables

Table 2-1. Bifenthrin water concentrations (± standard deviation) for steelhead trout after 4-day laboratory exposures 34
Table 2-2. Bifenthrin water concentrations (± standard deviation) for Chinook after 4-day laboratory exposures 34

CHAPTER 1

Introduction: An Evaluation of the Pyrethroid Bifenthrin and Climate Change in Relation to the San Francisco Bay Delta

Part 1: Introduction to Pyrethroids and Bifenthrin

Pyrethroids are synthetic derivatives of naturally occurring pyrethrin insecticides found in the extract from the flowers *Chrysanthemum cinerariifolium* that work by targeting the central nervous system. Bifenthrin is an example of a 3rd generation type I pyrethroid (Fig 1-1). The use of pyrethroids, including bifenthrin, has consistently increased over the past decade as organophosphate insecticides have been phased out in urban areas due to toxicological concerns in humans, especially children (Oros and Werner, 2005). Pyrethroids are now commonly used in urban areas (pest control), and on row crops (e.g., alfalfa, cotton, and lettuce), as well as orchards (Kuilvia and Hladik, 2008). Pet products and insect repellents for clothing also contain pyrethroids (Oros and Werner, 2005; Weston, 2010). Other uses include pest control in nurseries, landscaping, and construction sites (Spurlock and Lee, 2008).

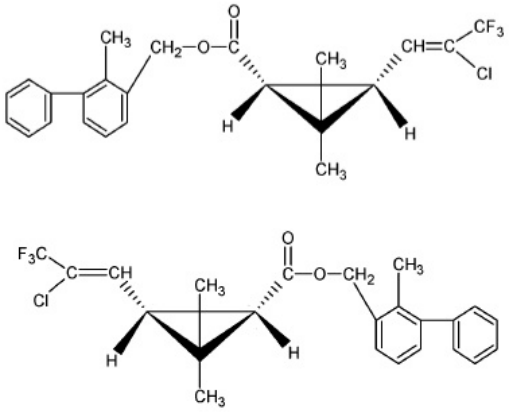

Figure 1-1. The chemical structure of bifenthrin enantiomers 1R-cis-bifenthrin (top) and 1S-cis-bifenthrin (bottom).

Bifenthrin was first registered for use by the United States Environmental Protection Agency (U.S. EPA) in 1985 (U. S. EPA, 1988). Similar to other pyrethroids, bifenthrin is used in agriculture (20%), commercial projects (40%), and in homes or gardens (40%) (Spurlock and Lee, 2008). In California agriculture, some of the main crops using bifenthrin include almonds, cotton, corn, tomatoes and pistachios. Its usage in agriculture in 2010 resulted in 354,390 lbs being used over 660,368 acres (CDPR, 2012). Some commercial products containing bifenthrin include Talstar, Maxxthor, Capture, Brigade, and Ortho Home Defense Max. In urban applications, pesticides are transported to surface water via the first flush (the first large runoff event in the winter), spring late-rainfall runoff events or tailwater return (direct runoff of irrigation return water), rice-field water release, and summer tailwater return in the Central, Sacramento, and San Joaquin Valleys of California (Kuilvia and Hladik, 2008; Oros and Werner, 2005). Urban runoff and water treatment facilities also are a major source for bifenthrin in surface water. Weston et al. (2010) found that residential outfalls, especially during the wet season, were the primary source of bifenthrin contamination of surface water. In residential outfalls, the frequency of detection of bifenthrin was 79%. Of those detections, 58% were at concentrations at or exceeding the LC50 of the aquatic invertebrate, *Hyallela azteca* (Weston et al., 2010). Weston et al. (2009) observed dry season median and maximum bifenthrin concentrations of 4.6 and 14.2 ng/L, respectively. Dry season median and maximum values were 17.3 and 29.7 ng/L in urban creeks. Given the continual output of pyrethroids through wastewater treatment plants and runoff, “pseudo-persistent”

exposure is also likely to occur in waterways (Weston et al., 2009). Pseudo-persistent chemicals are defined as compounds that are continuously added to the environment but also, through transformation or other process, are removed at the same time, resulting in persistent yet low environmental concentrations (Barceló & Petrovic, 2007; Kostopoulou & Nikolaou, 2008). The abiotic degredation of bifenthrin along with its high lipophilicity renders it easily removed from the watercolumn either by hydrolysis or sorption to sediments. In a study by the California Department of Fish and Game, 30 urban creek sites throughout California were sampled and bifenthrin was found at all locations in concentrations ranging from 2.19 to 219 ng/g dry weight in the sediment (Holmes et al., 2008). Concentrations of pseudo-persistent pollutants in waterways is low enough to avoid acutely toxic effects. However, chronic sublethal toxicity can result in developmental and reproductive endpoints that are more difficult to quantify and categorize.

Physical and Chemical Properties of Bifenthrin

Bifenthrin is found as a viscous liquid, crystalline solid, or waxy solid with a faint, slightly sweet odor (MSDS, 2011). It has a molecular weight of 422.9. At 25 °C, its vapor pressure is 1.81×10^{-7} mm/Hg, and Henry's law constant is 7.20x10-3 atm m^3/mol. The solubility of bifenthrin in water is relatively low at 0.1 mg/L. Furthermore, the octanol-water partition coefficient (Kow) is 1×10^6, causing bifenthrin to bind to organic carbon (Fecko, 1999).

Pyrethroids display stereospecificity in insecticidal action and environmental fate (Elliot et al., 1974; Nillos et al., 2010). Bifenthrin contains one chiral center, giving rise to 1R-*cis*-bifenthrin and 1S-*cis*-bifenthrin (Jin et al., 2010). Jin et al. (2010) investigated enantioselective effects on locomotor and developmental toxicity in embryonic-larval zebrafish (*Danio rerio*). 1R-*Cis*-Bifenthrin induced morphological abnormalities including pericardial edema and a curved body axis. No effects were seen at the highest concentration (300 μg/L) tested for 1S-*cis*-bifenthrin. Differential effects were also observed in locomotor activity. Larvae treated with 1R-*cis*-bifenthrin also showed decreased sensitivity to changes in light and dark. Such changes in behavior could result in increased predation or decreased success in reproduction (Jin et al., 2010). Another study by Wang et al. (2007) also observed enantioselective estrogenic activity.

Environmental Fate and Behavior

Given that bifenthrin is insoluble in water and its vapor pressure and Henry's law constant result in a low potential for volatilization into the air, there is potential for airborne transport if attached to drifting soil particles or as a spray as in during agricultural application (Fecko, 1999). The soil sorption coefficients (Koc) of bifenthrin range between 1.31×10^5 and 3.02×10^5 and indicate relatively tight binding to carbon in soil particles (Fecko, 1999). Bifenthrin also tends to bind to organic particulate materials in the water column (Oros and Werner, 2005). The Kow of bifenthrin may explain the bioconcentration factors (BCFs) observed in some animals, especially fish. For example, fathead minnows (*Pimephales promelas*)

exposed to 0.0037 µg/L bifenthrin had a BCF of 21,000 after 127 days and 28,000 after 254 days of exposure (Johnson et al., 2010).

In terms of abiotic degradation, hydrolysis of bifenthrin in buffered water occurs between pH 5 and 9. Photolysis studies in water and soil found that the half-lives were 408 and 96.9 days, respectively. In aerobic soil, the half-life of bifenthrin was 96.3 days while the anaerobic soil half-life was 425 days (Laskowski, 2002).

Biotransformation

The main routes of biotransformation of bifenthrin have been studied in rats and include hydroxylation via nicotinaminde adenine dinucleotide phosphate (NADPH) dependent cytochrome p450 enzymes, cleavage via esterases, or abiotically through hydrolysis. Phase I reactions can also be followed by conjugation reactions resulting in the elimination of the compound (Pesticide Residues in Food, 2009). Conjugates found in mammalian metabolism of pyrethroids are glucuronides, sulfates, and amino acid conjugates (Kaneko, 2011). After a 7-day exposure in rats, it was observed that most radiolabeled bifenthrin was removed in the urine (~21%) or feces (~69%) with a portion being converted to various metabolites. Some of the major metabolites in feces and urine include hydroxymethyl-bifenthrin, 4′-hydroxy-bifenthrin, 3′-hydroxy-methyl-bifenthrin, 4′-hydroxy-biphenyl acid, and 4′-hydroxy-biphenyl alcohol (Fig 1-2) (Pesticide Residues in Food, 2009).

The cytochrome p450 monooxygenase enzyme (CYP) system is the most dominant in terms phase I biotransformation responsible for catalyzing oxidation and reduction reactions. CYP's are heme-containing proteins that utilize NADPH to

reduce the iron of the heme group. O_2 then binds to the complex and a second electron is contributed by a reductase enzyme. This complex then binds the substrate which results in the cleavage of the oxygen, the uptake of two protons, and the formation of water. The final step is the generation of a hydroxyl radical that then binds to the substrate, returning the iron to the oxidized state. CYP carries out a wide variety of reactions including epoxidation, deaminations, and dehalogenations (Parkinson and Ogilvie, 2008).

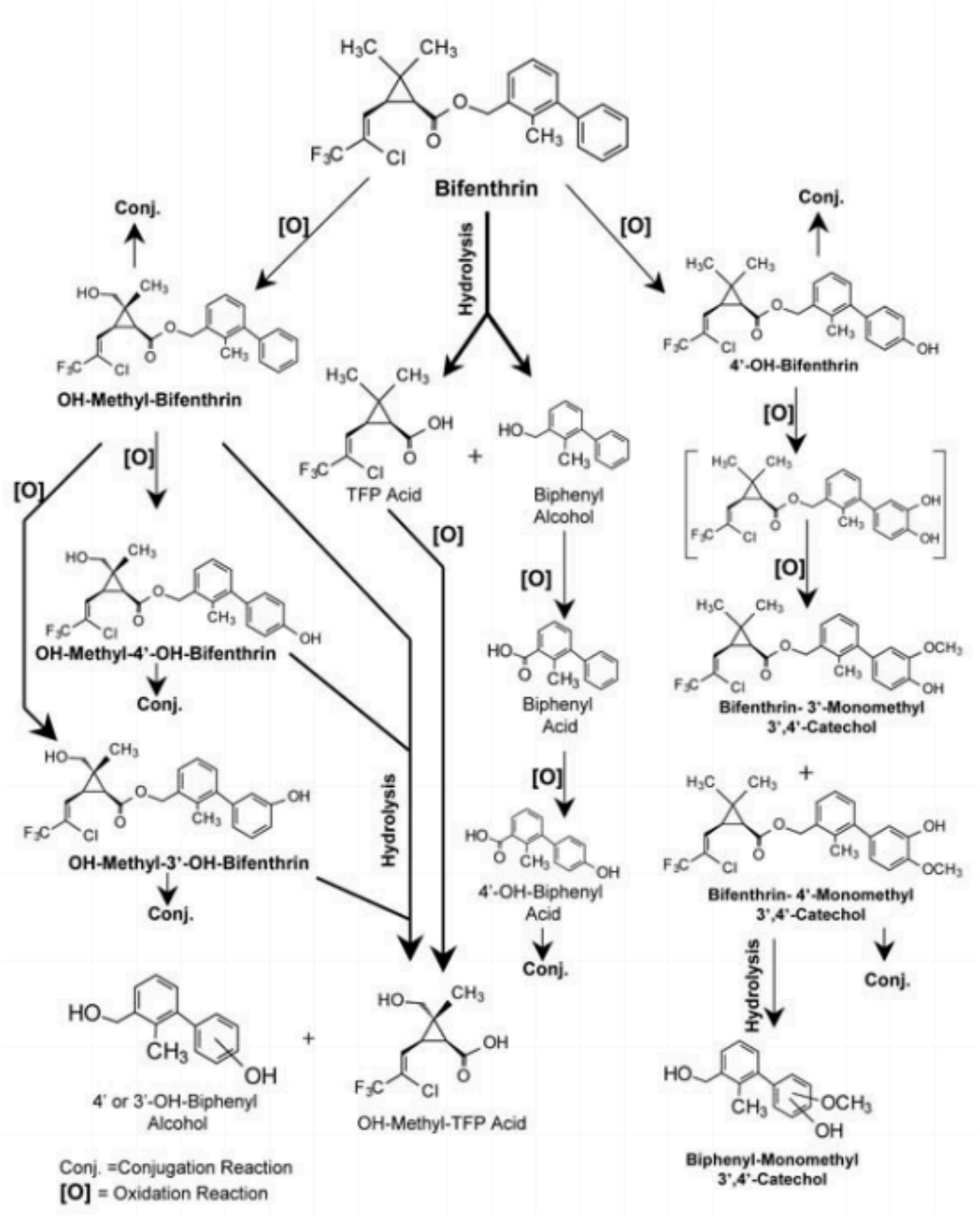

Figure 1-2. Proposed biotransformation pathway for bifenthrin in rats (Pesticide Residues in Food, 2009).

There are many different families of the CYP enzyme and each family contains isoforms that are species specific. Each CYP family differs in substrate specificity, induction, and localization. In humans alone, there are 57 different CYP enzymes coded by different genes (Parkinson and Ogilvie, 2008). One important family is the CYP3A subfamily which has a broad range of substrate specificities for both endogenous and exogenous compounds (Parkinson and Ogilvie, 2008). Bile acids, retinoids, and xenobiotic compounds such as pharmaceuticals and pesticides are substrates for CYP3A. In rat hepatic microsomes, CYP3A1 and CYP3A2 formed hydroxylated metabolites from several pyrethroids, including bifenthrin and permethrin (Scollon et. al., 2008). In humans, CYP3A4 was the most active form that converted pyrethroids into unknown metabolites (Scollon et. al., 2008). The CYP3A isoforms have been observed in including *Oncorhynchus mykiss* which possesses CYP3A27 in many tissues (Schlenk et al., 2008).

Mode of Action

Pyrethroids target the central nervous system by binding to voltage-gated sodium channels in neurons resulting in their prolonged depolarization, causing paralysis and even death. They also inhibit ATPase activity in some animals. One study investigated ATP hydrolysis (via ATPase and protein kinase phosphatase activity) in the American cockroach after exposure to the pyrethroids cypermethrin and permethrin. It was observed that both pesticides inhibited Na-Ca ATP hydrolysis both *in vitro* and *in vivo* (Clark and Matsumura, 1987). Another study found that cypermethrin resulted in irreversible noncompetitive inhibition of Na, K,

and Mg ATPases (El-Toukhy and Girgis, 1993). In this study, a single dose of 62.5 mg/kg body weight and repeated doses of 31.5 mg/kg every other day for 3 weeks were used. Percent inhibition was 59.3 and 70.8 for the single and repeated doses, respectively. Some pyrethroids also bind to the GABA receptor however this is not considered a major route of toxicity for type I pyrethroids including bifenthrin (Vijverberg and van den Bercken, 1990).

Acute Toxicity

Due to their rapid metabolism and excretion in mammals, pyrethroids tend to have lower toxicity in birds and mammals than fish (Leahey, 1985). The key to pyrethroid toxicity in aquatic ecosystems lies in the fact that pyrethroids are hydrophobic and bioavailable. In this way, pyrethroids can be acutely toxic to detritivorous organisms and fish (Oros and Werner, 2005). 96-Hour LC50 values for bifenthrin and permethrin in *O. mykiss* were found to be 0.15 and 0.62 µg/L respectively (U.S. EPA, 2002).

Chronic Toxicity

Exposure to pyrethroids results in estrogenic activity in some species of fish (Nillos et al., 2010; Wang et al., 2007;Tyler et al., 2000). Vitellogenin (VTG) is the precursor protein to egg-yolk normally found in females. In female fish, 17β-estradiol (E2) regulates VTG production in the liver (Sumpter and Johnson, 2005). Male fish and non-sexually mature juveniles may also produce VTG since they too have a receptor for E2. In this way, VTG translation and production in male fish and juveniles can act as biomarkers for estrogenic exposure (Sumpter and Johnson,

2005; Hutchinson, 2006).

Wang et al. (2007) studied uptake and estrogenic potential of bifenthrin enantiomers *in vivo* in male Japanese medaka (*Oryzias latipes*). Fish were exposed to 10 µg/L of either enantiomer for 10 days before measuring VTG levels in the livers where a 123-fold difference was observed in VTG production between 1S-*cis*-bifenthrin (highest) and 1R-*cis*-bifenthrin. The *in vitro* assay in this study observed a relative proliferative effect ratio of 74.2% and 20.9% for 1S-*cis*-bifenthrin and 1R-*cis*-bifenthrin, respectively in the human breast carcinoma MCF-7 cell proliferation assay suggesting the classic estrogen response pathway by way of the estrogen receptor. Stereoselective estrogenic activity, triggered by permethrin metabolites from enantiomers, was also observed by measuring VTG transcription (Nillos et al., 2010). This study entailed transcriptional measurements of VTG in *O.* mykiss primary hepatocyte cultures (*in vitro*) and VTG protein measurements in male medaka (*in vivo*) following permethrin treatment (10 µg/L). Statistically significant differences in the relative estrogenic potential was observed between enantiomers with 1S-*cis*- and 1S-trans-permethrin eliciting 2.5 and 1.3 times greater responses than their respective antipodes (Nillos et al., 2010). Hydroxylated metabolites were also found have more activity than the parent compounds suggesting biotransformation and cleavage products as a critical step in the activation of permethrin for estrogenic activity.

Tyler et al. (2000) also investigated endocrine disruption by 7 pyrethroids and some permethrin metabolites *in vitro* using genetically modified yeast

containing human estrogen and androgen receptor. Permethrin and fenpropathrin were observed to be weak estrogen agonists with potencies between 7 to 8 orders of magnituted less than E2 while bioallethrin, allethrin, cypermethrin and cyfluthrin showed antiestrogenic activity between 1000-fold and 10,000-fold less than that of the established antiestrogen, 4-OH-tamoxifen. Six of the 7 pyrethroids also displayed antiandrogenic activity. Metabolites of permethrin were also tested against the hormone receptors. 3-Phenoxybenzyl alcohol exhibited estrogenic and antiandrogenic activity while 3-phenoxybenzoic acid and permethrin cyclopropane acid both had antiestrogenic activity (Tyler et al., 2000).

Sex steroid concentrations in fish have also been affected by pyrethroids. A significant decrease in E2 and 11-KT was observed in catfish (*Heteropneustes fossilis*) after exposure to cypermethrin for 45 days at 20 μg/L (Singh and Singh, 2008).

Furthermore, a study by Kidd et al. (2007) observed almost total extinction of a population of fathead minnow stocked in an experimental lake and exposed during a 7 year study to a synthetic estrogen, 17α-ethynylestradiol (EE2) at 5-6 ng/L. High levels of EE2 led to the vitellogenin induction in males causing the formation of ova-testes in some animals along with lowered gonadosomatic indicies (GSIs) compared to reference fish, and testicular malformations (Kidd et al., 2007).

Findings in these studies suggest that bifenthrin may cause endocrine disruption resulting in estrogenic activity in the form of VTG production or change sex steroid levels.

Part 2: Introduction to the San Francisco Bay Delta and Salmonids

The San Francisco Bay Delta (SFBD) estuary system is home to a myriad of organisms including benthic invertebrates, endangered waterfowl, and many fish species. The estuary serves as a feeding ground for over a million migratory birds and also serves as a nursery for many species of juvenile fish (Cohen, 2000). Covering roughly 40% of California, it is also the largest estuary in western North America. Roughly half of the surface water supply comes from rain or snow in the Serra Nevada (Cohen, 2000). Much of this rain and snow is drained by the Sacramento and San Joaquin watersheds. In fact, each year, the Sacramento–San Joaquin watershed averages 30–40 km^3 of freshwater runoff from rain and snow (Knowles and Cayan, 2004).

Generally there are three different regions in the San Francisco Bay Estuary. The delta or upper bay are considered the lower salinity region and is the major source of freshwater in the system. The southern bay has much less freshwater input (only one-tenth as much as the northern bay) and generally has equivalent salinity as the ocean. Lastly, there is the Pacific ocean which is a source of not only oceanic water, but also marine life that is able to migrate from freshwater to saltwater and vice versa (Fig 1-3) (Cohen, 2000).

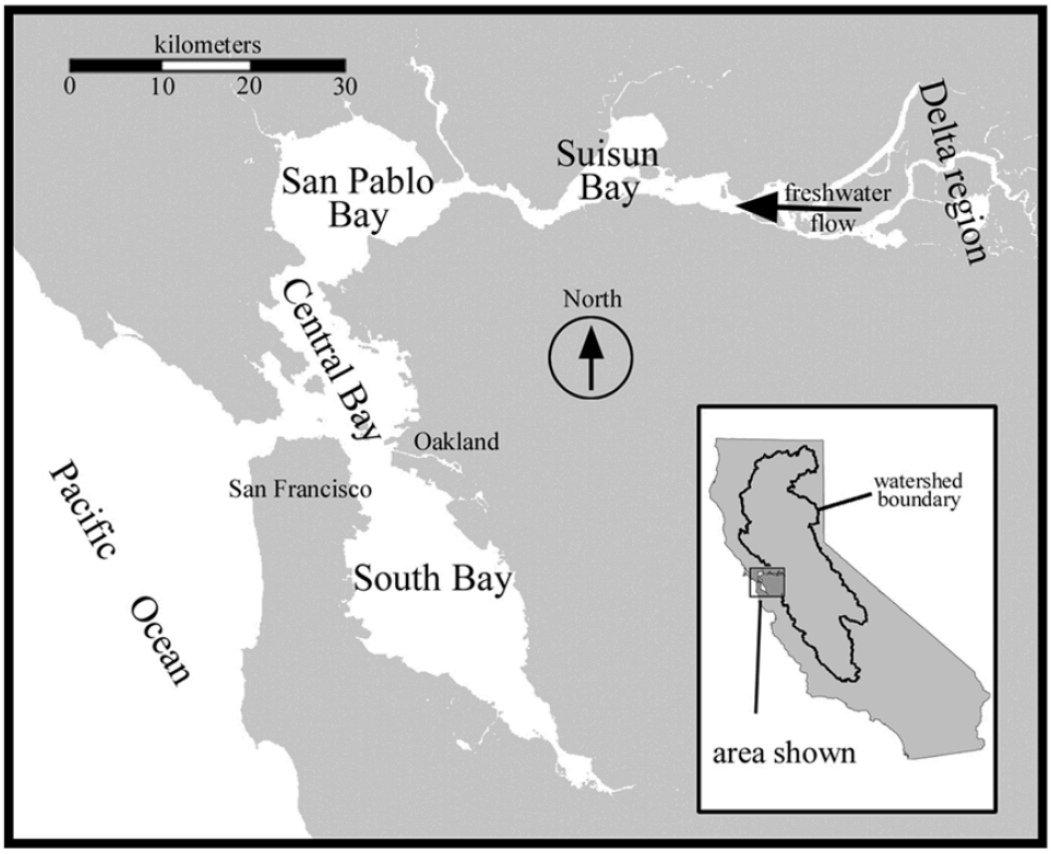

Figure 1-3. Map of the San Francisco Bay Delta Region (Knowles and Cayan, 2002).

Climate change and Hypersalinity in the San Francisco Bay Delta

The SFBD is a delicate balance of fresh and salt water. The mixing zone, where freshwater and saltwater meet, can move tens of miles upstream and downstream as river flows fall and rise (Cohen, 2000). Warm, dry summers and cool, wet winters lead to large changes in salinity throughout the year (Knowles and Cayan, 2002).

Climate change models predict a rise in temperature ranging from 1.4-5.8°C over the next 100 years (Knowles and Cayan, 2004). Increasing temperatures have

lead to the loss of alpine snow during the winter. It is expected that over the next 100 years, there will be a 60% reduction in snowpack (Knowles and Cayan, 2002; Knowles and Cayan, 2004; Howat and Tulaczyk, 2005). Increasing air temperatures have already led to earlier snowmelts causing freshwater flows to shift from spring to winter (Dettinger and Cayan, 1995). During the longer warmer seasons, much of the freshwater from snowmelts flushes out of the SFBD (Knowles and Cayan, 2004). As the inflow of snowmelt into the SFBD each year declines, seawater moves into the SFBD to replace it, leading to increased salinity during the dry season. With less and less inflow each year during the dry season, the salinity accumulates over time (Fig 1-4) (Short and Neckles, 1999).

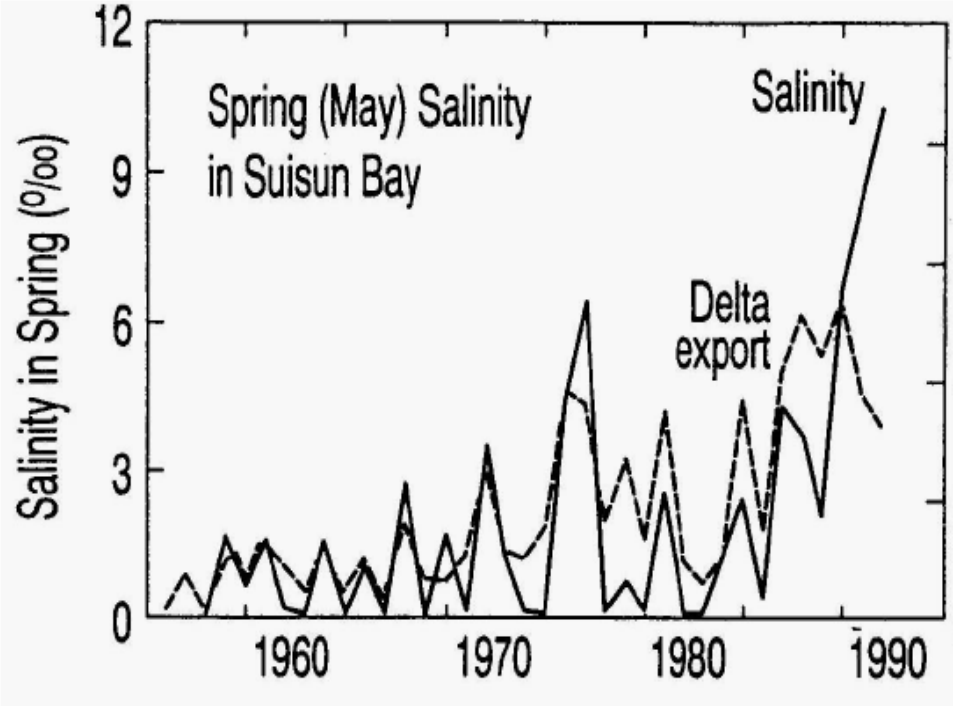

Figure 1-4. Salinity in Suisun Bay from 1941-1990 (Peterson et al., 1996).

Such an increase in salinity during the dry season could mean major impacts on the organisms of the SFBD (Short and Neckles, 1999). There are a large number of andramous, catadromous, and marine fishes that live in or frequent the SFBD (Hedgpeth, 1962). Moyle (2002) attributes the decline of pelagic fish populations to hypersaline conditions and modified water flow patterns. It is also expected that these conditions, along with increased temperatures, disturb spawning and larval transport (Bennett, 2005). Saline acclimation often results in physiological changes in affected species (i.e. anadromous fish) that enhance xenobiotic toxicity (Schlenk and Lavado, 2011). Lavado et al. (2009) observed that hypersalinity acclimation at 17 g/L increased the formation of fenoxon and fenthion sulfoxide from fenthion in rainbow trout. Hypersalinity, however, is not the only contributing factor to population decline. Human impacts on the SFBD system have also had lasting effects. It is especially detrimental that stressors including pesticide runoff and salinity changes occur in regions important for fish spawning and larval development (Brooks et al., 2012).

Salmonids of SFBD

Steelhead trout are the anadromous form of *Oncorhynchus mykiss,* also known as rainbow trout. They are an excellent example of a family of fish adopted for movement between both freshwater and saltwater along with other salmonids including *Oncorhynchus tshawytscha*, or Chinook salmon. Fry hatch in gravel streambeds where they remain for several months. Juveniles are referred to as

smolts and migrate downstream while feeding on insects, water fleas, amphipods and other crustaceans. Finally, after adjusting for life in saltier waters, the fish migrate to the ocean, where they remain for 2 to 4 years. Once mature, they travel back to their natal streams and spawn before dying (Cohen, 2000). Due to their migratory nature, steelhead trout and Chinook salmon traverse waterways throughout the SFBD. As previously mentioned, some of these spawning areas pass through 30 miles of urban development. Their migration through urban euryhaline waterways makes them ideal species to investigate the effects of salinity and pyrethroids in the estuary. Furthermore, pyrethroid pesticide usage has coincided with abrupt pelagic fish decline in these areas (Brooks et al., 2012)

Salinity effects on salmonid physiology

Smoltification is the establishment of strong hypoosmoregulatory capacity that takes place in anadromous fish species, including steelhead trout. During this process, the endocrine system drives a series of morphological, biochemical, physiological and behavioral adaptations that allow many salmonids to transition from freshwater to saltwater. This process involves increased salinity tolerance, a transition from territorial to schooling behavior, silvering of the body, and olfactory imprinting. During smoltification, one important endocrine pathway involves the increase in growth hormone (GH) and the steroid, cortisol. These in turn stimulate the development of chloride cells and cause changes in intestinal and gill osmoregulatory function (Björnsson et al, 2011).

Hypersaline acclimation increases cortisol levels in salmonids (McLean et al., 1997; Arjona et al., 2007). Cortisol induces the translation of CYP3A27 (Celander et al., 1999). In one study, expression of several CYP3A isoforms were also enhanced by hypertonicity in human derived cell lines and human primary colonic cells (Kosuge et al., 2007). Lavado et al. (2009) also demonstrated that hypersaline acclimation to 17 g/L induced CYP3A27 in *O. mykiss*. As demonstrated earlier, CYP3A has been shown to metabolize pyrethroids via hydroxylation (Scollon et al, 2009). It is these metabolites, which in turn can lead to estrogenic activity in fish (Wang et al, 2007; Nillos et al, 2010; Tyler et al, 2000). CYP3A is also important in steroid hydroxylation and clearance and therefore, changes in CYP levels may also affect sex steroid levels and associated pathways (Schlenk et al., 2008).

Hypothesis

Saline acclimation will induce an increase in CYP3A27 levels in salmonids. Since CYP3A produces hydroxylated pyrethroid metabolites in other vertebrates, and hydroxylated metabolites can cause estrogenic activity, an overall increase in estrogenic activity via VTG production will be observed in salmonids acclimated to hypersaline conditions. Changes in CYP levels may also affect circulating sex steroid levels. Furthermore, the enhanced biotransformation of bifenthrin will cause decreased acute toxicity in salinity acclimated fish. Overall, it is the objective of this study to investigate these claims and determine if estrogenic activity occurs salmonids at environmentally relevant concentrations of bifenthrin under hypersaline conditions as predicted by climate change.

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CHAPTER 2

American River Case Study: The Impact of Urbanization and the Pyrethroid Bifenthrin on Endocrine Disruption in Two Salmonid Species of the San Francisco Bay Delta

Abstract

Steelhead trout (*Oncorhynchus mykiss)* and Chinook salmon (*Oncorhynchus tshawytscha)* are both examples of salmonids adapted for movement between both freshwater and saltwater including the San Francisco Bay Delta. The American River, which is a part of this system, is a spawning and nursery habitat for these and other fish species and passes through over 30 miles of urban development that often supplies rainwater runoff containing pyrethroid pesticides to the waterways. In addition to being acutely toxic to aquatic non-target organisms, exposure to pyrethroids and their metabolites have been shown to have estrogenic effects in some fish species.

96-Hour laboratory exposures of bifenthrin (1 ng/L to 10 μg/L) were performed using steelhead trout and Chinook salmon to evaluate the effects of bifenthrin exposure on vitellogenin (VTG) protein levels and sex steroid (testosterone, 11-ketotestosterone, and 17β-estradiol) hormone concentrations. Both species were also exposed to American River storm water containing pyrethroids during a rain event with the same endpoints analyzed. No significant change in VTG or sex steroid levels was observed in any of the exposures in laboratory and field experiments. This suggests short-term low dose exposure to bifenthrin does not result in estrogenic activity in these two salmonid species.

Introduction

Steelhead trout *Oncorhynchus mykiss* and Chinook salmon (*Oncorhynchus tshawytscha*) have anadromous life histories. In each species, fry hatch in gravel streambeds where they remain for several months before migrating downstream as smolts. After adjusting for life in saltier waters, the fish migrate to the ocean, where they remain for 2 to 4 years. Once mature, they travel back to their natal streams and spawn (Cohen, 2000). Due to their migratory nature, steelhead trout and Chinook salmon traverse waterways including the San Francisco Bay Estuary system and its connecting delta. This area includes the American River located near Sacramento. The upper American River serves as a spawning area for each of these species of fish, but passes through over 30 miles of urban development, which provide a source of runoff that contains pyrethroid pesticides (Weston et al., 2009).

Historically, in the Central, Sacramento, and San Joaquin Valleys, pyrethroids were primarily used in agriculture (Oros and Werner, 2005). In California agriculture, some of the main crops using bifenthrin include almonds, cotton, corn, tomatoes and pistachios. Its usage in agriculture in 2010 resulted in 354,390 lbs being used over 660,368 acres (CDPR, 2012). Some pesticides can be transported to surface waters via the first flush (the first large runoff event in the winter), spring late-rainfall runoff events, tailwater return (direct runoff of irrigation return water), rice-field water release, and summer tailwater return (Kuilvia and Hladik, 2008). Urban runoff and water treatment facilities also are a major source for bifenthrin. Weston et al. (2010) found that bifenthrin was coming from an urban source in the

form of residential outfalls, especially during the wet season, after rainfall events. In these residential outfalls, the frequency of detection of bifenthrin was 79%. Of those detections, 58% were at concentrations at or exceeding the LC50 of the aquatic invertebrate, *Hyallela azteca* (Weston et al., 2010). Weston et al. (2009) observed dry season median and maximum bifenthrin concentrations of 4.6 and 14.2 ng/L, respectively. Dry season median and maximum values were 17.3 and 29.7 ng/L in urban creeks. Since wastewater treatment plants also discharge pyrethroids, it is possible that, "pseudo-persistent" exposure may occur in urbanized delta waterways. Pseudo-persistent and persistent pollutants have unique toxicological properties in that aqueous concentrations may not exceed acute toxicity thresholds, but continued exposure may lead to chronic sublethal effects that may impair developmental, endocrine, or reproductive endpoints. For example, the 96-hour LC50 for bifenthrin in *Oncorhynchus mykiss* is 150 ng/L (U.S. EPA, 2002) which is above the observed levels of bifenthrin in Northern California waterways (Weston et al., 2009). Chronic endpoints, however, are more difficult to quantify and categorize than direct lethality.

Recent studies have discovered that exposure to pyrethroids results in estrogenic activity in some species of fish (Nillos et al., 2010; Wang et al., 2007; Taylor et al., 2009). Vitellogenin (VTG) is an egg yolk precursor protein in oviparous fish species. In female fish, 17β-estradiol (E2) is the major hormone responsible for VTG production taking place in the liver (Sumpter and Johnson, 2005). Male fish and non-sexually mature juveniles may also produce VTG following exposure to E2

and other estrogens, thus production in male fish and juveniles can act as biomarkers for estrogenic exposure (Sumpter and Johnson, 2005; Hutchinson, 2006). Bifenthrin induced VTG in male Japanese medaka exposed to 10 μg/L concentrations for 10 days (Wang et al, 2007). However, typical aqueous concentrations of bifenthrin are in the ng/L range after a storm event (Weston et al, 2009; Weston et al, 2010).

The aim of this study was to evaluate the effects of bifenthrin exposure on estrogenic activity and steroid hormone concentrations in steelhead trout and Chinook salmon at environmentally relevant concentrations and during a storm water event to determine whether sublethal endocrine or reproductive effects occur in these two salmonid species that inhabit the American River.

Materials and Methods

Laboratory Fish Exposures

Juvenile *O. mykiss* (mean standard length 18.7 ± 2.2 cm and mean body weight 69.8 ± 19.9 g), hereon referred to as steelhead trout, were obtained from the Nimbus Hatchery (Folsom, CA). Upon acquisition, they were kept in a 530 L living stream tank by Fridge Units Inc (Toledo, OH) with carbon filtered municipal water at 11-12 °C. The fish were fed Silver Cup™ commercial feed (Murray, UT) every 48 hours and were kept on a 14-10 hour light-dark cycle. They were acclimated for approximately two weeks prior to use.

Individual steelhead trout were transferred to oxygenated 8 L replicate tanks. Fish were acclimated to these tanks for 48 hours prior to bifenthrin exposure. Replicate tanks (n=5-10) were exposed to nominal concentrations of 0.001, 0.01, 0.1, 1, 10 ug/L bifenthrin, 0.01% ethanol (EtOH) for the negative control, or 100 ng/L E2 for the positive control. Total water changes were performed every 24 hours for 96 hours. Fish were fed every 48 hours during the exposures just prior to the water changes to minimize the impacts of the bifenthrin adhering to the food particles. The same procedure was followed for juvenile *O. tshawytscha* (mean standard length 6.8 ± 0.56 cm and mean bodyweight 3.77 ± 0.92 g), hereon referred to as Chinook. Six fish were kept in each tank (n=5).

Field Exposures

Two locations were used for the field component of the study. The reference site was the Nimbus Hatchery in Folsom, California, hereon referred to as the

Nimbus site. The hatchery is operated by the Department of Fish and Game is located along the American River approximately 20 miles upstream of the downstream site. The hatchery also is upstream of the urbanized areas of Sacramento at the base of a dam (Folsom Reservoir) which is relatively free of pollution from urban runoff (Weston et al., 2009). The putative impacted site, Camp Pollock (Pollock site) is located near the American River-Sacramento River junction and is located downstream of downtown Sacramento and the surrounding suburbs.

The Nimbus site was equipped with large plastic raceways and a flow-through water system that provided individual tanks with running water from the American River and Folsom Reservoir. The temperature of the water is approximately 9°C in the winter months (Weston, unpublished data). Eight liter tanks were housed in the raceway, which also served as a water bath to maintain the tanks at a temperature comparable to the river. The Pollock site consisted of a temporary storage building housing water tables with flow-through water systems pumping water directly from the river. Similar to the Nimbus site, each water table provided individual tanks with running water (approximately 5 gallons per minute) and acted as a water bath to regulate the temperature.

Approximately 1 month prior to rainstorm events, replicate (n=10) 8 L tanks for each treatment (Nimbus and Pollock sites) were provided flow-through water lines at the Nimbus site where the fish were originally reared. Fish were fed daily and allowed to acclimate until one day prior to the rainstorm event. At this time, half

of the tanks were transported downstream for river water exposure, and the remaining fish were maintained at Nimbus under the same conditions at the downstream fish. Fish were exposed to runoff infused river water during and after the rainstorm event for approximately 4 days before the exposures were terminated.

Sample Collection and Analysis

At the end of the exposure period, blood was collected from fish using needles and heparinized syringes. Steelhead trout were dissected to determine sex by assessing macroscopic gonadal morphology. However, the small size of the Chinook prevented accurate sex determinations. Blood from the steelhead trout was separated by individual while blood from the Chinook was pooled for all individuals in each tank. The blood was centrifuged at 10,000 g and plasma was isolated and stored at -80 °C until analyzed. Plasma VTG protein levels were determined using a commercial *O. mykiss* VTG sandwich enzyme linked immunosorbant assay (ELISA) kit (Biosense Laboratories AS, NO) and the Coomassie Blue method with a commercial kit (Pierce Inc, Rockford, IL) with bovine serum albumin as the standard (Bradford, 1976). Testosterone (T), 17β-estradiol (E2), and 11-ketotestosterone (11-KT) steroid levels were determined using steroid specific commercial competitive ELISA kits by Cayman Chemical Company (Ann Arbor, MI). Prior to the steroid kit usage, plasma samples were extracted twice with approximately 3x diethyl ether and the organic layer collected and dried under a gentle stream of

nitrogen gas before being reconstituted in the steroid ELISA kit dilution buffer solution.

For the laboratory exposures, water samples from random tanks were taken on water change days to determine the measured bifenthrin concentrations for each treatment (Jiang et al., 2011; Gil-Garcia et. al., 2006). One liter of water was collected immediately from each tank after new water was added. From this sample, 500 mL was transferred to an amber 2 L bottle where methanol (MeOH) was added to a final 20% MeOH concentration. The mixture was percolated through solid phase extraction cartridges from Waters (Milford, MA) containing 360 mg of C18 silica (particle size 55-105 μm) at a flow rate of approximately 10 mL/min through a vacuum manifold system. The cartridges were preconditioned with 5 mL of MeOH, 5 mL of n-hexane, 5 mL of MeOH and 5 mL of Epure water. After the sample was passed through the cartridge, 200 mL of Epure water was passed through to eliminate salts and other contaminants from the cartridge. The cartridge was then dried for 30 minutes under vacuum before the retained analytes were eluted with 7 mL of hexane (Gil-Garcia et. al., 2006). The elution was dried down to near dryness and resuspended in 1 mL n-hexane before analysis by gas chromatography.

Quantification of the bifenthrin in the water extractions was carried out via gas chromatography using an Agilent 6890 GC equipped with a microelectron capture detector (Agilent, Wilmington, DE). Samples were introduced into the inlet at 250 ℃ in a pulsed splitless mode and the separation was achieved on a VF-5MS capillary column (30 m Å~ 0.25 mm Å~0.25 μm film thickness, with 10 m EZ-Guard

precolumn, Varian, Palo Alto, CA). The carrier gas was helium and the flow rate was 1.5 ml/min. The oven program was initially set at 80 ℃ for 1 minute, then increased to 300 ℃ at a rate of 15 per min, then held at 300 ℃ for 10 min. The detector temperature was set at 310 ℃ (Jiang et al., 2011). The mean retention time of bifenthrin was 15.59 minutes. The quantitation of bifenthrin was achieved through external calibration using standards of known concentrations. The detection limit was 0.005 μg/L.

Statistical Analysis

Statistical significance was assessed using one-way ANOVA (for three or more groups) or a two-sample t-test (for two groups). A p-value of less than 0.05 was considered statistically significant. If an overall significance was detected, Tukey's multiple comparison was performed to determine a difference among the groups. All data was analyzed prior to statistical analysis to ensure it met the criteria and assumptions for parametric tests. If data failed to meet these criteria, non-parametric tests including Kruskal-Wallis with Dunn's multiple comparison test or Mann Whitney were used (GraphPad Prism v 5.0; GraphPad Software Inc, San Diego, CA). For the VTG and steroid ELISA analysis, protein and/or steroid values below the minimum detection limit were considered to have 50% of the minimum detectable values (Noaksson et al., 2001; Burki et al., 2006).

Results

Laboratory Exposures-Bifenthrin Water Concentrations

The measured bifenthrin water concentrations were adjusted according to a recovery rate of 92% for the extraction method (Tables 2-1 & 2-2). One hundred percent mortality was observed in both steelhead and Chinook at the 10 µg/L exposure level. In the chase of the Chinook, the fish were dead before sufficient water samples could be collected. Recovery rate was determined by performing extractions on water samples of known bifenthrin concentrations and averaging the recovery rates of the different concentrations (Jiang et al., 2011).

Bifenthrin Concentration (µg/L)	
Nominal	Measured
0.001	0.006 ± 0.004
0.01	0.008 ± 0.002
0.1	0.044 ± 0.009
1	0.490 ± 0.036
10	4.886 ± 0.279

Table 2-1. Bifenthrin water concentrations (± standard deviation) for steelhead trout after 4-day laboratory exposures. A 92% recovery rate was applied to values determined by GC analysis to account for loss in during the extraction process.

Bifenthrin Concentration (µg/L)	
Nominal	Measured
0.001	0.002 ± 0
0.01	0.006 ± 0.002
0.1	0.085 ± 0.007
1	0.665 ± 0.174

Table 2-2. Bifenthrin water concentrations (± standard deviation) for Chinook after 4-day laboratory exposures. The 10 µg/L concentration for Chinook was not analyzed due to the lack of water samples after the rapid mortality of the fish. A 92% recovery rate was applied to values determined by GC analysis to account for loss in during the extraction process.

Laboratory Exposures-Vitellogenin

There was no significant difference in VTG levels among any of the bifenthrin treatment groups for both male and female steelhead trout (Fig 2-1). There was also no significant difference in VTG levels among any of the bifenthrin treatment groups for the Chinook (Fig 2-2). The VTG protein limit of detection was 0.39 ng/mL.

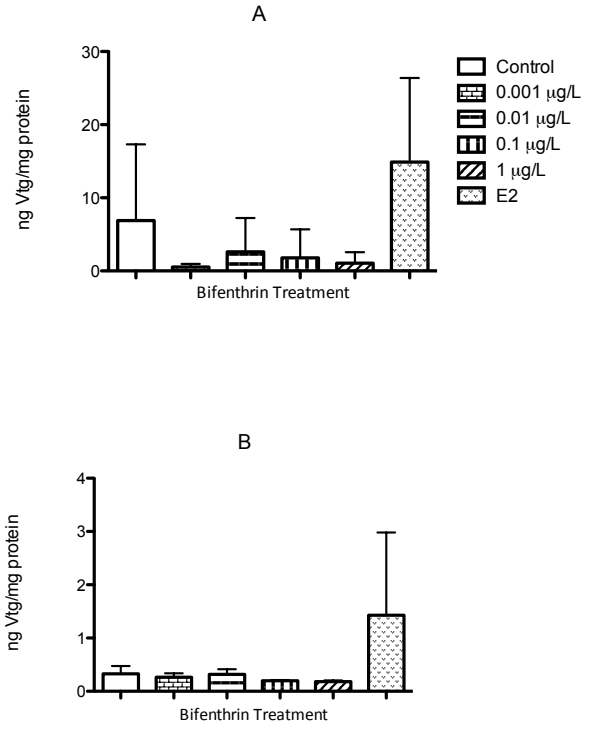

Figure 2-1. Effects of bifenthrin on vitellogenin plasma concentrations in female (A) and male (B) steelhead trout (*O. mykiss*) after 4-day laboratory exposures. No significant difference was observed in vitellogenin protein levels in both males and females in any bifenthrin treatment groups. Measurements that were below the limit of detection (0.39 ng/mL) were estimated using half of the detection limit.

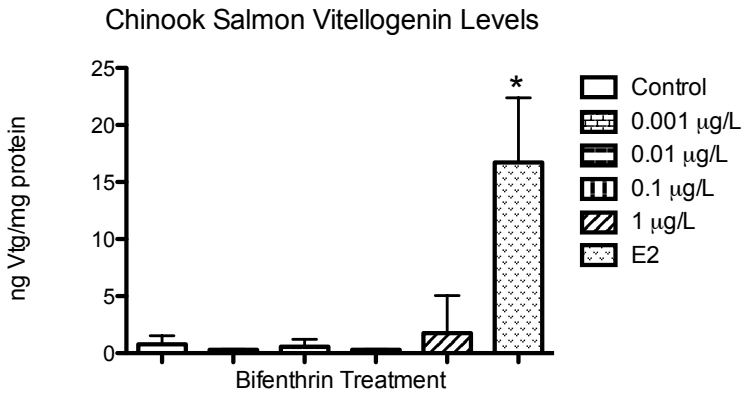

Figure 2-2. Effects of bifenthrin on vitellogenin plasma concentrations in Chinook salmon (O. tshawytscha) after 4-day laboratory exposures. No significant difference was observed in vitellogenin protein levels. Measurements below the limit of detection (0.78 ng/mL) were estimated using half of the detection limit. Significant differences was observed between the E2 positive control and the solvent control and is indicated by * (P=0.0216, Dunn's comparison test against the control).

Lab Exposures-Sex Steroids

There was no significant difference in T, E2, or 11-KT levels among any of the bifenthrin treatment groups for both male and female steelhead trout (Fig 2-3). Similarly, there was no significant difference in T, E2, or 11-KT levels among any of the bifenthrin treatment groups for Chinook (Fig 2-4).

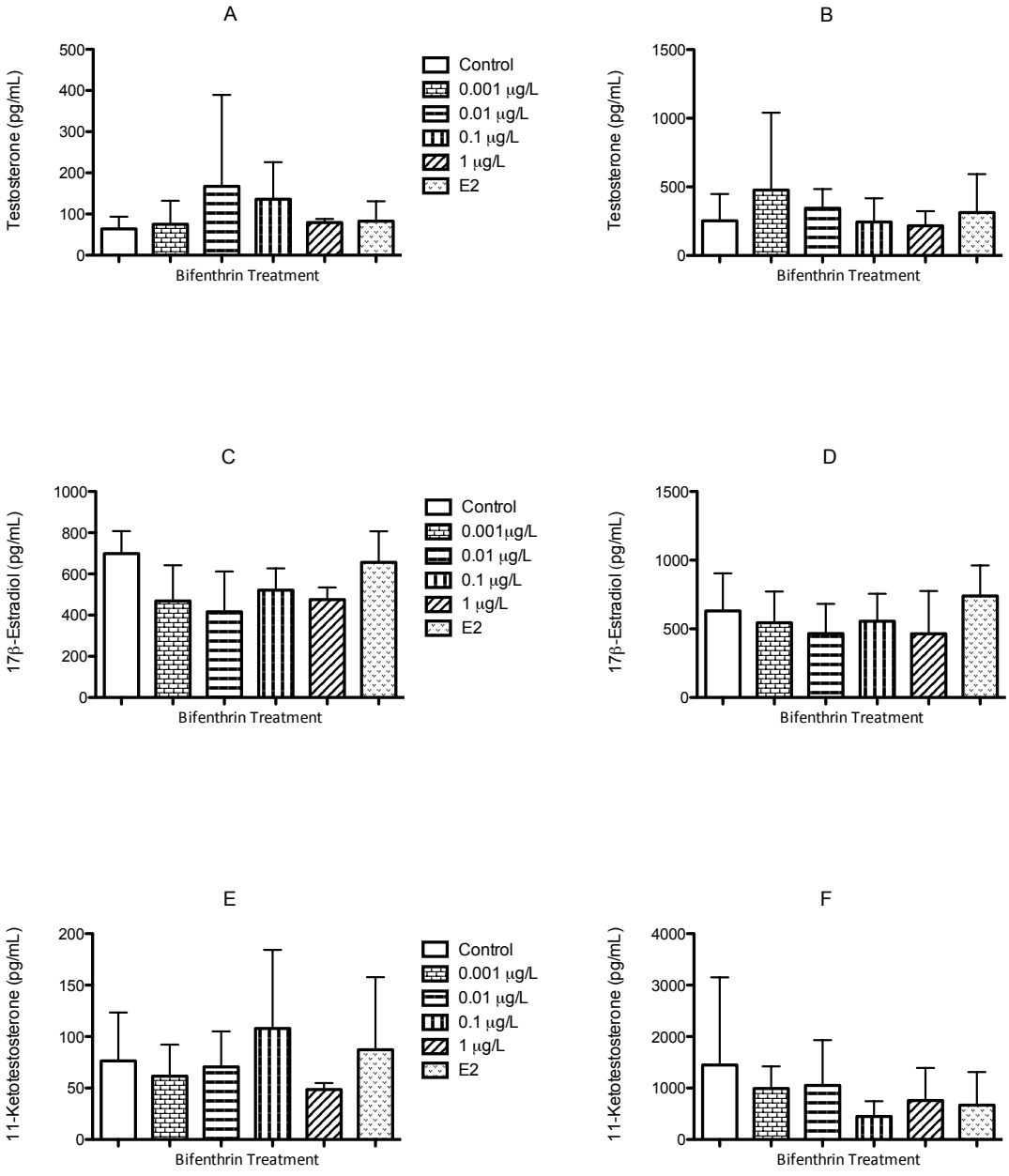

Figure 2-3. Effects of bifenthrin on plasma concentrations of sex steroids steelhead trout (*O. mykiss*) after 4-day laboratory exposures. (A) and (B) represent testosterone levels in females and males, respectively. (C) and (D) represent 17β-estradiol levels in females and males, respectively. (E) and (F) represent 11-keto-testosterone in females and males, respectively. Measurements below the detection limit were estimated using half the detection limit.

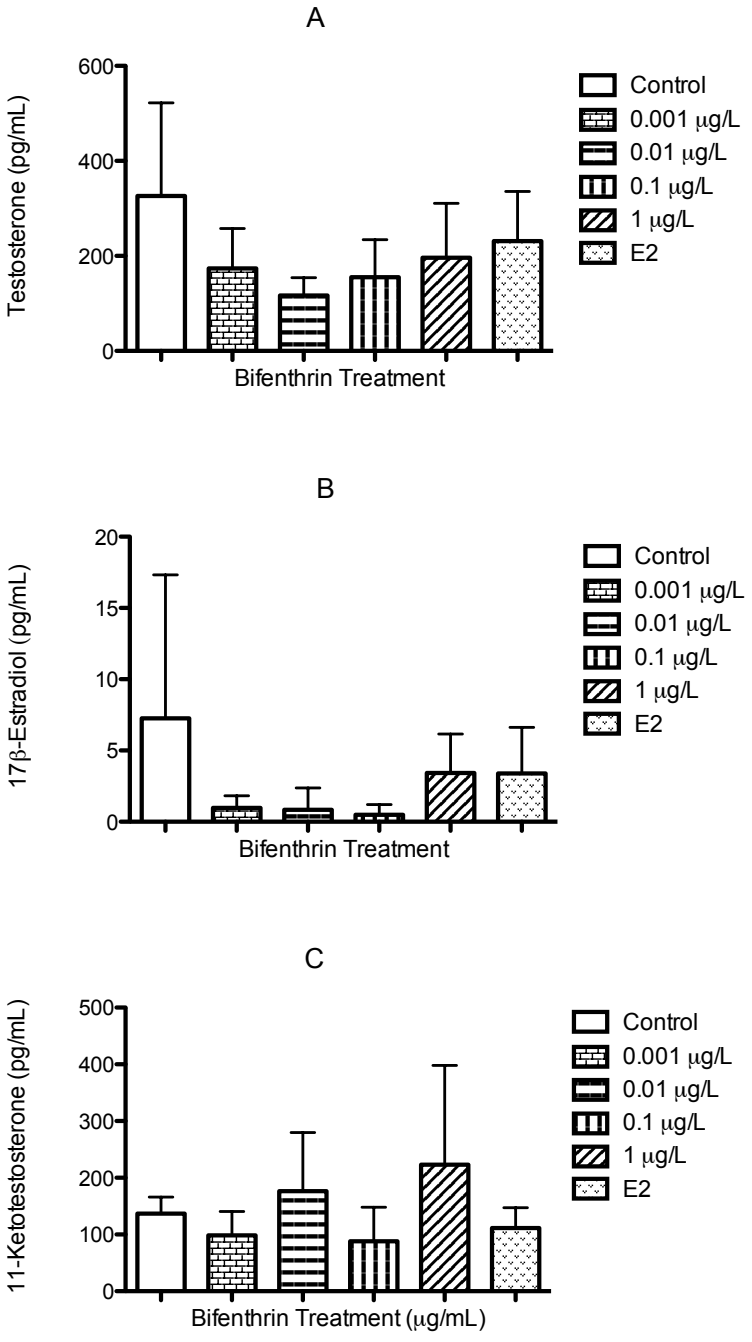

Figure 2-4. Effects of bifenthrin on plasma concentrations of testosterone (A), 17β-estradiol (B), and 11-keto-testosterone (C) in juvenile Chinook salmon (*O. tshawytscha*) after 4-day laboratory exposures. Measurements below the detection limit were estimated using half the detection limit.

Field Exposures-Vitellogenin

There was no significant difference in VTG levels among any of the river treatment groups for both male and female steelhead trout. The lack of an error bar in the female data for the Pollock group indicates that only a single female was identified in the treatment (Fig 2-5). There was also no significant difference in VTG levels among any of the river treatment groups for the Chinook (Fig 2-6). VTG protein limit of detection was 1.56 ng/mL.

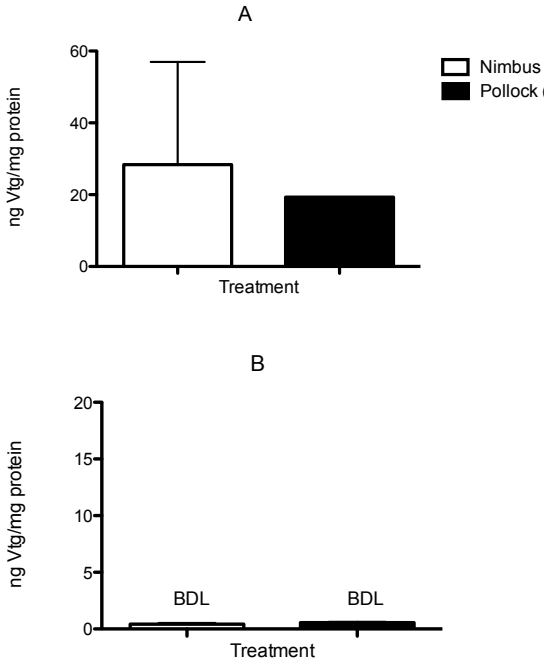

Figure 2-5. Vitellogenin plasma concentrations in female (A) and male (B) steelhead trout (*O. mykiss*) from field exposures. No significant difference was observed in vitellogenin protein levels in both males and females in any treatment groups. Measurements that were below the limit of detection (1.56 ng/mL) were estimated using half of the detection limit.

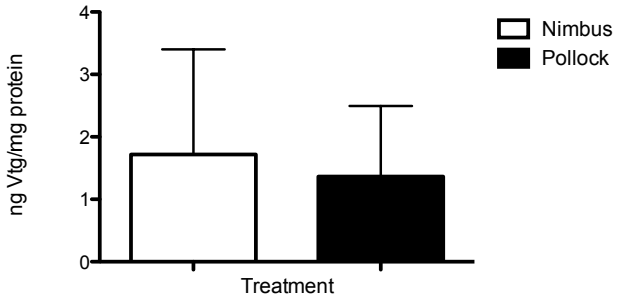

Figure 2-6. Pooled vitellogenin protein levels in Chinook blood plasma from field exposures. No significant difference was observed in vitellogenin protein levels. Measurements below the limit of detection (0.39 ng/mL) were estimated using half of the detection limit.

Field Exposures- Sex Steroids

There was no significant difference in sex steroid levels among any of the river treatment groups for both male and female steelhead (Fig 2-7). The T and 11-KT values in the female steelhead trout were all below the limit of detection. There was no significant difference in T, E2, or 11-KT levels among any of the treatment groups for Chinook (Fig 2-8).

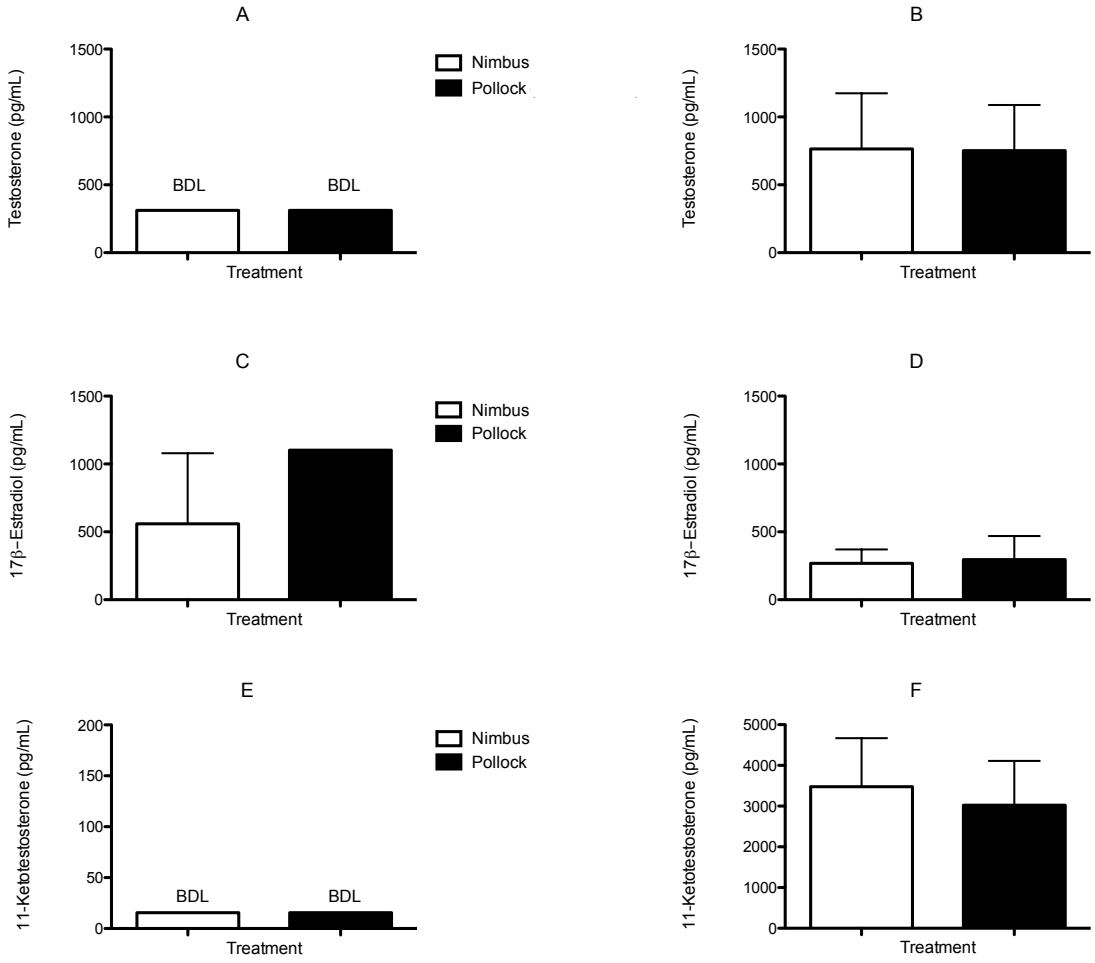

Figure 2-7. Effects of bifenthrin on plasma concentrations of sex steroids steelhead trout (*O. mykiss*) in field exposures. (A) and (B) represent testosterone levels in females and males, respectively. (C) and (D) represent 17β-estradiol levels in females and males, respectively. (E) and (F) represent 11-keto-testosterone in females and males, respectively. Measurements below the detection limit were estimated using half the detection limit.

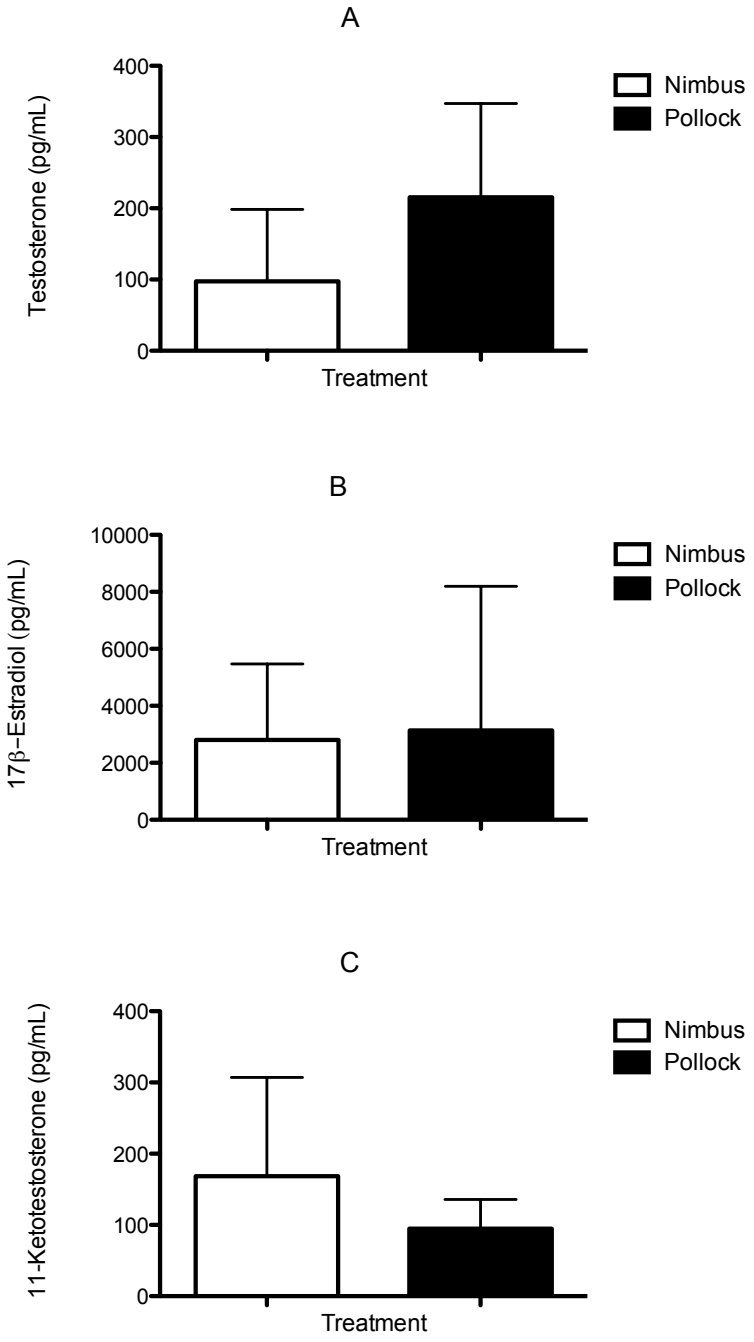

Figure 2-8. Effects of bifenthrin on plasma concentrations of testosterone (A), 17β-estradiol (B), and 11-keto-testosterone (C) in juvenile Chinook salmon (*O. tshawytscha*) after field exposures. Measurements below the detection limit were estimated using half the detection limit.

Discussion

Laboratory Exposures-Bifenthrin water concentrations

Random water samples were taken after each water change during the static exposure to determine the measured bifenthrin concentrations in the water. While most of the observed bifenthrin concentrations were below the expected values, measured concentrations were in the same order of magnitude as the nominal values. Due to the high water octanol coefficient (Kow), it is likely that some of the bifenthrin adhered to solid matter (food and waste) in the tanks as well as the walls of the aquaria themselves resulting in some deviation from the expected nominal values.

Vitellogenin

The 96-hour laboratory exposures and field exposures did not induce VTG protein in any of the bifenthrin treatment groups in neither the steelhead trout nor Chinook. In both species, the E2 positive control was successful in inducing VTG suggesting the pathway was indeed functional in both species. While this study failed to show estrogenic activity in the form of VTG protein induction, several other studies have observed these endocrine disrupting effects in fish from SFBD. Elevated levels of VTG were observed in juvenile striped bass (*Morone* saxitilis) collected from various sampling sites throughout the estuary (Spearow et al., 2011). A previous study also showed that water extracts from the Sacramento-San Joaquin River waterways of the San Francisco Bay Delta had estrogenic activity (Lavado et al., 2009). Water samples from 6 sites throughout the SFBD were extracted using

solid phase extraction and then exposed *in vitro* to rainbow trout hepatocytes for 24 hours. *In vivo* exposures were carried out by intraperitoneal injections on days 1 and 3 of a 7 day exposure. *In vitro* VTG mRNA and *in vivo* VTG protein levels in rainbow trout were estradiol equivalents up to 242 ng/L and 12 μg/kg, respectively.

Previous studies showed that the 2 bifenthrin enantiomers induced VTG in Japanese medaka (*Oryzias latipes*) exposed for 10 days at 10 μg/L (Wang et al., 2007). However, this concentration is much greater than those measured in the stormwater events or in ambient surface waters which are in the ng/L range (Weston et al., 2009). In Wang et al. (2007), VTG protein was measured and it was found that the 1S-*cis*-bifenthrin level averaged 1532 ng/mg protein while the 1R-*cis*bifenthrin averaged 12.45 ng/mg protein. While measurements in the current study were also made using VTG protein, mRNA expression may have been a more sensitive method for VTG measurement. VTG expression is transcriptionally regulated after E2 binds the estrogen receptor. The binding of E2 to the estrogen receptor is followed by dimerization with another receptor. This complex then translocates to the nucleus and binds to the estrogen response elements in the promotor region of the vitellogenin gene, resulting in transcription (Rempel and Schlenk, 2008).

Since mRNA expression precedes protein production, it may serve as an earlier indicator of estrogenic activity. Hemmer et al. (2001) observed dose dependent VTG mRNA synthesis during the initial 5 days of a 42 day exposure to different concentrations of the estrogen mimics nonylphenol (0.64 μg/L-42.7 μg/L),

methoxychlor (1.1-18.4 μg/L), and endosulfan (15.9-788 ng/L). After 5 days, the VTG mRNA levels remained relatively constant then diminished. VTG protein levels showed a dose dependent response up to day 42 of the exposure but often showed a delay in expression compared to the mRNA induction. This suggests that mRNA is more sensitive in the early days of an exposure. This study also suggests that in shorter term exposures, VTG protein may not be a suitable biomarker since adequate time may be necessary for bioactivation and subsequent estrogenic activity to occur, especially in compounds and metabolites that are already inherently lower in estrogenic potency. Many studies currently use VTG mRNA expression as an estrogenicity indicator with fathead minnow (*Pimephales promelas*) as the test organism (Kidd et al., 2007; Lattier et al., 2002). However, measurement of VTG mRNA also is not always an accurate indicator of VTG protein levels. A study by Folmar et al (2000) observed that exposures to E2, ethynyl estradiol and diethylstilbestrol in male sheepshead minnow (*Cyprinodon variegatus*), VTG mRNA levels did not correspond with VTG protein levels observed in 2 of the 3 other estrogens tested. Future studies may consider testing VTG mRNA synthesis for exposure concentrations with shorter durations of exposure.

Sex Steroids

Sex steroid concentrations in plasma from both steelhead trout and Chinook were unaffected by 4-day bifenthrin exposures. Sex steroid concentrations have been affected by other pesticides. Singh and Singh (2008) found a significant decrease in E2 and 11-KT levels in catfish (*Heteropneustes fossilis*) exposed for 45

days at 20 μg/L cypermethrin. Strong negative correlations of E2 to organochlorine pesticides, 11-ketotestosterone with phenols, and E2/11-KT ratios with total dissolved pesticides has been observed in common carp (*Cyprinus carpio*) sampled throughout the United States (Goodbred et al, 1997). One of the mechanisms proposed for sex steroid reduction is a reduction in gonadotropin levels, which in turn affects the production of steroids such as T (Singh et al., 1994). Another proposed mechanism is pesticide inhibition of aromatase, which is responsible for the conversion of T to E2 (Straube et al, 1999). Both of these proposed mechanisms focus on parts of the hypothalamic-pituitary-gonadal axis (HPG).

In this system, environmental cues are relayed to the hypothalamus and associated brain regions. Through a series of neurotransmitters, gonadotropin-releasing hormone, the primary neurohormone that controls reproduction, is released. This in turn stimulates the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that are responsible for sex steroid production (Thomas, 2008).

Due to their many functions in gametogenesis and sexual maturation, changes in sex steroid levels can have far reaching impacts in fish (Pandian and Sheela, 1995; Thomas, 2008). The importance of these sex steroids in early development (i.e., eyed eggs, alevin, and parr stages) of salmonids has been exemplified, in part, by their use in sex control, especially in aquaculture. Exposure during embryogenesis and early development to relatively high levels (2-50 mg E2/kg diet) of E2 can result in female sex reversal (Pandian and Sheela, 1995). T can

result in complete male sex reversal at doses of 55 mg T/kg diet (Pandian and Sheela, 1995). Kidd et al. (2007) significant declines of fathead minnow populations in experimental lakes dosed with environmentally relevant levels of the synthetic estrogen 17α-ethynylestradiol (EE2). EE2 had caused significant induction of VTG and intersex in many of the fish. Steroids play important roles in the timing and duration of reproductive processes including egg production in females and primary sexual characteristics in males (Thomas, 2008).

In conclusion, 96-hour laboratory exposures to environmentally relevant concentrations of bifenthrin did not affect estrogenic activity in the form of VTG protein induction or cause changes to sex steroid levels in either steelhead trout or Chinook. Field exposures of steelhead trout and Chinook to river rainwater suspected of containing bifenthrin also showed no effects. This data supports the conclusion that bifenthrin does not have endocrine disrupting effects in these two species after short term, low dose exposures.

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CHAPTER 3

Investigating Endocrine Disruption and Toxicity in *Oncorhynchus mykiss* Acclimated to Salinity and Exposed to the Pyrethroid Bifenthrin

Abstract

Bifenthrin is a pyrethroid pesticide commonly detected at ng/L concentrations in the San Francisco Bay Delta. It is acutely toxic in non-target aquatic invertebrates and salmonids. While acute lethality is of concern, far less understood are the sublethal effects on endocrine function. Previous studies have found that pyrethroids and their hydroxylated metabolites can result in estrogenic activity in the form of vitellogenin production and altered sex steroid levels. Salmonids also have the unique life history trait of moving from freshwater to saltwater which may enhance the formation of estrogenic hydroxylated metabolites of pyrethroids. Two populations of *Oncorhynchus mykiss* were acclimated to 0, 8, and 17 g/L salinity and then exposed them to 0, 0.1, and 1.5 μg/L bifenthrin for 14 days. The first population (Jess Ranch) was previously treated with 17β-estradiol (E2) to obtain a female population for the stocking of lakes and reservoirs in Southern California, while the second batch (Nimbus Hatchery) was hatched from wild trout which are released annually in San Francisco Bay Delta (SFBD) tributaries. Mortality, vitellogenin protein, and sex steroid levels were compared between the two populations. Livers from freshwater and saltwater animals were also used for *in vitro* microsomal incubations with bifenthrin to determine the percent conversion of the parent compound to metabolites. Compared to saltwater acclimated animals, mortality was significant higher in Jess Ranch fish maintained in freshwater exposed to the 1.5 μg/L bifenthrin treatment ($p<0.05$). However, in Nimbus fish, there were no significant differences in mortality between hypersaline

and freshwater animals treated with bifenthrin. There was no significant difference in vitellogenin levels in the plasma of the fish of either population in any of the treatments. A significant increase in E2 was observed in freshwater acclimated Nimbus fish exposed to 1.5 μg/L bifenthrin. Biotransformation studies showed that acclimation to saltwater had significantly reduced the conversion of bifenthrin to more polar metabolites in liver microsomes. Overall, no relationships were observed between biotransformation and mortality data in either freshwater or saltwater acclimated fish from both populations. However, this study indicated that embryonic exposure to E2 or other culturing practices can increase susceptibility to bifenthrin in freshwater and/or diminish protection by saltwater.

Introduction

Pyrethroids are a derivative of naturally occurring pyrethrin insecticides used in urban pest control and agriculture (Kuilvia and Hladik, 2008; Spurlock, 2008). Pet products and insect repellents for clothing also contain pyrethroids (Oros and Werner, 2005). Pyrethroids target the central nervous system by binding to voltage-gated sodium channels in neurons resulting in their prolonged depolarization, causing paralysis and death (Vijverberg and van den Bercken, 1990). In addition, pyrethroids inhibit Na, K, and Mg ATPase activity in some animals including fish (Roberts and Hutson, 1999; El-Toukhy and Girgis, 1993). The pyrethroid bifenthrin has consistently been measured in the ng/L range in Northern California waterways of the San Francisco Bay Delta (SFBD) (Weston et al., 2009).

Pyrethroids are generally considered safe for mammals. Due to their rapid metabolism and excretion from the body, pyrethroids tend to have lower toxicity in birds and mammals (Leahey, 1985). In contrast, bifenthrin has an LC50 in *Hyalella azteca* of 8 ng/L and 150 ng/L in *Oncorhynchus mykiss* (U.S. EPA, 2002). Sources of bifenthrin to surface water include urban runoff from storm events and wastewater discharge through treatment facilities (Weston et al. 2009). Given the continual output of pyrethroids into waterways through wastewater treatment plants, "pseudo-persistent" exposure is likely occur (Weston et al., 2009). "Pseudo-persistent" contaminants occur in waterways at concentrations that do not tend to have acute toxic effects. However, because of continual exposure, sublethal toxicity may occur, affecting developmental and reproductive endpoints that are more

difficult to quantify and categorize than direct lethality. Recent studies have discovered that exposure to pyrethroids results in estrogenic activity in some species of fish (Nillos et al., 2010; Wang et al., 2007; Beggel et al., 2011) and cell lines (Tyler et al., 2000). Hydroxylated metabolites of permethrin produced stereoselectively more estrogenic activity than the parent compound in liver microsomes of *O. mykiss* (rainbow trout) (Nillos et al., 2010). This study also observed that the formation of the metabolites was stereoselective with the 1S-*cis* enantiomer of permethrin having the greatest in vivo activity. Moreover, the 1S-*cis* enantiomer also had the greatest rate of conversion to hydroxylated metabolites. Estrogenic activity was measured using vitellogenin (VTG) production in male and sexually immature juveniles (Denslow et al., 1999).

VTG is the precursor protein to egg-yolk normally found in females. In female fish, 17β-estradiol (E2) regulates the transcriptional expression of VTG mRNA through activation of the estrogen receptor in the liver of oviparous animals (Sumpter and Johnson, 2005). Male fish and sexually immature juveniles may also produce VTG since they too have a receptor for E2. In this way, VTG production in male fish and juveniles can act as a biomarker for estrogenic exposure (Sumpter and Johnson, 2005; Hutchinson, 2006).

In addition to pesticide inputs, the SFBD also is facing threats due to global climate change. Increasing temperatures result in a loss of freshwater input into the SFBD in the form of snowmelt (Knowles and Cayan, 2002). Over time, this loss of freshwater input as well as freshwater removal for agriculture and transport to

Southern California has resulted in saltwater intrusion leading to increased salinity in the SFBD (Short and Neckles, 1999).

Saline acclimation is a normal life history strategy for salmonids in the SFBD. Saline conditions increase the hormone cortisol in the plasma of salmonids (McLean et al., 1997; Arjona et al., 2007). Cortisol in turn, induces CYP3A27 (Celander et al., 1999). CYP3A is the enzyme responsible for the hydroxylation and biotransformation of many xenobiotics, including pesticides (Parkinson and Ogilvie, 2008). As previously mentioned, hydroxylated metabolites of pyrethroids have the potential to have estrogenic effects. Thus, co-exposure to pyrethroids and hypersaline conditions could exacerbate estrogenic activities in areas where the combined exposure occurs.

CYP3A27 is also responsible for the hydroxylation and clearance of endogenous substances including sex steroid hormones. Major sex steroids in fish include testosterone (T), E2, and 11-ketotestosterone (11-KT) which are important in reproductive development among other things (Thomas, 2008). Changes in CYP3A27 or other CYP enzymes (i.e. aromatase) may affect steroid levels. Singh and Singh (2008) found a significant decrease in E2 and 11-KT levels in catfish exposed for 45 days to sublethal levels of cypermethrin. Negative correlations of E2 to organochlorine pesticides, 11-ketotestosterone with phenols, and E2/11-KT ratios with total dissolved pesticides has been previous observed in fish (Goodbred et al, 1997).

The objective of this study was to evaluate the toxicity of bifenthrin in two populations of *O. mykiss* in water and saltwater acclimation. In addition to acute toxicity, sublethal effects by measuring VTG production and sex steroid hormone concentrations will also be evaluated. Finally biotransformation studies using liver microsomes will be used to determine the relative conversion of bifenthrin to polar metabolites in fish undergoing saltwater acclimation.

Materials and Methods

Chemicals

Bifenthrin (99.1% purity, Z-cis-bifenthrin isomer mixture) was purchased from ChemService Inc (West Chester, PA). R-methyl(p)tolyl sulfoxide (MTSO) was obtained from Sigma Aldrich (St. Louis, MO). Ethanol, acetonitrile, n-hexane were all analytical grade (Fisher, Pittsburg, PA).

Fish acclimation and exposures

Juvenile *O. mykiss* (mean standard length 9.3 ± 1.0 cm and mean body weight 10.6 ± 3.4 g) were purchased from Jess Ranch Hatchery (Apple Valley, CA). These animals were exposed to an unknown concentration of E2 during the embryonic stage to produce an entirely female population (Pandian and Sheela, 1995). Upon acquisition, they were kept in a 530 L living stream tank by Fridge Units Inc (Toledo, OH) with carbon filtered municipal water at 11-12 °C. The fish were fed Silver Cup™ commercial feed (Murray, UT) every 48 hours and were kept on a 14-10 hour light-dark cycle. They were acclimated for approximately two weeks prior to use.

Fish were transferred to 8 L replicate tanks and acclimated to freshwater, 8, and 17 g/L salinity according to the procedures in Lavado et al. (2009). Briefly, fish were transferred to tanks starting at 4 g/L using a commercial salt mixture and acclimated for 48 hours (CrystalSea Marine Mix, Marine Enterprises International, Baltimore, MA). They were then transferred every 48 hours to 8, 12, and 17 g/L tanks until the desired salinity was achieved. They were kept at the final salinity for

1 week prior to bifenthrin exposure. The total salinity acclimation period lasted 14 days.

Bifenthrin exposures were carried out by exposing tanks acclimated to each salinity to the solvent control (ethanol 0.01%), 0.1 μg/L or 1.5 μg/L bifenthrin (n=3). Water changes and feedings were performed every 48 hours for 14 days. The same procedures were used for juvenile *O. mykiss* (mean standard length 9.6 ± 1.5 cm and mean body weight 10.6 g ± 3.4 g) obtained from the Nimbus Hatchery (Folsom, CA) (n=3).

Sample collection and analysis

Throughout the exposure period, mortality was recorded per tank every 24 hours. At the end of the exposure period, blood was collected from fish using needles and heparinized syringes. The blood was centrifuged at 10,000 g to obtain plasma which was then stored at -80 °C until analyzed. Plasma VTG protein levels were determined using an *O. mykiss* VTG sandwich ELISA kit (Biosense Laboratories AS, NO) and the Coomassie Blue method with a commercial kit (Pierce Inc, Rockford, IL) with bovine serum albumin as the standard (Bradford, 1976). The plasma was also used for sex steroid analysis by a collaborator, Kristy Forsgren. E2, T, and 11-KT steroid levels were determined using steroid specific commercial competitive ELISA kits by Cayman Chemical Company (Ann Arbor, MI). Prior to the steroid kit usage, plasma samples were extracted twice with approximately 3x diethyl ether and the organic layer collected and dried under a gentle stream of nitrogen gas before being reconstituted in the steroid ELISA kit dilution buffer solution.

Fish were dissected and livers collected for microsomal incubations. In the case of the Nimbus Hatchery fish, animals were sexed by comparing the macroscopic morphology of the gonads.

The concentrations of bifenthrin in water samples from the exposure tanks were determined using the solid phase extraction and gas chromatography coupled with electron capture detection (Jiang et al., 2011; Gil-Garcia et. al., 2006). Water samples from random tanks were taken on water change days to determine the measured bifenthrin concentrations for each treatment. One liter of water was collected from each tank and left undisturbed for several hours to allow solids to settle. Then 500 mL of this water was transferred to an amber 2 L bottle where methanol (MeOH) was added to a final 20% MeOH concentration. The mixture was percolated through solid phase extraction cartridges from Waters (Milford, MA) containing 360 mg of C18 silica (particle size 55-105 μm) at a flow rate of approximately 10 mL/min through a vacuum manifold system. The cartridges were preconditioned with 5 mL of MeOH, 5 mL of n-hexane, 5 mL of MeOH and 5 mL of Epure water. After the sample was passed through the cartridge, 200 mL of Epure water was passed through to eliminate salts from the cartridge. The cartridge was then dried for 30 minutes under vacuum before the retained analytes were eluted with 7 mL of hexane (Gil-Garcia et. al., 2006). The elution was dried down under a gentle stream of nitrogen gas and resuspended in 1 mL n-hexane before analysis by gas chromatography.

Quantification of the bifenthrin in the water extractions was carried out via gas chromatography using an Agilent 6890 GC equipped with a microelectron capture detector (Agilent, Wilmington, DE). Samples were introduced into the inlet at 250 ℃ in a pulsed splitless mode and the separation was achieved on a VF-5MS capillary column (30 m Å~ 0.25 mm Å~0.25 μm film thickness, with 10 m EZ-Guard precolumn, Varian, Palo Alto, CA). The carrier gas was helium and the flow rate was 1.5 ml/min. The oven program was initially set at 80 ℃ for 1 minute, then increased to 300 ℃ at a rate of 15 per min, then held at 300 ℃ for 10 min. The detector temperature was set at 310 ℃ (Jiang et al., 2011). The mean retention time of bifenthrin was 15.59 minutes. The quantitation of bifenthrin was achieved through external calibration using standards of known concentrations. The detection limit was 0.005 μg/L. Recovery rates of the extraction were determined by performing extractions on water samples of known bifenthrin concentrations. A recovery rate of 65.8% was observed for the 0.1 μg/L samples while a rate of 96.7% was observed for the 1.5 μg/L nominal concentrations.

Subcellular fractionation

Microsomes were isolated from livers of fish from freshwater and saltwater acclimations according to Lavado et al. (2009). Livers of fish from each tank were pooled and homogenized in 1:5 w/v cold 100 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4. The solution contained 100 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Homogenates were centrifuged at 12,000 g for 30 min. The supernatant was collected and then centrifuged at 100,000 g for 60 min to obtain microsomal

pellets. These pellets were resuspended in a small volume of the homogenization buffer with 20% (w/v) glycerol. Protein concentrations were determined by the Coomassie Blue method previously described (Bradford, 1976).

Bifenthrin Metabolism

Microsomes from the 0 and 17 g/L salinity acclimated control treatments were incubated with bifenthrin to determine conversion of the parent compound to polar metabolites. Briefly, 500 µg of protein were incubated with 500 µM bifenthrin, 2.5 mM nicotinaminde adenine dinucleotide phosphate (NADPH), and 100 mM Tris-HCl pH 7.4. Incubations were also carried out without NADPH and with boiled protein as negative controls. Samples (n=3) were incubated for a total of 90 minutes with NADPH added every 30 minutes. The reactions were stopped by adding an equal volume of acetonitrile. An internal standard of *R*-methyl(p)tolyl sulfoxide (1 mg/mL) was added to each sample after the incubation. The samples were then centrifuged for five minutes at 13,000 g and the supernatant collected and injected (40 µL) into the HPLC system.

HPLC analysis was carried out on a SCL-10AVP Shimadzu system equipped with a 250x4.6 mm Hypersil ODS C18 (5 µm) reverse phase column (Thermo Scientific, Waltham, MA). Separation of bifenthrin metabolites employed a gradient system elution at a flow rate of 1 mL/min with a mobile phase composed of (A) 90% acetonitrile and 10% water and (B) water brought to pH 1.7 with phosphoric acid. The run consisted of a 45 minute linear gradient from 100% A to 70% B. Peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 230 nm. Bifenthrin

was quantified by co-elution with authentic standard. The elution time of bifenthrin was 23.7 min.

Positive controls included phenobarbital induced rat liver supersomes from XenoTech (LLC, Kansas City, KS) and human liver microsomes that were a gift from Alan Rettie from the University of Washington. Incubations with fish microsomes were carried out at 25 °C while mammalian microsomal incubations were carried out at 37 °C. The integrated peak area of bifenthrin in the boiled protein controls was used as a baseline to determine the percent reduction of bifenthrin in incubations both with and without NADPH in humans, rats, fish.

Statistical Analysis

Statistical significance was assessed for some of the data using one-way ANOVA. A p-value of less than 0.05 was considered statistically significant. If an overall significance was detected, Tukey's multiple comparison was performed to determine a difference among the groups. All data was analyzed prior to statistical analysis to ensure it met the criteria and assumptions for parametric tests. If data failed to meet these criteria, non-parametric Kruskal-Wallis and Dunn's multiple comparison test were used. A two-way ANOVA's with factorial design was used when two factors were being investigated. If an overall significance was detected, a Bonferonni multiple comparison was used for the two-way ANOVA's. (GraphPad Prism v 5.0; GraphPad Software Inc, San Diego, CA).

Results

Bifenthrin water concentrations

The measured concentrations were adjusted according to recovery rates of 65.8% for the 0.1 µg/L samples and 96.7% for the 1.5 µg/L exposures. The measured concentration for the Jess Ranch fish at the nominal concentration of 0.1 µg/L was 0.250 ± 0.041 µg/L. The measured concentration for fish at the nominal 1.5 µg/L concentration was 1.072 ± 0.348 µg/L. The control treatment measured concentrations were below the detection limit of 0.005 µg/L. The measured concentration for the Nimbus Hatchery fish at the nominal concentration of 0.1 µg/L was 0.030 ± 0.016 µg/L. The measured concentration for fish at the nominal 1.5 µg/L concentration was 0.608 ± 0.182 µg/L. The control treatment measured concentrations were again below the detection limit of 0.005 µg/L.

Mortality

Jess Ranch fish showed no significant difference in survival at 8 and 17 g/L in all bifenthrin treatment groups. However, a significant decrease ($p=0.0336$) was observed at the 1.5 µg/L bifenthrin concentration with in the freshwater (0 g/L) treatment. Significant mortality was not observed in Nimbus fish (Fig. 3-1)

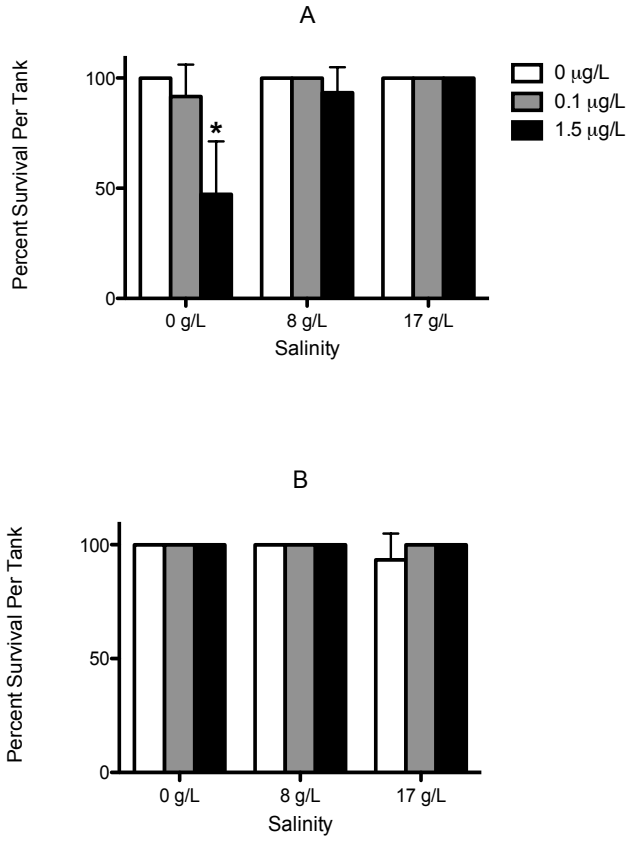

Figure 3-1. Survival of Jess Ranch (A) and Nimbus Hatchery (B) *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. Data as expressed as mean percent survival ± SD of fish per tank (n=5 fish per tank, 3 replicates). Significant differences in the 0 g/L salinity with respect to bifenthrin concentration are indicated by * ($P<0.05$, Dunn's multiple comparison test).

Vitellogenin

There was no significant difference in VTG protein levels in either Jess Ranch or Nimbus Hatchery fish. VTG protein values for steelhead trout were often below the limit of detection (0.39 ng/mL) and were estimated using 50% of the detection limit (Fig 3-2)

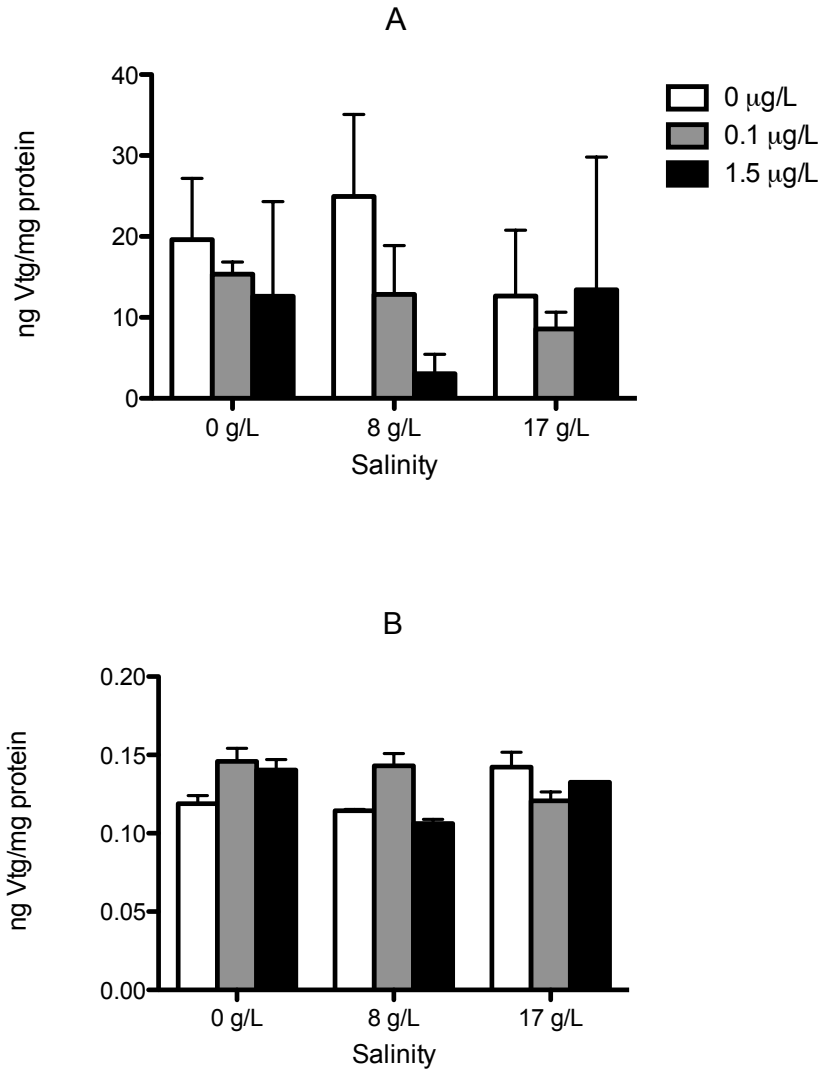

Figure 3-2. Vitellogenin plasma concentrations in Jess Ranch (A) and Nimbus Hatchery (B) *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. No significant difference was observed in Jess Ranch fish protein levels. Most Nimbus fish protein measurements were below the limit of detection (0.39 ng/mL) and thus estimated using half of the detection limit.

Sex Steroid Levels

There was no significant difference in T, E2 or 11-KT plasma levels in any treatment group for the Jess Ranch fish (Fig 3-3). There does, however appear to be a trend of decreasing E2 levels upon bifenthrin exposure at each salinity level (B).

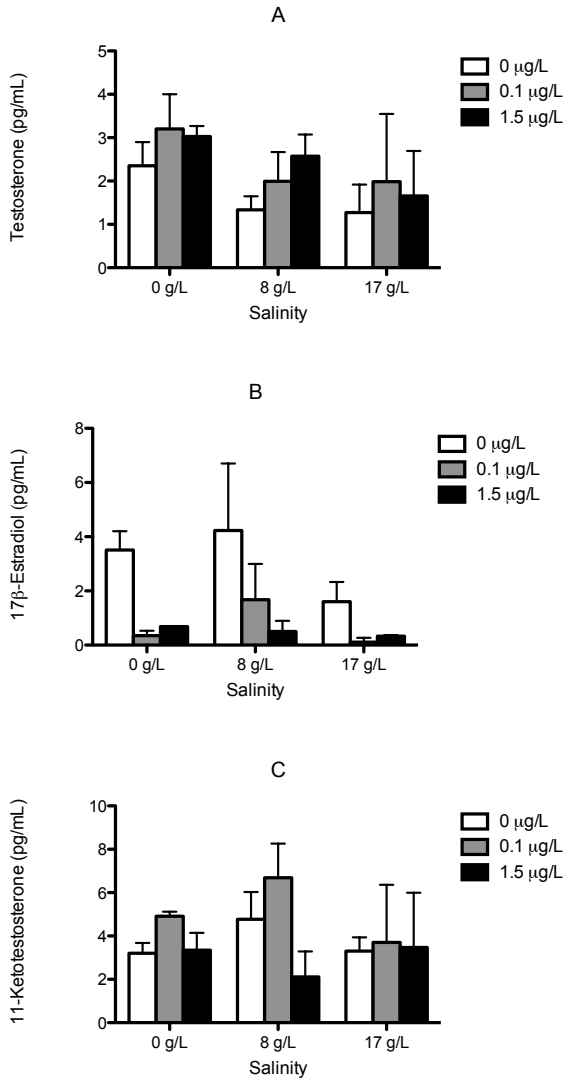

Figure 3-3. Sex steroid levels of testosterone (A), 17β-estradiol (B), and 11-ketotestosterone (C) in Jess Ranch *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. No significant difference was observed in any of the treatment groups.

For the Nimbus Hatchery fish, no significant difference was observed in T and 11-KT plasma levels. There was, however a significant ($p>0.05$) difference in E2 levels in the 0 g/L salinity acclimated fish exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations (Fig 3-4).

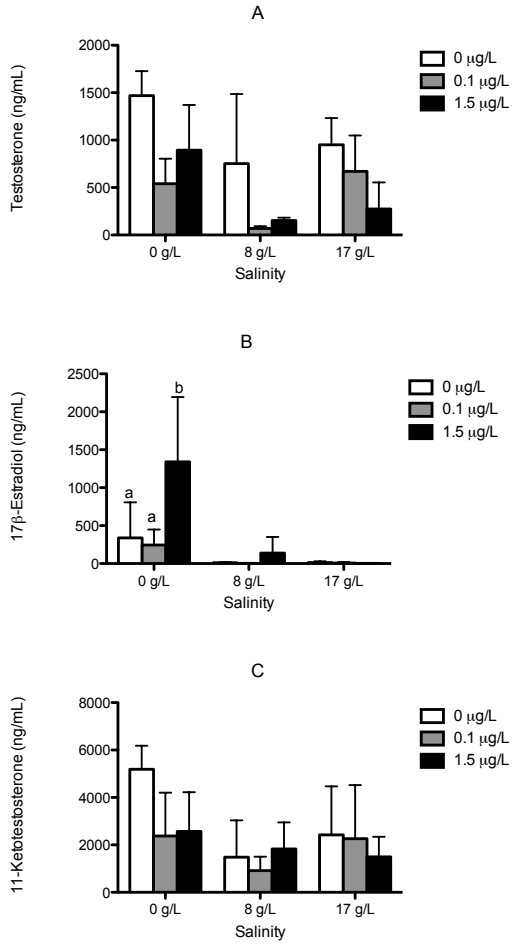

Figure 3-4. Sex Steroid levels of testosterone (A), 17β-estradiol (B), and 11-ketotestosterone (C) in Nimbus Hatchery *O. mykiss* in freshwater, 8, and 17 g/L salinity exposed to 0, 0.1, and 1.5 µg/L bifenthrin concentrations for 14 days. A significant difference was observed in the freshwater 0 and 0.1 µg/L bifenthrin exposure and the 1.5 µg/L exposure (Salinity-Bifenthrin p-value = 0.0007, bifenthrin p-value = 0.0009, salinity p-value < 0.0001, Bonferroni multiple comparison).

Bifenthrin Metabolism

NADPH-dependent conversion of bifenthrin to polar metabolites was higher in rat supersomes followed by human microsomes and then freshwater trout. Conversion was not detected in salinity acclimated fish from Jess Ranch or freshwater and saltwater acclimated fish from Nimbus (Fig 3-5). A t-test reported a significant difference between the freshwater and saltwater acclimated Jess Ranch fish (p-value=0.0271).

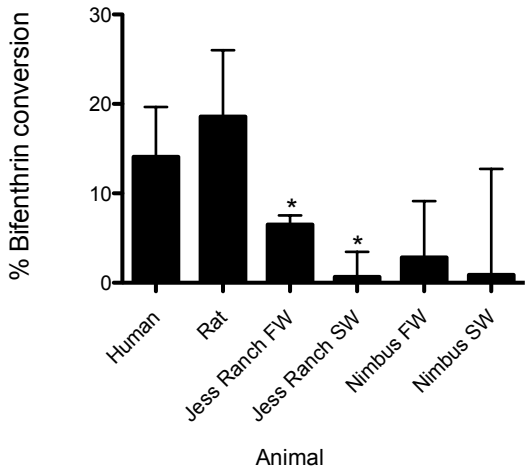

Figure 3-5. Percent conversion of bifenthrin in microsomal incubations containing NADPH compared to boiled protein controls (n= 3 replicate incubations per animal treatment). The * indicates a significant difference between the freshwater and saltwater acclimated Jess Ranch fish (p-value=0.0271, unpaired t-test).

Discussion

Laboratory Exposures-Bifenthrin water concentrations

The aim of the present study was to understand the lethal and sublethal effects of environmentally relevant bifenthrin exposure on juvenile *Oncorhynchus mykiss* acclimated to hypersaline conditions. The water concentration data revealed that measured concentrations were lower than the nominal values but in the same order of magnitude. The high water octanol (Kow) value of $1x10^{-6}$ likely resulted in bifenthrin adhering to biosolids in the water or with the sides of the aquaria (Fecko, 1999). Furthermore, during the water extraction process, it was discovered that the complete drying down of the sample after elution resulted in irreversible adhesion of bifenthrin to the sample tubes and thus needed to be avoided.

Acute Toxicity

Significant mortality occurred in the Jess Ranch fish exposed to the 1.5 µg/L in freshwater but no mortality was observed in Nimbus Hatchery fish. The initial hypothesis tested was that salinity acclimation detoxifies bifenthrin through enhanced biotransformation to non-toxic metabolites. Saltwater acclimation at 17 g/L has been shown to increase CYP3A27 in salmonids (Lavado et al., 2009). CYP3A carries out biotransformation of pyrethroids to unknown metabolites in mammals (Scollon et al., 2008). Increased levels of CYP3A in the saltwater acclimated fish then could potentially enhance the metabolism of bifenthrin to metabolites that do not cause neurotoxicity, thus reducing lethality.

Bifenthrin Metabolism

Liver microsomes from humans, rats, and freshwater fish (Jess Ranch) catalyzed NADPH-dependent turnover of bifenthrin. However, limited biotransformation was observed in either strain of fish and it was not induced by saltwater but diminished in Jess Ranch fish. A previous study performed a 7-day bifenthrin exposure in rats by giving single and or repeated doses of 4 or 35 mg/kg body weight. It was observed that most radiolabeled bifenthrin was removed in the urine (~21%) or feces (~69%) with a portion being converted to various metabolites. Some of the major metabolites in feces and urine included hydroxymethyl-bifenthrin, 4′-hydroxy-bifenthrin, 3′-hydroxy-hydroxymethyl-bifenthrin, 4′-hydroxy-biphenyl acid, and 4′-hydroxy-biphenyl alcohol and accounted for approximately 30% of the radiolabeled bifenthrin. In our study, the metabolites remain to be identified. Estrogenic metabolites of another pyrethroid, permethrin have been previously observed in *in vitro* and *in vivo* studies (Nillos et al., 2010; Wang et al., 2007;Tyler et al., 2000). Tyler et al. (2000) evaluated the affinity of permethrin metabolites for steroid receptors *in vitro* using genetically modified yeast containing human estrogen and androgen receptor. 3-Phenoxybenzyl alcohol exhibited estrogenic and antiandrogenic activity while 3-phenoxybenzoic acid and permethrin cyclopropane acid both had antiestrogenic activity. Nillos et al. (2010) observed that hydroxylated metabolites (4-hydroxy-permethrin) and ester cleavage products (3-phenoxyl benzyl alcohol 3-(4-hdroxy-phenoxy) benzyl alcohol) were found to increase VTG mRNA expression in primary

rainbow trout hepatocytes. Given that VTG expression was unchanged in the current study in both species under both salinity regimes, hepatic biotransformation of bifenthrin to less acutely toxic and more estrogenic metabolites does not appear to be a valid mechanism explaining the differences in toxicity.

Vitellogenin and Sex Steroids

VTG was unaltered by bifenthrin or hypersaline conditions. A previous study by Wang et al. (2007) observed a 123 fold increase in VTG mRNA expression in Japanese medaka exposed to 10 μg/L for 10 days. Another study on zebrafish found a 6 fold increase in VTG mRNA after a 72 hour exposure to 150 μg/L bifenthrin and an almost 12 fold increase after 96 hours (Jin et al, 2009). However, the concentrations of bifenthrin were in the μg/L range and the duration of exposures ranged from 4 to 10 days. While it was postulated that conversion to estrogenic metabolites was responsible for the estrogenic activity, another possible mechanism may be the enhanced biosynthesis or reduced clearance of E2. Although not statistically significant, there was a trend towards E2 reduction with increased salinity acclimation and bifenthrin exposure in the Jess Ranch fish. Singh and Singh (2008) found a significant decrease in E2 and 11-KT levels in catfish (*Heteropneustes fossilis*) exposed for 45 days at 20 *μ*g/L cypermethrin. The gonadosomatic index was also found to have decreased in these fish. Strong negative correlations of E2 to organochlorine pesticides, 11-ketotestosterone with phenols, and E2/11-KT ratios with total dissolved pesticides has been observed in common carp (*Cyprinus carpio*) sampled throughout the United States (Goodbred et

al, 1997). One of the mechanisms proposed for sex steroid reduction is a reduction in gonadotropin levels, which in turn affects the production of steroids such as T (Singh et al., 1994). Another proposed mechanism is pesticide inhibition of aromatase, which is responsible for the conversion of T to E2 (Straube et al, 1999). There was a significant increase in E2 in the Nimbus Hatchery freshwater acclimated fish exposed to 1.5 µg/L bifenthrin treatment. However, VTG was unchanged in the fish with elevated E2. One possible explanation for these data may be that a longer duration of exposure is necessary to enhance body burdens of E2 to elicit VTG protein formation. Consequently, VTGmRNA may have been a more sensitive measure. For example, Hemmer et al. (2001) observed dose dependent VTG mRNA synthesis during the initial 5 days of a 42 day exposure to different concentrations of the estrogen mimics nonylphenol (0.64 μg/L-42.7 μg/L), methoxychlor (1.1-18.4 μg/L), and endosulfan (15.9-788 ng/L). After 5 days, the VTG mRNA levels remained relatively constant then diminished. VTG protein levels showed a dose dependent response up to day 42 of the exposure and often showed a delay in expression compared to the mRNA induction. This study suggests that in shorter term exposures, VTG protein may not be a suitable biomarker since adequate time may be necessary for bioactivation and hence estrogenic activity to occur, especially in compounds and metabolites that are already inherently lower in estrogenic potential. Many studies currently use VTG mRNA expression as an estrogenicity indicator with fathead minnow (*Pimephales promelas*) as the test organism (Kidd et al., 2007; Lattier et al., 2002).

Differences in toxicity between the populations could not be explained by biotransformation. Thus, it is possible that differing culture methods may contribute to the Jess's Ranch sensitivity. Jess Ranch fish underwent embryonic exposure to estradiol (E2) in order to maintain a uni-gender stock of rainbow trout for annual replenishment only in freshwater reservoirs and lakes in Southern California. Exposure during embryogenesis and early development to relatively high levels (2-50 mg E2/kg diet) of E2 can result in female sex reversal (Pandian and Sheela, 1995). This is standard practice for stocking freshwater bodies of water that do not have temperature regimes appropriate for reproduction. In addition, although genetically capable, the fish never undergo saltwater acclimation. In contrast, Nimbus Hatchery fish are released in freshwater, but are allowed to undergo migration to saltwater as steelhead trout. One possible outcome of embryonic exposure to E2 may be the imprinting of animals and impairing osmoregulatory acclimation. E2 significantly reduces Na+/K+ ATPase activity in salmonids (Madsen and Korsgaard, 1989). Na+/K+ ATPase is a critical enzyme in salmonids undergoing saltwater acclimation, and is significantly induced during the smoltification process. (Björnsson et al, 2011). During smoltification growth hormone (GH) and cortisol increases stimulate the development of chloride cells whose function is driven largely by NA+/K+ ATPase (Björnsson et al, 2011). Chloride cells actively remove chloride ions by first creating a an electrochemical gradient outside of the cell using sodium potassium ATPase (Na+/K+ ATPase) which in turn causes chloride to enter the cell via a Na-K-2Cl cotransporter (Hill et al,

2008). Finally the chloride gradient inside cell allows diffusion of chloride out of the cell through a chloride channel (Fig 3-7).

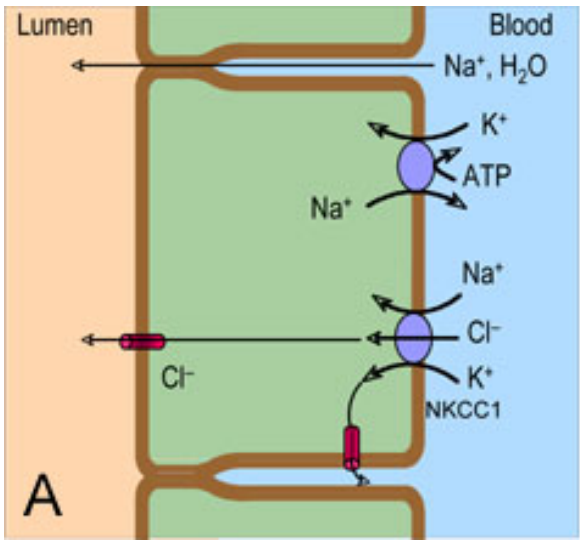

Figure 3-7. A diagram showing the function of a chloride cell (From http://surgicalresearch.bsd.uchicago.edu/faculty/matthews/index.html).

Since some pyrethroids can inhibit Na+/K+ ATPase (Roberts and Hutson, 1999), animals acclimated to hypersaline conditions may allow Na+/K+ ATPase to divert pyrethroids away from the voltage-gated sodium channel and protect against acute toxicity. Exposure to E2 during embryonic development could have resulted in diminished Na+/K+ ATPase which might remove the "buffer" in the Jess Ranch fish and explain the greater toxicity in freshwater. Additional studies are necessary to confirm this hypothesis.

In summary, the salinity enhanced acute toxicity of bifenthrin in Jess's Ranch fish but not in Nimbus fish was novel and requires additional study. Hepatic biotransformation does not appear to contribute to the detoxification of bifenthrin

in saltwater acclimated fish. The lack of VTG induction in either species under freshwater or saltwater conditions also do not support bifenthrin biotransformation *in vivo* and the formation of metabolites that have estrogenic activity. Further studies exploring the impacts of embryonic exposure to E2 and its effects on smoltification in salmonids is necessary to better understand the lethal and sublethal impacts of bifenthrin in salmonids.

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Conclusions

The purpose of this project was to determine the endocrine disrupting effects of the pyrethroid bifenthrin upon sublethal exposure in salmonids. It was also the goal of this project to determine the effects of bifenthrin coupled with hypersalinity acclimation as these conditions are relevant to anadromous salmonid populations in Northern California waterways.

The first project compared 96-hour laboratory and field exposures of bifenthrin and pyrethroid containing storm water runoff, respectively. There was no endocrine disruption in the form of vitellogenin induction or changes in sex steroid levels in either experiment both in steelhead trout and Chinook salmon.

The second project compared acute toxicity, vitellogenin induction, sex steroid levels, and bifenthrin biotransformation in two populations of *O. mykiss.* The first population (Jess Ranch) was previously treated with high levels of E2 to obtain uni-gender populations for predominantly freshwater stocking, while the second population (Nimbus Hatchery) was cultured without hormone treatment. The hormone (E2) treated population exhibited a significant decrease in survival at the highest bifenthrin treatment in freshwater. There was no significant difference in vitellogenin induction in either of the populations. A trend of decreased E2 levels was observed in the Jess Ranch fish upon bifenthrin treatment at each salinity. The Nimbus Hatchery fish, on the other hand showed a significant increase in E2 only at the highest bifenthrin treatment in freshwater. Biotransformation studies found little reduction of the parent compound in both freshwater and saltwater acclimated

fish of both populations when compared to the human and rat positive controls. This suggests that metabolism is not a major route of detoxification of bifenthrin in *O. mykiss*. Overall, it can be concluded that fish treated with E2 during early embryonic development are more sensitive to the toxic effects of bifenthrin but more research is needed to elucidate the mechanism of action.