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The Effects of Temperature, Salinity, and Bifenthrin on the Behavior and Neuroendocrinology of Juvenile Salmon and Trout

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of the requirements for the degree of

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by

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DEDICATIONS

I dedicate this dissertation to my mother, my first woman in science role model, who never let me forget my goals and showed me how to be strong. I also dedicate this dissertation to my grandmothers, who demonstrated to me true inner strength.

ABSTRACT OF THE DISSERTATION

The Effects of Temperature, Salinity, and Bifenthrin on the Behavior and Neuroendocrinology of Juvenile Salmon and Trout

by

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Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, June 2019
Dr. Daniel Schlenk, Chairperson

The San Francisco Bay-Delta in California has been experiencing increasing surface water temperatures, salt water intrusion, and intense runoff events that wash pyrethroids into waterways. Salmonid populations in the Bay-Delta have been declining, including the endangered Steelhead trout (*Oncorhynchus mykiss)* and Chinook salmon (*Oncorhynchus tshawytscha*). Previous studies suggest that estrogenic compounds can inhibit smoltification, and the pyrethroid pesticide bifenthrin has been shown to have estrogenic effects. Thus, the objective of my research was to investigate the effects of bifenthrin and climate change stressors on the neuroendocrine response of juvenile salmonids. Trout alevin and fry were exposed to 11^0C, 16.4^0C and 19^0C temperatures and then immediately challenged to sea water (SW) for 24 hours. Trout parr were slowly acclimated to SW and simultaneously exposed to the same temperature regime. Estradiol-17β, cortisol, triiodothyronine, and thyroxine hormone levels were measured in blood serum or whole animal homogenates using ELISAs. Brain growth hormone 1, brain gonadotropin-releasing hormone receptor 2, and gill Na+/K+ ATPase mRNA levels were

measured using qPCR. Alevin were more sensitive to salinity than temperature, and fry were more susceptible to increasing temperatures, thus demonstrating stage-dependent responses to climate change stressors. To evaluate the combined effects of bifenthrin and temperature, Chinook alevin, fry, and parr were exposed to the same temperature stressors for 10 days (alevin) or 14 days (fry and parr) and, in the final 96 hours of rearing, fish were exposed to 0, 0.15, or 1.5µg/L bifenthrin. The same endpoints were measured as in the trout, with the addition of quantifying testosterone levels, the expression of brain dopamine receptor 2α, and measuring brain dopamine levels. In addition, a predator avoidance Y-Maze behavioral assay was conducted on parr. Bifenthrin significantly impacted olfactory-mediated predator avoidance behaviors in parr. These results suggest that temperature may be impacting endocrine pathways differently at each juvenile stage because of the developmental timing of neuroendocrine signaling pathways. Together, these studies provide insight to how multiple stressors and sublethal pesticide exposure may have population-level impacts on Bay-Delta salmonid populations.

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Chapter 1: Introduction

Salmonids

Salmonids are a family of fishes that have significant ecological, economical, and cultural importance worldwide. Salmon and trout are especially important in the western regions on the United States where they are fished commercially and for sport fishing, and therefore are a valuable part of the economy in California, Oregon, and Washington states. In the state of California, the commercial salmon fishing industry was valued at 7.7 to 20.9 million dollars between the years 1996 and 2000 (Barrow, 2001). Sport and recreational fishing for Steelhead trout in the Sacramento River, CA was around 7.2 million dollars in those same years. It is evident that salmonid fisheries significantly contribute to the economy in the state of California, but they are also ecologically important to the state.

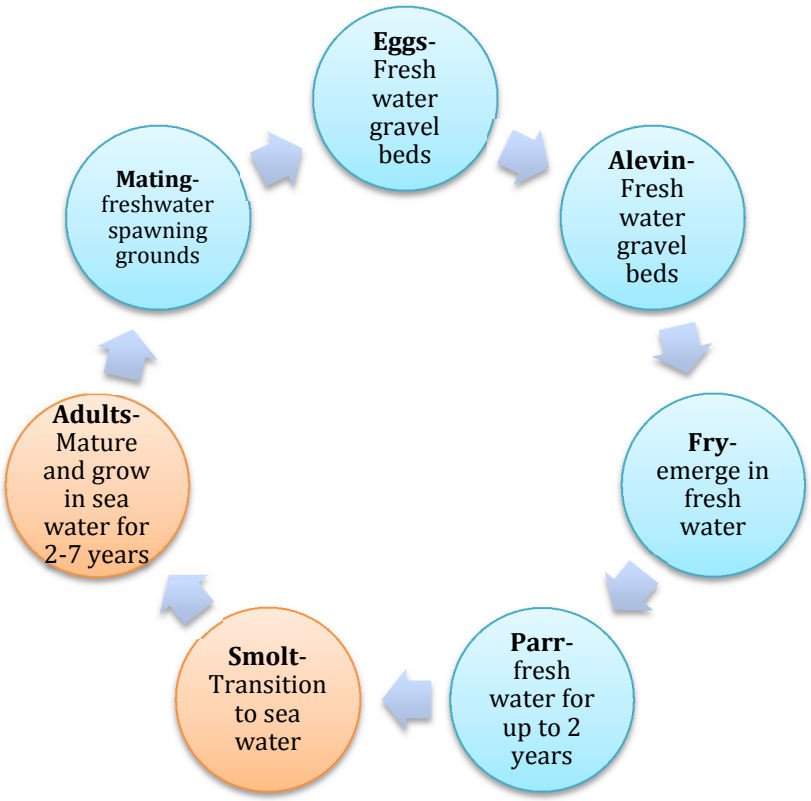

Figure 1.1, Typical lifecycle of anadromous salmonids. Life stages resident in freshwater are in blue, and seawater resident life stages are in orange.

Salmon and trout are anadromous fish, meaning that they inhabit freshwater ecosystems when they are young and then transition to seawater to grow and mature. After maturing in the ocean for several years, adult fish will return to their freshwater rearing grounds to spawn and complete the anadromous life cycle. Different species and populations of salmonids spend various amounts of time in freshwater habitats before transitioning to seawater (Fig 1). There are three juvenile stages that reside in freshwater habitats; alevin, fry, and parr. Alevin are the youngest juveniles and use their yolk sacks as a source of energy. After transitioning to the fry stage, juvenile salmonids swim freely, but mostly remain localized in freshwater areas. Following transition to parr, the oldest

juvenile stage, fish will eventually migrate downstream. The specific timing of parr migration downstream depends on the specific species and population, but movement downstream is the initiation of transition to seawater and smoltification (Moyle et al., 2017). During migrations between freshwater and seawater, fish will also transfer nutrients between aquatic and marine environments. Juvenile salmonids are important components of freshwater ecosystems because of their place in the food web, and adult salmonids are important in marine coastal food webs. Several salmonid species are semelparous since they only reproduce once in their lifetime. When semelparous fish return to freshwater native spawning grounds, they will subsequently die after reproducing, thus decomposing salmon bodies are a predominant source of essential nitrogen and phosphorus in often nutrient-limited freshwater systems (Naiman et al., 2002). In turn, the nutrients provided by the adult salmon will cause an increase in algal growth, which then boosts the growth of zooplankton and algae-consuming organisms in freshwater ecosystems (Quinn, 2005). Salmonids also serve a vital ecological niche as predator of macroinvertebrates and as prey for larger predatory species (Wipfli & Baxter, 2010). In these ways, salmonids are important for preserving the freshwater ecology for the other inhabitant species of freshwater ecosystems from primary producers to keystone predators.

Decline of California Salmon and Trout

Wild populations of coastal salmonids in California are drastically declining despite increasing effort to protect populations in recent decades. Several populations of

Chinook salmon (*Oncorhynchus tshawytscha*) and Steelhead trout (*Oncorhynchus mykiss*) are listed as threatened or endangered species under the Endangered Species Act (Barrow & Heisdorf, 2001; Moyle et al., 2017). Approximately 76% of salmonids in California have an elevated conservation status, and it is predicted that the other populations will likely have a more serious conservation status in the future (Katz et al., 2013; Moyle et al., 2017). The conservation status of the majority of California salmonid populations has shifted to critical and high conservation status in 2017 from lower (high/ moderate) conservation status in 2008 (Moyle et al., 2017). State and federal fish population surveys track adult fish counts and adult escapement, which is defined as the number of adult fish that escape angling/ predation and are able to return to spawn. Both Steelhead and Chinook escapements significantly declined over the past 50 years in the California Central Valley rivers. Natural Steelhead trout escapements have reduced to no spawning activity in several of the Central Valley rivers, and overall adult returns across the state are low (McEwan, 2001).

Human activity has been the major contributor to the decline of salmonid populations. Habitat destruction, including the damming of rivers, redirection of tributaries, and loss of riparian areas, causes physical barriers preventing salmonids from reaching their native spawning grounds and contributes to overall habitat loss. However, there are many other anthropogenic reasons for salmonid decline that are less apparent than physical habitat changes; urbanization, agriculture, and climate change are also significant contributors to increasing population declines (Katz et al., 2013). Urbanization, industrial uses of waterways, and agriculture cause increased turbidity and

pollutants to enter the estuary. The increased presence of pollutants as well as global changes in climate and weather patterns stresses both adult and juvenile salmonids, which leads to decreased populations (Katz, et al. 2013; McEwan, 2001). Ultimately, uncontrolled pollution and imminent onset of climate change has sublethal effects on salmonids, which already have stressful life history transitions from fresh water to sea water environments.

Smoltification

Anadromous salmon and trout will migrate to sea water in a process called smoltification in order to grow and mature in the ocean. The ability of salmonids to go through smoltification has ecological benefits, such as greater access to food and nutrients in the ocean and the opportunity to relocate to optimal environments. Juveniles undergo significant physiological and morphological changes during this life stage that are driven by hormonal changes. Although other cues, such as elongation of photoperiod (Fig 2), are known to initiate smoltification, salinity and temperature also induce of smoltification (Brauer, 1982; McCormick et al., 1998). Changes in salinity and temperature are closely linked with climate change, so normal environmental cues of smoltification will likely be altered as climate change progresses.

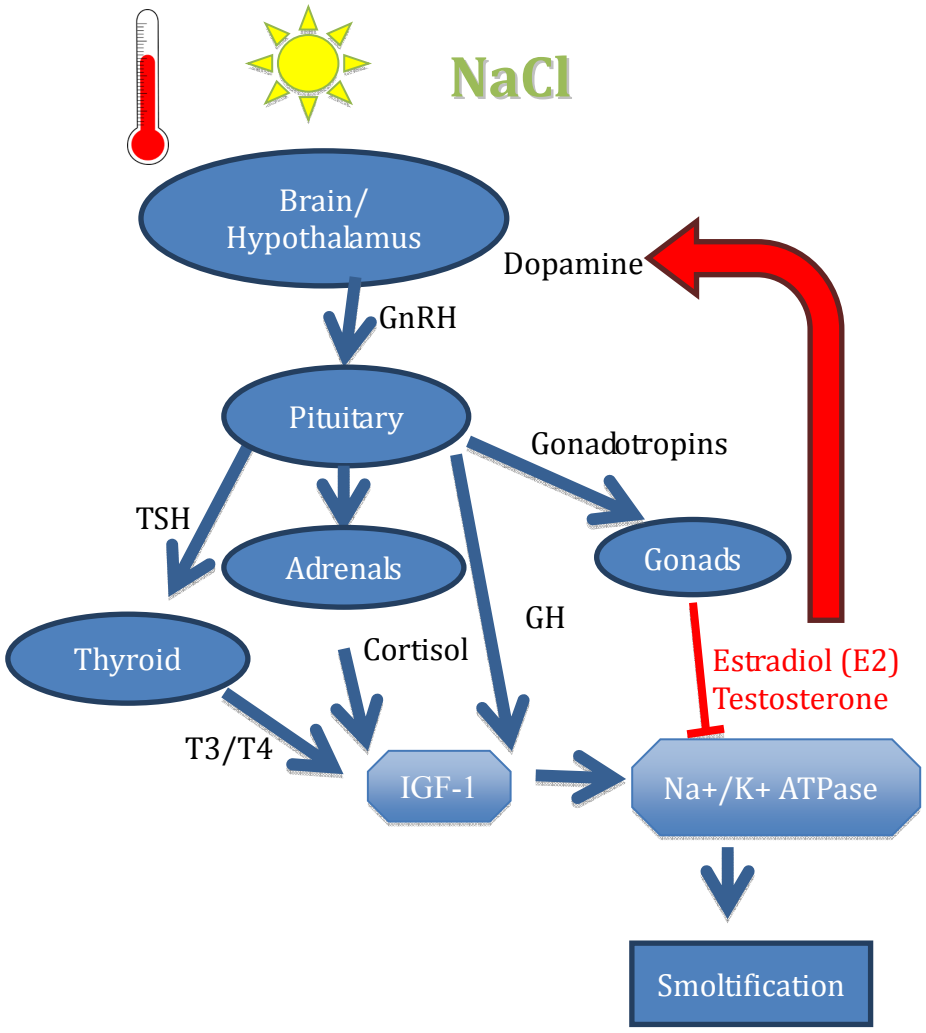

Figure 1.2, Environmental cues of smoltification and the involved hormonal pathways. (Adapted from Ikuta et al., 1987; Dickhoff et al, 1997; Bjornsson et al, 2011)

While smoltification is regulated through discrete endocrine pathways in year-old fingerling fish, there is little information about how early exposure to saline conditions can affect endocrine aspects of smoltification in pre-smolt life-stages. When salmon smolt, Na+/K+ ATPase (NKA) in the gills increases in order to increase osmoregulatory capabilities during the transition to sea water. In addition, cortisol, growth hormones, pituitary hormones, and thyroid hormones have been documented to be involved in

smoltification by upregulating Insulin-like Growth Factor I (IGF-1) that subsequently increased NKA in the gills (McCormick, 1996; Ban, 2004). The increase in thyroid hormones linked closely with growth and development in young fish (Specker, 1988; Power et al., 2001). The biologically functional triiodothyronine (T3) is transformed from precursory thyroxine (T4) hormone via the deiodinase enzymes (Sambroni et al., 2001). The thyroid axis is controlled by signaling in the hypothalamus of the brain, which then signals the pituitary gland to release thyroid stimulating hormone. This axis is subject to negative feedback loops for regulation, and elevated T4 levels are closely associated with induction of seawater tolerance and increased growth hormone levels (Ban, 2004). While an increase in thyroid hormones and subsequent increase in IGF-1 are characteristic of smoltification, the presence of sex hormones and estradiol (E2) diminish saltwater acclimation (Stefansson et al., 2008). Sex steroid hormones, like estradiol and testosterone, are also regulated by the hypothalamus and pituitary glands in the brain. This is called the Hypothalamus-Pituitary-Gonadal (HPG) axis, and it is a highly conserved pathway that is well studied in vertebrates. Estradiol and testosterone play a role in the negative feedback of the HPG axis to regulate activity of the HPG pathway and biosynthesis of sex steroid hormones. Sex steroids have been shown to even inhibit smoltification; studies with juvenile Atlantic salmon *(Salmo salar)* that were exposed to estradiol and estrogenic compounds did not smolt (Madsen et al., 1997). Effectively both estradiol and an estrogenic compounds inhibited smoltification (Madsen et al., 1997), and the same inhibition of smoltification by treatment of exogenous testosterone and estradiol was also observed in Masu (*Oncorhynchus masou*) salmon (Ikuta et al., 1987). This

suggests that the steroid hormone axis has the potential to cross-talk with other pathways during the smoltification process, and the HPG pathway can inhibit other important physiological changes associated with smoltification. Although sex steroid hormone levels decrease during smoltification, the HPG pathway does play a vital role in this process.

The nervous system integrates with endocrine pathways for regulatory functions often through the release of neurotransmitters. The dopaminergic system is an important part of the neuroendocrine pathway used in HPG hormone signaling. In teleosts, the dopaminergic system innervates the hypothalamus and pituitary glands to play a role in the feedback of the HPG axis (Levavi-Sivan et al., 2004; Dufour et al., 2005). Dopamine receptors are present on both the hypothalamus and pituitary glands, and dopamine receptor 2 (DR2) is an inhibitory G-protein coupled receptor that is activated in the HPG feedback in teleosts (Levavi-Sivan et al., 2004). DR2 activation inhibits the release of gonadotropin releasing hormone, which regulates downstream sex steroid biosynthesis. Thus, neuroendocrine pathways are necessary to help regulate the hormones that are integral to successful smoltification.

Previous studies have demonstrated that signaling and receptors within the pituitary gland are regulating salinity tolerance (Parhar & Iwata, 1996). However, some of the specific roles of pituitary hormones during pre-smoltification remain unknown, such as the role of adrenocorticotropic hormone and subsequent production of cortisol. Decreases in cortisol levels have been correlated with acclimation to seawater by increasing levels of growth hormones and Insulin-like Growth Factor I, which

upregulates Na+/K+ ATPase in fish gills (Richman & Zaugg, 1987; McCormick, 1996). Hormone-driven molecular changes result in physiological changes in the juvenile fish that are now called “smolts”. Smolts have a higher osmoregulatory capacity, increased silvering body color, and result in olfactory imprinting of nursery grounds. The previously discussed hormonal pathways are important for successful smoltification, but more research needs to be conducted on the effect of changing environmental cues on this process in juvenile salmonids.

Climate Change Stressors

With the onset of the changing global climate, many aquatic and marine ecosystems are at risk of experiencing increased environmental stress due to the sensitivity and ability of the resident organisms to adapt. Salmonids are particularly sensitive to environmental cues, such as temperature and salinity, which drive growth, smoltification, and the return of adult fish to spawning habitats. With the onset of climate change, the conservation status of many resident populations of salmonids in California are expected to increase by the end of the century, and the majority of populations are expected to reach critical status where they are at risk of extinction from the state of California due to climate change pressures (Moyle et al, 2017). Although recorded changes in climate may be good indicators of how ecosystems may be affected, it is the unknown future of climate change that poses serious threats to delicate ecosystems.

Global climate change causes surface water temperatures to increase and continue to rise without mitigation. For example, in California there is a projected increase by

approximately 4^0C in the annual temperatures of both the San Francisco Bay Delta and its drainage waterways (Cloern et al., 2011). Rising surface water temperatures have the highest increase in the summer months according to models using past monitoring data, and this coincides with development and growth of many juvenile salmonid populations (Kammerer & Heppell, 2013). There are several general uncertainties associated with the increase in surface water temperatures due to global climate change, including the rates of water temperature increases in each part of the Bay-Delta and the specific effect this will have on biological communities over the next century (Cloern et al., 2011). Different climate change models predict a range of rates and increased summer temperatures of surface water due to differences in model parameters, but these models all predict an increase in temperature in the Bay-Delta associated rivers by the end of the century. This raises concerns for the effects of chronic temperate increases on endangered fish species residing in the Bay-Delta (Wagner et al, 2011). Prolonged elevated temperatures adversely affect salmonid health by decreasing growth and fat reserves at sublethal temperature thresholds (Kammerer & Heppell, 2013). The same growth pathways involved in thermal stress response are involved in smoltification, but it is unknown how changing temperatures will affect the physiology of smoltification.

Climate change generated by humans is not only causing surface waters to warm, but it is also impacting sea level rise and salt water intrusion. The intrusion of saltwater into estuarine and fresh water environments is caused by rising oceans that results in hypersaline waterways. Hypersaline conditions are defined as a shift to higher salinity regimes than historically present in these waterways. This is a phenomenon that is

impacting many coastal fresh waterways around the world, and the San Francisco Bay-Delta has notably been experiencing hypersalinization and will continue to experience salt water intrusion as sea levels are predicted to rise by 80-110 cm by the end of the century (Cloern et al., 2011). The reduction in snowmelt and precipitation in drought years leads to decreased freshwater inputs to these waterways, thus contributing to seawater intrusion. Increasing surface water salinity is also potentially due to rising sea levels (Cloern et al., 2011; Shellenbarger et al., 2015). Together, the decrease in freshwater input and rising sea levels are ultimately causing estuaries and aquatic systems to become saltier. The hypersalinization of waterways is a complex phenomenon and is difficult to reverse by identifying a singular cause.

Hypersalinity can also results from urban and agricultural human activity, particularly in arid regions of the world. The areas surrounding the Bay-Delta are highly urbanized and support a high density of people living along this waterway. The tributaries and rivers feeding the Bay-Delta flow through the Central Valley of California, which is the agricultural mecca of the state. Both agricultural and private urban residence regions are highly irrigated, but crops and lawns do not absorb all of the dissolved salts in the water. As agricultural runoff and leachate increase salinity, this causes the receiving waterways to also become saltier (Canedo-Arguelles, 2013). The specific effects of climate change on the large network of Central Valley waterways can pose serious threats to resident organisms. Many euryhaline organisms in these ecosystems are adapted to a specific range of salinities during critical life stages, and shifting to higher salinity ranges from seawater intrusion could be detrimental to the physical and ecological dynamic of

the waterway. Specifically, juvenile salmonids may be susceptible to the environmental changes associated with climate change. As aforementioned, juvenile salmon and trout rely on temperature and salinity as environmental cues to begin smoltification. However, changing environmental conditions can cause juvenile salmonids begin smoltification earlier in order to adapt to hypersaline and warmer environments. It is ultimately unknown how juvenile salmonids, particularly the alevin and fry stages, may respond to intense environmental stress, as well as the timing of development of each life stage.

Contaminants in the Bay-Delta

Highly urbanized and agricultural regions are often polluted with manufactured chemicals. Pollution often occurs in aquatic systems near areas of high human activity, such as the Bay-Delta, which drains two agricultural valleys and has urbanized shorelines. Pesticides are applied to residential areas, public lands, and crops to curb pest activity. Pyrethroids are a class of insecticides that are of emerging concern in the environment due to the increased use in California since phasing out organophosphates pesticides (Spurlock & Lee, 2008; Fong et al., 2016).

Pyrethroids are synthesized pesticides commonly used as urban and agricultural insecticides. Pyrethroids are structurally similar to pyrethrin, a compound naturally produced by Chrysanthemums and other pyrethrum flowers. Chrysanthemums produce the natural insecticide pyrethrum to protect against insects, and pyrethrum contains two types of pyrethrin compounds. Pyrethrins have been historically used in agriculture as natural pesticides where the flowers are crushed and applied to the fields as a rudimentary

insecticide (Crombie & Elliott, 1961; Casida & Quistad, 1995), and chrysanthemums were specifically cultivated for the purpose of producing pyrethrin. The biosynthesis of pyrethrin occurs in the flower from the precursory pyrethric acid and is transformed into two different pyrethrin compounds; pyrethrin I and pyrethrin II. Crowley et al. (1962) found that pyrethric acid in flowers is synthesized from two dimethylallyl pyrophosphates that form cyclopropane rings. This addition synthesis is catalyzed by chrysanthemyl diphosphate synthase. From the cyclopropane structure of pyrethric acid, the flower can synthesize pyrethrin I and pyrethrin II. These natural pyrethrin compounds have low photostability, and therefore degrade quickly when applied as insecticides, so this led to the production of synthetic pyrethroids (Casida et al., 1983). The instability of the natural pyrethroid forms led to the investigation and synthesis of the pyrethroid compounds used as insecticides today. By substituting functional groups, the overall stability of pyrethroids is improved in soils, and that allows the insecticides to be more effective for longer periods of time (Crombie & Elliot, 1961).

Pyrethroids have largely replaced the use of DDT and other organophosphate pesticides in many regions of the world, including the United States. The endocrine disrupting and persistence of DDT posed severe environmental and human health effects, and thus initiated the production and use of alternative pest control compounds (Mnif et al., 2011). Initially organophosphate pesticides replaced the use of DTT; however, the use of organophosphates in agriculture and insect management were phased out as pyrethroids became the preferred method of pest management (Spurlock & Lee, 2008; Weston & Lydy, 2010). Pyrethroids have also become more widely used due to their low

cost, and relatively low toxicity compared to the EPA standards on organophosphate pesticides. Pyrethroids are available commercially in over 700 mixtures, and are commonly used year-round for urban use as well high use in the early spring for large-scale agricultural uses since the banning of organophosphates (Oros & Werner, 2005). In California, the use of synthetic pyrethroids, such as permethrin and bifenthrin, are also increasing in urban and agricultural areas. According to the California Department of Pesticide Regulation, permethrin had the fifth largest increase in number of applications between 1991-1996 of agricultural insecticides used in California, and permethrin was one of the highest used pesticides in 1996 for number of acres treated (Wilhoit et al., 1999).

Pyrethroids are heavily used for agricultural purposes in the San Francisco Bay-Delta and Central Valley regions compared to application rates in the rest of the state (USGS, 2016). However, pyrethroids have been detected in the Bay-Delta after rainfall events in concentrations acutely lethal to both fish and invertebrates, and the majority of all pyrethroids sampled were from urban sources rather than agricultural sources (Weston & Lydy, 2010). This exemplifies that a major source of pyrethroids may be from under regulated urban pest control in California. There are several Class I and Class II pyrethroids regularly detected in the Bay-Delta, and due to structural differences they each have different specific uses, environmental fates, and adverse effects.

Classes of Pyrethroids

Pyrethroids are structurally classified into type I and type II groups, with class II pyrethroids having an α-cyano-3-phenoxybenzyl group. The functional group distinction between the two classes affects the metabolic pathways and mode of toxicity of each pyrethroid compound. As aforementioned, there are two types of pyrethrin compounds, which are synthesized naturally in chrysanthemums. The main structural differences are that pyrethrin I has a methyl group whereas pyrethrin II has an ester in place of the methyl group. Pyrethrin I and II are both type I pyrethroid compounds due to the lack of this α-cyano-3-phenoxybenzyl group. Bifenthrin is also a type I pyrethroid and lacks the α-cyano group. In this research, we are specifically interested in bifenthrin due to its continuous presence in the San Francisco Bay-Delta.

Fate and Transport of Bifenthrin

Pyrethroids are often found in the San Francisco Delta and the freshwater rivers and streams that drain into the Bay-Delta. Weston & Lydy (2010) detected pyrethroids in agricultural canals feeding into the American River, San Joaquin River, and Sacramento River, as well as high concentrations (1.6-17.9 ng/L) in these rivers after rainfall events and in wastewater effluent. Studies by Weston and Lydy also found that urban runoff was the main source of pyrethroid insecticides in the receiving waterways, particularly bifenthrin, but treated waste water and agricultural drains were also major sources of pyrethroid contaminants (Table 1) (Weston et al, 2009; Weston & Lydy, 2010). Bifenthrin was the most frequently detected pyrethroid and was present in acutely toxic

concentrations to invertebrate amphipods (Weston & Lydy, 2010; Weston et al., 2009). Additionally, intense runoff events bring a variety of pesticides into the Bay-Delta, so aquatic organisms may be exposed to multiple pyrethroids and pesticides during these periods (Weston et al., 2015). The high detection rate raises concerns for the effects of the semipersistence of bifenthrin on nontarget aquatic organsisms.

Table 1, Maximum concentrations, frequency of detection, and frequency of EC50 exceedance of an invertebrate (*Hyalella aztecta*) of various synthetic pyrethroid compounds in the Bay-Delta. Results are categorized into urban runoff, publically owned treatment work water, and agricultural drains for sources of contaminants. Table adapted from Weston & Lydy (2010).

	Bifenthrin	Cyfluthrin	Cypermethrin	Lamba-cyhalothrin	Permethrin
source type	EC_{50}= 3.3[c]	EC_{50}= 1.9	EC_{50}= 1.7	EC_{50}= 2.3	EC_{50}= 21.2
Frequency of Detection (%)					
Urban runoff	79	55	33	45	61
POTWs [a]	39	6	6	17	33
ADs [b]	12	0	0	11	2
Maximum Concentration Measured (ng/L)					
Urban runoff	29.8	17.8	12.3	6.2	45.8
POTWs [a]	6.3	1.7	17.0	5.5	17.2
ADs [b]	5.8	0	0	17.5	10.3

[a] Publically owned treatment works
[b] Agricultural drainage
[c] EC_{50} of *Hyalella azteca* in ng/L

Bifenthrin, and other pyrethroids, are non-polar compounds with a water solubility of approximately 0.1mg/L and a Kow of $1x10^6$. The physical structure of bifenthrin makes this compound partition to sediment or organic matter in the environment. Bifenthrin partitioned quickly out of water to tanks, organic matter, and sediments, but can move into the water column when sorbed to resuspended sediments/ particulate matter (Fecko,

1999). Bifenthrin sorbs to organic matter easily due to a Koc range of 1.31-3.02 x 10^5. Bifenthrin is unlikely to contaminate groundwater because it sorbs strongly to both organic soils and particles and does not degrade easily when sorbed. However, bifenthrin can contaminate surface water during runoff events because particulates/ sediments easily wash into waterways and increased turbidity disturbs stream sediment.

When bifenthrin undergoes transformation or degradation in the environment, the major resulting product is 4-hydroxy-bifenthrin. This product is formed from photolysis, where a hydroxyl group is added to bifenthrin (Fecko, 1999). Other products can be formed from photolysis and hydrolysis, but these are pathways are less frequent. Although there is one primary bifenthrin transformation product, bifenthrin is highly stable in the parent form in the environment when bound to sediment or organic material. In water, bifenthrin has a half-life of approximately 276 days, suggesting that it is relatively stable to photolysis under light conditions and some hydrolysis. In addition to high application to sediments and soils, bifenthrin is also commonly used on concrete surfaces in urban settings. Like in soils, bifenthrin binds strongly to concrete surfaces and displays persistence after multiple rainfall events. Rainfall causes bifenthrin to runoff from these concrete surfaces in detectable concentrations, even months after application (Jiang et al., 2012). Bifenthrin residues can be found on concrete surface runoff after repeated rainfall events (Jiang et al., 2012). The continued application and the persistence of bifenthrin to adhere to soil, sediment, and concrete causes semi-persistence in nature because it is continuously inputted into the environment, particularly in aquatic systems.

Absorption and Exposure of Bifenthrin

Routes of exposure and absorption into the body differs between target and non-target organisms. Exposure to pyrethroids occurs typically through oral, dermal, and inhalation routes. However, for humans and other mammals oral exposure predominates through consuming produce treated with pyrethroids, such as fruits and vegetables. Some occupational inhalation and dermal exposure can occur when spraying pyrethroids (Chen et al., 1991). In rodents, exposure is typically oral, intravenous, or interperitoneal (Soderlund et al., 2002) due to their uses in laboratory studies focused on isolating the metabolic pathways and modes of toxicity of pyrethroids. Both classes of pyrethroids are lipophilic, and this allows them to pass through the intestine and enter the blood stream. If not metabolized, pyrethroids can then be distributed to fatty areas of the body, and target fatty myelin sheaths on neurons (Casida et al, 1983).

Bifenthrin Biotransformation

Mammals metabolize pyrethroids using a different phase I metabolic process than insects, allowing for rapid metabolism. Using a radiotracer technique to detect pyrethin I and II metabolites, Elliot et al. (1972) determined multiple metabolites including some that were rapidly oxidized presumably by cytochrome P450 (CYP450) enzymes. A study by Barr et al. (2010) measured urinary pyrethroid metabolites in randomized populations of humans of different age categories. The study found that most metabolites were hydrolysis products of the ester present in pyrethroid compounds. The compound 3-phenoxybenzoic acid, a metabolite of the benzyl alcohol segment of the parent

pyrethroids was detected in the highest quantities, indicating that most pyrethroids in humans undergo ester hydrolysis during phase I metabolism. Formation of oxidative metabolites can be catalyzed by CYP450 as well as aldehyde dehydrogenase (McCarthy, 2006; Barr et al. 2010). Glucuronide conjugation of oxidative metabolites can also occur. displays the metabolic pathways of pyrethroids in mammals. Ueda et al. (1975) found that enantioselective biotransformation can also occur dependent upon the *cis* or *trans* conformation of the pyrethroid. Steric hindrance of the metabolism of *cis* enantiomers may explain the higher toxicity relative to the *trans* isomers (Miyamoto, 1976). Although class I and class II pyrethroids have different substituents and functional groups, mammals typically metabolize compounds in both classes initially through ester hydrolysis by phase I metabolism. After phase I metabolic cleavage of the ester, the compounds may go through oxidation and dehydrogenation before being conjugated with sulfate or glucuronide. Further phase II metabolism (Glucuronide conjugation) largely depends on the specific isomer of the compound and the functional groups present. The ability to rapidly metabolize pyrethroids into less toxic forms allows mammals to be protected from the acute neurtoxicity of pyrethroids (Elliot et al., 1972), which contributes to the species-selective toxicity of pyrethroids.

In mammals, pyrethroid metabolites are excreted primarily through the urine, but also occur in the feces as well. The conjugated phase II pyrethroid metabolites are readily excreted in the urine, and pyrethroid metabolites that did not go through conjugation can be excreted in feces (Chen et al., 1991). However, in insects and fish with slower

metabolism of pyrethroids, the metabolites will remain in the body longer then be excreted as alcohols conjugated with glucuronide or sulfate (Casida et al., 1983).

Mechanisms of Toxicity of Bifenthrin

Acute Toxicity-

Pyrethroids are neurotoxins that affect proper neuronal function and signaling by binding to sodium channels. Class I pyrethroids typically cause tremors in exposed lab rodents, while class II pyrethroids primarily cause salivation and choreoathetosis, which is the convulsing of the abdomen and body writhing motions (Soderlund et al., 2002). Initially, mice and rats treated with pyrethroids convulsed and had tremors (Verschoyle & Barnes, 1972), but it was uncertain if the metabolites or the parent compound was causing the neurotoxic effects. However, Verschoyle and Barnes (1972) determined that the parent pyrethroids were the neurotoxic compounds due to the quick onset of tremors seen from the intravenous injection of pyrethroids in mice.

Pyrethroids also cause neurotoxic effects in insects, and can also affect neurons in other organisms. The first discovery of the mode of toxicity in insects used the voltage-clamp technique (Narahashi, 1971), which measures the flow of ionic current across a membrane. Changes in the membrane potential can be identified when a neuron perpetuates an action potential. The flow, or lack of flow, of ions needed for repolarization and depolarization for neuronal signal transduction can be determined by graphing the voltages across the membrane. Tippe et al. (1987) conducted studies using the voltage-clamp technique on the effects of various type II pyrethroids in neurons in

order to determine how pyrethroids cause neurotoxicity. The study found that pyrethroid exposure caused elongated period of depolarization and decreased action potential thresholds. Multiple signal firing was observed due to the delayed depolarization and lower thresholds. This suggested that the voltage-gated sodium channels remained open for a longer period of time, thus inhibiting normal resting potential and causing dysfunctional neuronal signaling.

Class II pyrethroids have typically been observed to be more acutely toxic to both insects and mammals, which is fundamentally due to the adverse effects of pyrethroids on voltage-gated calcium channels. Verschoyle and Barnes (1972) found that most type II pyrethroids cause choreoathetosis/ salivation in lab rodents, but type I pyrethroids did not cause this same toxicity. This suggested that type II pyrethroids acted through a different, or an additional, mechanism than type I pyrethroids. When the action potential moves down an axon and reaches the terminal end, the voltage-gated calcium channels are opened allowing calcium into the cell. Calcium then initiates the release of neurotransmitters into the synaptic cleft to propagate the signal to the next neuron or muscle fiber. The voltage-clamp technique can also be used to assess if pyrethroids affect calcium channels (Breckenridge et al., 2009). In the presence of type II pyrethroids, an increased uptake of calcium into the cell was observed due to the binding of type II pyrethroids to the voltage-gated calcium channels (Clark & Symington, 2007). Symington et al (2007) used tetrodotoxin, a neurotoxin that blocks the opening of voltage-gated sodium channels, to antagonize the hypothesized opening of sodium channels simultaneously with deltamethrin exposure in order to determine that the

alteration in calcium channel functions were the cause of this form of neurotoxicity. Administration of tetrodotoxin did not prevent neurotoxic effects, thus indicating that the α-cyano-3-phenoxybenzyl functional group contributes to the ability of type II pyrethroid to bind to voltage-gated calcium channels.

The acute toxicity of bifenthrin differs between families of organisms. Toxicity may be related to the occurrence of esterase within organisms. The oral LD50 in rats is 375 mg/kg (Crop Protection Handbook Volume 100). In *Daphnia magna*, the 48-hour LC50 is 0.86ug/L (Brausch et al., 2010). In rainbow trout (*Oncorhynchus mykiss*) the 96-hour LC50 is 0.00015 mg/L, (Tomlin, 2011). It is difficult to compare LD50 with LC50 because the nominal concentration can differ from the measured concentration due to sorption to the tanks and the routes of exposure are different. However, the doses of bifenthrin that the rats in the study receive are unrealistic of environmental levels, whereas the concentrations that the aquatic organisms were exposed to have been recorded in water samples. The vast difference in lethal concentrations, as well as the sublethal responses to bifenthrin, between species may demonstrate differences in metabolic capabilities for these organisms.

Chronic Toxicity-

Early research on pyrethroids shows that exposure lengthens the period of cell depolarization, yet there was still the question of how pyrethroids interacted with voltage-gated sodium channels. Lombet et al. (1988) found that pyrethroids exposure in rats cause modulation of the sodium channels in in rat brains. Using other neurotoxicants,

such as tetrodotoxin, to enhance and compete with the effects of pyrethroids on sodium channels, the researchers found that pyrethroids allosterically bind to sodium channels. The alpha-subunit of brain sodium channels was determined as a binding location for pyrethroids, which then inhibit the proper functioning of the sodium channel to pump sodium ions across the membrane and return to a normal membrane potential (Trainer et al., 1997). However, the allosteric binding to sodium channels does not cause permanent neuronal damage to mammals. Humans and rodents are protected by rapid metabolism by ester hydrolases that allow for the reversal of acute bifenthrin toxicity (Soderlund et al., 2002). However, prolonged exposure to pyrethroids has the potential for extended damage, especially in sublethal doses to non-mammalian species.

Toxicity to Non Target Organisms

Insect pests are the target organisms for pyrethroid insecticides. However, as previously stated, insects and other invertebrates are more sensitive to pyrethroid compounds largely due to the differences in their metabolism and inability to rapidly convert pyrethroids to less toxic metabolites. Insects typically lack ester hydrolases, which cleave the ester in pyrethroids resulting in acidic and alcohol metabolites (Casida et al, 1983). Instead, insects metabolize the substituents of pyrethroid compounds by CYP450 oxidation. The metabolism in insects and other invertebrates is slower and less efficient than in mammals (Soderlund et al., 2002). Since insects metabolize pyrethroids primarily through oxidation by CYP450 enzymes (Casida et a., 1983), inhibiting CYP450s enzymes causes significantly slower pyrethroids metabolism. Inhibiting

metabolism keeps the absorbed pyrethroids in a toxic form in the body longer, thus increasing the lethality of pyrethroids on invertebrates. Therefore, pyrethroids are commonly used in a mixture with piperonyl butoxide, a CYP450 inhibitor (Casida et al, 1983). The application of pyrethroids with piperonyl butoxide allows pyrethroid insecticide to be more effective and have a lower application rate.

Fish are some of the non-target organisms that are affected by wastewater and urban/ agricultural runoff into waterways. Pyrethroids have a higher acute toxicity to fish compared to mammals due to the unique toxicokinetic dynamics within fish compared to mammals. Fish have lower carboxylesterase activity compared to other vertebrate organsisms (Haya, 1989). Exposure to pyrethroids in waterways occurs orally, dermally, and through the gills. Fish can be constantly exposed to sublethal concentrations of pyrethroids in the aqueous environment, whereas exposure in mammals can be intermittent. Bifenthrin has a recorded bioconcentration factor of approximately 6000 in many fish due to its lipophilicity (Fecko, 1999), allowing rapid partitioning into fish. The metabolic and detoxification pathways of fish also differ slightly from mammals (Kaviraj & Gupta., 2014). Aquatic and marine systems are typically cooler than land surfaces, and there is evidence that pyrethroids are more toxic in lower temperatures likely due to the lower rate of metabolism (Narahashi, 1971). Like invertebrates, fish have lower carboxyesterase activity, which cleaves the ester group in pyrethroids, so oxidation by CYP450 is the major route of metabolism (Demoute, 1989). As in insects, this makes the metabolism of pyrethroids slower so that the toxic parent form is present within the body for longer periods of time.

Aquatic invertebrates are also adversely affected by pyrethroid runoff. *Hyalella azteca*, a commonly found amphipod in many regions of the country, is susceptible to pyrethroids with LC50 values in the ng/L concentration range. Chart 1 shows that the majority of water samples collected from the Bay-Delta watershed contain levels of bifenthrin above the EC50 or LC50 of this species. Persistent exposure to this pesticide can threaten the population of these organisms that serve as food for many consumers in the ecosystem. Many other invertebrates that serve as prey to salmonids can be at risk to runoff events that contain high levels of bifenthrin. Weston et al. (2015) found that after a runoff event into the American River, *H. azteca* mortality correlated with bifenthrin concentrations. Mesocosm studies found that bifenthrin directly reduced invertebrate survival and also indirectly caused a trophic cascade where species diversity was significantly altered after the invertebrate die-off (Rogers et al., 2016). In this way, bifenthrin could cause indirect toxicity to fish through adversely affect the prey of the fish.

Many studies investigating the effects of pyrethroids on fish species measure the activity of sodium-potassium ATPase (Na+/K+ ATPase/ NKA) and the transcription of the subunits of NKA to determine how and if pyrethroids cause osmoreglatory stress. Na+/K+ ATPase is vital for maintaining the ionic balance in fish, particularly those in brackish or sea water. Class I pyrethroids adversely affect Na+/K+ ATPase in fish by binding to the channels and altering ionic balance (de Assis et al. 2009; Riar et al., 2013). In addition to osmoregulatory stress, Werner et al. (2002) found that fish in freshwater exhibited decreases in reproductive endpoints when exposed to a class II pyrethroid.

These findings could be related to the recently observed endocrine disrupting effects of pyrethroids (discussed below). As in humans, biomarkers of pyrethroid exposure in fish are being developed and standardized to assess fish exposure (Kaviraj & Gupta, 2014).

Endocrine Disruption

Over the past few decades, research has emerged investigating the potential for pyrethroids and their metabolites to have endocrine disrupting effects. Endocrine disrupting compounds are those that affect the endocrine system through hormonal pathways and cause adverse physiological and/or behavioral effects (Kime, 1998). Endocrine disruptors can be endogenous or exogenous compounds, and both can have negative effects on proper endocrine function through modulation of molecular pathways. Awareness for the potential of emerging contaminants to have endocrine disrupting effects has risen in recent years. Brander et al. (2012) reported that class I pyrethroids (permethrin and bifenthrin) activated estrogen receptor activity, but was unsure if the parent compound or the metabolites were causing the effects. Zebrafish exposed to bifenthrin exhibited similar molecular effects as zebrafish estrogen receptor α (ERα) knockdown zebrafish also exposed to bifenthrin, suggesting that bifenthrin may not be binding or acting directly upon ERα (Bertotto et al., 2019). Therefore, bifenthrin may be acting indirectly on the HPG axis and estrogen biosynthesis. Forsgren et al. (2013) found that exposure to bifenthrin caused a reduction in the biosynthesis of estradiol and testosterone in juvenile rainbow trout. Overall, bifenthrin has been shown to induce changes in estrogenic pathways, reproduction, and transcription of a variety of genes

related to sex steroid pathway signaling in multiple fish species and at multiple life stages (Brander et al., 2016). The alterations of the estradiol and testosterone pathways may affect gonad development, and therefore affect reproduction and may diminish populations.

Molecular biomarkers are used in both laboratory and field studies to determine if fish have been exposed to endocrine disrupting toxicants. An example of a biomarker used to assess endocrine disruption within male oviparous animals is the occurrence of the egg-yolk precursor, vitellogenin, as an indicator for estrogen exposure. Juvenile trout exposed to bifenthrin exhibited increased estradiol and vitellogenin, following exposure to 0.15 and 1.5 ng/L bifenthrin for 14 days (Crago & Schlenk, 2015). Increases in both estradiol and vitellogenin in juvenile fish suggest endocrine alterations, but the impacts on reproduction and population are unclear. Studies using *Menidia beryllina* have found reduced fecundity and decreased choriogenin, an important protein in egg formation, with exposure to bifenthrin (Brander et al., 2016).

Endocrine disruptors may act directly on hormone receptors by mimicking hormones or blocking the receptors, but modulation of neuroendocrine feedback loops may be an additional cause for endocrine disruption. Sublethal doses of pyrethroids have been shown to modulate the dopaminergic system through altered expression of transporter and receptor mRNAs (Gillette & Bloomquist, 2003; Kung et al., 2015). As aforementioned, the dopaminergic system is vital in regulating the HPG axis in teleosts. Crago & Schlenk (2015) found a trend towards decrease of Dopamine Receptor 2A when

juvenile rainbow trout were exposed to bifenthrin for two weeks, and this correlated with a trend towards an increase in estradiol levels.

In humans, 3-phenoxybenzoic acid (3-PBA) is a major pyrethroid metabolite, and 3-PBA concentrations can be measured noninvasively in urine. metabolite in the urine correlated with elevated blood serum levels of luteinizing hormone, which is necessary in the regulation and production of other sex hormones. 3-PBA levels excreted in the urine were inversely related to estradiol levels in males (Han et al., 2008). The study by Han et al. (2008) study did not investigate fertility, reproductive abilities, or long-term hormone levels, but the alterations of hormone levels correlated with increased 3-PBA suggests that 3-PBA affects steroid synthesis and has the potential to be an endocrine disruptor. The sublethal impacts of widespread chronic pyrethroid exposure on human populations are largely unknown.

Fish Behavior

Fish olfaction is necessary for survival throughout the entire lifespan of the animal. Proper olfactory functions allow fish to find food, avoid prey, find mates, and return to their native spawning habitat. The olfactory organs in fish appear as rosettes in the olfactory cavities on either side of the rostrum. Rosettes are innervated with overlying layers of sensory nerves. The olfactory epithelium covers the surface of neuronal cells that form synapses with the central nervous system, and chemical odorants activate signal first at the olfactory epithelium (Zielinski & Hara, 2006). The dopaminergic system is capable of regulating the fish olfactory bulb, and can elicit a response in both immediate

behaviors and in longer-term physiology through neuroendocrine pathways (Kermen et al., 2013). Environmental stimulus through odorant detection causes a cascade of responses at the behavioral level (Figure 1.3). Disruption at any point through the cascade could negatively impact behaviors associated with that stimulus and can have negative effects at the population level (Scott & Sloman, 2004; Tierney et al., 2010). Salmonids exposed to organophosphates and other pesticides have resulted in compromised olfactory functions and associated behaviors (Moore et al., 2007; Maryoung et al., 2015). Fish exposed to pyrethroids have demonstrated impaired olfactory responses to mating odorant cues, which resulted in impaired reproductive behaviors and physiology (Moore & Waring, 2001). Bifenthrin has also been linked to general neuronal damage and inflammation of the brain in rats (Gargouri et al., 2018), so this suggests that bifenthrin can impact memory and decision-making capabilities as well. In *M. beryllina,* exposed to bifenthrin at picomolar concentrations, predatory avoidance behaviors of were negatively impacted compared to control fish (Frank et al., 2018). Transcriptomic changes in genes related to neuromuscular functions in Fathead minnow (*Pimephales promelas)* correlated with impaired swimming performance behavior when exposed to 0.07- 0.14 μg/L bifenthrin (Beggel et al., 2011). Disruption of important fish behaviors and locomotion can lead to population-level effects (Baldwin et al., 2009; Macneale et al., 2014). Therefore, pyrethroids have the potential to disrupt vital fish behaviors and cause adverse effects on the entire organism.

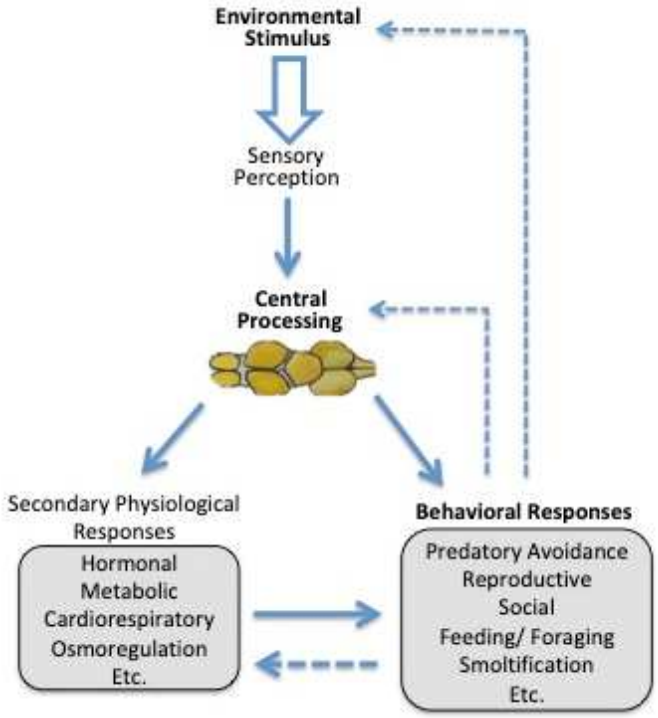

Figure 1.3, Behavioral response cascade in fish from the environmental stimulus to the behavioral outcomes (figure adapted from Scott & Sloman, 2004)

Combined Contaminant and Climate Change Stressors

It is known that the toxicity of environmental agents may be changed in seawater with varying risks from the contaminant to organisms that live in saline and estuarine environments (Hall & Anderson, 2005). Additionally, there is an increasing risk of toxicological stress to organisms to seawater alone (Voorhees et al., 2013), which may be enhanced in near shore environments due to climate change (Schiedek et al., 2007). Increased toxicity of pesticides in salmonids following hypersaline conditions has been observed in recent studies (Riar et al., 2013; Maryoung et al, 2015). Similarly, pre-exposure to pesticides and endocrine disrupting compounds can affect the ability of salmon to smolt and survive in seawater (Moore & Waring, 2001; Moore et al., 2007),

which may have adverse effects on the survival of salmonids. Warmer water temperatures have also been shown to increase the toxicity of pollutants to aquatic organisms through altering the uptake, toxicokinetics, and elimination of the contaminants (Noyes & Lema, 2015). Consequently, there may be a higher potential for adverse effects of pyrethroids in aquatic organisms with warming waters due to climate change. In a study with *M. beryllina* exposed to two temperatures and bifenthrin, fish exhibited altered sex ratios reared at higher temperatures, and more offspring had developmental deformities and fewer offspring viable (DeCourten & Brander, 2017). This suggests that the combination of increasing temperatures and bifenthrin can have persisting population-level impacts. Overall, more research needs to be conducted to understand how contaminants can affect fish in a changing climate.

Research Objectives

Juvenile salmonids are facing a changing environment caused by human activity. It is known that temperature, salinity, and bifenthrin can all cause mortality to young fish when they are exposed to excessively high levels. However, the sublethal effects of these combined stressors remains largely unknown for the ecologically and economically important endangered salmon and trout populations in the San Francisco Bay-Delta. The purpose of this research is to investigate these sublethal endpoints of combined stressors through the following objectives:

1. To understand the effects of increasing temperature and hypersalinity on the endocrinology of juvenile salmonid alevin, fry, and parr.

2. To understand how increasing temperatures affect the toxicity of bifenthrin on the neuroendocrinology of juvenile salmonid alevin, fry, and parr.
3. To understand if bifenthrin and climate change stressors will have negative effects on the olfactory behaviors of juvenile salmonids by impairing the olfactory system through modulation of the dopaminergic system.

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Chapter 2: The Effects of Temperature and Salinity on the Endocrinology of Smoltification in Juvenile Rainbow/Steelhead Trout (*Oncorhynchus mykiss*)

Abstract

The San Francisco Bay Delta is experiencing seasonally warmer waters and salt water intrusion into historically freshwater ecosystems due to climate change. Steelhead/rainbow trout (*Oncorhynchus mykiss*) are resident in the Bay-Delta from juvenile development through the smoltification process. Due to sea level rise, premature hypersaline acclimation may co-occur with increased temperatures on pre-smolt juveniles. To evaluate the interactive effects of salinity and temperature on juvenile life stages of salmonids, rainbow trout alevin (3 days post-hatch) were exposed to 11^0C, 16.4^0C and 19^0C temperatures for 10 days and then challenged for 24 hours to 32 ppt seawater (SW). Similarly, fry (4 weeks post-hatch) were exposed to 13^0C, 16.4^0C and 19^0C temperatures for two weeks and then challenged to SW. Estradiol-17□ (E2), cortisol, triiodothyronine (T_3), and thyroxine (T_4) were measured in blood serum and whole animal homogenates using Enzyme Linked Immunnosorbent Assays. Transcripts of gill Na+/K+ ATPase β (NKAβ), brain Growth Hormone I (GH1), and brain Gonadotropin-Releasing Hormone Receptor 2 (GnRH2) were also measured. Alevin exhibited a significant temperature-dependent decrease in survival and fry showed a temperature-dependent decrease in condition factor. Gene expression of NKAβ, GH1, and GnRH2 was significantly decreased in all SW challenged alevin, and a significant decrease in GnRH2 expression was observed in fry with temperature. Alevin T3 and T4 concentrations were significantly increased with increasing temperature. There was a

temperature-dependent increase in E2 of fry, but not in alevin. The results of this study demonstrate that increasing temperature and SW exposure may adversely affect the survival and SW acclimation of alevin and fry stages of salmonids, and that the tolerances of younger juvenile stages should be considered when assessing the response of salmonid populations to climate change stressors.

1. Introduction

In marine ecoystems, global climate change generated by humans causes physical and chemical changes that include sea level rise, acidification, and increasing sea surface temperatures. Increased surface water temperatures are occurring along the coast of California and are predicted to increase over the next century (Cloern et al, 2011). Projected increases in the annual and summer month temperatures of the San Francisco Bay-Delta, a heavily populated coastal region, and the rivers feeding into the Delta indicate resident organisms may be adversely affected (Wagner et al, 2011). Climate change is already driving an ecological shift for euryhaline fish that reside in the Bay-Delta and San Francisco Estuary (Feyrer et al., 2015)

The intrusion of saltwater into fresh water environments results in hypersaline waterways (Xiao et al., 2014). Hypersaline conditions tend to be characterized as a shift to higher salinity regimes compared to current and past salinity ranges. Annual average surface water salinities have been increasing in the Bay-Delta since the mid 1980s due to decreases in freshwater input from drought, manipulation of Bay-Delta hydrology, and rising sea levels (Hutton et al., 2017; Shellenbarger et al., 2015). Euryhaline organisms in

estuarine systems are adapted to a specific range of salinities during critical life stages, and shifts to higher salinity from seawater intrusion could be detrimental, particularly in stages that may not experience osmotic changes during early life stages.

Salmonid species are economically and ecologically important components of estuarine and freshwater ecosystems. After spawning in freshwater systems, subsequent decomposition of the moribund animals transfers necessary nutrients from the ocean to inland aquatic regions (Naiman et al., 2002). Salmonids also serve a vital ecological niche as predators of macroinvertebrates and as prey for larger predatory species (Macneale et al., 2014). During their transition from freshwater to saltwater in estuarine areas, salmonids undergo significant physiological and morphological changes during smoltification. The animals are referred to as "parr" during this developmental stage, and as they become "smolts" specific hormone and environmental signaling is required that will eventually allow the juvenile fish to utilize the higher abundance of food in marine systems to enhance growth and return for spawning (McCormick, 1996).

Along the West Coast of the United States, populations of Steelhead trout *(Onchorynchus mykiss*) have become endangered from both overfishing and habitat destruction (O'Farrell & Satterthwaite, 2015). However, other factors may also be contributing to the decline including the adversity of osmotic and thermal stress in premature life stages due to climate change. Generally, steelhead become smolts after approximately 1 year in open estuarine systems, but it is likely that exposure to elevated temperatures may be more prominent and have greater impact in shallow freshwater areas where the fish reside at earlier developmental stages. Prior to parr, animals are

characterized as alevin and fry. Alevin are the youngest juvenile stage and are classified as immediately post-hatch through yolk sac absorption. Fry are the intermediate juvenile stage and range from approximately one month through 5-6 months. While smoltification is regulated through discrete endocrine pathways in fingerling fish, there is little information about how exposure to increased temperatures at earlier life stages can affect endocrine pathways that are important in later development.

When salmonids undergo smoltification, Na+/K+ ATPase (NKA) in the gills increases in this transition to saltwater for increased ion regulation. Cortisol, growth hormone, pituitary hormones, and thyroid hormones are also involved in initiating smoltification throughout the process. Gonadotropin releasing hormone levels increase during smoltification, while sex steroid (estradiol and testosterone) levels decrease and can even inhibit successful smoltification (Parhar & Iwata, 1996; McCormick, 1996). The hormone levels in the Hypothalamus-Pituitary-Gonad axis (HPG axis) are subsequently changed in response to environmental cues to smolt via negative feedback regulation. In addition, the Hypothalamus-Pituitary-Thyroid (HPT) axis is also involved in smoltification with increases in thyroxine (T4) and triiodothyronine (T3) thyroid hormones (McCormick, 1996). Increasing thyroid hormone levels induce Insulin-Like Growth Factor-I, which, in turn, help increase levels of NKA for enhanced osmoregulation in seawater (Stefansson et al., 2008). Elevated cortisol levels also increase the activity of NKA in the gills as juvenile fish transition to seawater (McCormick, 1996). Multiple overlapping endocrine pathways are necessary for successful smoltification, and subsequent survival. Although other cues, such as longer

daylight hours, are known to initiate smoltification (Naiman et al., 2002) the objectives of our study will focus on how salinity and temperature can affect smoltification and salmonid endocrine function and survival.

To better understand the impacts of climate change on steelhead trout, the present study will investigate the effects of increasing temperature on hormonal pathways associated with smoltification and growth in early life stages of the steelhead model, rainbow trout, at alevin and fry stages. We hypothesize that increased temperature will negatively impact smoltification hormonal pathways in pre-fingerling salmonids potentially leading to reduced acclimation to seawater, which may impact population growth.

2. Methods

2.1 Test Organisms

Alevin (3 days post hatch, 0.089 g ± 0.0063 and 2.35cm ± 0.18) and fry (4 weeks post hatch, 5.08 g ± 0.41 and 7.86 cm ± 0.2) of *Oncorhynchus mykiss* were obtained from Jess Ranch (Apple Valley, CA). Alevin were acclimated to laboratory conditions for three days in static systems at 12^0C on a 14:10 light/ dark cycle. Fry were acclimated to laboratory conditions for one week in Living Stream systems at 12^0C on a 14:10 light/ dark cycle. Fry were fed approximately 2% of their body weight daily (Oncor Fry floating trout pellet, Skretting, Toole, UT). Alevin were not fed because yolk sacs were still present. All fish were handled and treated in accordance with the approved

Institutional Animal Care and Use Committee protocols at the University of California, Riverside (animal use protocols 20130010).

2.2 Experimental Design

2.2.1 Alevin Exposures

Alevin were subjected to three temperature regimes for 10 days; 13 ± 0.24^{0}C, 16.4 ± 0.2 ^{0}C, and 19 ± 0.2^{0}C (N=36 for each temperature). Fish were exposed individual 8L glass aquaria (N=12 for each tank, and three tanks per temperature) and chilled or heated to the respective temperature with external heating and chilling units. 50% water changes were conducted every other day. The optimal rearing temperature is approximately 16.4 ^{0}C for juvenile Steelhead growth (Marine & Cech 1998; Richter & Kolmes, 2005); however, there is little specific information on the optimal temperature for juvenile Steelhead trout in the Central Valley regions. We used 16.4^{0}C as our optimal temperature because Steelhead are the anadromous form of rainbow trout. The recorded temperatures according to the USGS surface water temperature monitoring station in Fair Oaks, CA for the latest spawning season in the American River corresponding to the Steelhead juvenile developmental periods are approximately 11-13^{0}C for alevin and fry. The projected mean daily maximum water temperature at the end of the century is approximately 19^{0}C in the San Joaquin river (Wagner et al., 2011), so 19^{0}C was used for the highest temperature exposure. The current recorded temperatures, the optimal temperatures, and the 50-year projection temperatures were used for the exposures. The current recorded temperatures,

the optimal temperatures, and end of century projection temperature were be used for the exposures.

After the 10 day temperature exposure, fish were challenged to a 24 hour seawater exposure at 18 parts per thousand (ppt) sea water, which is the salinity that fish would be exposed to moving from the Delta into the Bay. Three tanks (N=12 per tank) reared at 13^0C were kept in fresh water as controls. Fish were then sacrificed using MS222, and tissues were harvested for molecular analysis and stored at -80^0C. Mortality was recorded throughout the exposure, and the average masses and lengths were measured at the beginning and end of treatment.

2.2.2 Fry Exposures

Fry were reared at 13 ± 1.1^0C, $16.4 \pm 0.28\ ^0$C, and 19 ± 1.02^0C (n=12 for each temperature) for two weeks (due to the longer developmental period of the fry stage compared to alevin). After the lab acclimation period, 3 replicates of 4 fish each were transferred to 20 L glass aquaria. Individual glass aquaria were placed in a water table chilled or heated to the respective temperature with external heating and chilling units. Fish were fed 2% of their body weight every other day, and 50% water changes were done every other day. Similarly to the alevin exposures, fry were subjected to a 24-hour seawater challenge following the two-week temperature exposures. 3 tanks (N=8 per tank) reared at 13^0C were kept in fresh water as controls. Fish were sacrificed in 300 mg/L MS-222 (Sigma Aldrich, St. Louis, MO). Tissues were harvested for molecular analysis, snap frozen in liquid nitrogen, and frozen at -80^0C.

2.3 *Hormone Quantification*

Enzyme-Linked Immunsorbent Assays (ELISA) were used to determine the concentrations of alevin whole-body thyroid hormones (T3/T4), cortisol, and estradiol (E2). ELISA assays were also used to determine the concentrations of fry thyroid hormones (T3/T4), cortisol, and 17β-estradiol (E2) in muscle. Estradiol kits were sourced from Cayman Chemical (Ann Arbor, MI), and T3, T4 and cortisol kits were sourced from Genway Biotech (San Diego, CA). Whole alevin and fry muscle were homogenized in a lysis buffer with 1uM phenylmethylsulfonyl fluoride (Sigma Aldrich, St. Louis, MO), centrifuged, and the supernatant used for ELISA analyses. Separate whole alevin and fry muscle tissue were homogenized in the Cayman Chemical ELISA buffer, centrifuged, and then the supernatant was extracted 3 times with methylene chloride. Fry muscle was harvested and extracted according to methods developed by Guest et al. (2016). The samples were dried under N_2 and reconstituted with the ELISA buffer to be used in E2 ELISA analyses. Standard curves (6 point for Genway assays and 8 point for Cayman assays) for calculating hormone levels were run for each analysis. Data was normalized by tissue mass.

2.4 Gene Expression

Real-time quantitative PCR was used to assess the expression of Na+/K+ ATPase β (NKAβ) transcripts in fry gill tissue and alevin head tissues, which regulate ion concentration in the gills and increase during smoltification. qPCR was also used to determine the relative expression of Growth Hormone I (GH1) and Gonadotropin

Releasing Hormone Receptor II (GnRH2) in fry brain and alevin head tissue. RNA was extracting using the SV Total RNA Isolation Kit (Promega, Madison, WI), and RNA was specked on a Nanodrop ND-1000 (ThermoFischer Scientific) with 260/280 ratios above 2.0 and 260/230 ratios between 1.90-2.1. RNA integrity was further confirmed on a 1% agarose gel by the presence of two solid 28S and 18S bands. cDNA was synthesized with normalized volumes of total RNA (1µg) using the Reverse Transcription System (Promega, Madison WI) with random primers following the manufacturer protocol. SSO Advanced Universal SYBR Green Supermix (BioRad, Hercules, CA) was used as the quantification dye for qPCR and reactions were done in 20µL volumes. Elongation Factor 1a (EF1α) and 18S were selected as reference genes because of their high stability in multiple salmonid tissues (Olsvik et al., 2005). GnRH2 primers sequences were obtained from Crago et al. (2015), and GH1, 18S and EF1α primer sequences were the same as those used in Giroux et al. (2019). Primers for qPCR were originally designed using Rainbow trout sequences and manufactured by Integrated DNA Technologies (San Diego, CA). Optimization and qPCR protocols were followed as described in Giroux, et al. (2019), and all primer efficiencies were within 99.8-101% as calculated from 5-point cDNA dilution curves. qPCR was run on a CFX Connect Real-Time PCR Detection System (BioRad) with the following protocol: 95^0C for 3 min, and 40 cycles of 10 s at 95^0C and 30 s of 56^0C. A melting curve was run after the PCR protocol with continuous fluorescent measurement. PCR products were run on a 1.25% agarose gel to confirm target amplicon lengths. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method with both reference genes averaged together.

2.5 Statistics

A power analysis (alpha= 0.05) was run to determine an appropriate sample to reduce the number of fish used in this study. Shapiro-wilk's and Levene's tests were run on all data to test normality and variances. Data was log-transformed as necessary to fit normality assumptions. One-way ANOVAs were used to determine the significance of the hormone and qPCR results. A Tukey's post-hoc was run to compare the significant differences between treatment groups. Survival tests were run on the survival data to determine significance between survival curves of each treatment.

3. Results

3.1 Survival and Condition Factor

Alevin and fry survival were significantly lowered with increasing temperatures after the 24 hour sea water challenge compared to the fresh water controls. In alevin, there was 0% survival of fish reared at 19^0C, which was significantly lower ($p<0.001$) compared to a 97% survival rate in the fresh water controls (Fig 1A). In fry, significant differences were not observed between the fish reared at 11^0C in the fresh water control or those challenged with sea water. There was a significant 50% reduction in survival ($p=0.0121$) in fry reared at 19^0C compared to the fresh water controls and 11^0C sea water challenged- fry (Fig 1B).

Condition factor (CF) remained unchanged in alevin and fry in the different temperature treatments. However, there were significant increases in CF in both alevin and fry in the FW controls compared to all of the SW challenged fish (Fig 2A-B).

3.2 Hormone Analysis

Cortisol levels were significantly affected by temperature in alevin ($p<0.001$), but not in fry. All alevin challenged with SW had significantly elevated average whole-body cortisol levels compared to the FW controls. Additionally, there was a temperature-dependent decrease in cortisol in the SW challenged alevin, with an approximately 3-fold decrease in cortisol concentration between the alevin reared in 13^0C and 19^0C. In fry, the data was not significant at $p<0.05$, and average muscle cortisol levels were consistent among treatments.

E2 levels were significantly lowered in all alevin challenged to SW, and estradiol levels increased with temperature in fry. In alevin, estradiol levels were 5.8-7.4- fold lower in the SW challenged fish in all temperature exposures compared to the fresh water controls ($p<0.01$). In fry, there was no difference between the homogenate E2 concentrations in fresh water control fish compared to the 19^0C exposed fish. However, there was a significant temperature-dependent increase in estradiol levels in the SW challenged fish.

In alevin, both T3 and T4 data were significantly altered ($p<0.0001$), with a significant increase in thyroid hormones in all SW challenged exposed fish compared to the FW controls ($p<0.001$). T3 increased by 1.7 to 2.2 fold in the SW treated alevin compared to the FW controls. There was also a significant increase ($p<0.001$) in T4 levels of alevin with increasing temperature with 7.7 fold, 9.9- fold, and 15.8 fold increases in the SW challenged alevin exposed to 11, 16.4, and 19^0C treatments, respectively, compared to the FW controls. T3 concentrations in fry were not

significantly different ($p>0.05$), but fry T4 concentrations were significantly increased ($p<0.0001$), with 4-5 fold higher T4 levels in all SW challenged-fish compared to the FW controls.

3.3 Gene Expression

Temperature significantly decreased the expression of GnRH2 and NKAβ in the SW challenged-fry ($p=0.011$ and $p<0.0001$, respectively). Fry GnRH2 expression was approximately 75% lower in the 19^0C fish compared to the 11^0C fish challenged with SW. GH1 expression was not significantly changed in fry (Fig 6A). In alevin, GH1, GnRH2, and NKAβ mRNA expression were significantly ($p<0.001$) lowered in all SW challenge treatments compared to the FW controls (Fig 6C).

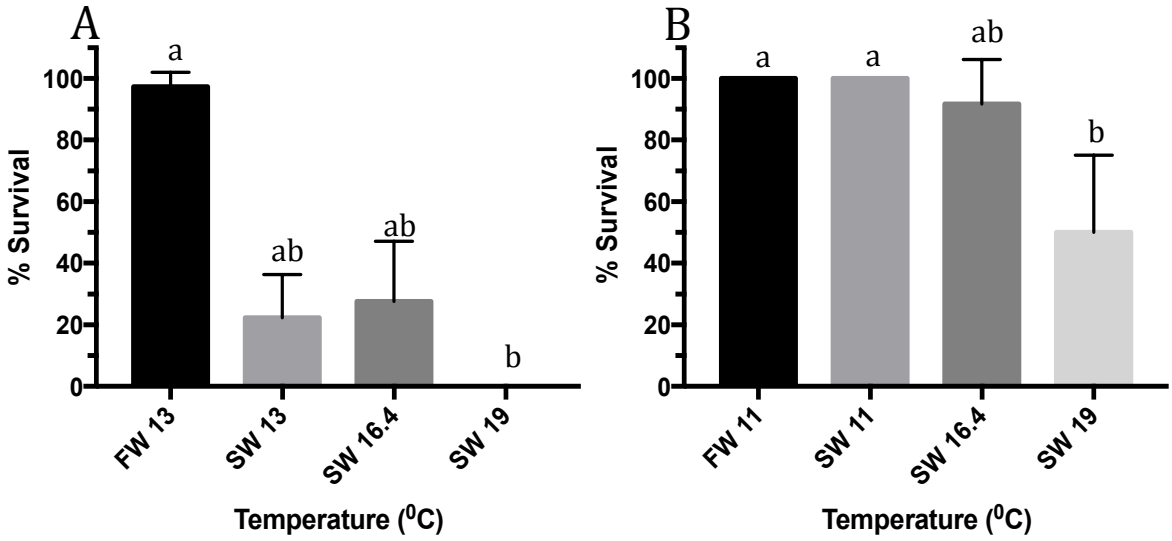

Figure 2.1, Percentage of A) alevin survival after a 24 hour seawater challenge (SW) following 10 day acclimation to 13^0C, 16.4^0C and 19^0C with a fresh water control (FW) and B) fry survival after a 24 hour seawater challenge following 14 day acclimation to 11^0C, 16.4^0C and 19^0C with a fresh water control. Each value represents the mean of 3 replicates ± SEM . Letters indicate significant differences among the survival curves ($p<0.05$).

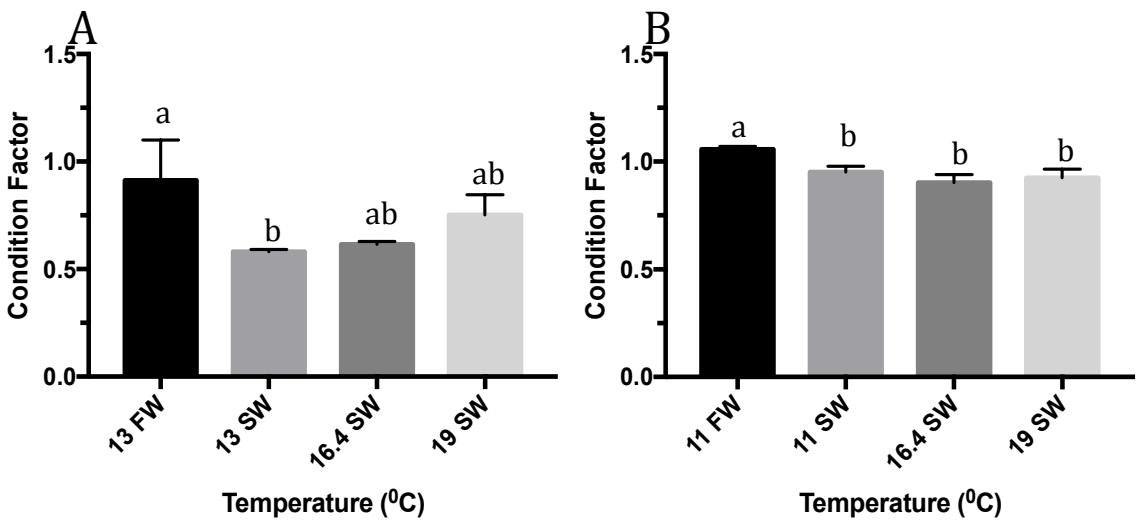

Figure 2.2, Condition factor of A) alevin exposed to 13^0C, 16.4^0C, and 19^0C for 10 days and post sea water (SW) challenge with a 13^0C fresh water (FW) control and B) fry exposed to 13^0C, 16.4^0C and 19^0C for 14 days and post sea water challenge with a 13^0C fresh water control. Each value represents the mean of all biological replicates ± SEM. Data are significant (ANOVA $p< 0.001$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

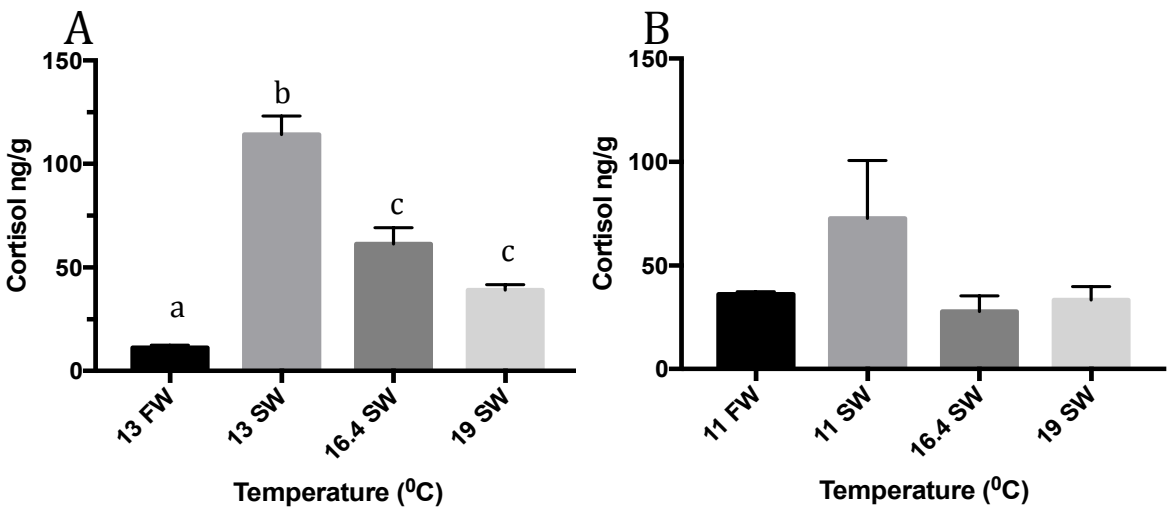

Figure 2.3, Whole-body cortisol (ng/ g) in A) alevin exposed to 13^0C, 16.4^0C and 19^0C for 10 days and post sea water (SW) challenge with a 13^0C fresh water (FW) control, and B) muscle cortisol (ng/ g fish) in fry exposed to 11^0C, 16.4^0C and 19^0C for 14 days post sea water challenge with a 11^0C fresh water control. Each value represents the mean ± SEM. Alevin data are significant (ANOVA $p< 0.001$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

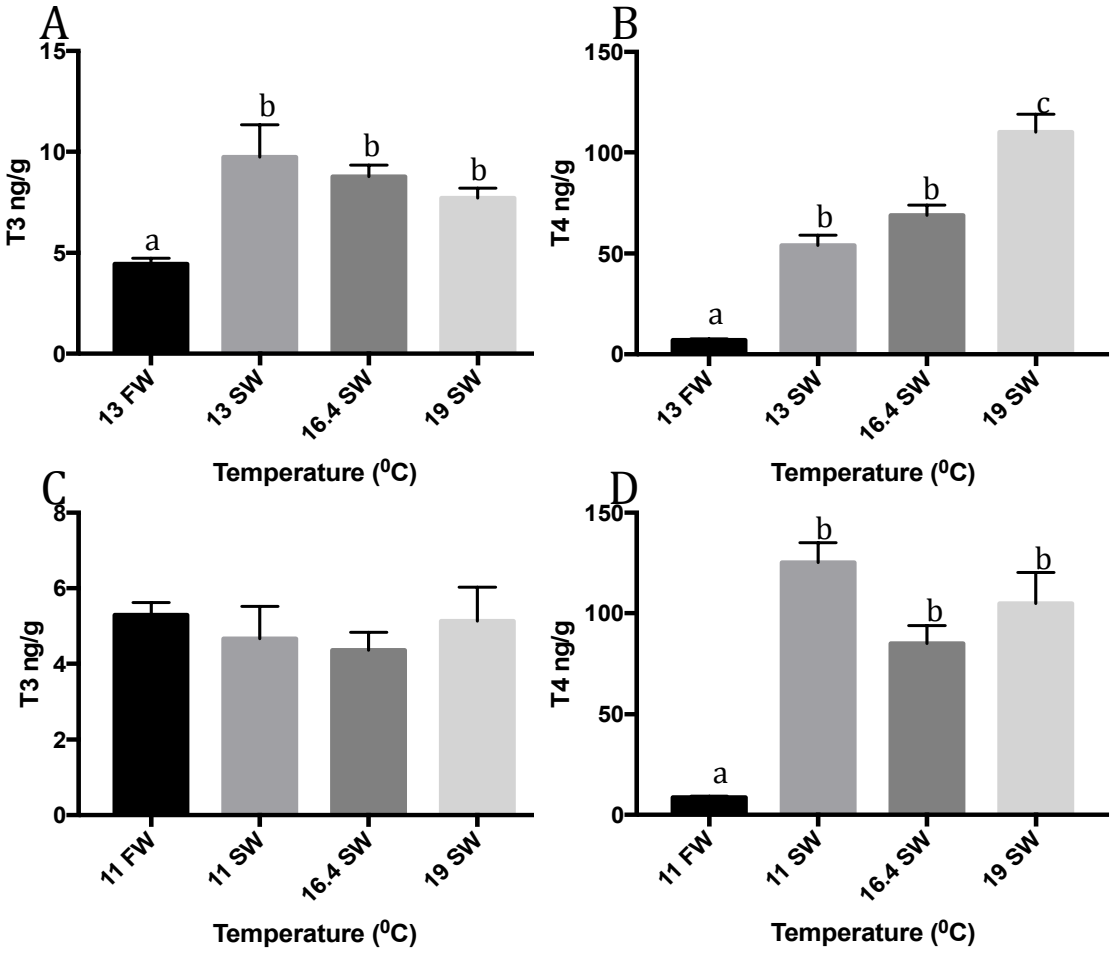

Figure 2.4, T3 and T4 levels (ng/g) in alevin and fry. A) Whole body alevin T3 and B) whole body alevin T4 in fish exposed to 13^0C, 16.4^0C and 19^0C for 10 days and post sea water (SW) challenge with a 13^0C fresh water (FW) control. C) Fry muscle T3 and D) fry muscle T4 in fry exposed to 11^0C, 16.4^0C and 19^0C for 14 days post sea water challenge with a 11^0C fresh water control. Each value represents the average ± SEM. Data are significant (ANOVA $p< 0.001$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

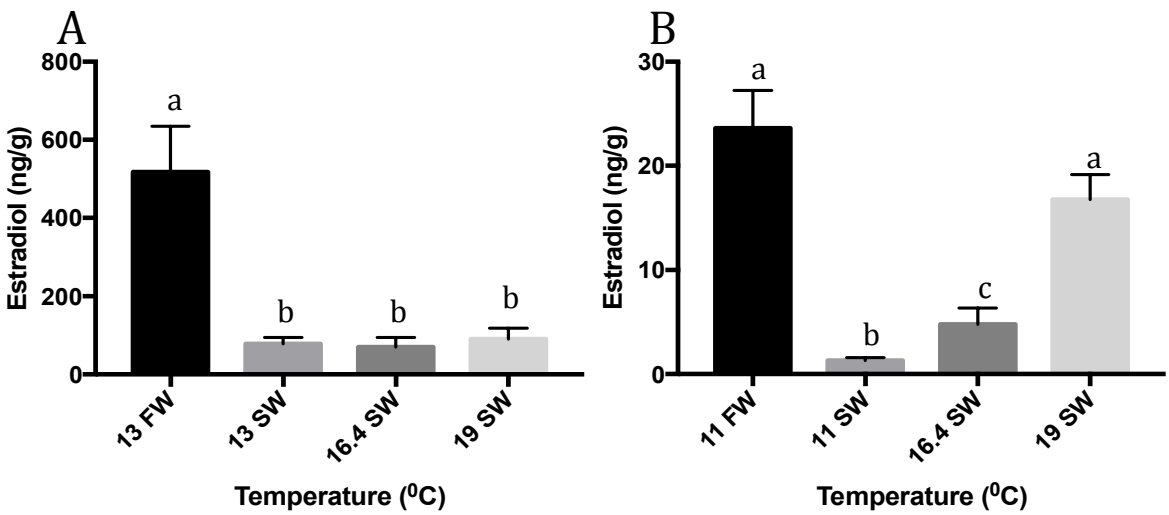

Figure 2.5, Whole-body E2 (ng/ g) in A) alevin exposed to 13^0C, 16.4^0C and 19^0C for 10 days and post sea water (SW) challenge with a 13^0C fresh water (FW) control, and B) muscle E2 in fry exposed to 11^0C, 16.4^0C and 19^0C for 14 days and post sea water (SW) challenge with a 11^0C fresh water (FW) control. Each value represents the average ± SEM. Data are significant (ANOVA $p< 0.001$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

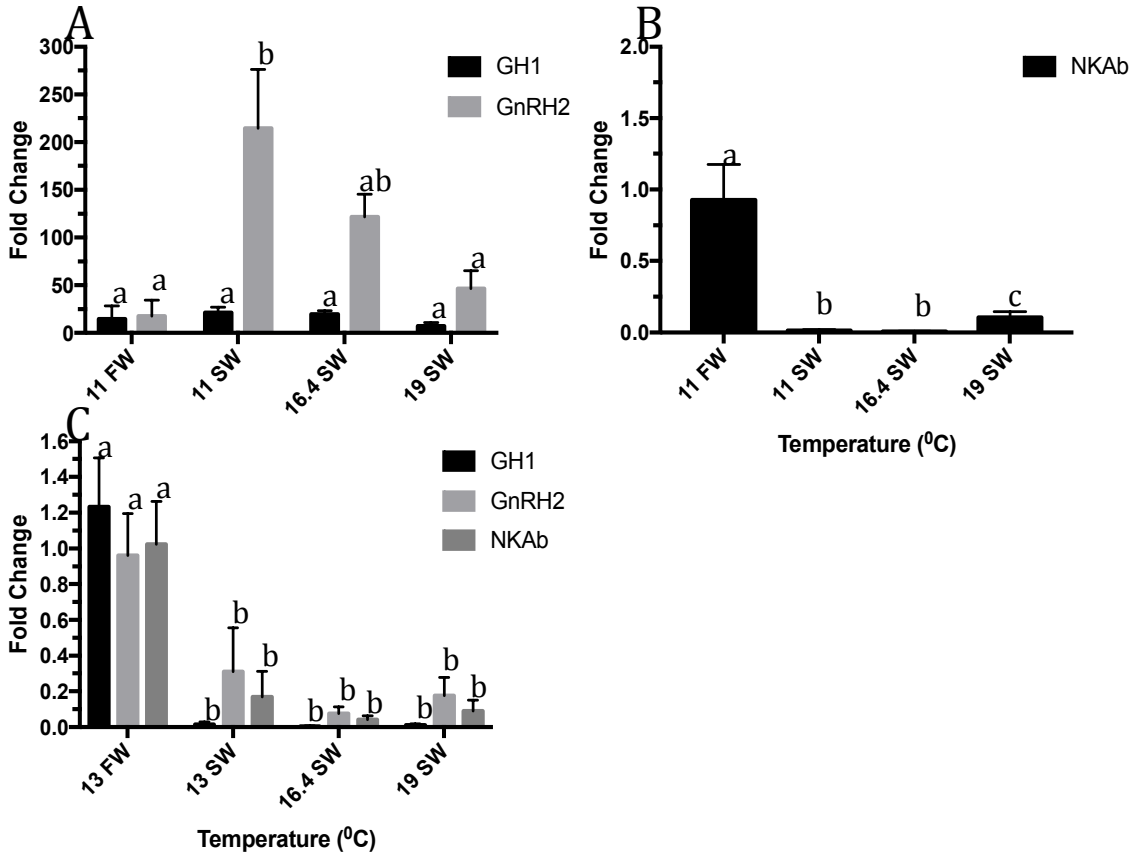

Figure 2.6, Log fold change of A) fry brain GnRH2 and GH1 and B) and fry gill NKA β exposed to 11^0C, 16.4^0C and 19^0C for 14 days, and post sea water (SW) challenge with a 11^0C fresh water (FW) control. C) Alevin head GnRH2, GH1, and NKAb exposed to 13^0C, 16.4^0C and 19^0C for 10 days post sea water (SW) challenge with a 13^0C fresh water (FW) control. Each value represents the mean ± SEM. Data are significant (ANOVA $p< 0.001$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

4. Discussion

In the current study, we exposed two stages of juvenile rainbow trout to increasing temperatures in order to understand how increasing surface waters caused by global climate change can affect seawater adaptation and endocrine functions. In the Bay-Delta, juvenile salmonids may be experiencing increasing thermal shifts in water temperature at younger life stages, and this study found that temperature does adversely impact sea water adaptation in fry. Additionally, we demonstrated that developmental stage does have an impact on HPG and HPT responses to increasing thermal stress.

Temperature played a critical role in survival at the alevin and fry juvenile stages after sea water challenge, and also affected fish condition. The lower survival of alevin at higher temperatures indicates that alevin are a more sensitive juvenile stage than fry to environmental stressors associated with climate change. Increasing temperature adversely affected SW acclimation due to the decreased survival seen after the 24 hour SW challenge in higher temperatures in both alevin in fry. The FW control and sea water challenged fry reared at 11^0C both had 100% survival rates, but a significant temperature-dependent decrease in survival in 19^0C reared-fry indicate that increasing temperature significantly impacts mortality at this life stage more than the stress of premature salinity exposure. The decrease in survival in alevin and fry with increasing temperatures above 13^0C is consistent with other studies on salmon and trout populations in the Pacific Northwest, which showed decreased survival and growth in multiple young salmonid species as they were exposed to a thermal critical maximum. These data suggest that the California populations of salmonids also exhibit similar thermal tolerances (Richter &

Kolmes, 2005). Most studies investigating the impacts of salinity and temperature on juvenile salmonids use the parr, or pre-smolt, stage, rather than the younger alevin and fry stages. However, understanding the mortality associated with the thermal and salinity tolerances of alevin and fry are crucial for evaluating how climate change may impact the survival of endangered salmonid populations in California.

In the alevin and fry stages, successful growth is an important factor in survival. In the current study, condition factor was used as a proxy for growth. Condition factor (CF) trended towards an increase in alevin with temperature, but significantly decreased in fry with increasing temperature. The increase in alevin CF corresponds with an increase in both T3 and T4 in alevin. Alevin T3 and T4 follow the same trend, indicating that T4 is being produced and subsequently converted to the biologically active T3 by deiodinase 2 (Sambroni et al., 2001). However, these same patterns were not observed in fry because T4 was significantly elevated in all SW challenged fish reared in all temperature groups compared to the FW control, and T3 levels were unchanged between groups. Thus, thyroid hormones may have different roles in each developmental stage as hormonal pathways continue to mature. This could be due to the rapid growth of alevin during this short life stage (Tagawa & Hirano, 1987). Additionally, maternal transfer of thyroid hormones may be driving the increase in T3 and T4 as alevin begin to produce endogenous thyroid hormones (Specker, 1988; Power et al., 2001). Larval fish begin producing endogenous thyroid hormones as their yolk sac is resorbed and the fish start actively feeding in the transition from alevin to fry stages (Power et al., 2001). It is well characterized that thyroid hormones play a role in thermal acclimation to cooler

temperatures in parr (Zak et al., 2017). Thus, the observed decrease of CF in fry despite elevated levels of T4 suggests that T4 may play a role in thermal acclimation as fry reach their upper critical thermal limits rather than playing a role in the growth axis. The function of T4 in parr may shift from promoting growth at optimal temperatures to primarily aiding acclimation to higher temperatures.

Increasing temperatures negatively affected the ability of fry to acclimate to SW through the HPG and cortisol axes. Higher temperature exposures resulted in a significant increase of E2 in fry. Elevated E2 levels in fry may have decreased GnRH receptor expression in the SW challenged fish because increases in E2 causes a decrease of GnRH through an HPG axis feedback loop (Alvarado et al., 2016). There was also a trend towards a decrease of cortisol levels in fry with increasing temperature. However, when salmonids undergo smoltification there is typically a decrease in E2 and an increase in cortisol (Bjornsson et al., 2011). In the current study, the opposite relationship between E2 and cortisol was observed, thus suggesting that higher temperatures may alter this hormonal interaction and prevent SW acclimation and subsequent smoltification. This was consistent with the observation of a decrease in NKAβ in gill of fry challenged with SW. Increases in gill NKA are necessary for osmoregulation as fish prepare to transition to SW (McCormick, 1996; Bjornsson et al., 2011). Unchanged cortisol levels may lead to a lack of NKA mRNA expression and subsequent activity (Richman & Zaugg, 1987; McCormick & Bern, 1989). The relationships between cortisol and NKA deserve further study in juvenile life stages of salmon and trout.

The SW challenge negatively impacted alevin through multiple endocrine pathways. Alevin NKAβ levels did not significantly change with temperature and alevin at higher temperatures also had higher mortality after the SW challenge. There have been few studies on the endocrinology and development of the alevin life stage, and there is little information about the potential for alevin to produce NKA for seawater acclimation in anadromous salmonids. Alevin E2 levels did not significantly change between temperature treatments, but there was a significant difference between the FW controls and the SW treated fish. However, there was a temperature-dependent decrease in GnRH2 receptor expression in alevin that did not correspond with the observed E2 levels. This suggests that the HPG axis in alevin may not be significantly impacted by increasing temperature because the HPG feedback loop is not fully developed since alevin do not have functioning gonads at this life stage (Vizziano et al., 2007). As with E2 measurements, there was a temperature-dependent decrease in alevin cortisol levels, so it is possible that temperature negatively impacts upstream synthesis of all steroid-based hormones in alevin. Further studies are needed to confirm this hypothesis.

It is evident from this research that alevin and fry trout respond to increasing temperature and SW exposure differently, and alevin are more sensitive to the SW challenge whereas fry are more sensitive to increasing temperatures. Differences in alevin and fry hormonal responses to environmental stressors have been observed in previous studies (Giroux et al., 2019). Some pathways are more highly affected in alevin compared to fry likely due to the timing of development and physiological purpose of the hormonal pathways measured in the current study, such as the HPT and HPG axes.

5. Conclusions

Few studies have investigated the biological implications of increasing temperature of coastal sea water on anadromous fish and their ability to osmoregulate at earlier life stages. Juvenile salmonids are found in coastal systems worldwide, and the survival of entire populations depends on the successful smoltification of juveniles. Our results demonstrated that increasing temperature affects the endocrine pathways of alevin and fry differently, and that alevin are more susceptible to osmotic stress than fry. Understanding how saltwater intrusion and varied temperatures may impair smoltification in premature life stages will help protect other anadromous fish populations with the rapid onset of climate change, and provide a comprehensive understanding of anadromous fish endocrinology and development.

Acknowledgements

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Chapter 3: The effects of two climate change effects (temperature and salinity) on the endocrinology of endangered juvenile Steelhead trout (*Oncorhynchus mykiss*)

Abstract

The San Francisco Bay-Delta is a vital ecosystem for populations of anadromous salmonids. As the southernmost range for the endangered Steelhead trout (*Oncorhynchus mykiss*), changes in habitat due to climate change can have drastic effects on individuals during sensitive life stages. Surface water temperature and salinities in the Bay-Delta are predicted to increase over the next century, and the sublethal effects of multiple climate change stressors on juvenile life stages of salmonids are understudied. Steelhead parr were exposed to three temperatures (13.0 ± 0.18, 16.4 ± 0.45, and 19.0 ± 0.63^{O}C) and then half of the fish were acclimated to 32 ppth sea water (SW) over 16 days. 17β – estradiol, testosterone, cortisol, triiodothyronine, and thyroxine hormone levels were measured using ELISAs after seawater acclimation. Messenger RNA of growth hormone 1 (GH1), and gonadotropin-releasing hormone receptor 2 were measured in the brain along with Na+/K+ ATPase (NKA) α and β in the gill. Condition factor significantly decreased with increasing temperature and salinity. GH1 expression in the brain significantly increased in the SW exposed fish at higher temperatures. NKA expression diminished following exposure to salinity and increased temperatures. These results demonstrate that changes in temperature and salinity can have adverse effects on hormonal pathways in presmolt salmonids, which may have significant impacts on populations in areas susceptible to climate change such as the San Francisco Bay Delta.

I. Introduction

In coastal systems worldwide, the rapid onset of climate change may have significant impacts on native populations of anadromous salmon and trout. In northern California, salmonid populations in the San Francisco Bay-Delta watershed have been declining, and 47% of the salmonid species found in California are federally listed as endangered or threatened, including Steelhead trout (*Oncorhynchus mykiss*). California is the southern range limit for the majority of anadromous salmon and trout on the West Coast of the United States and 78% of these species are predicted to become extinct in the state by the end of the century due to a combination of pollution, habitat degradation, and climate change (Katz et al., 2012; Moyle et al., 2017). The specific threats of climate change facing salmonids in the San Francisco Bay-Delta include increasing surface water temperatures and sea water intrusion (Katz et al., 2012). In addition to higher water temperatures (i.e 20^0C by 2050), the continued decline of fresh water inputs from decreased precipitation by the end of the century will cause salinities within nearshore environments to also increase (Cloern at al., 2011; Wagner et al., 2011). Thus, juvenile salmonids will be simultaneously exposed to increasing temperatures and hypersaline environments within the next century, so it is necessary to understand how climate change stressors might impact salmonids, particularly during susceptible juvenile life stages which are dependent upon hormonal regulation for acclimation to environmental conditions critical to survival.

Steelhead trout undergo a transition from freshwater to seawater during smoltification in order to complete their anadromous life cycle. Smoltification of parr, the

most advanced juvenile stage, is triggered by critical environmental cues, including increases in temperatures and/or salinity (Stefansson et al., 2008). As a hormone-driven process, smoltification is dependent upon an increase in sodium-potassium ATPase (NKA) in the gills for increased osmoregulation as parr smolt and adapt to sea water. Increases in growth hormone (GH) and thyroid hormones [triiodothyronine (T3) and thyroxine (T4)], promote an increase in Insulin-like Growth Factor 1 (IGF-1) which upregulates NKA in the gills (Dickhoff et al., 1978; McCormick,1996; Bjornsson et al., 2011). Cortisol also increases during smoltification and aids osmoregulation through increases in chloride cell differentiation (Richman & Zaugg, 1987). Significant increases in gill NKA are commonly used as a marker for successful smoltification (Bjornsson et al., 2011; Handeland et al., 2013)

Although thyroid hormones, GH, and cortisol help mediate smoltification, the Hypothalamus-Pituitary-Gonadal axis (HPG) and the resulting sex steroid pathways are reduced during this process (Stefansson et al., 2008). Parr treated with exogenous testosterone, 11-ketotesosterone, and estradiol delayed smoltification, indicating that sex steroid hormones can impair parr osmoregulation (Ikuta et al., 1987; Madsen et al., 1997). The impact of temperature on hormonal pathways during smoltification has not been well studied. It is known that thyroid hormones play a vital role in the acclimation of salmon to colder water (Zak et al. 2017), but the role of the HPG axis, and the Hypothalamus- Pituitary- Thyroid (HPT) axis, and growth hormone axis in warm water acclimation has not been well-characterized.

In the San Francisco Bay-Delta, juvenile salmonids will not only be exposed to the singular stress of increasing temperatures, but according to models, they will also be simultaneously exposed to higher salinity. The combination of these stressors may alter the endocrinology and long-term survival of salmonid parr. Consequently, the objective of our study was to evaluate the effects of increasing temperature and salinity on Steelhead trout at the susceptible stages of smoltification.

Steelhead parr were maintained at three different temperatures and then subjected to increased salinity from <1.5 to 32ppth sea water over 16 days. Survival and condition factor were measured as well as sex steroid, cortisol, and thyroid hormone concentrations. In addition, expression of NKA mRNA in gill, gonadotropin releasing hormone receptor 2 (GnRH2) in brain, and growth hormone 1 (GH1) in brain were also measured. Results of this study will provide data to better understand the impacts of climate change on organisms that are dependent upon environmental cues for sustainable populations.

II. Methods

Chemicals

Instant Ocean Sea Salt (Instant Ocean, Blackburg, VA) was used for hypersaline exposures. Advanced Universal SYBR Green for qPCR was obtained from Bio-Rad Laboratories, Hercules, CA. All other chemicals were purchased from Fisher Scientific (Hampton, NH) or Sigma Aldrich (St. Louis, Mo)

Exposures

Steelhead trout parr (approximately 10 months post hatch) were obtained from the CA Department of Fish and Game Nimbus Fish Hatchery (Oroville, CA). Fish fork length was 8.34 ± 1.1 cm and 6.62 ± 2.29 g. Fish were housed in Living Stream systems (Frigid Units, Toledo, OH) at 12^{O}C on a 12:12 light/ dark cycle and fed Ocor Fry pellets (Skretting, Tooele, UT) ad libitum daily. Parr were exposed consistently to three temperatures, 13.0 ± 0.18, 16.4 ± 0.45, and 19.0 ± 0.63^{O}C for 16 days in static 20L aquaria. 13.0^{O}C was used as the control temperature because that is the current temperature in the Bay-Delta, 16.4.0^{O}C is the optimal temperature for steelhead parr growth (Moyle, 2002), and 19.0^{O}C is the predicted end of century temperature in the Bay-Delta (Wagner et al., 2011). Three fish were placed in each tank with 8 tanks per temperature. Every other day, the salinity in half of the tanks at each temperature (n=4) was increased by 4 ppt until reaching 32 ppt on day 16. The other half of the tanks were kept in aerated system water (<1.5ppth). Water changes were done every other day to maintain water quality and increase salinity. External chilling and heating units regulated temperatures. Survival was recorded throughout the exposure, and condition factor was calculated at the conclusion of the experiment. All fish were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (#20130010) at the University of California, Riverside.

Hormone Analysis

Blood serum samples from each tank were pooled together (n=3) and caudal peduncles were extracted for thyroxine, triiodothyronine, cortisol, estradiol (E2), and testosterone. ELISA kits for thyroxine, triiodothyronine and cortisol were sourced from Genway Biotech (San Diego, CA), and E2 and testosterone kits were sourced from Cayman Chemical (Ann Arbor, MI). Blood serum (30 µL) was transferred to test tubes and extracted 3 times with of 1 mL methylene chloride (MeCl). Resulting extracts were lyophilized in a water bath at 42^{O}C under N_2. E2 ELISA buffer (300µL) from each kit was used to reconstitute the samples, and samples were stored at -20^{O}C until analysis. Due to low blood volume from the size of the juvenile fish, the caudal peduncle was substituted for thyroid analysis following the extraction protocol outlined in Guest et al. (2016). Based on the mass of the sample, a 5X volume of lysis buffer was added with 1 µM PMSF. After a thirty-minute incubation on ice, the samples were homogenized then centrifuged at 15,000g for twenty minutes at 4^{O}C. The supernatant was removed and subsequently stored at -20^{O}C until use for TH analysis.

Assays were run according to manufacturer instructions and standard curves for all hormone analyses were provided with the kits. Six-point standard curves were run for the TH and cortisol assays, and eight-point curves were run for the E2 and testosterone assays. A Spectra Max Plus 384 plate reader was used to measure the absorbance of the TH and cortisol assays at 450 nm and 405 nm for the E2 and testosterone ELISAs.

Gene Expression

Brain RNA was extracted using the RNeasy Lipid Mini Kit and gill tissue was extracted using the RNeasy Mini Kit (Qiagen, Germany). All RNA samples were specked on a NanoDrop 1000 or 2000 instrument (Thermo Fisher Scientific, Waltham, MA) to ensure 260/280 ratios above 2.0 and 260/230 ratios between 1.9-2.1. RNA quality was further confirmed on a 1.5% agarose gel by the presence of two bands (28S and 18S). cDNA was synthesized from 1 μg RNA using the Reverse Transcription System (Promega, Madison, WI) kit in 20 μL reactions using Oligo(dT) primers .

The target transcripts for sodium-potassium ATPase α and β (NKAα and NKA β) from the gill, and gonadotropin releasing hormone receptor 2 (GnRH2), and growth hormone receptor 1 (GH1) from the brain, were optimized with the reference genes EF1α and 18S RNA. The percent efficiency for all primers ranged from 98.2%- 100.5%. GnRH2 primers sequences were sourced from Crago et al. (2015), NKAβ primers were sourced from Riar et al. (2013), and GH1, 18S, and EF1α primer sequences were the same as those used in Giroux et al. (2019). Primer optimization and qPCR protocols were followed as described in Giroux, et al. (2019). PCR protocols were run on a CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA) with the following parameters: 95^0C for 3min, and 40 cycles of 10s at95^0Cand 30 s of 55^0C, and a subsequent melting curve with continuous fluorescent measurement. Target amplicons were confirmed on a 1.5% agarose gel. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method with EF1α and 18S averaged reference genes.

Statistical Analysis

All data was tested for normality using Shapiro-Wilk's test and homogeneity of variance using Levene's test. Two-way ANOVAs were used to determine the significance of the gene expression data, followed by Tukey's HSD post-hoc analysis for pairwise-comparison between all treatments. Data was log transformed as necessary to meet normality assumptions for ANOVAs. A multivariate survival analysis was used on the survival data to determine significance between survival curves of each treatment. Fish reared at 11^{O}C and kept at 0ppth salinity were used as the controls in each analysis. All statistical analyses were conducted in R studio.

III. Results

Survival and Condition Factor

Temperature and salinity did not significantly affect the survival of Steelhead parr. The highest mortality occurred in the 19.0^{O}C and 32ppth acclimated fish with an average survival rate of 58% (Table 1). Condition factor significantly decreased in fish exposed to 19.0 ^{O}C in both salinities compared to the control 11.0 ^{O}C reared fish (Fig 1). There was a 29% decrease in the fish exposed to 32ppth salinity and reared at 16.4 ^{O}C compared to the 0ppth fish at the same temperature (Fig 1).

Hormone Analysis

Temperature had a significant impact on both T3 and T4. There was a significant 28% increase in T3 in the FW control groups at 16.4^{O}C and 19.0^{O}C compared to the 11

^{O}C controls (Fig 2A). T3 data was significant at p=0.02 for the interaction of salinity and temperature. Temperature only had an impact on T4, but treatments with higher salinity did not affect T4 concentrations. There was a significant increase in T4 in all fish exposed to 16.4 ^{O}C and 19.0 ^{O}C, regardless of salinity, when compared to all 11.0 ^{O}C fish. T4 values was significantly altered by temperature at p=0.0044.

Serum testosterone levels were significantly affected by both temperature and salinity (p=0.0085), but E2 concentrations were not significantly changed by salinity or temperature treatments (Fig 3A). Testosterone concentrations were significantly lower in fish exposed to 32 ppth and reared at 16.4^{O}C and 19.0^{O}C compared to the 32ppth fish at 11.0^{O}C (Fig 3B). Salinity significantly impacted cortisol levels (0=0.0063), but temperature did not have an effect on serum cortisol (Fig 3C).

Gene Expression

Temperature and salinity significantly affected the relative expression of GH1 in brains. GH1 expression increased by approximately 2.2 fold in the 16.4 ^{O}C and 19.0 ^{O}C reared fish acclimated to 32 ppth compared to the controls (Fig 4A). While temperature did not significantly impact the expression of GnRH2, acclimation to sea water significantly (p=0.0134) increased GnRH2 expression compared to all FW control animals (Fig 4B). Gill NKAα and NKAβ transcripts were not significantly affected by either temperature or salinity (Fig 4C-D). Expression of both NKAα and NKAβ were diminished (approximately 12.5- 51.1 fold lower) in the 19 ^{O}C parr acclimated to FW control and 32 ppth salinity when compared to the 16.4^{O}C treated fish.

Table 3.1, Average percent survival of Steelhead exposed to 13.0, 16.4 and 19.0^{O}C and acclimated to 0 or 32 ppth seawater [a]		
Rearing Temperature (^{0}C)	Salinity (ppth)	Average Percent Survival [b]
13.0	<1.5ppth[c]	100 ± 0.0
	32	92 ± 0.167
16.4	<1.5ppth	100 ± 0.0
	32	100 ± 0.0
19.0	<1.5ppth	100 ± 0.0
	32	58 ± 0.419
[a] N=3 individuals per tank [b] N=3 tanks used to calculate mean % survival [c] System water consistently measures below 1.5ppth salinity		

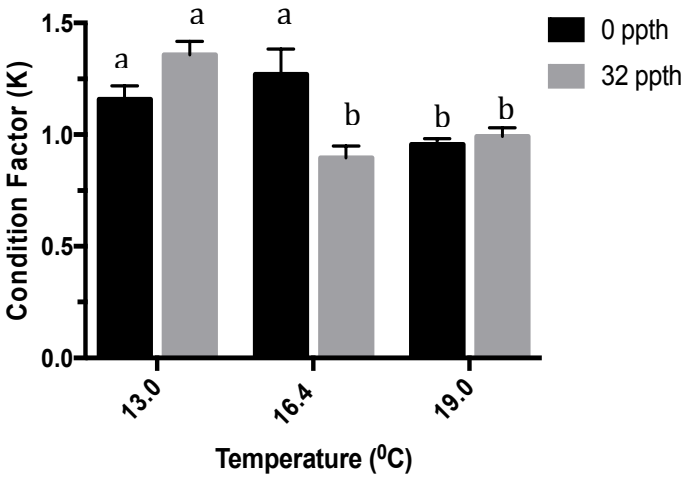

Fig 3.1, Condition factor (K) Steelhead parr exposed to 13.0, 16.4, and 19.0^{0}C and acclimated to 0 or 32 ppth salinity. Bars represent the mean ± SEM (N=7-12). Data are significant (ANOVA $p < 0.05$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

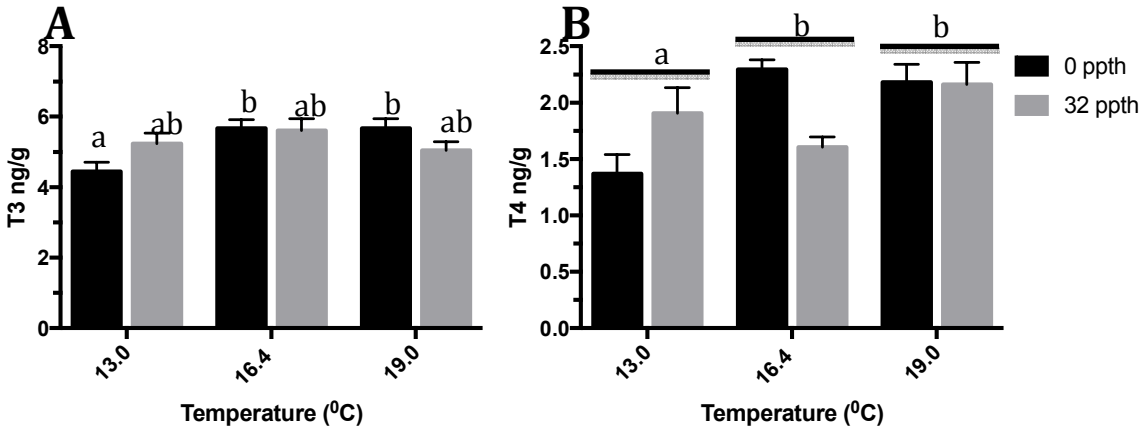

Fig 3.2, Levels of A) T3 (ng/g tissue) and B) T4 (ng/g tissue) in Steelhead exposed to 13.0, 16.4, and 19.0^{0}C and either 0 or 32 ppth salinity. Bars represent the mean ± SEM (N=5-6). Data are significant (ANOVA $p< 0.05$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

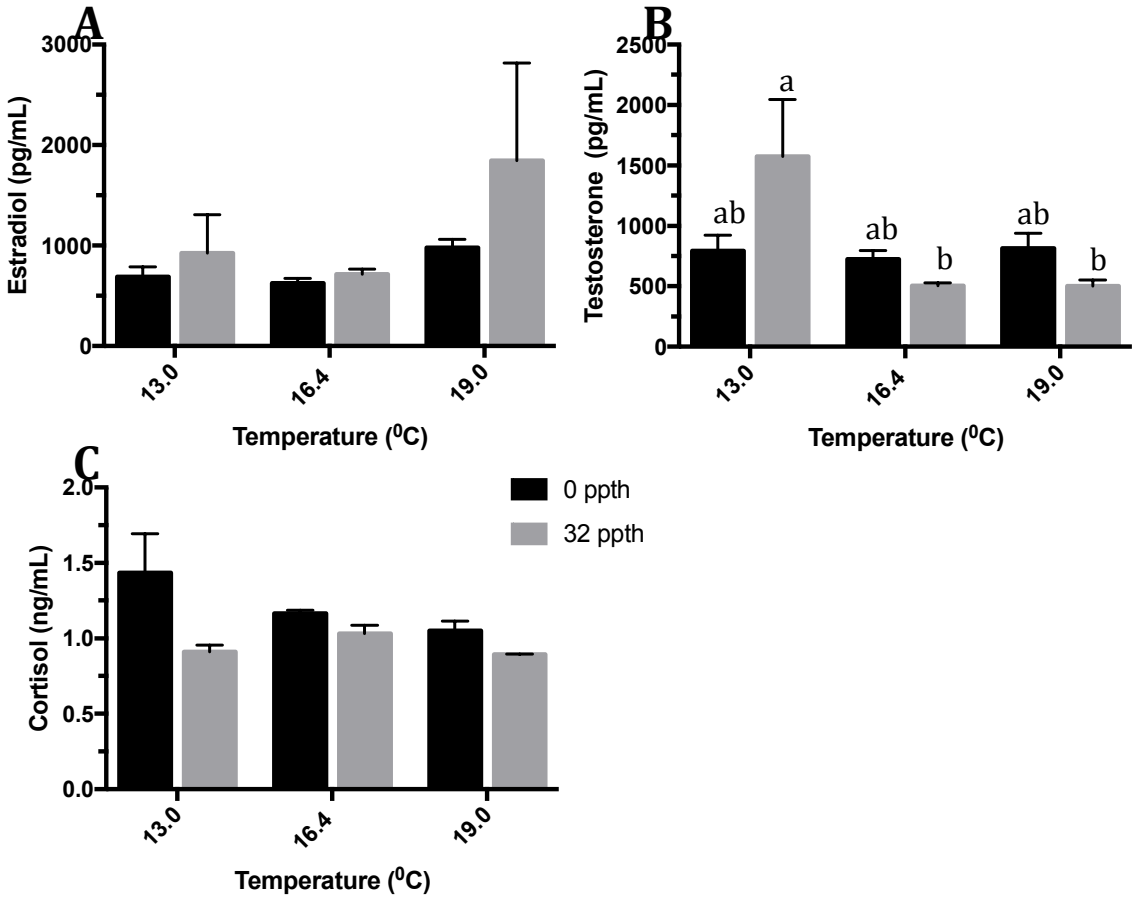

Fig 3.3, Levels of A) E2 (pg/mL), B) testosterone (pg/mL), and C) Cortisol (ng/mL) in serum from Steelhead exposed to 13.0, 16.4, and 19.0^{0}C and either 0 or 32 ppth salinity. Bars represent the mean ± SEM (N=3-4 samples pooled by tank). Data are significant (ANOVA $p< 0.05$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD. Estradiol data was not significant.

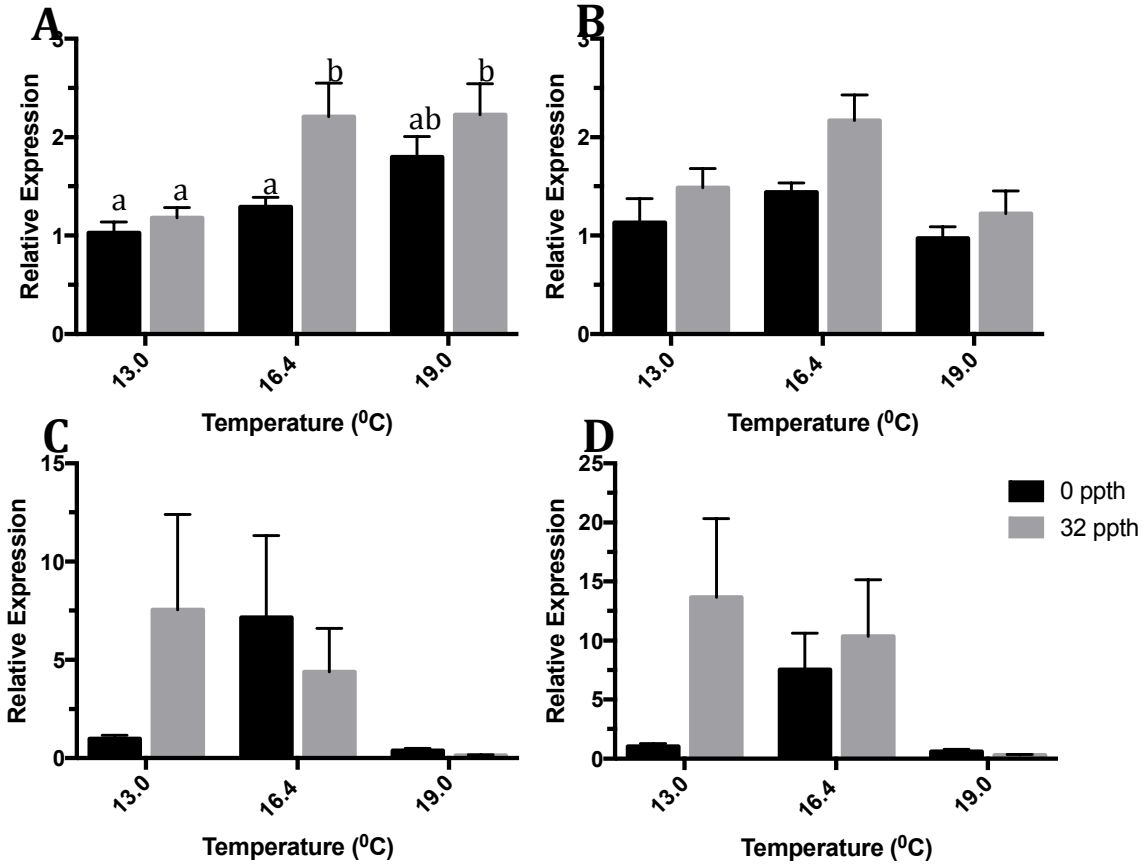

Fig 3.4, Relative expression of A) brain GH1, B) brain GnRH2, C) gill NKAα , and D) gill NKAβ in serum from Steelhead exposed to 13.0, 16.4, and 19.0^{0}C and acclimated to either 0 or 32 ppth salinity. Bars represent the mean ± SEM (N=5-6). Data are significant (ANOVA $p< 0.05$), and letters denote significance ($p<0.05$) between groups using Tukey's HSD.

IV. Discussion

The current study investigated the impacts of the climate change stressors of temperature and salinity on the endocrine pathways of Steelhead trout parr. Although the environmental stressors did not significantly impact survival, overall condition declined with increasing temperatures. Condition factor has been recommended as a metric of overall fish health, so a decrease in condition factor due to both increasing temperatures

and the combination of high temperatures and salinity acclimation demonstrate steelhead parr may be undergoing stressful conditions that affect fish health. Chinook salmon (*Oncorhynchus tshawytscha)* from the Bay-Delta have experienced lower growth and impaired smotlification when reared at high temperatures (Marine and Cech, 2002). If fish are struggling to maintain homeostasis and survive under environmentally stressful conditions, then it is unlikely parr are using energy resources to grow and undergo smoltification (Clark & Nagahama, 1977).

Thyroid hormones were significantly increased with temperature. Although salinity did not increase T3 levels, temperature significantly increased T3 in the FW fish. Similarly, temperature increased T4 levels regardless of salinity treatment. These patterns are consistent with TH profiles from successful smoltification in Atlantic (*Salmo salar*) and Coho (*Oncorhynchus kisutch*) salmon (Ebbeson et al., 2000; Bjornsson et al., 2011). Temperature and salinity also increased GH1 expression in the brain, which occurs during smoltification (Bjornsson et al, 2011; Handeland et al., 2013). Both THs and GH lead to the upregulation of NKA in the gill. However, NKA mRNA did not increase in Steelhead parr with exposure to increasing salinity and this correlated with a strong trend in a decrease in survival in fish exposed to higher temperatures and acclimated to 32 ppth salinity. Steelhead parr will move to cooler, less stressful waters despite the bioenergetic tradeoff of relocating, and adult steelhead mortality can occur at temperatures exceeding 25^0C (Moyle et al., 2017). Therefore, other molecular pathways may be adversely impacted by environmental stressors, and subsequently could lead to the inability of parr to produce NKA for successful osmoregulation.

Testosterone is another hormone that was significantly increased by both temperature and salinity, but estradiol levels trended towards a decrease with increasing exposure to each factor. Aromatase converts testosterone to E2, and temperature can impact aromatase activity and expression in many teleost fish species (Piferrer & Blazquez, 2005). It is possible that temperature significantly increased the activity of aromatase to produce more E2 from testosterone (Watts et al., 2005). E2 and exogenous estrogenic compounds inhibit smoltification (Madsen et al, 1997), but the effects of testosterone on smoltification are largely unknown. Testosterone significantly decreased with temperature in the SW exposed fish, which appears to correlate with the increase in E2 at both salinities at the highest temperature exposure. The HPG pathway for sex steroid synthesis is decreased during smoltification (Stefansson 2008), but we observed that testosterone did not decrease, thus indicating that steroid biosynthesis overall was not affected. Additionally, GnRH2 is important in regulating the feedback of sex steroid biosynthesis, and GnRH2 expression remained unchanged with increasing temperature and salinity, Thus, these results indicated impairment of the HPG pathway during duel temperature and salinity exposure.

Cortisol levels remained unchanged following exposure to increasing temperature and salinity, which could be due to chronic stress resulting from exposure. Cortisol plays a role in increasing chloride cell abundance and enhancing the efficiency of NKA in the gills (Richman & Zaugg, 1987). Chronic stress from consistently increasing salinity and high temperatures could lead to cortisol exhaustion as found in Atlantic salmon exposed to chronic stress (Madaro et al., 2015). The fish in the current study were reared for 16

days at consistent temperatures, so it is possible that fish exposed to higher temperatures and salinity may also experience chronic stress by the end of the study and therefore also cortisol exhaustion.

NKA mRNA in the gill exhibited an insignificant increase following temperature and salinity treatment. However, at 19 ^{O}C in FW and SW treatments both gill NKAα and NKAβ expression decreased, which is consistent with studies evaluating thermally stressed Atlantic salmon where NKA activity was decreased in fish that were acclimated to warmer temperatures at an accelerated speed compared to those acclimated at a slower rate (Zydlewski et al., 2005). Production of NKA is a bioenergetically costly process for fish, so NKA activity and expression is lowest at optimal temperatures and salinities (Imsland et al., 2002; Yang et al., 2018). Therefore, it is possible that production of NKA is too energetically costly for the fish reared at the highest temperature treatment, and this is consistent with the significantly lowered condition factor metrics. Additional studies are need to confirm this hypothesis. Overall, the combination of higher salinity and temperature prevented the upregulation of NKA mRNA and the hormonal profiles characteristic of smoltification.

V. Conclusion

Salmonids are encountering a multitude of anthropogenic stressors related to climate change, and it is imperative to assess the combined effects of multiple stressors on sensitive life stages. The parr- smolt transition is an important milestone in anadromous fish life history, and the current study aimed to understand how increasing temperatures and salinity would impact the vital hormonal pathways associated with

successful smoltification. We found that overall fish health was negatively impacted by the combination of increasing temperature and salinity. Additionally, higher temperatures led to altered HPG and HPT pathways, as well as lowered expression of NKA. This study is important for assessing the future risk of climate change on sensitive and endangered fish populations in the Bay-Delta, and for understanding the best management practices for Steelhead to mitigate adverse effects

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Chapter 4: The Effects of Bifenthrin and Temperature on the Endocrinology of Juvenile Chinook Salmon

Abstract

The San Francisco Bay-Delta is experiencing seasonally warmer waters due to climate change and receives rainstorm runoff containing pyrethroid pesticides. Chinook salmon (*Oncorhynchus tshawytscha*) inhabit the affected waterways from hatch through smoltification, and thus juvenile fish may experience both pyrethroid and warmer water exposures. The effects of higher temperatures and pesticide exposure on pre-smolt Chinook are unknown. To improve understanding of the potential interaction between temperature and pesticide exposure on salmonid development, juvenile alevin and fry were reared in 11ºC, 16.4ºC and 19ºC fresh water for 11 days and two weeks, respectively, and exposed to nominal concentrations of 0, 0.15, and 1.5 µg/L bifenthrin for the final 96 hours of rearing. Estradiol-17β (E2), testosterone, triiodothyronine, and thyroxine levels were measured in whole body homogenates using hormone-specific Enzyme-Linked Immunosorbent Assays (ELISAs). Brain gonadotropin-releasing hormone receptor (GnRH2), dopamine receptor 2A, and growth hormone mRNA levels were measured using qPCR. Results showed significantly decreased survival and condition factors observed with increasing temperature in alevin. Alevin thyroid hormones increased significantly with temperature, but fry thyroid hormones trended towards a decrease at lower temperatures with increasing bifenthrin exposure. There were

significant reductions in fry testosterone and E2 at 11ºC with increasing bifenthrin treatments, and significant changes in *GnRH2* and *GH1* gene expression in both alevin and fry, indicating potential disruption of hormonal and signaling pathways.

1. **Introduction**

Global climate change is causing surface water temperatures to rise in coastal California as well as increasing the intensity of rainfall events, specifically in the San Francisco Bay-Delta in Northern California (Wagner et al., 2011). The highly urbanized area around the Bay-Delta is a significant source of pesticides associated with storm event-induced runoff loads into the waterways (Weston & Lydy, 2010). Pyrethroids have been frequently detected in surface waters in the Bay-Delta watershed, and of all the pyrethroids detected, bifenthrin is often the predominant compound with concentrations up to 3.79 μg/L (Weston & Lydy, 2010; Siepmann & Holm, 2000). Bifenthrin use has been steadily increasing in the state of California, with approximately 370,000 lbs reported use in 2015 for both agricultural and urban sectors (CPDR, 2015). The high frequency of detection of bifenthrin in surface water and sediments raises concerns of its effect on nontarget aquatic organisms. Bifenthrin is acutely toxic to insects through binding to sodium channels and prolonging neuronal depolarization, which leads to fatal invertebrate neurotoxicity (Bradberry et al., 2005).

Bifenthrin has been shown to elicit estrogenic effects in ng/L concentrations, causing the inappropriate production of downstream sex steroids and expression of egg yolk proteins in male and juvenile fish associated with the endocrine pathway (Wang et

al., 2007; Brander et al., 2012; Forsgren et al., 2013; Crago & Schlenk, 2015; Brander et al., 2016). In teleost fish, bifenthrin has been shown to affect the dopaminergic pathway, which plays a vital role in the regulation of estrogen homeostasis (Crago & Schlenk, 2015; Bertotto et al., 2017). Previous studies with zebrafish have demonstrated that bifenthrin may alter endocrine responses depending on the developmental timing of neuroendocrine pathways in fish (Bertotto et al., 2017). The exact mechanism by which bifenthrin affects endocrine pathways in fish is unclear, particularly at different developmental time points.

In addition to increased exposure to contaminants, euryhaline organisms that reside in the Bay-Delta, such as the endangered Chinook salmon (*Oncorhynchus tshawytscha*), are also exposed to increasing annual and seasonal temperatures that cause increased thermal and osmotic stress (Hasenbein et al., 2013). Surface water exhibits the highest temperature increase in the summer months reaching temperatures over 25^0C according to models using past monitoring data (Wagner et al., 2011; Cloern et al., 2011), and this coincides with development and growth of juvenile salmonid populations (Kammerer & Heppell, 2013). Temperature can also influence the growth and rearing time of salmonids within the San Francisco Bay-Delta and alter migratory behaviors (Carter, 2005).

Salmonids are commonly found in estuaries during smoltification - the physiological and morphological transition of fish from fresh water to salt water. However, there has been little research on salmonids exposed to increasing temperatures in earlier juvenile stages. Salmonids advance through the alevin, fry, and parr juvenile

life stages, and parr typically undergo smoltification after a year. In the current study, we use winter-run Chinook salmon, which spawn in the winter months and develop from the alevin through the fry stage as surface water temperatures seasonally increase. Additionally, spring and fall-run Chinook spawn as surface water temperatures increase through the spring and summer months in the Bay-Delta watershed. Spawning is dependent on timing of adult Chinook returning to fresh water, and this largely depends if adults are fall, winter, spring, or summer run Chinook. Returning adults consistently spawn around the time of year of their own hatch.Increasing temperature is a cue for juvenile salmonids to migrate downstream and smolt (Brauer, 1982). Due to warming surface waters, juvenile salmon may experience the environmental cue to smolt at earlier stages. Smoltification is a hormone-driven process that involves alterations in cortisol, growth hormones, pituitary hormones, sex steroids, and thyroid hormones (McCormick, 1996; Ban, 2004). The thyroid hormones triiodothyronine (T3) and thyroxine (T4) increase during smoltification through stimulation of the hypothalamus-pituitary-thyroid (HPT) axis, which can be regulated by environmental cues such as salinity, temperature, and photoperiod (Blanton & Specker, 2007; Dickhoff et al, 1978). The feedback of the hypothalamus-pituitary-gonadal (HPG) axis and the production of sex steroids are regulated by the dopaminergic system in teleost fishes (Dufour et al., 2005; Levavi-Sivan et al., 2004). Dopaminergic neurons innervate the hypothalamus and decrease the release of gonadotropin-releasing hormone (GnRH), which furthers reduces the release of gonadotropins and subsequently sex steroid hormones from the gonads (Levavi-Sivan et

al., 2004). The potential impacts of thermal stress coupled with contaminant exposure on early salmonid life stages are largely unknown.

The overall objective of this study was to investigate the effects of pyrethroid exposure and increasing water temperatures on the survival and endocrinology of juvenile Chinook salmon through HPG and HPT axis endpoints. Chinook salmon were used because they are vital components of the complex ecosystem within the San Francisco Bay-Delta. This research will help explain the effects bifenthrin may have on the endocrinology of early life stages, and evaluate the potential impacts climate change can have on salmonid survival and restoration. We hypothesize that bifenthrin can negatively affect the sex steroid, dopaminergic, and thyroid hormone pathways and the adverse effects of bifenthrin may be exacerbated by the stress of increasing temperatures.

2. Methods

Test organisms

Alevin (29 dph) and fry (68 dph) Chinook salmon were obtained from the CDFW Feather River Fish Hatchery (Oroville, CA). Alevin (mass=0.288g ± 0.037) were acclimated to laboratory conditions for three days in static systems at 12^0C on a 14:10 light/ dark cycle. Fry (mass=0.887g ± 0.184) were also obtained from the Feather River Hatchery and acclimated to laboratory conditions for one week in flow-through Living Stream systems (Frigid Units). Fry were fed BioClark's Starter (Bio-Oregon) twice a day ad libitum. All fish were handled and treated in accordance with the approved

Institutional Animal Care and Use Committee protocols at the University of California, Riverside (animal use protocols 20130010)

Experimental design

Alevin exposures. After laboratory acclimation, 216 alevin were divided randomly into individual 8L static glass aquaria (2 replicates per treatment, N=16 individuals per tank) and subjected to three temperature regimes for 11 days; 11^0C, 16.4 ^{0}C, and 19^0C. Water was chilled or heated to the respective temperature with external heating and chilling units. Alevin were exposed for 11 days because we wanted to ensure that the fish still retained their yolk sacs in this short life stage for the duration of the exposure. In the final 96 hours (4 days) of the temperature exposure, fish were also simultaneously co-exposed to bifenthrin (purity >98%, Chem Service) in concentrations of 0, 0.15, and 1.5 µg/L with ethanol as the vehicle control. Bifenthrin concentrations were chosen from a range of reported measured concentrations during runoff events and have been used in previous studies (Forsgren et al, 2013; Crago & Schlenk, 2015). Alevin were given 6 days to acclimate to each temperature regime and then exposed to bifenthrin for 96 hours in order simulate exposure from an intense pesticide runoff event and increased surface water temperatures throughout the next century. The low 11^0C temperature represents the current temperature during spawning, 16.4^0C is the spring time water temperature, and 19^0C is the minimum predicted end of century temperature in the Bay-Delta (Wagner et al., 2011). Bifenthrin stock solutions were prepared to administer 1mL of solution per 1L of tank water. A 50% water change was conducted daily to refresh the bifenthrin

treatment. Fish were starved during the final 96 hours of exposure to prevent bifenthrin sorption to food and organic matter. Fish were then euthanized using 0.3g/L MS222 (Sigma Aldrich), and tissues were harvested, snap frozen in liquid nitrogen, and stored at -80^{0}C. Mortality was recorded throughout the exposure, and the average masses and fork lengths were measured at the end of the treatment.

Fry exposures. After lab acclimation, 144 fry were then divided randomly into individual 20L static glass aquaria (2 replicates per treatment, N=8 individuals per tank) and were then exposed to three temperature regimes for 14 days; 11^{0}C, 16.4 ^{0}C, and 19^{0}C. As with the alevin exposures described above, the fry were also exposed to 0, 0.15, and 1.5 µg/L bifenthrin with ethanol as the vehicle control for the final 96 hours of the temperature exposure. Water changes of 50% were carried out daily during exposure. Fish were then euthanized using MS222, tissues were harvested as described above, and masses and fork length were taken at the beginning and end of the experiment.

Bifenthrin water chemistry

Water samples were taken from bifenthrin treated fry tanks at 0 and at 96 hours after bifenthrin exposure. Water samples at time 0 were taken immediately after the first volume of bifenthrin was added. The samples were collected in 1L amber glass bottles and stored at 4 ºC, and were processed within two weeks of sampling. Extractions were preformed in glass separatory funnels with the individual water sample after the addition of 10 g of burned NaCl. Methylene chloride (ThermoFischer Scientific) (50mL) was added to the funnel and shaken for 2.5 min. After phase separation, the methylene

chloride layer was filtered through a layer of dried Na_2SO_4 into a round bottom flask. The same extraction was repeated for three consecutive times, and the combined extract was dried on a Rotevaporator to approximately 1 mL. The flask was rinsed with hexane and the sample further dried under N_2 to dryness, and the final sample was reconstituted in 1mL hexane. Samples were run on a GC/MS (5973N/ 6890N, Agilent Technologies) and a 7-point standard curve was used to determine concentrations. PCB 206 was used as the recovery surrogate and was added to each sample before extraction.

Hormone quantification

Competitive enzyme-linked immunosorbent assays (ELISA) were used to determine the concentrations of triiodothryonine (T3), thyroxine (T4), estradiol (E2), and testosterone in whole-body extracts of alevin and fry. Testosterone and E2 kits were sourced from Cayman Chemical, and T3 and T4 kits were sourced from Genway Biotech. Whole alevin and fry were homogenized in a lysis buffer with 1μM PMSF, centrifuged at 12,000g at 4^0C, and the supernatant used for the T3 and T4 ELISA analyses. Homogenate supernatant (250μL) was extracted for sex steroid hormones 3 times with methylene chloride. The samples were dried under N_2 and reconstituted with the E2 ELISA buffer. All buffers and reagents were prepared with ultra-pure water and mixed according to respective kit protocols. Standard curves for calculating hormone levels were run for each analysis, and were provided with the kits. Plate absorbances were detected on a Spectra Max Plus 385 microplate reader (Molecular Devices) at 450 nm for the thyroid hormone ELISAs and 405 nm for the E2 and testosterone ELISAs.

Gene expression

Real-time quantitative PCR (qPCR) was used to assess the expression of *Growth Hormone I* (*GH1*), *Gonadotropin Releasing Hormone II* (*GnRH2*), and *Dopamine Receptor 2a* (*DR2a*) in fry brain (the same individuals used for hormone analysis) and a subset of alevin head tissues. Genes were selected because of the role of each receptor in the development and smoltification of salmonids through the HPG, growth axis, and dopaminergic system, respectively. The expression of *Deiodinase II* (DIO2) in fry brain was also assessed using qPCR to further characterize the stressor effects to the thyroid axis observed in fry. Total RNA was extracted using the RNeasy mini extraction kit (Qiagen), and brain tissue was extracted with trizol and chloroform to aid in the extraction of RNA from lipid tissue. RNA quality and concentration were checked using a Nanodrop ND-1000 (ThermoFischer Scientific) and ratios of 280/260 and 260/230 were all between 1.85-2.1. RNA integrity was confirmed by the presence of distinct 28S and 18S bands on 1% agarose gels. Complementary DNA (cDNA) was synthesized with 1μg of total RNA using the Reverse Transcription System (Promega) with random primers according to the kit protocol to result in 20 μL of cDNA. qPCR was run using primers designed for Rainbow trout (*Oncorhyncus mykiss*) from Integrated DNA Technologies (PrimerQuest tool) (Supplemental Table 1) and SSO Advanced Universal SYBR Green Supermix (BioRad) was used as the quantification dye. Protocols were optimized for primer concentration and 1 μL of cDNA was used per well. The samples run on a CFX Connect Real-Time PCR Detection System (BioRad) with the following protocol: 95^0C for 3 min, and then 40 cycles of 10 s at 95^0C and 30 s of 56^0C. A melting

curve was preformed after the PCR protocol with continuous fluorescent measurement. PCR products were run on an agarose electrophoresis gel to confirm target amplicons. Elongation Factor 1α (EF1α) was used as the housekeeping gene in fry, and both EF1α and 18S were used as housekeeping genes in Alevin. Percent efficiency curves were calculated for all primers using a 5-point serial dilution; DIO2 (97%), EF1α (98.9%), DR2A (97.8%), GH1 (98.1%), GnRH2 (97.9%). Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Differences between tank replicates were analyzed using a t-test, and no significant differences between replicates at each treatment were observed. All data were tested for normality using a Shapiro-Wilk test, and Levene's tests were run to determine homogeneity of variance. Since the data were not normal, then generalized linear models (GLM) fitted to a Poisson distribution were used to determine the significance of the hormone and qPCR data for both alevin and fry. A Tukey's post-hoc was run to compare the significant among between treatment groups if $p < 0.05$ for each variable and for the interaction of bifenthrin and temperature. Multivariate survival tests were run on the survival data to determine significance between survival curves of each treatment. All statistical analyses were conducted in R studio.

3. Results

Bifenthrin water chemistry

Measured bifenthrin and recovery values are reported as averages of 0 and 96-h samples (Table 1). There was approximately 200% higher measured concentrations of bifenthrin in the 0.15 µg/L nominal treatment between 0 and 96 hours. In the 1.5 µg/L treatment, measured values were 75% higher than nominal concentrations. Recovery values were calculated from PCB 206 and ranged between 118 and 137%.

Survival and condition factor

For all comparisons 11^0C and the ethanol vehicle controls were used as an optimal control. Alevin survival significantly decreased by 34.1% at the 19^0C exposure compared to the optimal 11^0C controls (Supplemental Fig 1A). At 16.4^0C, the1.5 µg/L bifenthrin treatment was associated with a 23.5% decrease in alevin survival compared to the vehicle control. Although bifenthrin and temperature did not significantly impact the survival of fry, there was a trend towards a decrease in fry survival after the 1.5 µg/l bifenthrin exposure in all temperature treatments (Supplemental Fig 1B).

Alevin condition factor significantly decreased by an average of 7.7% in all 19^0C exposures regardless of bifenthrin exposure when compared to 11^0C exposures (Fig 1A). Similarly, fry condition factor significantly decreased with exposure to 19^0C compared to the 11^0C exposures (11% and 12% respectively). There was also a significant decrease (9.5%) in condition factor in fry exposed to 11^0C and 1.5µg/L bifenthrin compared to the vehicle controls (Fig 1B).

Thyroid hormone measurements

Whole-body T3 and T4 levels followed the same patterns in alevin after bifenthrin and temperature exposure increasing by an average of 105% and 70.5%, respectively, from all 19^0C exposures compared to the 11^0C treated fish (Fig 2A-B). Additionally, there was a 77.3% and 67.7% increase in the concentrations of T3 in alevin at the 16.4^0C temperatures treated with 0.15 and 1.5 µg/L bifenthrin compared to the controls. Fry T3 levels trended towards an increase and T4 levels significantly increased ($P<0.05$) at the 16.4^0C and 19^0C exposures with increasing bifenthrin exposure (Fig 2C-D). In contrast, T3 levels in the 11^0C exposed fry showed a decreased trend and T4 levels significantly decreased ($P<0.05$) with increasing bifenthrin concentrations. T3 levels showed a decreased trend and T4 significantly decreased ($P<0.05$) with increasing temperature exposures in the vehicle control treated fry.

Steroid hormones measurements

Testosterone in alevin treated at 19^0C was significantly increased 1.7-fold compared to the controls (Fig 3A). Temperature also significantly increased testosterone levels 2.7 and 4.4-fold in 16.4°C exposures following treatment with 0.15 and 1.5 µg/l, respectively, compared to the 11°C exposed alevin in the same bifenthrin treatment. Additionally, testosterone levels were significantly increased in the 19°C exposures in the 0.15 and 1.5 µg/l bifenthrin treatments when compared to the 11°C alevin (3.5 and 6.7-fold, respectively). Bifenthrin significantly elevated alevin testosterone levels 1.0-fold at the 19°C and 1.5 µg/l bifenthrin treatment compared to the vehicle controls. There was

also a significant temperature-dependent increase in E2 levels in alevin in the 16.4°C and 19°C exposed fish in all bifenthrin treatments (79% and 187% average increase, respectively) (Fig 3C). However, bifenthrin did not have a significant effect on the alevin E2 levels.

Fry testosterone was significantly decreased in the 11°C treatment with 0.15 and 1.5 µg/L bifenthrin exposure (46.3 and 10.7-fold, respectively) compared to the control (Fig 3B). Similarly, fry E2 significantly decreased in the 11°C treatment with exposure to 0.15 and 1.5 µg/L bifenthrin (34.5 and 36.4-fold, respectively) compared to the control (Fig 3D). There was a significant increase in E2 levels at the 16.4°C and 19°C temperatures with exposure to 0.15 µg/L (96.4 and 52.9-fold, respectively) and 1.5 µg/l (37.4 and 61.9-fold, respectively) bifenthrin when compared to 11°C exposed fry in the same bifenthrin exposures. There was also a significant increase in testosterone levels at the 16.4°C and 19°C temperatures with exposure to 0.15 µg/L (99.9 and 92.8-fold, respectively) and 1.5 µg/l bifenthrin (9.17 and 33.5-fold, respectively) when compared to fry at 11°C exposed to the same bifenthrin concentrations.

Relative expression of brain transcripts

Bifenthrin significantly affected alevin *GnRH2*, *GH1*, and *DR2A* mRNA expression; however, temperature had a more significant affect on the expression of the same genes in fry. There was a significant increase in transcripts of *GH1* (Fig 4D) and *DR2A* (Fig 4F) in fry exposed to 11°C and 0.15 and 1.5 µg/l bifenthrin when compared to the control treated fish in the same temperature. *GnRH2* mRNA expression was

significantly increased 10.0-fold in alevin exposed to 11°C and 1.5 µg/l bifenthrin compared to controls (Fig 4A). Relative expression of the targeted genes did not significantly change in alevin exposed to 16.4°C and 19°C when treated with bifenthrin. In fry, there was a 1.9 and 1.8-fold increase *GH1* in both the vehicle control and 0.15 µg/l bifenthrin exposed fish, respectively. Similar to alevin, both 0.15 and 1.5 µg/l bifenthrin significantly increased *GnRH2* mRNA expression in fry at 11°C compared to the controls (Fig 4B). *DIO2* mRNA was not significantly affected by treatments, but a trend of an increase was observed with increasing temperature in the ethanol control treated fry (P=0.117) (Fig 5).

Table 4.1. Measured mean concentrations of bifenthrin in fry exposures for 0 and 96 hours[a]

Nominal Bifenthrin Water Concentration (µg/L)	Average Measured Bifenthrin ± SD (µg/L)	% Recovery ± SD
0	< LOD	131.0 ± 25.83
0.15	0.4749 ± 0.1835	118.2 ± 34.65
1.5	2.63 ± 1.751	137.1 ± 22.99

[a] N= 12 for bifenthrin aquaria, and N=4 aquaria for vehicle controls.

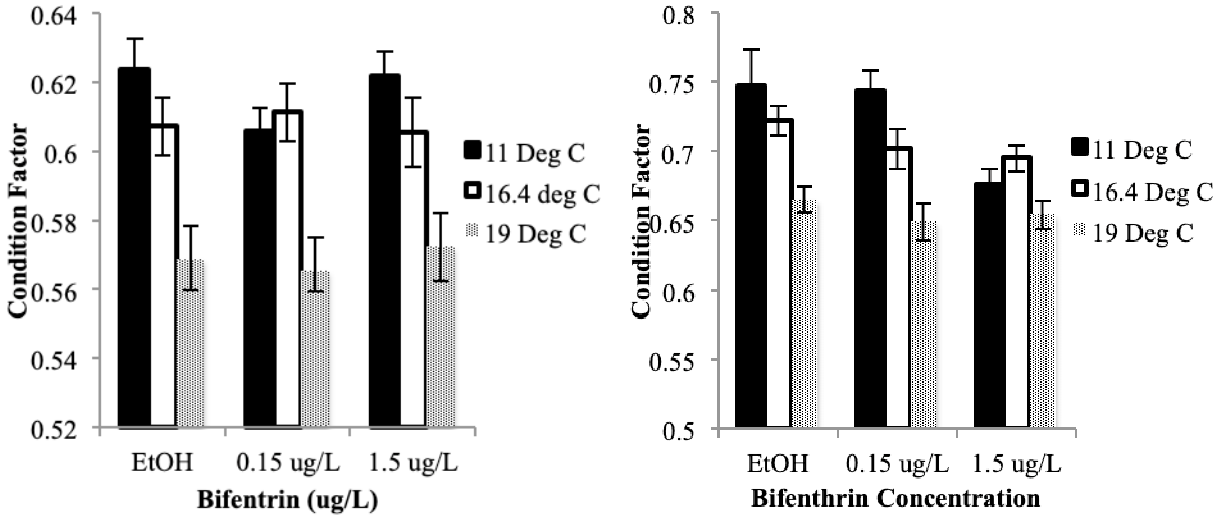

Fig. 4.1. Condition factor of (A) Chinook alevin and (B) Chinook fry exposed to 11, 16.4, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean of 32 individual alevin and 16 individual fry ± SE. Same letters denote no significance among treatments with Tukey's post-hoc ($p<0.05$)

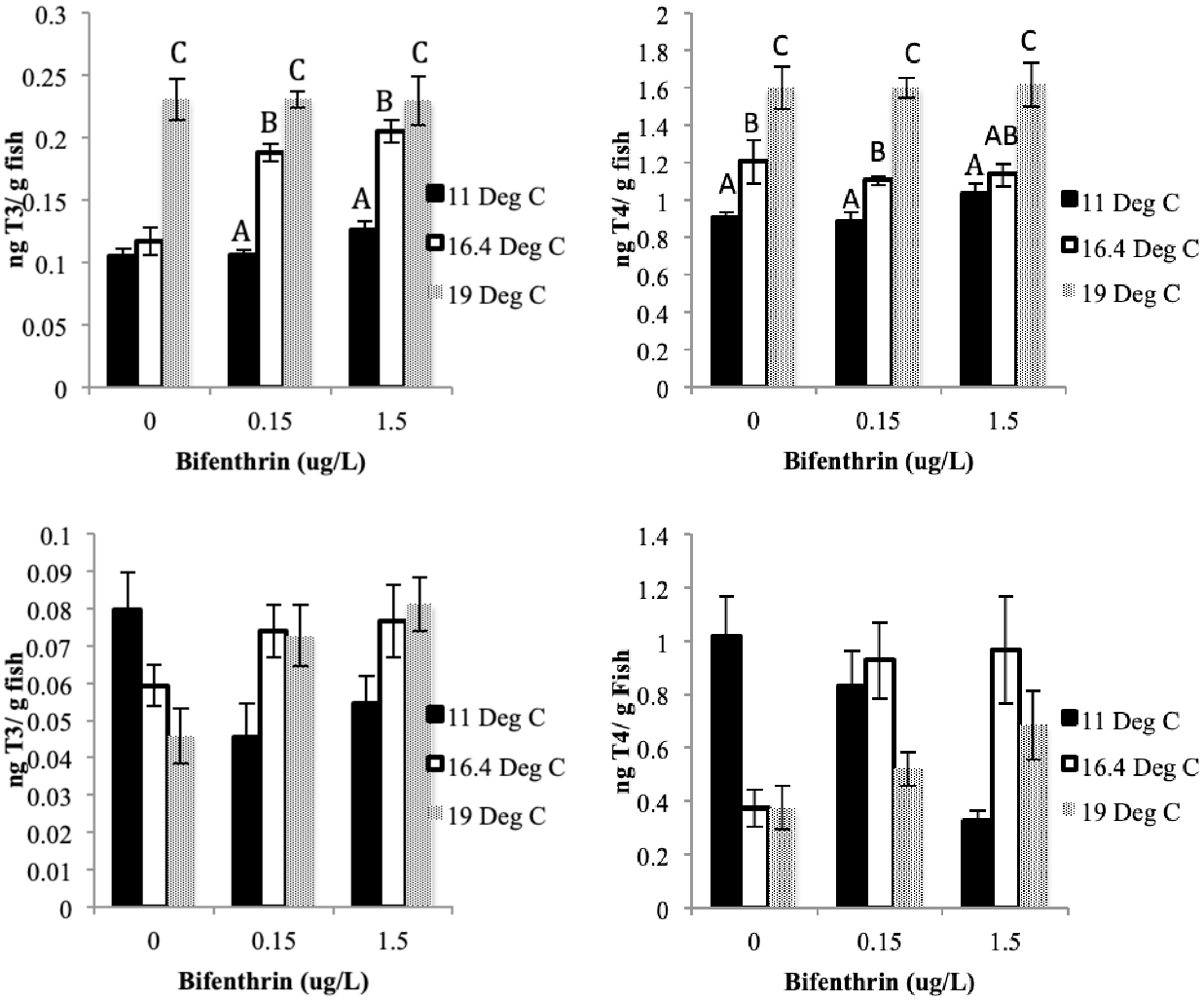

Fig. 4.2. Levels of whole-body (A) T3 (ng/ g fish) and (B) T4 (ng/g fish) in Chinook alevin and whole-body (C) T3 (ng/ g fish) and (D) T4 (ng/g fish) in Chinook fry exposed to 11, 16.4, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE (N=7). Same letters denote no significance among treatments with Tukey's post-hoc ($p<0.05$). Data were not significant in fry T3 (GLM $p> 0.05$).

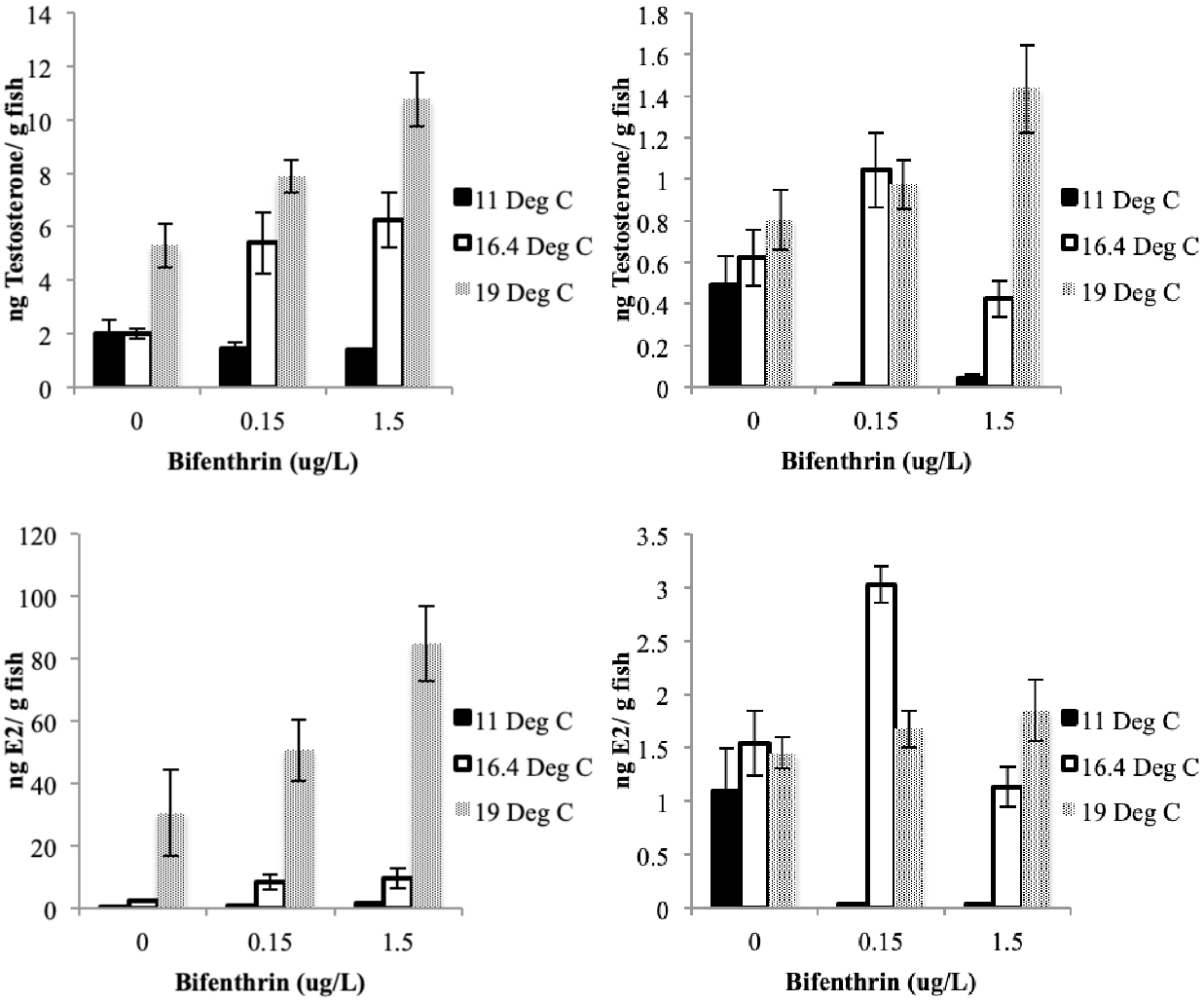

Fig. 4.3. Levels of whole-body testosterone (ng/ g fish) in Chinook (A) alevin and (B) fry and whole-body estradiol (ng/ g fish) in Chinook (C) alevin and (D) fry exposed to 11, 16.4, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE (N=7). Same letters denote no significance among treatments with Tukey's post-hoc ($p<0.05$).

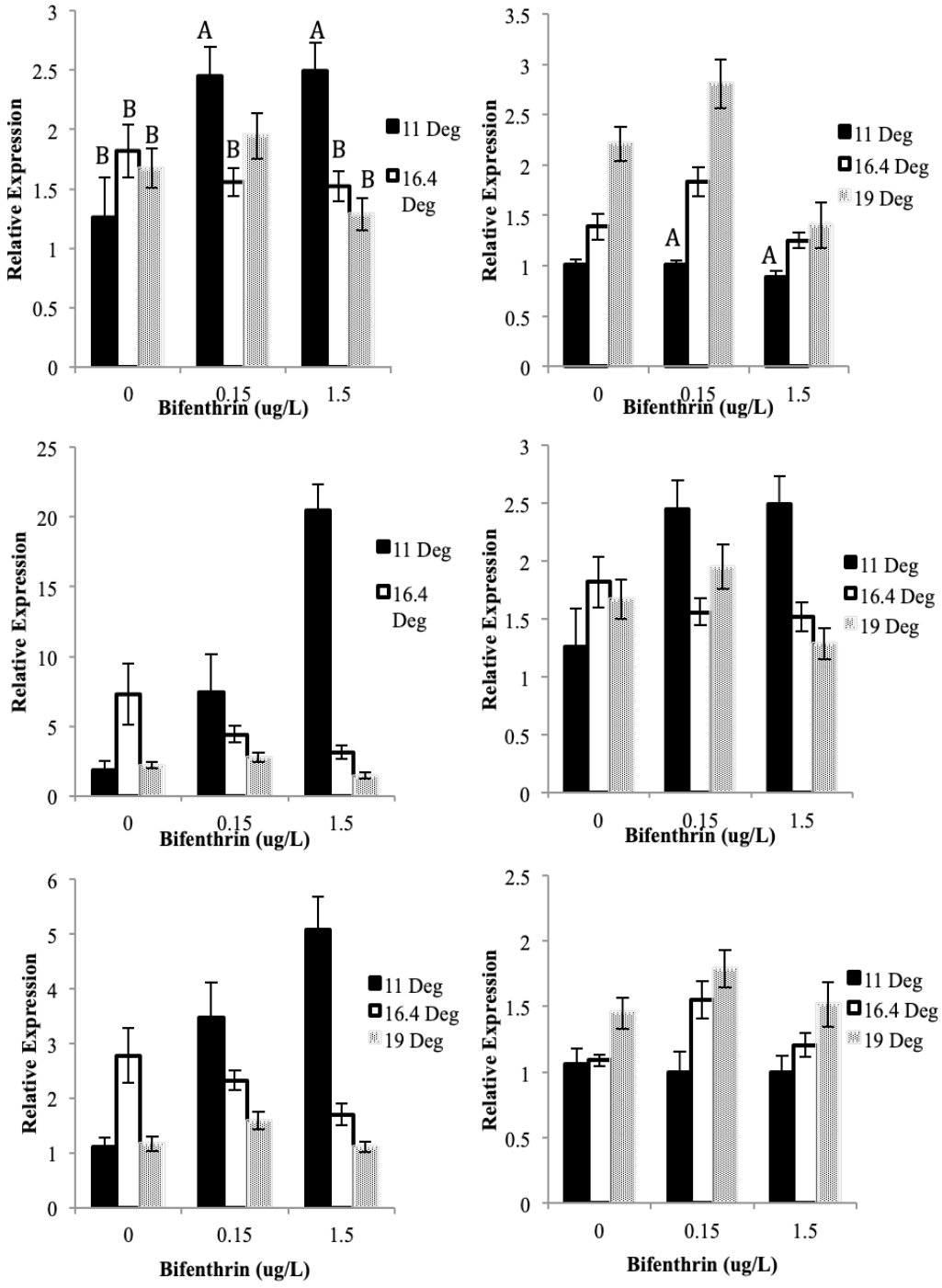

Fig. 4.4. Relative Expression of brain GH1 in Chinook (A) alevin and (B) fry, GnRH2 in (C) alevin and (D) fry, and DR2A expression in (E) alevin and (F) fry exposed to 11, 16.4, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE (N=8). Same letters denote no significance among treatments with Tukey's post-hoc ($p<0.05$).

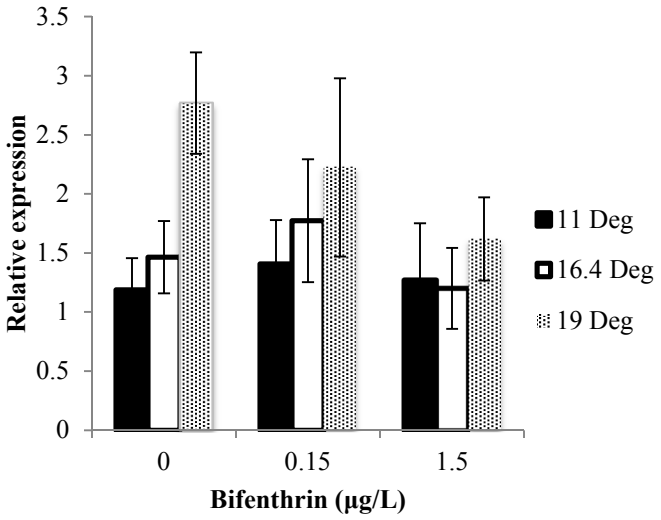

Fig. 4.5. Relative Expression of DIO2 expression in Chinook fry exposed to 11, 16.4, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE (N=8). The data were not significant (GLM p>0.05).

4. Discussion

The San Francisco Bay-Delta is experiencing the effects of climate change as surface water is seasonally warming (Wagner at al., 2011). Juvenile endangered Chinook salmon (*Oncorhynchus tshawytscha*) inhabit affected waterways as alevin, fry, and parr. Due to enhanced intensity of storm events, bifenthrin concentrations have been observed in surface waters of the Bay-Delta comparable to those used in this study, in particular the 0.15 µg/L treatment (Siepman & Holm, 2000). Although bifenthrin is not acutely toxic to humans and mammals, many studies have demonstrated that bifenthrin has adverse sublethal effects in fish (Brander et al. 2016). Thus, juvenile fish may experience increased seasonal temperatures during bifenthrin runoff events. Consequently, the

effects of these combined stressors were investigated through controlled lab exposures of Chinook salmon at two developmental stages in the current study.

Survival and thyroid hormone effects

Increasing temperature without bifenthrin caused a significant decrease in alevin survival and condition factor. In fry, there was no significant mortality observed in any treatment, but there was a significant decrease in condition factor in fish exposed to 19°C in the control and 0.15 ug/l bifenthrin treatments. Increased mortality, decreased fish health, and decreased growth with exposure to increasing temperatures due to climate change have been documented in juvenile teleost fish. Fish from cold and temperate waters experience faster development leading to early hatching and increased metabolic vulnerability in juveniles when exposed to upper thermal limits (Pankhurt & Munday, 2011). When juvenile Chinook salmon were exposed to 21–24°C at the upper limit of their thermal tolerance, significantly lower condition factors and growth rates were observed (Marine & Cech, 2004). A decrease in condition factor in both alevin and fry at the 19°C temperature exposure in our study suggests that the predicted end of century surface water temperatures in the Bay-Delta can negatively impact juvenile salmonid health and survival. Additionally, bifenthrin decreased fry condition factor at the 1.5 µg/l concentration in salmon exposed to both 16.4^0C and 19^0C temperatures, indicating bifenthrin may also impact the growth axis in fry during this period where rapid growth normally occurs and is critical for the successful survival and timing of salmonid smoltification.

The decrease in fry condition factor due to increasing temperature may be related to the trending decrease of both thyroid hormones. Thyroid hormones, specifically T3, play a major role in rapid growth and development in juvenile salmonids (Tagawa & Hirano, 1987; Farbridge & Letherland, 1992; Dickhoff et al., 1997). In alevin, T3 and T4 followed the pattern of increasing concentration after exposure to both increasing bifenthrin and temperature. Higher T4 levels and low T3 levels suggest that T4 is not converted into T3 by deiodinase, specifically in the fry stage. The hypothalamus of the brain signals the pituitary gland to release thyroid-stimulating hormone, which subsequently releases the precursory thyroxine (T4) to be converted by deiodinase to the biologically functional triiodothyronine (T3) (Ban, 2004). We observed that alevin T3 and T4 increased with temperature, so bifenthrin and temperature may not be impacting deiodinase activity at this life stage since T3 and T4 were not measured in inverse concentrations. If we observed an increase in T3 and a decrease in T4, this would suggest that deiodinase is converting T4 to T3, which was observed as a trend at the high temperature exposures in fry. There is evidence that larval fish use maternal thyroid hormones in growth and development until they develop their own thyroid follicles to produce T4 (Blanton & Specker, 2004). Alevin may not be producing endogenous T3 and T4 at this stage, so healthy control fish may be using more of the maternal hormones for growth compared to the bifenthrin and high temperature treated alevin. Less chemical and environmental stress may allow the alevin to grow and develop faster than stressed fish, and this is seen in the condition factor results.

To further investigate our thyroid hormone results, we measured the relative expression of deiodinase2 (DIO2) mRNA to determine if bifenthrin and/ or temperature had an impact in fry. A trend toward an increase of deiodinase mRNA expression with increasing temperature was consistent with lower T4 levels at the 16.4^0C and 19^0C exposures compared to the 11^0C exposures. DIO2 expression also trended towards a decrease with increasing bifenthrin concentration at the 19^0C exposure, which also corresponds to the same decreasing trends observed in fry T3/T4. Thyroid hormone levels change throughout salmonid development, and T4 levels typically decrease as T3 levels increase during periods of rapid growth after yolk absorption due to the formation of thyroid follicles in older juvenile stages (Tagawa & Hirano, 1987). However, studies with other teleost fish have found that increasing temperature does not directly affect deiodinase activity; rather, the levels of altered thyroid hormones are due to temperature impacts upstream of thyroid hormone conversion (Van Den Burg et al., 2003). Contrasting these results, a decrease in DIO2 was observed in zebrafish embryos exposed to nominal concentrations of 1, 3 and 10 μg/L of bifenthrin (Tu et al. 2016). The impacts of temperature and bifenthrin on DIO2 in fish are unclear and suggest multiple crosstalk hormonal targets that may undergo equally subtle changes. For example, increasing sublethal temperatures have been shown to promote the increase of thyrotropin-releasing hormone and trigger crosstalk with cortisol pathways that can also regulate downstream thyroid hormone production in fish (Van Den Burg et al., 2003). Alternatively, molecular docking studies have indicated that bifenthrin can effectively bind to thyroid hormone receptors in zebrafish (Tu et al., 2016). Additional studies exploring crosstalk

linkages with other hormonal systems involved in thermoregulation within poikilothermic organisms is necessary to identify the role of the HPT axis in bifenthrin toxicology.

Sex steroid effects

The most notable response in the HPG axis endpoints examined in the present study was the significant decrease in estradiol and testosterone at the 11^0C temperature with increasing concentrations of bifenthrin in fry. Aromatase (CYP19A1) converts testosterone to estradiol in both fish brain (CYP19A1b) and gonadal tissues (CYP19A1a) (Piferrer & Blázquez, 2005), and previous studies have shown that aromatase enzyme activity is reduced by temperature in fish (Shen & Wang, 2014). Although salmonid sex differentiation is genetically determined (rather than through temperature-dependent sex determination), higher temperature exposures can lead to masculinization in many other teleost species by decreasing aromatases (Piferrer & Blázquez, 2005). However, fry E2 and testosterone followed the same pattern of decreasing levels in 11^0C and increasing levels in 16.4^0C and 19^0C with bifenthrin exposure in our study, and we observed no significant differences in steroid levels in control fry in each temperature treatment. The decrease of the sex steroid hormones at optimal temperatures suggest upstream modulation of the HPG axis by bifenthrin in the sex steroid biosynthetic pathways because the activity of aromatases in fry do not appear to be affected by either temperature or bifenthrin, as evidenced by the same profiles of E2 and testosterone. Cholesterol-based hormones, such as pregnenolone and progesterone, are synthesized to

estradiol or testosterone in both the brain and gonads by a series of cytochrome P450s and hydroxysteroid dehydrogenases enzymes (Sanderson, 2006) so it is possible that bifenthrin could target other components of steroid biosynthesis signaling upstream of estradiol synthesis.

The stage-dependent effects of diminished sex steroids between fry and alevin may be attributed to the incomplete development of gonads at the earlier larval stage. Rainbow trout become sexually dimorphic around 35 days post fertilization (Vizziano et al., 2007), which is comparable to the same alevin stage in Chinook salmon. Although salmonids are sexually differentiated at the alevin stage, sex steroid synthesis is localized to a group of somatic cells in the precursory gonads (Vizziano et al., 2007). The gonads in juvenile salmonids are still developing through smoltification; therefore, gonad and HPG development may be more sensitive to stress from temperature compared to older, mature fish. Life stage has been shown to be important with regard to the estrogenic effects of bifenthrin with greater estrogenic activity noted in juveniles relative to embryos in zebrafish (Bertotto et al., 2017). Temperature increases the rate of development and growth of juvenile salmonids as long as there is ample food availability (Marine & Cech, 2004; Brett et al., 1969); however, as juvenile fish approach their critical thermal maximum, nutrient conversion and basic physiological processes are impaired (Brett et al., 1969). If young salmonids are rapidly developing due to warmer temperatures, there could be a threshold where excessive temperatures lead to developmental impairment specifically in the HPG pathways. The differences we observed in E2 and testosterone levels with exposure to bifenthrin may be due to incomplete development of the HPG

pathway in earlier life stages, and differences observed due to temperature may be attributed to basic changes in the rate of development. Whatever the mechanism, it is clear that temperature impacted the stage-dependent effects of bifenthrin, which raises concern for how temperature will affect the toxic mechanisms of other potential endocrine-disrupting compounds in aquatic systems.

Dopamine receptor effects

Dopaminergic signaling is a critical regulator of gonadotropin release in the HPG axis. Dopamine has an inhibitory effect on GnRH hypothalamic neurons by binding directly to dopmamine 2 receptors (DR2A), and thus prevents the release of GnRH and the cascade of downstream HPG axis signaling (Gopurappilly et al., 2013; Levavi-Sivan et al., 2004). *GnRH2* was chosen as an endpoint in the present study because of its role in the neuroendocrine system and in fish sensory behavior, and it is well characterized in salmonids (Wootten & Smith, 2014). Overall, dopamine prevents release of gonadotropins and subsequently lowers E2 and testosterone levels (Dufour et al., 2005). Given the altered concentrations observed in the temperature and bifenthrin treatments, the role of the dopaminergic system was evaluated. Expression of *DR2A* mRNA was unchanged with bifenthrin exposure in fry at the higher temperature exposures, but increased with bifenthrin exposure at 11^0C. In contrast, *GnRH2* mRNA was lower in control fry at 11^0C compared to the higher temperatures where there was a significant increase with bifenthrin. Similar to fry, there was also a significant increase in alevin *DR2A* mRNA with bifenthrin exposure at 11°C, indicating that bifenthrin has

concentration-dependent effects on *DR2A* mRNA expression at optimal temperatures at both the alevin and fry life stages. Although DR2A expression was not significantly changed in alevin exposed to 19^{O}C in any bifenthrin treatment, E2 and testosterone levels significantly increased with increasing bifenthrin compared to the other temperatures. This suggests that negative feedback through DR2A interactions on hypothalamic neurons to reduce GnRH levels may not be occurring at lower temperatures, but disruption may be occurring at higher temperatures and with bifenthrin exposure. Ratios of dopamine and its metabolite DOPAC have been shown to increase in juvenile zebrafish exposed to bifenthrin (Bertotto et al., 2017). While this did not correlate with significant changes in estradiol, this does indicate that bifenthrin can possibly impact dopamine production or metabolism. Another variable is the relationship between DA and *DR2A* expression. It is unclear whether DR2A undergoes autoregulation by DA or its metabolites. Thus, to assume a direct relationship between DA and its receptor is unfounded. In addition, control of the HPG axis by the dopaminergic system may not be fully developed in salmon alevin and fry compared to older juvenile stage animals (Levavi-Sivan et al., 2004; Crago & Schlenk, 2015). If the dopaminergic system was actively regulating the production of sex hormones in alevin through negative feedback, then we should observe an increase in DA, which would presumably increase DR2A due to an increase of GnRH to potentially lower the elevated sex hormone levels in the higher temperature exposures. However, this correlation was not observed, so the developing dopaminergic system may not be fully capable of regulating HPG feedback in alevin, or

temperature may be disrupting other HPG feedback processes. Clearly, additional study is needed to evaluate the stage-dependent regulation of HPG in teleosts.

Growth hormone receptor effects

An additional hormonal pathway that regulates fish condition and/or growth is that controlled by growth hormone. Bifenthrin had concentration-dependent effects on *GH1* mRNA expression at optimal temperatures at the alevin life stage. Growth hormone is critical to the development of juvenile fish and eventually to the smotification of juvenile salmonids (Melamed et al., 1998; Nilsen et al., 2007). Growth hormone stimulates thyroid hormone activity in trout (MacLatchy and Eales, 1990), and GnRH and sex hormones partially regulate GH during sexual development in fish (Bjornsson et al., 2002). Both decreasing thyroid hormones and increasing GH levels may play a role in the regulation of GH receptor in developing vertebrates by increasing GH receptor expression (Schwartzbauer & Menon, 1998). We observed that bifenthrin and temperature impact growth and expression of *GH1* in alevin, but temperature only significantly increases T3 levels in alevin. If T3 was independently regulating *GH1* expression, we would expect to see lowered *GH1* expression in all 11^{O}C treated fish. However, we did not see this correlation. In fry we observed an increase in *GH1* expression with temperature, but T3 levels were lower in 11^{O}C bifenthrin treated fish. This suggests that in the later life stage the increase in *GH1* expression could be due to a subtle decrease in T3 and possibly due to an increase in GH. Especially in alevin, GH

may be a potential link in the crosstalk between HPG and HPT pathways due to the rapid growth at these earlier stages of overall development.

Summary and conclusions

Although we observed lethality due to increasing temperature and bifenthrin in alevin, the results showed a range of adverse sublethal effects in both the alevin and fry life stages. Increasing temperature and bifenthrin concentrations may have negative impacts to juvenile salmon. Altered reproduction, viability, and growth were several of the adverse transgenerational effects observed from low (1 ng/L) bifenthrin and increased temperature exposures in *Menidia beryllina* (DeCourten & Brander, 2017). Similarly, we found that subchronic exposure to increasing temperature and bifenthrin can adversely affect growth as well as thyroid and estrogenic signaling pathways in developing juvenile salmonids. Although exposures to bifenthrin and elevated temperature do not cause direct mortality on all stages of juvenile salmonids (Weston et al., 2015; Crago & Schlenk, 2015), the adverse sublethal effects on endocrine and reproductive pathways may have ongoing population-level effects (Forsgren et al, 2013; Baldwin et al., 2009). The results of this study indicated that climate change and contamination of man-made chemicals may affect endangered salmon populations, and demonstrate the need to consider the developmental stage of fish when determining the risks of climate change and chemical stressors.

Acknowledgements

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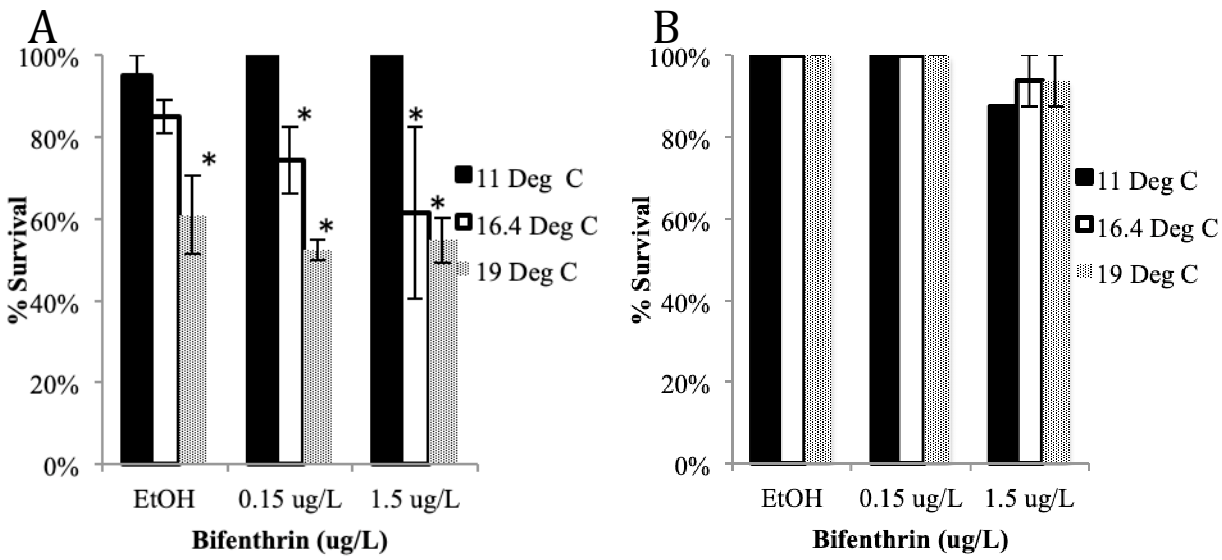
Supplemental Data



Supplemental Fig. 1 (Fig 4.6). Percent survival of (A) Chinook alevin and (B) Chinook fry exposed to 11^0C, 16.4^0C and 19^0C and 0, 0.15, and 1.5 ug/L bifenthrin for 11 and 14 days, respectively. Asterisk denotes significance ($p< 0.01$) using Dunnett's post hoc analysis compared to 11^0C at each bifenthrin treatment.. Each value represents the mean of 2 replicates ± SE.

Supplemental Table 1 (Table 4.2), Primers used for qPCR of alevin and fry brain

Target Gene	Primer (Forward)	Primer (Reverse)	NCBI Acc. Number
18S	CGTCGTAGTTCCGACCATAAA	CCACCCACAGAATCGAGAAA	XM_021615674
EF1α	GAGACCCATTGAAAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	XM_021561980
GnRH2	GATCGCAAACTTCTGCTTGATG	ATACGACCAGTGCTGAGAGA	NM_001124280
GH1	CGAGCTGGACATGGAGGAGC	GGGGACAGTCTAGGAGGCA	XM_021601256
DR2a	CTCCTCCATTGTGTCCTTCTAC	GCTTTGTGTTCACCCGTTTC	NM_001124372
DIO2	GAGTCGCTTTTAGTCTGG	ACTGAGATGGCAGACTGAAGGACT	NM_001124268

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Chapter 5: The Effects of Temperature and Bifenthrin on the Endocrinology and Behavior in Parr of Chinook Salmon (*Oncorhynchus tshawytscha*)

Abstract

Coastal California has been experiencing the effects of non-chemical and chemical anthropological stressors through respective increases in surface water temperatures and rainstorm-derived runoff events of pyrethroid pesticide movement into waterways such as the San Francisco Bay-Delta. Salmonid populations in the Bay-Delta have been dramatically declining in recent decades. Therefore, the aim of this study was to investigate the effects of bifenthrin, a pyrethroid insecticide, and increasing water temperatures on targeted neuroendocrine and behavioral responses in Chinook salmon (*Oncorhynchus tshawytscha*) parr (10- month post-hatch). Parr were reared at 11ºC, 16.4ºC, or 19ºC for 14 days and, in the final 96 hours of rearing, exposed to nominal concentrations of 0, 0.15, or 1.5 µg/L bifenthrin. A predatory avoidance Y-Maze behavioral assay was conducted immediately following exposures. Parr were presented a choice of clean or odorant zones, and locomotive behavior was recorded. Thyroid hormones (T3 and T4), estradiol, and testosterone were quantified within plasma using ELISAs, and the expression of brain hormone and dopamine receptor genes were also evaluated by qPCR. Brain dopamine levels were analyzed by LC/MS. No significant changes were observed in brain transcripts or plasma hormone concentrations with bifenthrin or increasing temperature. However, temperature did significantly lower brain dopamine levels in fish reared at 19ºC compared to 11ºC controls, but was unaltered by

bifenthrin treatment. In contrast, parr reared at 11ºC and exposed to 1.5 µg/L bifenthrin spent significantly less time avoiding a predatory odorant compared to vehicle controls reared at 11ºC . The 16.4ºC and 1.5 µg/L-treated fish spent significantly more time in the neutral arm compared to the odorant and clean arms, as well as spending significantly less time in the clean arm compared to the 11ºC control fish. These results suggest that temperature and bifenthrin may be adversely impacting predator-avoidance behavior, which may not be related to dopaminergic responses.

I. Introduction

Pyrethroid insecticides are commonly used for agriculture and urban purposes throughout the United States. In California, pyrethroid use has been increasing as organochlorines and organophosphates have been phased out over recent decades (Epstein et al., 2000). Although pyrethroids are not acutely toxic to mammals at the concentrations applied for pest control, they are acutely toxic to aquatic organisms such as invertebrates and fish (Haya et al, 1989). Following runoff events from storms, pyrethroids are frequently detected in water samples from the San Francisco Bay-Delta (Bay-Delta) watershed. Bifenthrin is the most frequently detected pyrethroid in the San Francisco Bay Delta (Weston & Lydy, 2010), and its increased use in urban locations has been associated with the decline of endangered pelagic fish populations (Fong et al, 2016).

The Bay-Delta is a critical rearing ground for the endangered anadromous fish, Chinook salmon (*Oncorhynchus tshawystscha*). Juvenile salmonids live in fresh water

during the alevin, fry and parr life stages. The transition from freshwater to seawater, termed smoltification, occurs after the parr stage, and is largely initiated by environmental cues such as warming water temperatures (Zydlewski et al., 2005; Bjornsson et al., 2011). Given the importance of temperature in the initiation of smoltification, climate change may have significant impacts on the transition from fresh to salt water. Juvenile salmonid populations in the Bay-Delta are experiencing warmer waters from climate change both annually and seasonally, and surface water temperatures are predicted to rise above 19ºC by the end of the century (Wagner et al., 2011).

Smoltification is a hormone-driven process that results in an increase in sodium-potassium ATPase (NKA) in the gills of fish, which allows them to adapt to higher salinities. Multiple hormone axes regulate the physiological changes of smoltification. For example, cortisol, thyroid hormones [thyroxine (T4) and triiodothyronine (T3)], and growth hormone all increase Insulin-like Growth Factor I (IGF-1), which upregulate gill NKA during smoltification (McCormick, 1998; Nilsen at al, 2007; Stefansson et al., 2008; Bjornsson et al., 2011). Conversely, the levels of sex steroid hormones, such as estradiol and testosterone, decrease during smoltification through negative regulation of the hypothalamus-pituitary-gonadal (HPG) axis (Bjornsson et al., 2011). Estradiol, testosterone, and other estrogenic compounds inhibit smoltification and production of gill NKA in salmon and trout parr through the HPG axis (Ikuta et al., 1987; Madsen et al., 1997). Therefore, it is possible that other endocrine disrupting compounds may affect the ability of parr to smolt.

Bifenthrin has been shown to have estrogenic effects in fish (Wang et al. 2007; Brander et al., 2012). Following acclimation to hypersaline conditions and increasing temperatures, fish exposed to bifenthrin can also show enhanced estrogenic responses (Forsgren et al., 2013; Riar et al., 2013; Decourten & Brander, 2017). Estrogen biosynthesis is a key component of the HPG axis and is negatively regulated by the dopaminergic neurons in gonadotropes to prevent the release of gonadotropin-releasing hormone from the hypothalamus (Dufour et al, 2005). Tyrosine hydroxylase expression, an important dopamine precursor, and dopamine receptor 2A (DR2A) expression were decreased in juvenile salmonids exposed to 0.15 and 1.5 µg/L bifenthrin (Crago & Schlenk, 2015). Thus, bifenthrin may induce estrogenic effects through modulation of the dopaminergic pathway.

The dopaminergic system also plays a vital role in teleost olfactory sensation and processing (Hoglund et. al., 2005). Salmon rely on olfaction to find food, mate, return to fresh water as adults, and to avoid predators. The ability of fish to detect and avoid predators is necessary for their survival both as juveniles and as adults (Laberge & Hara, 2001). Sublethal concentrations of common pesticides have negatively affected predator-avoidance behaviors and olfactory function in salmonids, which were predicted by various models to cause adverse population-level effects (Tierney et al., 2010; Baldwin et al., 2009; Maryoung et al., 2015).

The purpose of this study was to evaluate the impacts of temperature and bifenthrin on the dopaminergic and endocrinology of salmonid parr through analyzing hormone levels and gene expression of the HPG and hypothalamus-pituiatry-thyroid

(HPT) axes. Predator-avoidance behaviors were also assessed to determine whether there were relationships among endocrine responses and potential olfactory impairment.

II. Methods

Chemicals

Bifenthrin (98% purity) was purchased from ChemService (West Chester, PA), and then dissolved in ethanol ((99.9%); Decon Laboratories) to make a working stock of 0.3 g/L. Tricaine methanesulfonate (MS-222; 98% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Tauracholic acid (TCA, >95%) was purchased from Sigma Aldrich (St. Louis, MO). Methylene chloride (≥99.9%), hexane (≥99.9%), acetone (≥99.9%), ethanol, and formic acid (OPTIMA grade) were purchased from Fisher Scientific (Hampton, NH). Dopamine-1,1,2,2-d_4 hydrochloride (DA-d4 standard) was purchased from Sigma Aldrich (St. Louis, MO) as an internal standard for UPLC quantification of dopamine.

Test Organisms

Juvenile Chinook salmon were obtained from the Feather River Hatchery (CA Department of Fish and Wildlife, Oroville, CA) and reared in Living Stream (Frigid Units, Toledo, OH) systems for 9 months and held at 12ºC and 12 h:12 h light-dark cycles. Fish were approximately 10 months old and had an average mass of 23.7 g ± 0.094 and length of 12.76 cm ± 0.02. Salmon were fed Oncor Fry (Skretting, Tooele, Utah) fish pellets ad libitum daily. Water quality tests for ammonia, nitrate, nitrite, pH,

and hardness were performed weekly. All fish were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (#20130010) at the University of California, Riverside.

Experimental Design

Fish were acclimated to three temperatures, 11 ± 0.36ºC, 16.4 ± 0.13ºC, and 19 ± 0.18ºC for two weeks. The temperatures were selected because they are representative of the current, optimal rearing temperature (11ºC), and predicted end of century temperature in the Bay-Delta (19ºC), respectively (Marine & Cech 2004; Richter & Kolmes, 2005; Wagner et al., 2011). Fish exposed to 11ºC were used as the control temperature because this is the average current temperature during the late fall. In the final 5 days (96 hours), fish were simultaneously co-exposed to 0, 0.15, and 1.5 µg/l bifenthrin. Three tanks containing three fish each were used for each treatment group (N=81 total fish). Ethanol (0.001%) was used as a vehicle control. Water changes (50%) were conducted every other day prior to bifenthrin exposure, and then conducted daily to renew bifenthrin treatment and maintain water quality. Fish were housed in static 20-L glass aquaria, and external chilling and heating units were used to regulate temperature. Immediately following exposures, individual fish length and mass were recorded for condition factor calculations.

Bifenthrin Water Chemistry

Bifenthrin water samples were collected in 1-L amber bottles from a subset of tanks in each treatment at 0, 24, 48 and 96 hours during the bifenthrin exposure. Samples from 0 and 48 hours were collected immediately after water changes with renewed bifenthrin solutions, and samples from 24 and 96 hours were collected immediately before water changes with bifenthrin renewal. Samples were stored at 4ºC in 1-L amber bottles and processed within 12 days of collection. Bifenthrin extraction and measurements were performed according to Giroux et al. (2019) and PCB126 was used as a recovery standard. Briefly, individual water samples underwent liquid-liquid extractions three consecutive times in glass separatory funnels using methylene chloride. The methylene chloride extracts were condensed on a Rotevaporator and then further dried under N_2. Samples were reconstituted in 1 mL of hexane and analyzed with a GC-MS/MS (5973N/6890N; Agilent Technologies, Santa Clara, CA) to determine bifenthrin concentrations. A laboratory blank extraction was performed with every round of extraction to ensure no contamination from glassware.

Hormone Quantification

Thyroid hormones (T3 and T4), estradiol, and testosterone were quantified using Enzyme-Linked Immunosorbent Assays (ELISAs). Serum was used directly for T3 and T4 assays (GenWay Biotech Inc., San Diego, CA). For estradiol and testosterone, a 3X methylene chloride extraction was conducted on individual plasma samples. After evaporation of the solvent under N_2, the residue was reconstituted in 300 µL of ELISA

buffer (Cayman Chemical, Ann Arbor, MI) for estradiol and testosterone ELISAs (Cayman Chemical, Ann Arbor, MI) as optimized in Giroux et al., 2019. Standard curves and measurements were performed according to individual kit instructions .

Gene Expression

Gonadotropin releasing hormone receptor 2 (GnRH2), growth hormone 1 (GH1), and dopamine receptor 2α (DR2α) mRNA levels were analyzed using qPCR. RNA was extracted from brain tissues using the Qiagen RNeasy Lipid kit (Hilden, Germany). RNA integrity was confirmed on 1% agarose gels by the presence of 16S and 18S bands, and samples were quantified using a NanoDrop 2000 (Thermo Fischer, Waltham, MA). All 260/280 and 260/230 values were >1.85. cDNA was synthesized from 1 μg of RNA using random primers in the Promega Reverse Transcription kit (Madison, WI). qPCR was conducted according to the same methods and GHR and GnRH2 primers from Giroux et al. (2019). DR2α were designed from Rainbow trout DR2α RNA (Genbank: NM_001124372.2) using IDT PrimerQuest Design Tool (Integrated DNA Technologies, Coralville, IA) and then blasted using NCBI Primer Blast to confirm primer specificity. EF1α and 18S were used as housekeeping genes (Giroux et al, 2019). All primers were optimized for concentration and products confirmed by gel electrophoresis.

Brain Dopamine Measurements

Parr brains were extracted for dopamine following the protocol described in Bertotto et al. (2019). Briefly, individual brain samples were homogenized in 1% formic acid with

deuterated dopamine as an internal standard. Samples were centrifuged at 12,000g at 4ºC for 5 minutes and the supernatant was transferred to a new tube. The extraction was repeated for a total of three times. Solid phase extraction of the supernatant was performed using Strata-X 8B-S100-UBJ cartridges (Phenomenex, Torrance, CA) in a vacuum manifold, and samples were eluted from the column with 3 mL of 0.1% formic acid in methanol/acetonitrile 50:50 (v/v). Samples were dried under a gentle stream of N_2, reconstituted in 1% formic acid in e-pure water, and syringe filtered using a PP 0.2-μm filter (Tisch Scientific, North Bend, OH) before storage at -20ºC.

Samples and 8-point standard curves were analyzed on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) coupled with a Waters Micromass Triple Quadrupole mass spectrometer (qQq) equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA). An ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 μm) (Waters, Milford, MA) was used for analyte separation at 40ºC. Mobile phase A consisted of 0.1% formic acid in DI water and mobile phase B consisted of 0.1% formic acid in MeOH/MeCN (1:1, v/v). The system was primed with mobile phase A and 5 μL of each sample was injected for analysis. Peaks were detected and integrated using TargetLynx XS software, and sample concentrations were normalized to initial brain tissue mass.

Olfactory Behavioral Assay

Olfactory functions were assessed using a predator-avoidance behavioral assay that allowed fish to sense a predatory odorant and choose to swim in the freshwater

section or the odorant section (Maryoung et al., 2015; Tierney et al. 2010; Rehnberg et al. 1985). Predator-avoidance tests were preformed in a Y-Maze tank to test olfactory functions immediately following the conclusion of the 96-hour bifenthrin exposure. Behavioral tests were (Supplemental Fig.1). Taurocholic acid (TCA) was used a predatory odorant because it has been shown to elicit predator-avoidance responses (Tierney et al., 2010). In each treatment, a maximum of 8 fish were used for the behavioral analysis. Fresh water chilled to the respective temperature of exposure for each individual fish was constantly pumped through the Y-maze at a rate of 3.6 L/min. Individual parr were acclimated to the Y-Maze in the central area for 5 minutes with the gates closed. TCA at 0.1 M was pumped continuously into the Y-Maze at a rate of 3.6 mL/min for the next 5 minutes while the gates were still closed. The gates were then lifted and individual fish were allowed to swim freely for 5 minutes with the TCA continuously pumping. Fish behavior was recorded during 5 minutes of free swimming using a GoPro Hero 3+ action camera. The Y-maze was thoroughly rinsed two times with fresh water and refilled with the appropriate temperature water before each behavioral trial. The odorant arm was alternated between each trial in order to eliminate spatial bias between fish. The total number of visits to each arm, duration of time spent in each arm, and final location of each fish was determined from video recordings.

Immediately following behavioral analysis, individual fish were immediately sacrificed by immersion in 300 mg/L MS-222 for 2 minutes and tissues/serum were harvested immediately after each behavioral trial. Serum was separated by centrifugation

at 5,000g for 10 minutes. All tissues and serum were snap frozen in liquid nitrogen and stored at -80ºC.

Statistics

Levene's test was run to determine variance and Shapiro-Wilk's test was run to determine distribution normality. Since the distribution of hormone and qPCR data were not normal, multivariate Generalized Linear Models (GLMs) using a poisson distribution were used to test the significance of these results. Tukey's post-hoc was run to compare the significant differences between each treatment group. A multivariate survival test was run on the survival data to determine significance between survival curves of each treatment.

A two-way ANOVA was run to analyze brain dopamine data. A three-way ANOVA was used to analyze the interaction of bifenthrin, temperature, and zone choice for the time spent in each zone and the percentage of fish in each zone from the predator-avoidance behavioral assay. A Tukey's post-hoc test was subsequently run to compare between treatment groups.

III. Results and Discussion

Water Chemistry

There were no significant differences between measured bifenthrin concentrations in each temperature treatment at each time point, so reported bifenthrin concentrations are averages of all temperature samples for each bifenthrin treatment at each time point

(Supplemental Table 1). Average measured bifenthrin concentrations for all time points were 102.7% and 113.1 % of the nominal 0.15 and 1.5 μg/L concentrations respectively. Bifenthrin was not detected in the vehicle control samples (<LOD, 0.1 ng/L).

Survival and Condition Factor

Survival significantly decreased with temperature at the 19ºC exposures in all bifenthrin treatments ($p<0.05$) relative to controls (Supplemental Figure 2A). When compared to the 11ºC treatment groups, survival decreased in the 19ºC exposures by 33%, 45%, and 89% at the 0, 0.15, and 1.5 μg/L treatments, respectively (Supplemental Figure 2A). In the 16.4ºC treatment group, survival was 100% for both the control and the 0.15 μg/L treatments, and survival was lowered by 33% in the 1.5 μg/L treatment. Survival was 100% in the 11ºC control group. Condition factor was not significantly changed with either bifenthrin or temperature treatments (Supplemental Fig 2B). However, there was a trend towards a decrease in condition factor with bifenthrin exposure in both the 16.4ºC and 19ºC temperature treatments.

Hormone Analysis

Thyroid hormone levels were not significantly affected ($p<0.05$) by either temperature or bifenthrin treatments in parr (Supplemental Figures 3A,B). There was a trend of increasing T4 levels in the 11ºC and 16.4ºC-exposed parr with increasing bifenthrin exposure relative to controls, as well as trends towards increasing T4 with temperature. Teststerone and E2 data were not significantly affected ($p<0.05$) by either

temperature or bifenthrin treatments (Supplemental Fig 4A,B). However, there was a trend towards an increase in E2 levels with increasing bifenthrin in salmon reared at 16.4ºC. Due to high mortality, hormone analyses for the fish exposed to both 1.5 μg/L and 19ºC were not obtained.

Gene Expression

There were no significant differences in relative expression of GnRH2, GH1, or DR2α (Fig. 1) in any treatment relative to vehicle controls ($p>0.05$). All values are reported as the mean of N=8. The interaction of bifenthrin and temperature did not significantly alter expression of measured genes, and the impacts of bifenthrin and temperature stressors separately had no significant impacts.

Brain Dopamine Analysis

Temperature significantly lowered brain dopamine levels (Fig. 2) in the 19ºC-reared fish compared to the 11ºC- reared fish, regardless of bifenthrin treatment. As bifenthrin did not significantly alter dopamine levels, the post-hoc analysis only compared temperature treatments.

Behavioral Assay

High mortalities of fish exposed to 19ºC in all bifenthrin treatments prevented accurate behavioral assay measurements at this temperature; therefore, only behavioral data from the 11ºC and 16.4ºC treatments were reported in the current study. No fish

from the 11ºC group in the vehicle control and 0.15 µg/L bifenthrin treatments remained in the odorant arm at the end of the behavioral assay, but significantly more fish were found in the odorant arm of the 11ºC and 1.5 µg/L bifenthrin treatment compared to the control (Fig. 3A). Additionally, there were no significant differences ($p<0.05$) between the percentages of fish found in each zone within the 16.4ºC treatment groups (Fig. 3A). Both vehicle control and 0.15 µg/L bifenthrin treatment groups reared at 11ºC spent significantly less time (95%) in the odorant arm of the Y-Maze compared to the clean arm, and they only spent an average of 4% of time in the odorant arm (Fig. 3B). The fish reared at 11ºC and exposed to 1.5 µg/L bifenthrin spent 91% more time in odorant arm compared to vehicle controls in the same temperature group. There were no significant differences in the amount of time spent in each zone between fish from the 11ºC and 1.5 µg/L bifenthrin treatment and the fish reared at 16.4ºC vehicle control and 0.15 µg/L bifenthrin treatment (range 70-120 seconds). The 16.4ºC and 1.5 µg/L bifenthrin-treated fish spent significantly more time in the neutral arm compared to the odorant and clean arms, as well as spending significantly less time in the clean arm compared to the vehicle and 11ºC control fish.

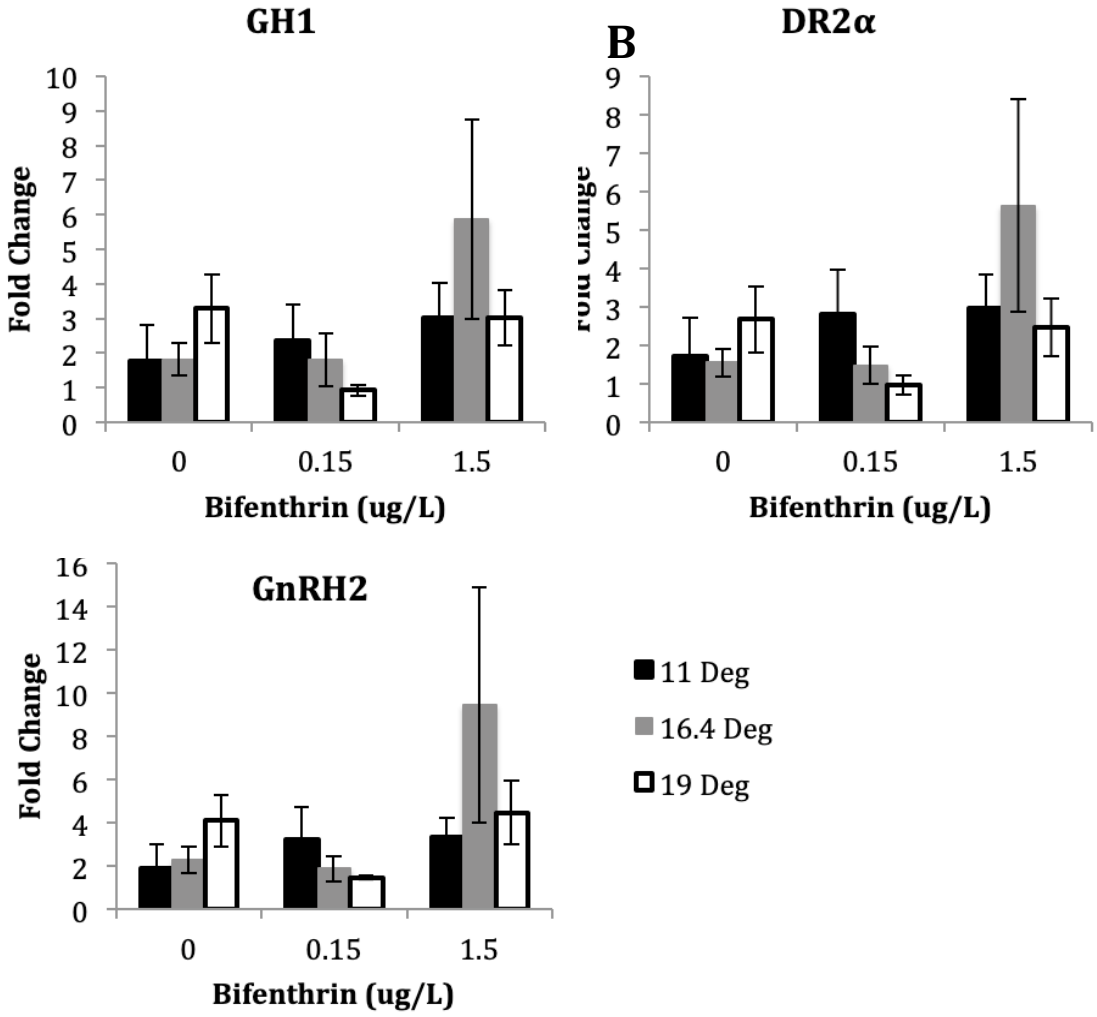

Fig. 5.1. Relative Expression of brain GH1(A), DR2α (B), and GnRH2(C) in parr exposed to 11ºC, 16.4°C, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, N=5-6. Data were not significant (GLM p> 0.05).

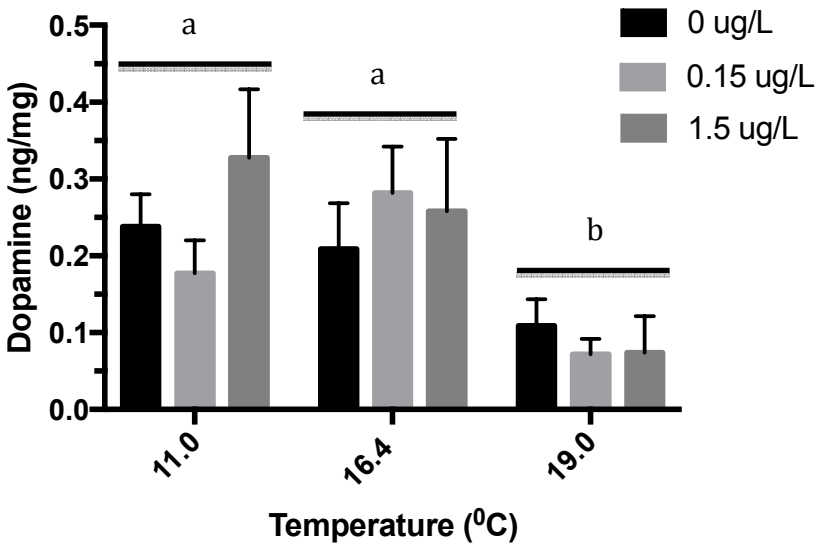

Fig. 5.2. Dopamine levels (ng/mg tissue) in parr brain exposed to 11°C, 16.4°C, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, N=5-6. Data was significant (ANOVA p> 0.05) for the effects of temperature, and letters denote significant changes between temperature treatment groups.

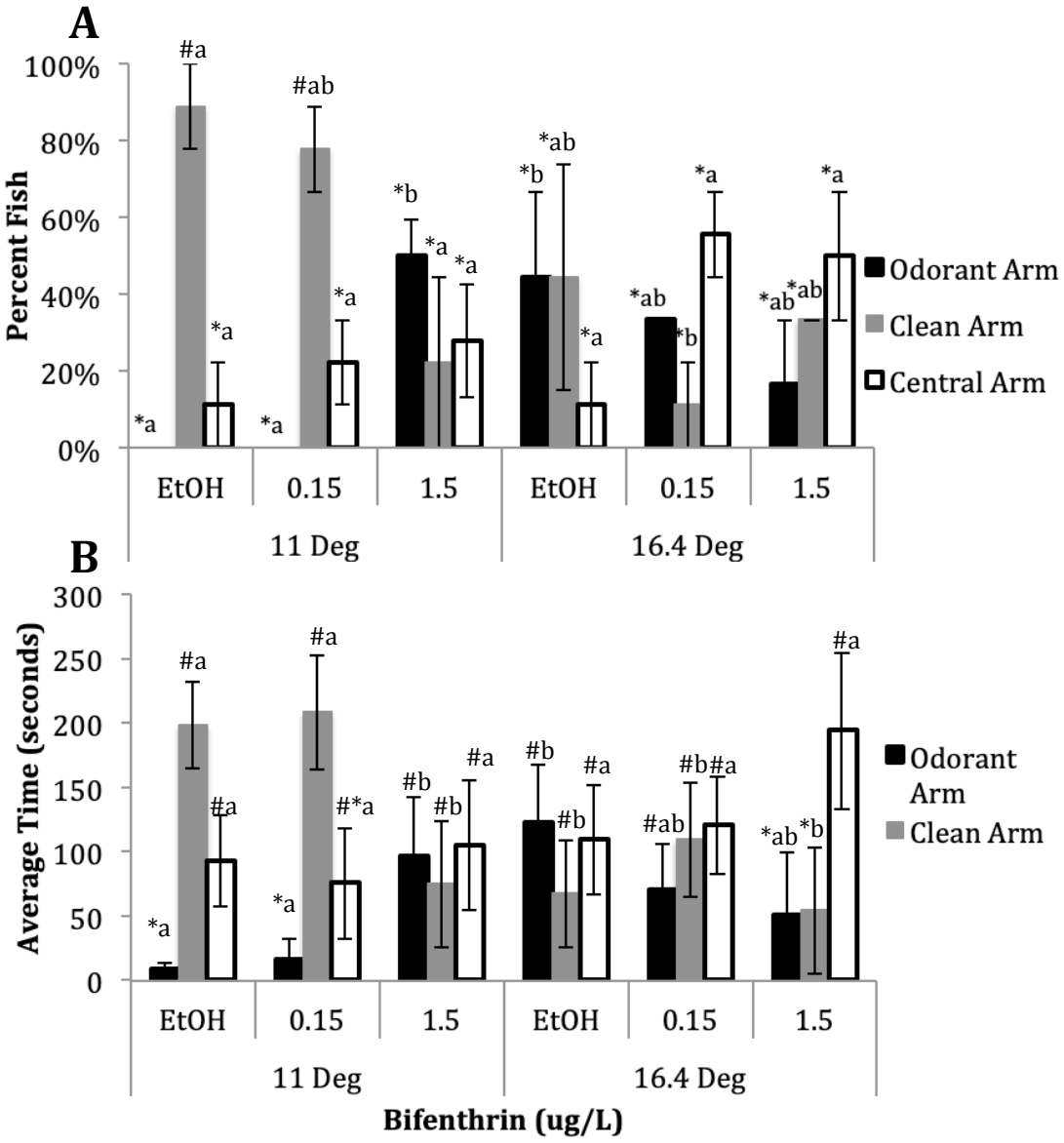

Fig. 5.3. (A) Number of individual parr in each of the odorant, clean, or central Y-Maze areas exposed to 11°C and 16.4°C and 0, 0.15, 1.5 µg/L bifenthrin, and (B) average time of parr in the odorant, clean, or central Y-Maze areas exposed to 11°C and 16.4°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, N=5-7. Data was significant at $p<0.05$ (Three-way ANOVA). Symbols denote significant differences between areas (Odorant, Clean, Central) within each treatment group, and letters denote significant differences between treatments within each area (Tukey's $p<0.05$).

IV. Discussion

Chinook parr can be exposed to contaminant and climate change stressors in the Bay-Delta before transitioning to seawater. Previous studies with bifenthrin indicated alterations in sex steroid hormones derived from the HPG axis (Forsgren et al., 2013; Crago & Schlenk, 2015; Giroux et al., 2019). In contrast to studies performed in other salmonids at different life stages, estradiol and testosterone levels were not significantly changed with either temperature or bifenthrin treatments, and sex hormone levels were lower than in fry, a younger juvenile stage, exposed to bifenthrin and increasing temperatures in a similar experiment (Giroux et al, 2019). Decreased concentrations of sex hormones, particularly E2 and testosterone are characteristic of smoltification (Bjornsson et al., 2011). While thyroid hormone and cortisol levels increase to promote osmoregulatory capabilities as salmonids transition to sea water, sex steroid hormone production is limited through the HPG pathway during smoltification. The developmental and endocrine pathway differences between each juvenile stage in fry and parr could contribute to the apparent differences in responses to both temperature and bifenthrin. Early development of sex-steroid producing tissues in salmonids begins during the fry stage, but gonad maturation continues into adulthood after smoltification (Vizziano et al., 2007). At the parr stage, gonads and the HPG axis are more developed compared to earlier juvenile stages, and the developmental stage of gonads can impact the production of sex steroid hormones.

While thyroid and growth hormones are essential for growth during the fry stage, these same hormones are fundamental in adaptation to seawater and the physiological

changes during the smoltification process (Ebbesson et al., 2000; Blanton & Specker, 2007). No significant changes were noted in Chinook salmon parr with any treatment in the current study. Treatment with zebrafish to higher temperatures increased T3, but under hypothyroid conditions lower levels of T3 did not significantly impair behavior or metabolic responses in fish acclimated to warmer temperatures (Little et al. 2013). Thus, thyroid hormones help regulate temperature acclimation in teleosts, but may not be the only mechanism for thermal acclimation to warming waters. Bifenthrin has been shown to bind to thyroid hormone receptors and cause differential expression of thyroid hormone axis genes in zebrafish (Tu et al., 2016). If bifenthrin binds to TH receptors, there could be a decrease in the overall thyroid hormone axis response in parr due to negative feedback of the hypothalamus-pituitary- thyroid axis. Thus, the lack of changes observed in T3 and T4 may be due to the antagonistic interaction of bifenthrin and temperature.

The lack of observed increases in E2, testosterone, and GnRH2 receptor in the present study indicate that bifenthrin may not be directly interacting with the estrogenic pathway and the associated feedback cascade at this life stage or concentration. Potent estrogenic compounds, as well as excessive E2 exposure, are known to inhibit the salt water acclimation and prevent increases in gill NKA through suppression of IGF-1 and the thyroid hormones (McCormick et al, 2005; Madsen et al., 1997). To our current knowledge, the current study is among the first to address the effects of a potential endocrine disruptor on thermal acclimation in salmonids. Additionally, previous studies have found that bifenthrin, in the same concentrations used in this study, increased levels

of E2 in rainbow trout (Forsgren et al., 2013; Crago & Schlenk, 2015). Fish exposed to ng/L concentrations, approximately 100-fold lower compared to the 0.15 μg/L bifenthrin concentrations used in the current study, also had significantly higher estradiol levels in an estuarine fish species, *Menidia beryllina* (Brander et al., 2012). The current study showed that treatment with bifenthrin did not affect the relative expression of GnRH2 or DR2α, two important genes encoding receptors in the regulation of HPG pathway hormones and signaling. Therefore, the lack of an increase in sex steroid hormones and expression of GnRH2 in parr exposed to bifenthrin suggests that bifenthrin may not be acting through the estrogen biosynthesis pathway, but rather another pathway at this specific life stage.

Bifenthrin has been shown to alter tyrosine hydroxylase and dopamine receptor gene expression, as well as levels of dopaminergic metabolites, in teleosts (Crago & Schlenk, 2015; Bertotto et al., 2018). Dopamine and other catecholamines in the brain fluctuate during predatory response behaviors (Hoglund et al., 2005). However, in this study, bifenthrin had no effect on the expression of dopamine receptor nor brain concentrations of dopamine or its metabolites. Although temperature may alter dopaminergic control of behavior, this pathway may not be a critical target for bifenthrin in Chinook salmon parr.

The most significant finding in the current study was that increasing temperature and bifenthrin exposure at the highest bifenthrin concentration significantly altered predatory avoidance behaviors in the Y-Maze assay. The salmon maintained in control conditions only entered into the odorant arm to investigate the novel area, but fish would

subsequently leave upon sensing the predatory odorant. Therefore, the control fish responded appropriately to the predatory cue. The alteration of olfactory-driven behaviors between vehicle- and bifenthrin-exposed fish indicate that bifenthrin may modulate the neurophysiology of parr brains, impair olfactory bulbs, or affect sensory signaling via neurotransmitters. Bifenthrin has been demonstrated to be acutely neurotoxic to fish at mg/L concentrations (Haya, 1989), but in sublethal doses bifenthrin exhibits differences in toxicity on larval fish locomotor activity due to the enantioselectivity of this compound (Jin et al., 2010). Thus, the uptake and composition of each enantiomer of bifenthrin could affect individual fish behaviors. A study done in zebrafish found that prolonged exposure to 10 ng/L bifenthrin altered predatory response behaviors even after a 5-day recovery period, thus indicating that subchronic bifenthrin exposure may have long-term neuromodulatory effects in fish (Frank et al., 2018). The same study found that ryanodine receptor and associated calcium signaling genes were significantly changed during and after exposure to bifenthrin (Frank et al., 2018). These results suggest that bifenthrin may have neurophysiological effects at a number of molecular targets.

Impaired olfactory functions can result in individual fish death, and subsequently population-level effects. Salmonid parr with completely nonfunctional olfaction were shown to be unable to properly smolt (Stabell et al., 1985). Sublethal pesticide exposure has been previously linked to impaired neurotransmission, olfaction, and behavioral effects, which was predicted to have significant impacts on salmonid populations (Baldwin et al., 2009; Tierney et al., 2010). Although the current study did not model population- level effects from the observed behavioral alterations due to bifenthrin

exposure, this information can be used to predict adverse outcomes of susceptible salmonid populations exposed to subchronic levels of bifenthrin at various temperatures. Predatory avoidance behaviors are complex and involve multiple sensory systems, including olfactory sensation.

V. Conclusion

The results of the current study demonstrate that bifenthrin and temperature stressors significantly impact the survival and predatory avoidance behaviors in parr stages of Chinook salmon both separately and in combination as stressors. However, impacts on neuroendocrine endpoints of the HPG and HGT axes and dopaminergic control are unclear. Additional studies measuring other neurotransmitter levels and signaling pathways are necessary to investigate the specific mechanisms behind the impaired behavioral responses observed in the current study.

Acknowledgements

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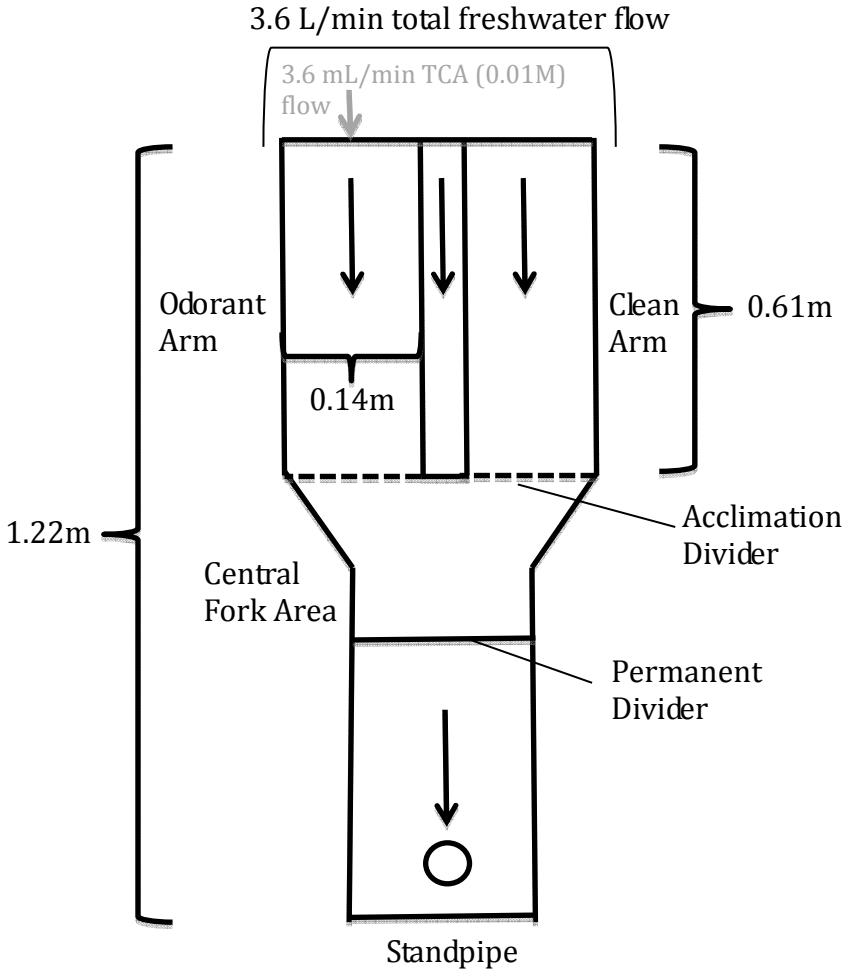
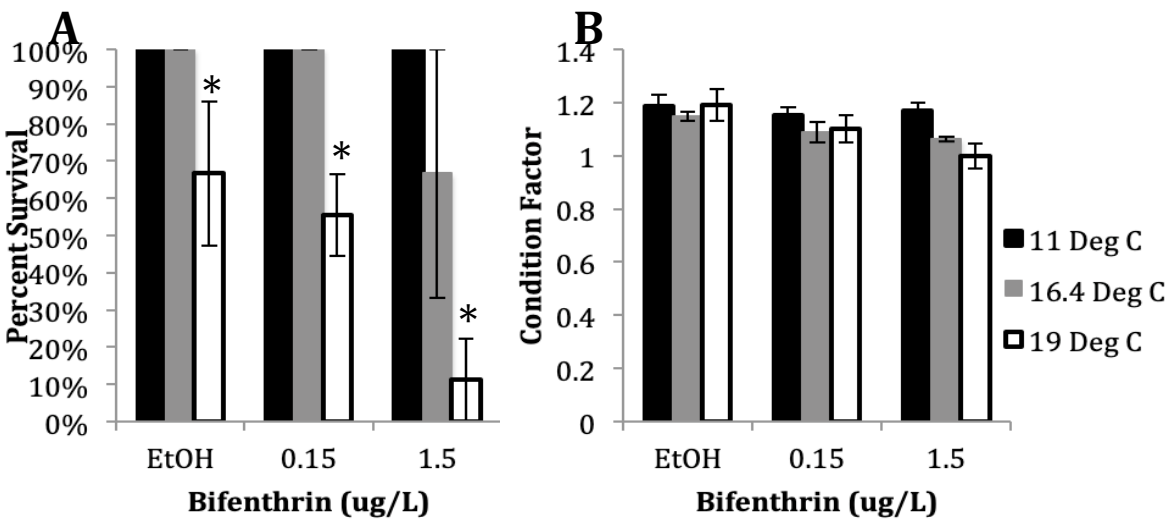
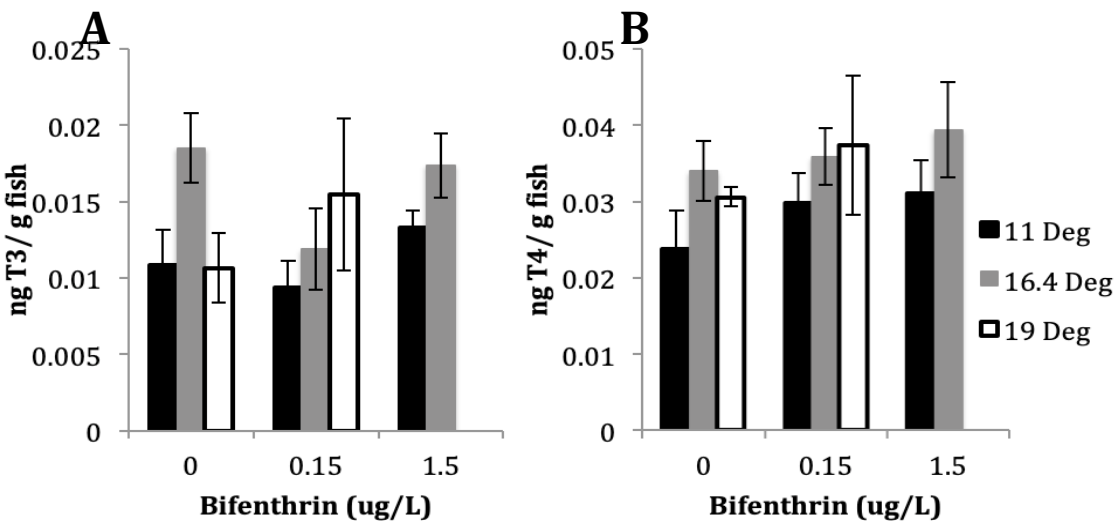
Supplemental Figures



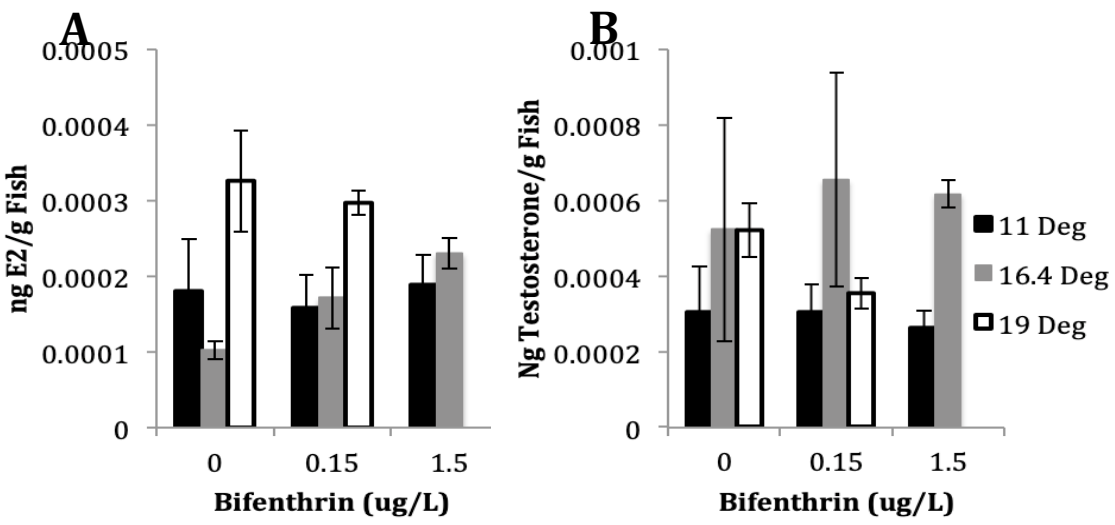
Supplemental Fig. 5.1 (Figure 5.4). Diagram of a Y-Maze tank adapted from Rehnberg et al. (1985). There are two arms (one odorant and one clean) and a central fork area where the fish are allowed to swim.



Supplemental Fig. 5.2 (Figure 5.5). (A) Percent survival and (B) Condition factor of parr exposed to exposed to 11°C, 16.4°C, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, and N=9. Survival data was significant by survival test ($p<0.05$), and * indicates significant differences compared to 11°C treatments in the same group. Condition factor data was not significant (GLM $p> 0.05$).



Supplemental Fig. 5.3 (Figure 5.6). Levels of serum (A) T3 (ng/ g fish) and (B) T4 (ng/g fish) in parr exposed to 11°C, 16.4°C, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, N=5-6. Data was not significant (GLM p> 0.05).



Supplemental Fig. 5.4 (Figure 5.7). Levels of serum (A) estradiol (ng/ g fish) and (B) testosterone (ng/ g fish) in parr exposed to 11°C, 16.4°C, 19°C and 0, 0.15, 1.5 µg/L bifenthrin. Each value represents the mean ± SE, N=5-6. Data was not significant (GLM $p > 0.05$).

Supplemental Table 5.1. Measured mean concentrations of bifenthrin in all temperature exposures at 0, 24, 48, and 96 hours.[a]

Nominal Concentration (µg/L)	0 hr BF (µg/L)	24 hr BF (µg/L)	48 hr BF (µg/L)	96 hr BF (µg/L)	Average Measured Bifenthrin ± SD (µg/L)	% Recovery ± SD
Control	<LOD	<LOD	<LOD	<LOD	<LOD	72.893 ± 9.577
0.15	0.260	0.103	0.177	0.077	0.154 ± 0.095	77.060 ± 8.444
1.5	2.752	0.862	2.587	0.585	1.696 ± 1.036	78.671 ± 3.287

[a] N= 6 for bifenthrin aquaria, and N=8 aquaria for vehicle controls.

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Chapter 6: Conclusions

Sensitive populations of salmon and trout in the San Francisco Bay-Delta have been declining in recent decades. Alevin, fry, and parr, the juvenile life stages, which reside in the Bay-Delta will likely experience the effects of multiple pesticide contaminants and climate change stressors, such as increasing salinity and temperature. As climate change rapidly progresses, juvenile anadromous fish may be experiencing warmer surface water temperatures and higher salinity at earlier developmental stages. Additionally, juvenile fish may periodically experience pesticide runoff events associated with increased storm intensity because they inhabit the Bay-Delta from hatch through smoltification. The combination of climate change and pyrethroid stress has been suggested to be the primary causes of salmonid population decline in the Bay-Delta ecosystem (Fong et al., 2016). Bifenthrin is the most frequently detected pyrethroid pesticide in the Bay-Delta, and is present in ug/L concentrations after runoff events (Siepmann & Holm, 2000; Weston & Lydy, 2010). Bifenthrin is acutely neurotoxic to fish and invertebrate species because these organisms lack carboxylesterases to metabolize bifenthrin into less toxic metabolites (Haya, 1989). However, at sublethal concentrations bifenthrin has been shown to have estrogenic and thyroid axes disrupting effects (Crago & Schlenk, 2015; Bertotto et al, 2018; Tu et al, 2016).

Juvenile salmonid growth, development, and smoltification are all partially regulated by hormonal pathways. Since bifenthrin is known to adversely impact endocrine pathways, this raises concern for the effects of bifenthrin runoff on juvenile

salmon and trout hormones and successful smoltification. Smoltification typically involves the thyroid hormone (TH) axis, growth hormone (GH) axis, and cortisol axis to initiate increases in gill Na^+ /K^+ ATPase (NKA) for adaptation to sea water (Stefansson et al., 2008; Bjornsson et al., 2011). Sex steroid biosynthesis is also reduced during smoltification, and estrogenic compounds have been shown to inhibit successful smoltification (Madsen et al., 1997; Stefansson et al., 2008). Therefore, it is prudent to understand the effects of climate change stressors and bifenthrin on the endocrine pathways in juvenile salmonids. In my research, this was accomplished through three main aims: 1) Understanding how temperature and salinity impact the endocrinology of juvenile *Oncohynchus mykiss*;2) Understanding how temperature and bifenthrin stressors impact neuroendocrine pathways in juvenile Chinook salmon; and 3) If bifenthrin and temperature affect juvenile salmon olfactory behaviors.

To evaluate the effects of both temperature and salinity on juvenile salmonids, alevin, fry, and parr were exposed to a range of current and predicted Bay-Delta temperatures. Alevin and fry were challenged to salt water for 24 hours, and parr were acclimated to seawater conditions. High temperatures and salinity significantly reduced alevin and fry survival, and alevin TH axis was also impacted by temperature, but this did not occur in the fry. Alevin and fry were negatively impacted by temperature in different endocrine pathways, and alevin were more susceptible to osmotic stress compared to fry, as observed by higher alevin mortality after the sea water challenge. In parr, smoltificaiton was not induced at high temperature and salinity treatments as evidenced by low expression of NKA in the gills and lowered overall fish health (condition factor).

Thus, knowing the effects of thermal and salinity exposure during each life stage is necessary for determining the susceptibility of each juvenile stage to multiple climate change stressors.

To evaluate how temperature impacts the toxicity of bifenthrin, juvenile Chinook alevin, fry, and parr were exposed to the same temperature regimes as in aim 1, and also exposed to bifenthrin for 96 hours, which simulates a pyrethroid runoff event after a storm. Each life stage responded to both bifenthrin and temperature differently. While the thyroid hormone axis was upregulated in alevin with both increasing temperature and bifenthrin, this response was not exhibited in fry. Conversely, sex steroid hormones decreased in fry exposed to increasing bifenthrin, but increased in alevin with bifenthrin exposure. Due to the minimal alteration of E2, testosterone, dopamine receptor, and GnRH2 expression, it appears that bifenthrin is not significantly impacting the HPG axis in parr through dopaminergic alterations. However, temperature decreased dopamine levels and also significantly altered the effects of bifenthrin on alevin and fry hormones. Many of the observed HPG and HPT responses to bifenthrin and temperature could be due to the timing of gonad and the thyroid gland development in juvenile salmonids, as well as the varied functions of each hormone throughout development (Specker et al., 1988; Tagawa & Hirano, 1997; Vizziano et al., 2007). Therefore, this research highlights the fundamental hormonal and developmental differences between alevin, fry, and parr stages reared at optimal temperatures, as well as understanding how bifenthrin and temperature can affect normal endocrine functions at each life stage.

To further evaluate the combined effects of bifenthrin and increasing temperature on behavior, a predatory avoidance assay was preformed on Chinook parr. A Y-maze allowed fish to choose between an area with a predatory odorant cue and a clean area. Control parr successfully avoided the predatory cue. However, bifenthrin-exposed parr showed no preference for either the predatory odorant or clean area. This suggests that bifenthrin may be adversely affecting fish olfaction and the parr cannot differentiate between clean and predatory areas. Additionally, higher temperatures altered the behavioral responses of bifenthrin exposed fish compared to the lower temperature groups, suggesting that temperature can exacerbate the adverse effects of bifenthrin to fish olfaction. Impaired fish olfaction can negatively impact entire populations through reducing normal behaviors such as feeding, smolting, and avoiding predation (Tierney et al., 2010). Since salmon behavior is adversely impacted by bifenthrin, then this data can be used in formulating Adverse Outcome Pathways (AOPs) to link molecular initiating events to population-level outcomes.

This research has provided valuable information for evaluating the impacts of multiple stressors on sensitive fish and for future management of threatened salmon and trout populations in coastal California. Few studies have examined the effects of pollutants on the alevin and fry stages. To my knowledge, this research is the first to evaluate the effects of a pyrethroid pesticide on all three juvenile stages, and the first to evaluate the combined effects of multiple environmental and chemical stressors on alevin and fry. Determining differences in sensitivity and endocrine responses is vital for understanding how susceptible each life stage is to anthropogenic stressors. Future

management practices should consider the sublethal effects of combined stressors on each life stage in order to successfully manage populations. The data generated from this research can be directly used in risk assessments for evaluating the risk of the interaction of climate change stressors and pyrethroid pollution within the Bay-Delta ecosystem.

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