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UNIVERSITY OF CALIFORNIA
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Impacts of Hypersaline Acclimation on Chlorpyrifos Toxicity to Salmonids

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

Doctor of Philosophy

in

Environmental Toxicology

by

Lindley Anne Maryoung

December 2014

Dissertation Committee:
Dr. Daniel Schlenk, Chairperson
Dr. David Eastmond
Dr. Jay Gan

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The Dissertation of Lindley Anne Maryoung is approved:

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University of California, Riverside

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Dedication

This dissertation is dedicated to my parents, Victor and Terese, and my sister, Hilary. My family has been incredibly supportive throughout my entire education and their encouragement has been instrumental in the completion of this work.

ABSTRACT OF THE DISSERTATION

Impacts of Hypersaline Acclimation on the Toxicity of Chlorpyrifos to Salmonids

by

Lindley Anne Maryoung

Doctor of Philosophy, Graduate Program in Environmental Toxicology

University of California, Riverside, December 2014

Dr. Daniel Schlenk, Chairperson

As part of their unique life cycle, most Pacific salmonids transition from freshwater to saltwater, requiring various adjustments in physiology. However, molecular mechanisms underlying this transition are largely unknown. Additionally, acclimation to hypersaline conditions enhances the acute toxicity of certain thioether organophosphate and carbamate pesticides in some species of euryhaline fish, yet sublethal impacts have been far less studied. The current study aimed to determine underlying molecular mechanisms of Pacific salmonid smoltification, as well as determine how hypersaline acclimation impacts acute and sublethal toxicity of a common organophosphate pesticide, chlorpyrifos (CPF). A transcriptomics approach was used to assess differential gene expression in coho salmon (*Oncorhynchus kisutch*) liver, gills, and olfactory rosettes after salinity acclimation and found that the majority of the altered genes were tissue and

salinity concentration dependent. From the few shared genes, a potential osmosenor was identified. Osmotic signal transduction cascades were also impacted in the three tissues. Salinity acclimation was then coupled with CPF to determine impacts on acute toxicity. Time to death of rainbow trout (*Oncorhynchus mykiss*) by CPF was more rapid in freshwater than in hypersaline water (16 ppt). Salinity acclimation did not impact metabolism, precipitation, or acetylcholinesterase inhibition of CPF. In contrast, mRNA expression of certain neurological targets was upregulated in saltwater acclimated fish, consistent with diminished neuronal signaling which may protect fish from cholinergic overload associated with acetylcholinesterase inhibition. Sublethal experiments, which are more environmentally relevant, were conducted to determine impacts on olfaction. Combined acclimation and exposure to CPF impacted rainbow trout olfaction at the molecular, physiological, and behavioral levels. Concurrent exposure to hypersalinity and 0.5 µg/L CPF upregulated four genes that inhibit olfactory signal transduction. At the physiological level, hypersalinity and chlorpyrifos caused a decrease in sensory response to the amino acid L-serine and the bile salt taurocholic acid. Combined acclimation and exposure also negatively impacted behavior and reduced the avoidance of a predator cue (L-serine). Overall, these results will be very useful in risk assessment strategies evaluating compounds of this nature in estuarine environments and freshwater environments that may be altered by hypersaline stress or rising sea levels.

Table of Contents

Chapter 1: Introduction	1
Salmonids	2
Hypersalinity	6
Chlorpyrifos	11
Hypersalinity and Pesticides	29
Olfaction	32
Behavior	38
Hypotheses	45
References	47
Chapter 2: Differential Gene Expression in Liver, Gill and Olfactory Rosettes of Coho Salmon (<i>Oncorhynchus kisutch</i>) after Acclimation to Salinity	60
Abstract	60
Introduction	62
Materials and Methods	64
Results	69
Discussion	79
References	92
Chapter 3: Impacts of Hypersaline Acclimation on the Acute Toxicity of the Organophosphate Chlorpyrifos to Salmonids	98

Abstract	98
Introduction	99
Materials and Methods	102
Results	111
Discussion	115
References	122
Chapter 4: Sublethal Toxicity of Chlorpyrifos to Salmonid Olfaction After Hypersaline Acclimation	127
Abstract	127
Introduction	128
Materials and Methods	133
Results	141
Discussion	150
References	158
Chapter 5: Summary and General Conclusions	163
References	168

List of Tables

Table 1.1. Behavioral measures and acetylcholinesterase activity of juvenile coho salmon after exposure to chlorpyrifos.	43
Table 2.1. Primer sequences used to qPCR validation.	69
Table 2.2. Genes differentially expressed in all salinities in two tissues.	71
Table 2.3. Significant gene ontology terms for liver.	72
Table 2.4. Significant gene ontology terms for gills.	73
Table 2.5. Significant gene ontology terms for olfactory rosettes.	74
Table 3.1. Signal transduction target gene primers used in this study.	110
Table 3.2. Median lethal time (LT ₅₀) in hours for juvenile rainbow trout acclimated to either freshwater (< 0.5 ppt) or hypersalinity (16 ppt) and exposed to one of five nominal chlorpyrifos concentrations.	112
Table 3.3. <i>In vitro</i> liver and gill chlorpyrifos metabolism detoxification ratios for juvenile rainbow trout, median <i>in vitro</i> AChE inhibition values for juvenile rainbow trout brains, and bioavailability of chlorpyrifos in aqueous media after 48 hours.	113
Table 4.1. Signal transduction target gene primers used in this study.	138
Table 4.2. Average chlorpyrifos concentration in exposure tank water immediately after dosing.	141

List of Figures

Figure 1.1. Diagram of anadromous salmon lifecycle.	3
Figure 1.2. Adult abundance of winter-run Chinook salmon from the Sacramento River.	5
Figure 1.3. Mean monthly salinity for May at (A) Fort Point and (B) Pittsburg, CA, with water export from the delta shown as a percentage of annual inflow.	8
Figure 1.4. Comparison between geographic distribution of threatened and endangered salmonids in the western United States and the study area of the USGS NAWQA program.	12
Figure 1.5. Structure of the organophosphate chlorpyrifos.	13
Figure 1.6. Degradation pathways for chlorpyrifos in the environment.	16
Figure 1.7. The biotransformation pathways for chlorpyrifos.	21
Figure 1.8. Diagram of acetylcholine based neurotransmission and acetylcholinesterase inhibition by organophosphates.	25
Figure 1.9. Survival curves of coho salmon exposed to four concentrations of phorate acclimated to freshwater (A) and hypersalinity (B).	31
Figure 1.10. Concentrations of pesticides measured in the environment compared to concentrations of odorants needed to cause a detectable olfactory response.	33
Figure 1.11. Overview of the fish brain with emphasis on the olfactory system.	34
Figure 1.12. Diagram of the dual second messenger pathways in olfactory signal transduction.	35
Figure 1.13. The olfactory pathway for processing of odorants or contaminants which can be impacted at different steps causing different responses.	36
Figure 1.14. Percent olfactory response as compared to controls for juvenile	37

coho salmon exposed to three concentrations of chlorpyrifos.

Figure 1.15. Sequence of events leading to behavioral responses.	39
Figure 1.16. Behavioral measures, (A) spontaneous rate and (B) mean total strikes in first minute, of juvenile coho salmon after exposure to chlorpyrifos.	43
Figure 2.1. Venn diagrams for differentially expressed genes in gills, liver, and olfactory rosettes after acclimation to low, medium, and high salinity as compared to freshwater controls.	70
Figure 2.2. Relative gene expression for coho salmon tissues (liver, gills, and olfactory rosettes) acclimated to freshwater or high (32 ppt) salinity.	76
Figure 3.1. Inhibition curves for AChE inhibition in hypersaline and freshwater acclimated rainbow trout brain homogenates.	114
Figure 3.2. Relative gene expression of calcium calmodulin-dependent protein kinase II delta (CAMKII), chloride intracellular channel 4 (CLC4), and G protein alpha i1 (Gi1) in juvenile rainbow trout brains after acclimation to freshwater or hypersalinity (16 ppt).	115
Figure 4.1. Olfactory response to L-serine (10^{-4} M) of juvenile rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos.	143
Figure 4.2. Olfactory response to TCA (10^{-4} M) of juvenile rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos.	144
Figure 4.3. Relative gene expression of five signal transduction related genes in olfactory tissue of juvenile rainbow trout acclimated to freshwater or hypersalinity for fourteen days and exposed to chlorpyrifos.	146
Figure 4.4. Behavioral response (a – avoid, b – no choice, c – no prefer) to L-serine (10^{-7} M) of juvenile rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos.	148

Chapter 1: Introduction

Salmonids are important species in the Pacific Northwest for economic and ecological reasons; however, population declines have caused many to be listed as threatened or endangered. Many salmonids are anadromous, moving between freshwater and saltwater at different stages in their life cycle (Quinn, 2005). This unique life history strategy makes salmonids especially sensitive to natural and anthropogenic alterations of their habitats. Salmonid habitats can be impacted in several ways as many migration routes go through urban and agricultural areas near estuaries.

One way salmonid habitat can be impacted is through hypersalinity. Increased salinity can result from natural processes, as well as anthropogenic-related factors, such as saline runoff from agriculture and reduced freshwater inputs from climate change (reviewed in Cañedo-Argüelles et al., 2013; Knowles and Cayan, 2004). Increased salinity can co-occur with chemical insult, such as pesticide runoff. In the United States, pesticides and salinization both rank among the top 15 causes of impairment to streams (U.S. EPA, 2012b). One particular pesticide of interest is the organophosphate chlorpyrifos. Chlorpyrifos was one of the most heavily applied pesticide active ingredients in 2007 and has been detected in salmonid waterways at concentrations up to 3.7 µg/L (Grube et al., 2011; Xuyang et al., 2012). Chlorpyrifos is also of particular interest because it is acutely toxic to fish through acetylcholinesterase inhibition, as well as sublethally toxic through impacts on behavior (reviewed in Barron and Woodburn, 1995).

Studying co-exposure of hypersalinity and chlorpyrifos is of particular interest because previous studies on hypersaline acclimation and exposure to certain thioether organophosphates and carbamates showed an enhancement in acute toxicity. Sublethal effects have been far less studied. Olfaction is critical for salmonids as it is used in kin recognition, predator avoidance, reproductive behavior, and homing. Impacts on olfaction can have detrimental effects (reviewed in Laberge and Hara, 2001). Thus, understanding how these two factors, hypersalinity and chlorpyrifos, affect both acute and sublethal toxicity to salmonids is important in protecting these threatened and endangered species.

Salmonids

Salmonids are critical species in the western United States, particularly in the Pacific Northwest, for economic and ecological reasons. Salmonids comprise one of the most valuable commercial fishery resources in the United States. According to the Wild Salmon Center 2009 report, it was estimated that in 2007 the worldwide total ex-vessel value from salmon commercial fisheries was \$818 million and the first wholesale level value was estimated to be \$2.2 billion (Wild Salmon Center, 2009). In 2010, landings alone brought in \$555 million for Pacific salmon in the United States (NMFS, 2011).

Along with the benefits salmonids provide to humans, both economically and culturally, they also play critically important roles for other organisms in the ecosystem. Salmonids exhibit unique life histories in that many are anadromous, moving between freshwater and saltwater at different life stages, with many species homing to their native area to reproduce. As depicted in Figure 1.1, anadromous salmon hatch in freshwater

rivers or streams. They spend various amounts of time in freshwater, depending on species, before migrating through the brackish water of estuaries to eventually reach the ocean. Once in the ocean they continue to grow and mature before finally returning to freshwater to reproduce (Quinn, 2005).

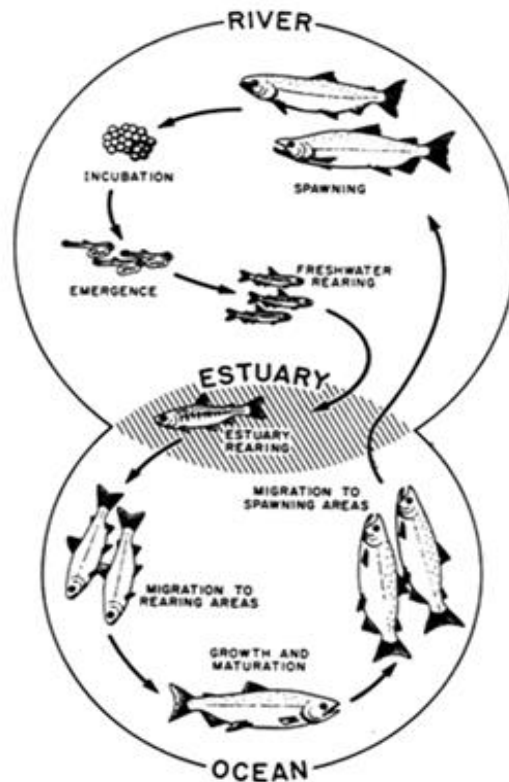


Figure 1.1. Diagram of anadromous salmon lifecycle. Figure from Five Counties Salmonid Conservation Program (<http://www.5counties.org/salmoncycle.htm>; link accessed on December 11, 2014).

Many salmonids are also semelparous, reproducing only once during their life history and dying soon after reproducing. Since salmonids return to their natal streams for reproduction and die quickly thereafter, they transfer millions of kilograms of salmon flesh to often nutrient poor freshwater ecosystems (Quinn, 2005). Through this process

salmon are able to move marine derived nitrogen and phosphorous to freshwater systems. The return of salmon to the rivers of the Pacific Northwest makes a significant contribution to the flora and fauna of terrestrial and riverine ecosystems. Salmon carcasses strewn along riverbeds and in river channels provide abundant food and nutrients to the animals and plants in those ecosystems. The wide range of animals that rely on the regular availability of spawning salmon reinforces the importance of anadromous fish as a component of wildlife ecology and an important element in the biodiversity in the Pacific Northwest (Willson and Hallupka, 1995).

Although anadromous life history has benefits for salmonids, such as greater access to food resources, it also requires many physiological changes. For example, they alter osmoregulatory physiology, patterns of drinking, energy storage, urination, color, and shape. During the transition state, salmonids are not confined to one area; they can be found in freshwater preparing for migration, migrating in freshwater, or in the marine environment near shore. Although natural, the unique life cycle of salmonids makes them especially sensitive to natural and anthropogenic alterations of their habitats (Quinn, 2005).

Alarmingly, on the west coast of the United States, formerly abundant salmonid species have experienced dramatic declines in population during the past several decades. For example, winter-run Chinook salmon (*Oncorhynchus tshawytscha*) in the Sacramento River, which is the only extant winter-run Chinook salmon population, have heavily declined since the mid-1960s (Figure 1.2). This overall decline in salmonids will undoubtedly have major economic and ecological impacts. One example is that as salmon

populations decline, so does the amount of marine-derived nitrogen and phosphorus entering the freshwater ecosystems. The historical biomass of salmon returning annually to the Pacific Northwest (Washington, Oregon, California, and Idaho) was estimated to be between 160 and 226 million kg based on records from 1866 to 1920. The biomass of fish returning to the same area, based on records from 1970 to the mid-1990s, was estimated at around 11.8 to 13.7 million kg, suggesting that only 6-7% of the marine derived nutrients previously delivered to the Pacific Northwest was reaching those streams (Gresh et al. 2000).

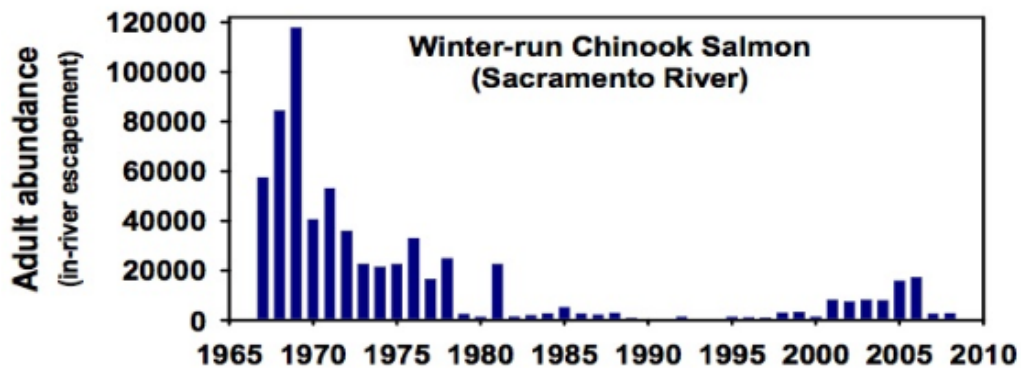


Figure 1.2. Adult abundance of winter-run Chinook salmon from the Sacramento River. Figure from the Bay Institute of San Francisco (<http://www.c-win.org/c-win-recommends-delta-flow-criteria-state.html>; link accessed on December 10, 2014).

Due to the ecological importance of salmonids, population declines have created immense concern over their protection and propagation. In the Western United States, salmon populations have been listed under the Endangered Species Act (ESA) for protection (Quinn, 2005). As of 2012, the National Oceanic and Atmospheric Administration (NOAA) listed five populations of salmonids as endangered and twenty three as threatened in the Pacific Northwest (NOAA, 2012). Understanding how

environmental and anthropogenic factors impact salmonid populations is critical for assessing risk and finding ways to protect these organisms.

Hypersalinity

Salmonid habitats can be impacted in several ways as many migration routes go through urban and agricultural areas near estuaries. One way salmonid habitats can be impacted is through increased salinity. In the United States, salinization is ranked among the top 15 causes of impairment to streams and rated as equally important as pesticide input (U.S. EPA, 2012b).

Hypersalinity is a global environmental phenomenon that affects many different aspects of aquatic life (Williams, 2001a, b). Commonly occurring in surface waters from arid and semi-arid zones, salinization can cause alterations in the chemical composition of natural water resources, such as lakes, rivers, and groundwater potentially leading to loss of biodiversity as native species can be replaced by halotolerant species (Williams, 2001a, b).

Salinity can be impacted by various natural and anthropogenic factors. In rivers and streams, in the absence of anthropogenic impacts, salinity is influenced by three main sources: (1) weathering of the catchment, a function of the geology of the catchment and precipitation, (2) sea spray in coastal areas, and (3) dissolved salts in rainwater resulting from evaporation of seawater. In semi-arid and arid regions with seasonally hot or dry climates, dissolved ions can be concentrated by evaporation and transpiration. Thus, natural salinity in rivers and streams is a complex combination of climate, geography of

catchment, distance from the sea, topography, and vegetation (reviewed in Cañedo-Argüelles et al., 2013).

One of the main anthropogenic causes of hypersalinity in rivers is irrigation and rising groundwater tables. This is especially problematic in arid and semi-arid agricultural regions where crop production consumes large quantities of water. In these areas salt concentrates and soil water become more saline as crops only absorb a fraction of the salt of the irrigation water. Salts may then be leached out through runoff and enter surface waters (reviewed in Cañedo-Argüelles et al., 2013).

Climate change is another anthropogenic factor that can play a role in causing hypersaline conditions in certain areas, which also serve as habitat for salmonids. One particular location where this type of alteration in salinity may be found is San Francisco Bay, California. San Francisco Bay is one of the largest estuarine systems in the United States and its watershed covers more than 130,000 km². Water inputs into the system include rain and snowmelt, with 40% of annual input coming from snowmelt. The salinity regime of the system is highly variable during the year with two distinct seasons. The summers are warm and dry while the winters are cool and wet, resulting in a change of as much as 10 parts per thousand (ppt) seasonally. Salinity in this region has also been shown to vary annually with an increase in 3 – 4 ppt since 1941 (Figure 1.3; Knowles and Cayan, 2004).

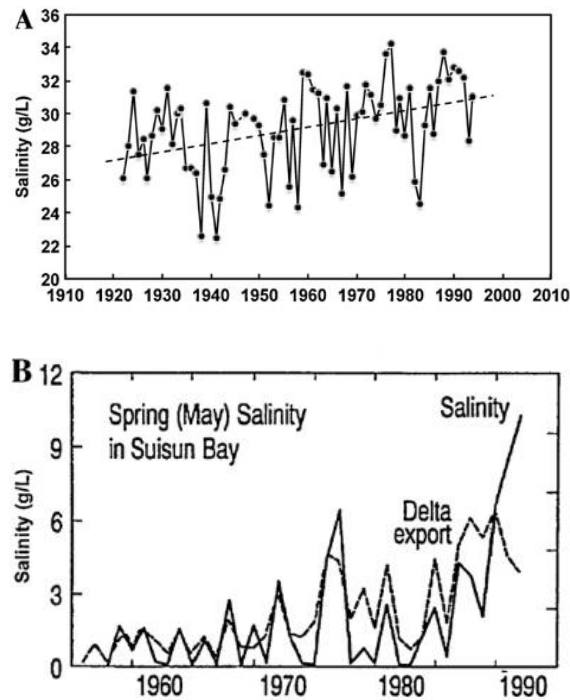


Figure 1.3. Mean monthly salinity for May at (A) Fort Point (Stahle et al., 2001) and (B) Pittsburg, CA (Peterson et al., 1996) with water export from the delta shown as a percentage of annual inflow.

The San Francisco Bay estuarine system relies heavily on artificial (reservoirs) and natural (snowpack) freshwater storage making it highly susceptible to impacts of climate change. The Earth's climate has a strong influence on the hydrologic cycle which controls the strength, timing, and volume of freshwater runoff to coastal ecosystems. As various models indicate a rise in temperature ranging from 1.4 to 5.8 °C over the next 100 years, salinity in this area could be greatly affected. As temperature rises, the alpine snow volume may decrease resulting in loss of snowmelt input into the estuarine system. Climate models for the region estimate a reduction in snowpack of up to 60% over the next 100 years (Knowles and Cayan, 2002, 2004; Howat and Tulaczyk, 2005).

As mentioned previously, many salmonids are anadromous and thus naturally transition between freshwater and saltwater. To successfully undergo this transition salmonids go through smoltification establishing a strong hypoosmoregulatory capacity. During smoltification, morphological, biochemical, physiological, and behavioral adaptations, driven by the endocrine system, allow salmonids to transition from freshwater to saltwater. These adaptations include elevated salinity tolerance, silvering of body color, olfactory imprinting, elongation of the caudal peduncle, changes in hemoglobin isoforms, and a transition from territorial to schooling behavior (reviewed in Hoar, 1988; Winans and Nishioka, 1987; Fyhn et al., 1991; McCormick et al., 1998). These changes improve fitness for the salmonid pelagic, marine life-stage.

During this process, one endocrine pathway involved includes an increase in growth hormone and the steroid, cortisol. The development of chloride cells is then stimulated which enhances $\text{Na}^+ \text{K}^+$ ATPase activity altering intestinal and gill osmoregulatory function (reviewed in Björnsson et al., 2011). Another pathway involves thyroid hormones, which are elevated during smoltification and are suggested to play a role in morphological and behavioral changes (reviewed in Björnsson et al., 2011). Prolactin has a more complicated role in smoltification, being elevated in early smolt development and decreasing at the peak of smolting (Young et al., 1989).

Although studies have been conducted on smoltification, there are still several aspects of Pacific salmonid smoltification that are currently unknown, particularly the regulation of smoltification at the molecular level. Seear et al., (2010) conducted a transcriptomic study of Atlantic salmon (*Salmon salar*) smoltification in brain, gill, and

kidney tissues to try to elucidate some of the molecular mechanisms involved in smoltification regulation. They found that the gills had the highest number of differentially expressed genes and that differentially expressed genes in the three tissues were mostly involved in growth, metabolism, oxygen transport, or osmoregulation (Seear, et al., 2010). Many of the genes that were upregulated complimented previous findings from other studies on salmonid smoltification physiology and biochemistry. Pacific salmon have also been the subject of a transcriptomics study of migration, although the focus was on the migration from the ocean to freshwater (Evans, et al., 2011). Thus, there is still a need to understand the molecular responses of Pacific salmon during smoltification.

Transcriptomic studies of other euryhaline fish undergoing salinity acclimation have also been conducted. Evans and Somero (2008) performed a time course acclimation of goby, *Gillichthys mirabilis*, to hyper and hypo osmotic stress in gills. The dominant class with the most differentially expressed genes was cell signaling. Many of the genes they found to be differentially regulated had no previous reported role in osmotic stress adaptation, highlighting the value of the transcriptomic approach.

Although the transition from freshwater to saltwater is natural for salmonids, steeper gradients of hypersaline conditions can force salmonids to adapt more rapidly and impact the timing and life stage of salinity adaptation, as fish may experience higher salinity further upstream than normal. Since acclimation to hypersaline conditions increases cortisol levels (McLean et al., 1997; Arjona et al., 2007), and cortisol induces cytochrome P450 (CYP) 3A27 (Celander et al., 1999), alterations in steroid clearance or

xenobiotic biotransformation may occur. In rainbow trout (*Oncorhynchus mykiss*) acclimated to hypersaline conditions of 17 ppt, hepatic CYP3A27 was up regulated (Lavado et al., 2009b). Cortisol has also been shown to inhibit adenylyl cyclase in another euryhaline fish, tilapia (*Oreochromis mossambicus*) (Borski et al., 2002). Adenylyl cyclase plays an important role in osmosensory and neuronal signal transduction in euryhaline fish (reviewed in Fiol and Kultz, 2007). Since salinity significantly alters physiological responses in salmon, it is unclear how this influences stress due to anthropogenic insults such as contaminants.

Chlorpyrifos

Another way in which salmonid habitats can be impacted is through chemical insult, which can co-occur with hypersalinity. As mentioned previously, in the United States pesticides rank among the top 15 causes of impairment to streams (U.S. EPA, 2012b). Pesticides applied to agricultural crops, forests, residential areas, and public lands can runoff into aquatic environments and have been detected in various water ways used by salmonids for migration, rearing, and spawning (Wentz et al., 1998). On a national scale, a ten year study was conducted by the U.S. Geological Survey's National Water-Quality Assessment Program (USGS NAWQA) and found that pesticides were detected throughout much of the year, and more than 90 percent of the time, in streams with urbanized watersheds (Figure 1.4; Gilliom, 2007).



Figure 1.4. Comparison between geographic distribution of threatened and endangered salmonids in the western United States and the study area of the USGS NAWQA program. Shaded areas are the freshwater ranges for Endangered Species Act listed salmonid populations and dashed lines show the boundaries of the water quality study where pesticide concentrations were measured in surface waters. Figure from Leatz et al., 2009.

One particular class of pesticides, organophosphates (OPs), is of major concern in the United States as well as Canada. For example, out of the ten most frequently occurring pesticides in British Columbia's Nicomekl River in 2004, which included four classes of pesticides, OPs made up 86.9% by mass. The list of the top ten OPs included

chlorpyrifos, diazinon, and parathion (Tierney et al., 2008). Chlorpyrifos is especially important because in 2007 it ranked first as the most commonly used organophosphate insecticide active ingredient (Figure 1.5; Grube et al., 2011).

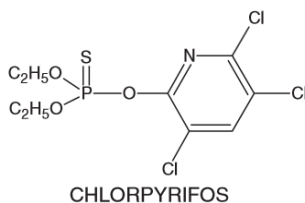


Figure 1.5. Structure of the organophosphate chlorpyrifos. Figure modified from Sams et al., 2004.

Chlorpyrifos, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)phosphorothioate, production began in 1962 and was first registered for use by the United States Environmental Protection Agency (EPA) on July 1, 1965 (reviewed in Barron and Woodburn, 1995; U.S. EPA, 2006). It is used to control foliage and soil borne insect pests on a variety of food crops including fruits, nuts, vegetables, and grains, and on non-food sites such as golf course turf, industrial sites, greenhouses, nurseries, sod farms, and wood products (U.S. EPA, 2012a). Chlorpyrifos has been banned for residential use except in the form of containerized baits (U.S. EPA, 2006). In 2007 chlorpyrifos was ranked fourteenth as the most commonly used conventional pesticide active ingredient in the agricultural market sector with between seven and nine million pounds used (Grube et al., 2011).

In 2007, a petition was filed by the Natural Resource Defense Council and the Pesticide Action Network North America requesting that the EPA revoke all tolerances and cancel all registrations of chlorpyrifos. In response to the petition, the EPA has

lowered the maximum aerial application rates from 6 pounds per acre to 2 pounds per acre, as well as developed new mitigation measures to include buffer zones for ground and aerial applications around sensitive sites. As of 2013, the EPA found that the claims in the petition do not warrant revoking tolerances or canceling registrations for chlorpyrifos. The EPA still intends to complete a comprehensive risk assessment of chlorpyrifos on human health, including both dietary risk and occupational risk (U.S. EPA, 2012a).

Physical and Chemical Properties –

Chlorpyrifos occurs as a colorless to white crystalline solid (U.S. EPA, 2006; Tomlin, 2006). It gives off a mild mercaptan (thiol) odor (Tomlin, 2006; Lewis, 1998). The molecular weight of chlorpyrifos is 350.6 g/mol (U.S. EPA, 2006). At 25°C, its vapor pressure is 1.87×10^{-5} mmHg, and at 25°C, its Henry's law constant is reported as either 4.2×10^{-6} atm·m³/mol or 6.7×10^{-6} atm·m³/mol, depending on the experimental technique used (Tomlin, 2006; U.S. EPA, 1999). The water solubility of chlorpyrifos is 1.4 ppm at 25°C (Packard, 1987). The log Kow is 4.7 - 5.3 (De Bruijn et al., 1989; McDonald et al., 1985). The solid sorption coefficient (Koc) ranges between 360 to 31,000 depending on soil type and environmental conditions (Smegal, 2000).

Environmental Fate and Transport –

Chlorpyrifos released into the environment has the potential to partition into soil, air, water and biota. The chemical properties of chlorpyrifos, such as its low water solubility, allow it to strongly bind organic contents of soils, where it is relatively immobile (U.S. EPA, 1999; Kamrin, 1997). In soils, chlorpyrifos is quite stable with

reported half-lives ranging from 7 – 120 days. In the atmosphere chlorpyrifos degrades to chlorpyrifos oxon through reaction with photochemically produced hydroxyl radicals. The atmospheric vapor half-life is 4.2 hours (U.S. HSS, 2005). Given its hydrophobicity, chlorpyrifos does not easily partition from soil to water. Thus, chlorpyrifos in runoff is not likely the result of dissolved chlorpyrifos, but rather from soil-bound chlorpyrifos from eroding soil (U.S. EPA, 1999). Once chlorpyrifos enters water systems, volatilization can be a significant mechanism of dissipation from certain surfaces such as pond water even though chlorpyrifos has an intermediate vapor pressure of 1.87×10^{-5} mmHg at 25°C (reviewed Racke, 1993; U.S. EPA, 1999). Field assessments have been conducted to determine the runoff potential of chlorpyrifos. Overall, the absolute quantities of chlorpyrifos in surface waters are quite small compared to the quantities applied. Field-scale studies have been conducted in multiple states, with various plot sizes, application rates, and rainfall patterns. Out of seven different studies, the amount of chlorpyrifos detected as runoff in terms of the percentage applied ranged from 0.0 to 1.0 percent, with the greatest single runoff event releasing 7.9 g/ha into surface water (reviewed in Racke, 1993).

As exposure in aquatic environments is the focus of this project, chlorpyrifos degradation in this environmental compartment will be discussed in further detail. In aquatic environments, chlorpyrifos can be degraded by three main processes: hydrolysis, photolysis, and microbial degradation resulting in various metabolites (Figure 1.6). Hydrolytic cleavage of chlorpyrifos can occur on one of three ester bonds. These candidates for cleavage include two tertiary alkyl ester bonds and one phosphate ester

(pyridyl) bond. All three ester bonds may be hydrolyzed from the observance of four major hydrolysis products of chlorpyrifos. Zidan et al., (1981) found both 3,5,6-trichloro-2-pyridinol (TCP) and O-ethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate (desethyl chlorpyrifos) in distilled water and water extracts of sterile loamy and loamy sand soils. Batzer et al., (1990) also observed these products in river water. The pH and temperature of the medium altered the relative proportion of each metabolite generated (McCall, 1985). At neutral pH (7.68) and elevated temperature (70 - 80°C), the major metabolites were ethanol and desethyl chlorpyrifos, with a smaller quantity of TCP and diethylthiophosphate.

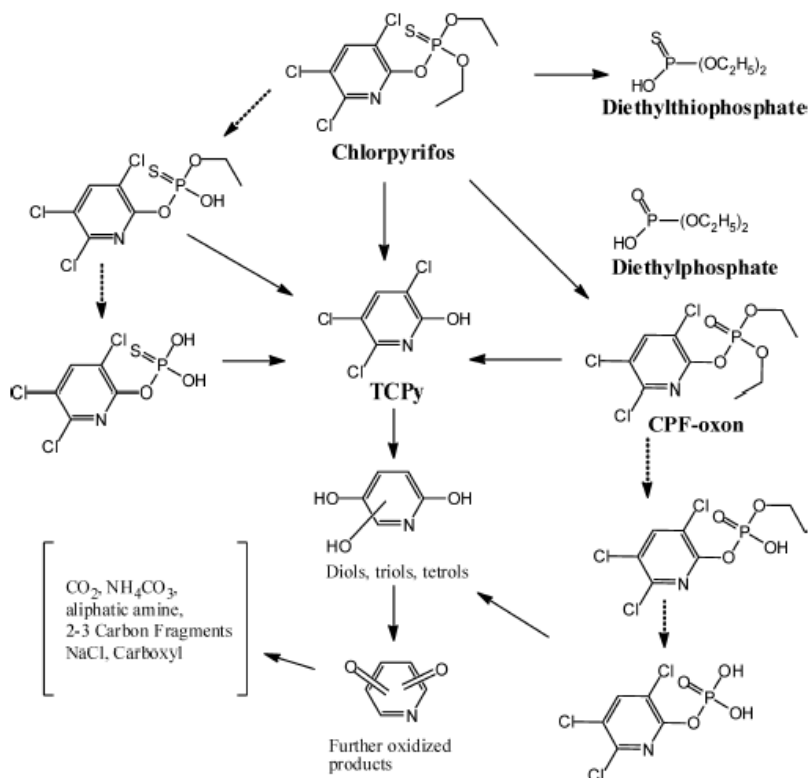


Figure 1.6. Degradation pathways for chlorpyrifos in the environment. The dashed arrows depict theoretical metabolites that have not been definitively identified as environmental degradation products. Figure from Eaton et al., 2008.

For chlorpyrifos, both neutral and alkaline hydrolysis mechanisms are involved in the formation of the four products and the kinetics of the reactions also depend on pH of the media. For neutral hydrolysis of chlorpyrifos, a classical SN₂-type displacement reaction occurs with a nucleophilic attack of water at the carbon of the ethoxy groups resulting in the formation of ethanol and desethyl chlorpyrifos. For neutral hydrolysis, pH is not a determining factor. For alkaline hydrolysis of chlorpyrifos, a nucleophilic attack of the hydroxide ion at phosphorus occurs and obeys second-order kinetics (Macalady and Wolfe, 1985). Thus, pH plays a major role in this reaction. As pH increases, the reaction greatly accelerates, which is consistent with chlorpyrifos hydrolysis (reviewed in Racke, 1993). The rate of hydrolytic reactions of chlorpyrifos are relatively slow when compared to other important dissipative processes in the aqueous environment, such as volatility, microbial degradation, and photolysis; however, they do indicate some contribution to the overall degradation rate (reviewed Racke, 1993).

Chlorpyrifos also undergoes transformation through photolytic degradation. Sunlight exposure to chlorpyrifos in solution, on surfaces, and in the atmosphere may cause photolysis. Chlorpyrifos can undergo both direct and indirect photolytic degradation. In direct photolysis, the compound absorbs ultraviolet (UV) radiation and then interacts with environmental reactants or itself to undergo transformation. Chlorpyrifos and TCP are both able to absorb UV radiation. During indirect photolysis, humic or inorganic substances absorb sunlight and activated forms interact with the compound or produce oxygen radicals or peroxides that interact with the compound

(reviewed in Racke, 1993). Half-lives for chlorpyrifos in aqueous media range from 7.8 to 108 days depending on pH, temperature and light source (reviewed in Racke, 1993).

Photoproducts of chlorpyrifos have been postulated, but few have been identified in the environment. TCP is a photodegradative metabolite of chlorpyrifos; however, in aqueous solutions it is not generally observed (Meikle et al., 1983; McCall, 1985; Batzer et al., 1990). TCP is less photolytically stable than chlorpyrifos, which may explain why it is not as frequently detected in aqueous solutions. Studies on photodegradation of TCP in aqueous buffer identified 14 photoproducts; however, definitive structures were not reported due to their oxidative instability. It was hypothesized that a series of partially dechlorinated pyridine-based diols and triols were being formed (Smith, 1966). Batzer et al., (1990) identified a suite of photoproducts of chlorpyrifos in buffered, distilled water. The majority of the products were small organic acids. Trace amounts of dichlorinated phosphorothioate isomers were also formed. After 21 days, oxamic acid, oxalic acid, 2-chlorosuccinic acid, maleamic acid, succinamic acid, and E- and Z-2-chlorobut-2-enedioic acid were identified. These products suggest that the end result of aqueous photodegradation is destruction of the pyridyl ring. The significance of photolysis in impacting dissipation of chlorpyrifos in the environment is unclear; however, it has been shown to be a pathway of degradation in the laboratory under controlled conditions (reviewed Racke, 1993).

Chlorpyrifos can also be degraded in the environment through microbial biotransformation. There are two main pathways of microbial degradation for organic chemicals: catabolism and cometabolism. Catabolism involves degradation during which

the compound or a part of the compound is completely mineralized and the energy or nutrient gained aids in cell growth. For cometabolism, the degradation of the compound does not benefit the organism; instead, the compound is incorporated in some metabolic pathway during the normal metabolic activities of the microorganism. Cometabolism is the primary metabolic pathway involved for chlorpyrifos metabolism in microorganisms. There is some debate as to the extent of the role microorganisms play in metabolizing chlorpyrifos in the aquatic environment. Schmimmel et al., (1983) concluded that microorganisms play an important role in metabolism based on laboratory studies with aqueous solutions and sediments. However, Sharom et al., (1980) and Walker et al., (1988) concluded that microorganisms essentially have no role in the breakdown of chlorpyrifos in aqueous environments.

Pharmacokinetics –

In fish, pharmacokinetic studies show that chlorpyrifos is rapidly absorbed from aqueous media and extensively metabolized (reviewed in Barron and Woodburn, 1995). For example, Barron et al. (1993) showed that peak levels of chlorpyrifos in channel catfish (*Ictalurus punctatus*) blood occurred 1 to 2 hours after waterborne exposure. Uptake clearance values of chlorpyrifos in fish have been reviewed by Barron and Woodburn (1995) and range from 2 mL g⁻¹ hr⁻¹ for eels (*Anguilla Anguilla*) (Douglas and Bell, 1990) to 57.4 mL g⁻¹ hr⁻¹ for three-spined stickleback (*Gasterosteus aculeatus*) (Deneer 1994). For rainbow trout, the uptake clearance value was 14.4 mL g⁻¹ hr⁻¹ (Murphy and Lutenske 1986). Barron and Woodburn (1995) also reviewed elimination half-life values in fish, which ranged from 13.9 hr in three-spined stickleback (Deneer

1994) to 81.5 hr in eels (Douglas and Bell, 1990). The elimination half-life value for rainbow trout was 66 hr (Murphy and Lutenske 1986). Barron et al. (1993) examined tissue distribution of chlorpyrifos in channel catfish after chlorpyrifos exposure. The highest concentration of chlorpyrifos was found in fat, while muscle had the lowest concentration of the tissues studied. Gills, liver, and trunk kidney had higher concentrations than blood, brain, and spleen (Barron et al., 1993).

In eukaryotes, chlorpyrifos undergoes desulfuration to produce chlorpyrifos oxon or dearylation to produce TCP and diethylthiophosphate (Figure 1.7). Formation of chlorpyrifos oxon is a bioactivation pathway as it is a much more potent acetylcholinesterase (AChE) inhibitor. The parent compound by itself is unable to inhibit AChE to any significant extent (Chambers, 1992). Conversion of chlorpyrifos to chlorpyrifos oxon is primarily cytochrome P450 (CYP) mediated. Formation of TCP is the detoxification pathway, which can be CYP mediated as well. Specific isoforms of CYP have different abilities to produce each product. Heterologously expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 catalyzed chlorpyrifos metabolism. CYP2C19 had the highest activity for dearylation, while CYP2B6 had the highest activity for desulfuration. CYP3A4 had a high activity for both reactions, but overall had a higher activity for dearylation (Tang et al., 2001). Chlorpyrifos oxon can also be inactivated by esterases, such as paroxonase, to form TCP.

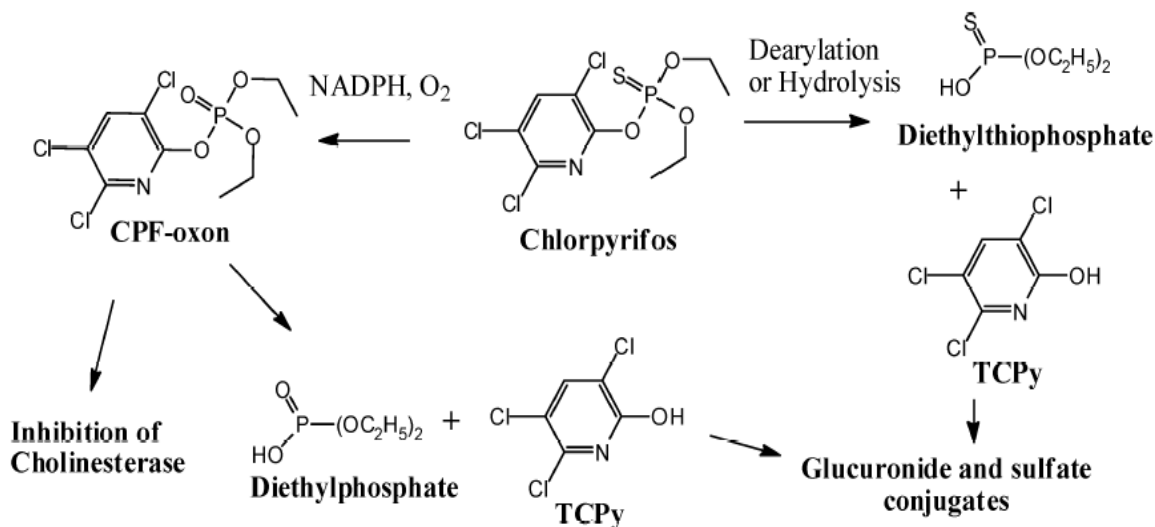


Figure 1.7. The biotransformation pathways for chlorpyrifos. Figure from Eaton et al., 2008.

Chlorpyrifos can undergo glutathione conjugation mediated by glutathione S-transferases. Glutathione conjugation allows for excretion of the chlorpyrifos conjugate through the bile. Previous studies in mice showed the presence of glutathione conjugates of chlorpyrifos in livers, suggesting that this pathway may also play a role in overall metabolism (Fujioka and Casida, 2007). Additionally, chlorpyrifos can undergo glucuronidation conjugation mediated by UDP-glucuronosyl transferases also allowing for excretion through the bile and urine. In channel catfish (*Ictalurus punctatus*), the glucuronide TCP conjugate was the major metabolite in bile and urine (Barron et al., 1991; Barron et al. 1993).

Specifically for fish, biotransformation of chlorpyrifos results in the formation of TCP, methoxytrichloropyridine, and glucuronide conjugates (Barron et al., 1991). Goldfish have been shown to produce at least four metabolites (TCP as the major

metabolite and desethyl chlorpyrifos, desethyl chlorpyrifos oxon, and TC phosphate as minor metabolites) (Smith et al., 1966). After chlorpyrifos exposure, mosquitofish (*Gambusia affinis*) were shown to contain 50% parent compound, 29% TCP, and 21% unidentified polarized metabolites (Metcalf, 1974). Another study using mosquitofish found TCP and two unidentified polar metabolites after chlorpyrifos exposure (Hedlund, 1973). In guppies (*Poecilia reticulata*) and channel catfish, excretion of parent chlorpyrifos was limited, indicating that biotransformation is the dominant elimination pathway (Welling and de Vries, 1992; Barron et al., 1993).

For salmonids in particular, Lavado and Schlenk (2011) compared *in vitro* biotransformation of rainbow trout and coho salmon (*Oncorhynchus kisutch*) in liver, gills, and olfactory tissues. Biotransformation of chlorpyrifos was observed in liver and gills as chlorpyrifos-oxon and TCP were detected, but not in olfactory tissues. TCP formation was higher than chlorpyrifos-oxon in both species in liver and gills. Chlorpyrifos biotransformation was higher in liver than in gills in both species. Coho had lower transformation activities in liver and gill than rainbow trout (Lavado and Schlenk, 2011).

Accumulation and Bioconcentration –

Bioconcentration factor (BCF) values for chlorpyrifos are generally lower than predicted from equilibrium partitioning due to biotransformation and partitioning onto dissolved organic carbon in aqueous media (reviewed in Barron and Woodburn, 1995). Chlorpyrifos degradates are expected to have lower BCF values than the parent due to greater polarity (reviewed in Barron and Woodburn, 1995). Barron and Woodburn (1995)

reviewed BCF values in different species of fish in fresh and salt water. In freshwater, BCF values ranged from 100 in bluegill (*Lepomis macrochirus*) (Eaton et al., 1985) to 1680 in fathead minnow (*Pimephales promelas*) (Jarvinen et al., 1983). For rainbow trout BCF values ranged between 725 for muscle fillet and 1370 for whole body (Murphy and Luterske 1986). Species, exposure concentration, and exposure conditions influenced BCF values (reviewed in Barron and Woodburn, 1995). Exposure concentration showed no relationship to BCF in certain studies, however in other studies BCF increased with increasing concentration (reviewed in Barron and Woodburn, 1995). Toxicity at higher concentrations of exposure may impact concentration dependence of BCFs as chlorpyrifos biotransformation may be inhibited at higher concentrations allowing for slower elimination (Deneer 1993, 1994).

Varó et al., (2002) conducted a food chain bioaccumulation study with *Artemia* and Spanish toothcarp (*Aphanius iberus*), both of which are euryhaline. Through dietary exposures, fish accumulated chlorpyrifos concentrations of up to 5.9 ng/g. Bioaccumulation in fish was lower than in *Artemia*, showing no biomagnification. The biomagnification factor (BMF) decreased continuously throughout the bioaccumulation phase of the study. Biotransformation of chlorpyrifos or physicochemical characteristics may explain the lack of biomagnification (Varó et al., 2002).

Mode of Action –

Chlorpyrifos, along with other organophosphates, is acutely toxic with acetylcholinesterase (AChE) inhibition as the primary mode of action. During neurotransmission, acetylcholine (ACh) is released into the synaptic cleft from a pre-

synaptic neuron. ACh receptors on the post-synaptic membrane bind ACh initiating signal transduction through the post-synaptic neuron, glandular cell or myocyte. After the signal, AChE hydrolyzes ACh into choline and acetic acid. As these products do not stimulate the post synaptic membrane, synaptic transmission is effectively terminated. If AChE is inhibited, it is not able to break down ACh which can lead to accumulation of ACh at synaptic junctions leading to over stimulation (Figure 1.8). Organophosphates inhibit AChE by phosphorylating the enzyme at its esteratic site through an electrophilic mechanism. The serine hydroxyl moiety in the esteratic site of the enzyme is phosphorylated in a manner similar to the acetylation of AChE. The acetylated enzyme rapidly breaks down to produce acetic acid and the regenerated enzyme; however, the phosphorylated enzyme is highly stable. Depending on the groups attached to the phosphorous atom, the enzyme can either be reversibly inhibited or irreversibly inhibited (Fukuto, 1990). Binding of the organophosphate to the enzyme causes the serine hydroxyl group to be blocked by a phosphoryl moiety preventing the enzyme from hydrolyzing ACh. Chlorpyrifos contains a P=S moiety, which causes the parent compound to be a fairly poor anticholinesterase. However, after undergoing biotransformation to chlorpyrifos oxon the P=S is converted to a P=O moiety. The P=O bond is more polarized than P=S as oxygen has a higher electronegativity than sulfur. The P=O bond facilitates attack on the phosphorus by nucleophilic agents, such as the serine hydroxyl of AChE (Fukuto, 1990).

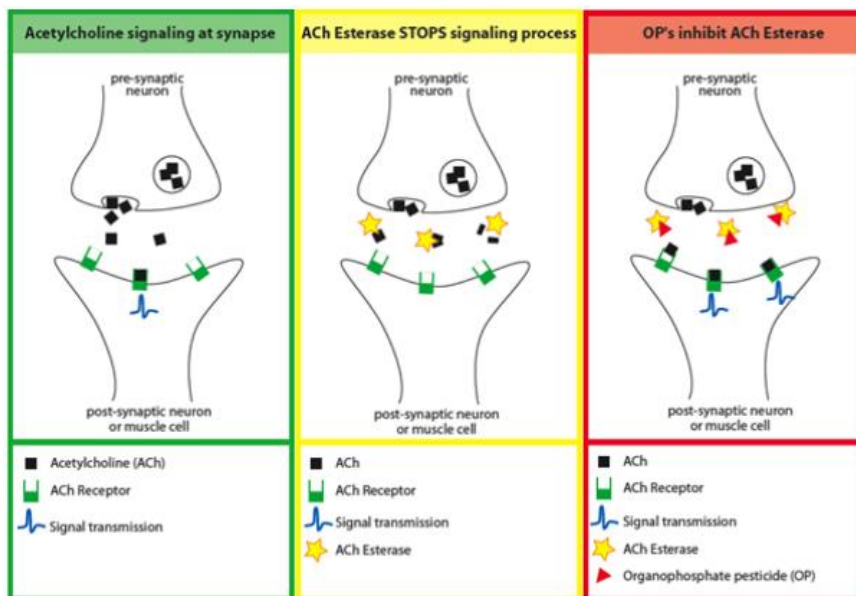


Figure 1.8. Diagram of acetylcholine based neurotransmission and acetylcholinesterase inhibition by organophosphates. Figure from the Pediatric Environmental Health Specialty Unit, Department of Environmental and Occupation Health Sciences at the University of Washington, 2007.

Acute Toxicity –

Acute toxicity tests have been performed for a variety of freshwater and saltwater fish, typically employing 96-hr LC_{50} tests. Chlorpyrifos has been found to be acutely toxic at concentrations ranging between 0.5 to 1000 $\mu\text{g/L}$ for freshwater and saltwater fish. Freshwater bluegill (*Lepomis macrochirus*) and saltwater silverside (*Menidia sp.*) are the most sensitive with LC_{50} s of 1.7 - 10 $\mu\text{g/L}$ and 0.5 - 3.0 $\mu\text{g/L}$, respectively. Freshwater mosquitofish, certain cyprinid species, the channel catfish, and the saltwater sheepshead minnow (*Cyprinodon variegatus*) are the most resistant fish species with LC_{50} values greater than 100 $\mu\text{g/L}$ (reviewed in Barron and Woodburn, 1995). Juvenile rainbow trout (between 0.6 - 3.0 g) maintained in freshwater had LC_{50} values of < 1 - 51 $\mu\text{g/L}$ (reviewed in Barron and Woodburn, 1995). Several factors may affect toxicity

including exposure conditions, formulations, source and size of fish, and water quality (reviewed in Barron and Woodburn, 1995).

TCP, the main chlorpyrifos degradate, does not cause cholinesterase inhibition and is classified as having low to moderate toxicity to aquatic and terrestrial biota. The 96-hr LC₅₀ values for TCP for bluegill and rainbow trout were three orders of magnitude greater than for chlorpyrifos (reviewed in Barron and Woodburn, 1995). Acute toxicity tests have also been performed for aquatic invertebrates, terrestrial invertebrates, reptiles and amphibians, birds, and wild mammals which have been reviewed by Barron and Woodburn, (1995). Acute toxicity in mammals has also been reviewed by Eaton et al., (2008).

Chronic Toxicity –

Chronic toxicity of chlorpyrifos has been studied in early life stage studies, as well as full life cycle studies in fish. Early life stage studies focus on embryo or larval stages through juvenile life stages, while full life cycle studies evaluate the larval stage through reproduction of first generation larvae. For early life stage studies, the lowest observable effect concentration (LOEC) ranged from 0.12 µg/L to 7.2 µg/L for fathead minnow (*Pimephales promelas*) and sheepshead minnow (*Cyprinodon variegatus*), respectively (reviewed in Barron and Woodburn, 1995). For a life cycle study with fathead minnows, growth was significantly reduced when fish were exposed to 1.2 µg/L for a complete life cycle. When exposed to 0.63 µg/L for the same amount of time, maturation and reproduction were reduced. From the same study, second generation fish showed reduced growth after exposure to 0.12 µg/L (Jarvinen et al., 1988). With survival

as the most sensitive endpoint measured, another fathead minnow life cycle study found a LOEC of 1.1 $\mu\text{g/L}$ (Mayes et al., 1993). Occasionally, survival was equal to or exceeded growth as the most sensitive endpoint; however, growth was typically the most sensitive measure for chronic toxicity of chlorpyrifos on fish. Chronic toxicity tests have also been performed for microorganisms, plants, aquatic invertebrates, terrestrial invertebrates, reptiles and amphibians, birds, and wild mammals which have been reviewed by Barron and Woodburn, (1995). Chronic toxicity in mammals with an emphasis on potential human exposure and neurodevelopment has been reviewed by Eaton et al., (2008).

Besides impacts on growth and survival, chlorpyrifos has also been shown to have sublethal impacts on behavioral avoidance, temperature preference, and cause biochemical alterations of fish (reviewed in Barron and Woodburn, 1995). Other sublethal endpoints have been examined in different species, which have not yet been studied in fish. For example, Song et al., (1997) focused on the impact of chlorpyrifos on the adenylyl cyclase signaling cascade in neonatal rats. Sublethal effects of chlorpyrifos on postnatal days 1 – 4 or 11 – 14 were evaluated in forebrain, cerebellum, and heart. Chlorpyrifos exposure resulted in deficits in multiple components of the adenylyl cyclase cascade in all three tissues. Deficits included expression and activity of adenylyl cyclase itself, function of G-proteins that link neurotransmitter and hormone receptors to cyclase activity, and expression of neurotransmitter receptors that act through this cascade (Song et al., 1997).

Chlorpyrifos Detection in Salmonid Habitats –

Chlorpyrifos is frequently detected in surface waters which provide habitat for various aquatic species, even though usage has declined in recent years. Chlorpyrifos has been detected in surface waters of Washington and California and from 2009 to 2011, samples from salmon-bearing waterways had chlorpyrifos exceeding water quality criteria (EPA's chronic National Recommended Water Quality Criteria, chronic invertebrate assessment criteria, and or chronic water quality standard (0.04 µg/L)) (Sargeant et al., 2013). For example, at the Spring Creek location, concentrations of chlorpyrifos were over the criteria guidelines on seven different occasions during the three year period and it was detected in 17% of samples (Sargeant et al., 2013). At this location, chlorpyrifos concentrations did not meet the chronic standard or assessment criteria for two to three consecutive sampling weeks each year between 2009 and 2011. For the Brender Creek location, concentrations of chlorpyrifos were deemed to be of acute and chronic concern for aquatic life for this sampling period (Sargeant et al., 2013).

Additionally, in a recent monitoring study in California, chlorpyrifos was detected in 17.7% of samples, with 9.9% exceeding water quality criteria between 2006 and 2010, with the maximum detected concentration of 3.7 µg/L in 2007 in the San Joaquin region (Xuyang et al., 2012). Anderson et al., (2014) detected chlorpyrifos in all surface water samples analyzed from the Santa Maria estuary, which provides refuge and habitat for endangered steelhead trout (*Oncorhynchus mykiss*). Elevated chlorpyrifos concentrations accounted for water-associated toxicity to *Ceriodaphnia dubia* and *Hyalella azteca* in the majority of samples from their study. Chlorpyrifos, along with several pyrethroid pesticides, also contributed to sediment toxicity to *H. azteca* from the same area. The

authors noted that concentrations of chlorpyrifos detected in their study were comparable to concentrations used in previous studies to demonstrate effects on salmonids (Anderson et al., 2014; Scholz et al., 2000; Moore and Waring, 2001; Sandahl et al., 2004). Additionally, Smalling et al., (2013) detected chlorpyrifos in 100% of starry flounder (*Platichthys stellatus*) and staghorn sculpin (*Leptocottus armatus*) tissues, sand crab (*Emerita analoga*) and water samples taken from the Santa Maria estuary. It was also detected in sediment in 89 % of samples.

Hypersalinity and Pesticides

Since hypersaline conditions and organophosphates can both occur in salmonid habitats there is potential for concurrent exposures and toxicological interactions. A review on the influence of salinity on the toxicity of various classes of chemicals to aquatic organisms found a direct correlation between organophosphate toxicity and salinity in 65 percent of the studies reviewed. This trend held true for both fish and invertebrates (Hall and Anderson, 1995). More specifically, for several euryhaline species, including salmonids acclimated to hypersaline conditions, certain thioether organophosphates and carbamates have shown significant enhancement of acute toxicity (reviewed in Schlenk and Lavado, 2011).

Studies on Japanese medaka (*Oryzias latipes*) exposed to hypersaline conditions and aldicarb, a carbamate insecticide, showed salinity enhanced aldicarb toxicity (El-Alfy and Schlenk, 1998). This enhancement in acute toxicity was postulated to be due to upregulation of flavin-containing monooxygenases (FMOs) which biotransform aldicarb

to aldicarb sulfoxide, a more potent AChE inhibitor (El-Alfy and Schlenk, 1998). Wang et al., (2001) examined the effects of hypersaline acclimation on aldicarb toxicity in rainbow trout and striped bass (*Morone saxatilis*). Salinity acclimation significantly enhanced the acute toxicity of aldicarb in rainbow trout, but not in striped bass. FMO mRNA expression was upregulated in rainbow trout liver, gill, and kidney microsomes, but not in striped bass suggesting that the difference in toxicity may be attributed to biotransformation by FMO (Wang et al., 2001).

The relationship between hypersaline acclimation and acute toxicity in euryhaline fish after organophosphate exposure has also been evaluated using fenthion and phorate. Bawardi et al. (2007) examined the impacts of concurrent exposure with fenthion in rainbow trout, striped bass, and tilapia (*Oreochromis mossambicus*). Acute toxicity was significantly enhanced in rainbow trout and striped bass and a trend toward greater toxicity was observed in tilapia. In this study FMO did not appear to contribute to the enhanced toxicity, although CYP may have played a role. However, the overall mechanism could not be fully predicted due to the complexity of multiple oxidation pathways and potential metabolites (Bawardi et al., 2007). Further studies on fenthion toxicity in hypersaline acclimated rainbow trout determined that CYP3A27 was primarily involved in the enhancement of fenthion activation in hypersaline acclimated fish with FMO contributing in a limited capacity to initial sulfoxidation (Lavado et al., 2009b).

Lavado et al., (2011) examined the differences in toxicity of another organophosphate, phorate, after hypersaline acclimation in coho salmon (*Oncorhynchus kisutch*). Phorate was found to be more than 30 times more potent in hypersaline

acclimated fish than in freshwater fish (Figure 1.9). The mechanism responsible for the difference was suggested to be differential expression and or catalytic activities of Phase I enzymes, including CYP and FMO, which enhanced oxidative metabolism (Lavado et al., 2011). Furthermore, Lavado et al., (2014) studied the expression and activity of certain CYP (1A, 2K1, 2M1, and 3A27) and FMO (A and B) enzymes in coho salmon gill, liver, and olfactory tissues after hypersaline acclimation. CYP2K1, CYP2M1, CYP3A27, and FMO A were induced in the three tissues after salinity acclimation, while CYP1A was down regulated in liver and FMO B was down regulated in all three tissues, supporting the idea that salinity acclimation can alter xenobiotic metabolism (Lavado et al., 2014). Although impacts on acute toxicity from pesticide exposure after hypersaline acclimation have been examined for various compounds in multiple species, sublethal endpoints have been far less studied.

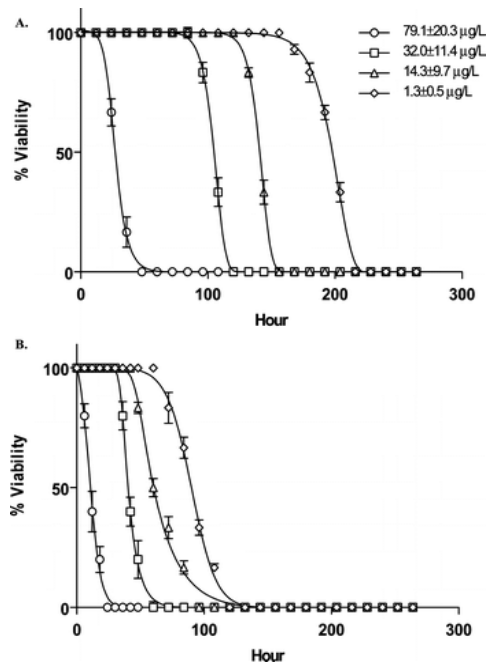


Figure 1.9. Survival curves of coho salmon exposed to four concentrations of phorate acclimated to freshwater (A) and hypersalinity (B) from Lavado et al., 2011.

Olfaction

Salmonids use olfaction to detect chemical cues that provide crucial information about food, predators, reproductive status of mates, environmental contamination, and natal streams of imprinting (reviewed in Laberge and Hara, 2001). Through olfaction, directional, conditional, tactical, and genetic information can be signaled to fish. Thus, impairment of the olfactory system can have detrimental effects. Exposure to environmentally realistic concentrations of common pollutants, such as pesticides and metals, can impair olfaction and interfere with life history processes that impact individual survival and reproductive success in several species of fish (reviewed in Tierney et al., 2010).

Fish olfaction detects five major classes of chemical odorants: amino acids, gonadal steroids, bile acids, prostaglandins, and nucleotides (reviewed in Laberge and Hara, 2001). The anatomy of fish olfactory systems allows for recognition of these critical odorants; however, it also leaves fish vulnerable to harmful chemicals in their environment. Dissolved contaminants can interact with the olfactory neurons as readily as odorants especially since many contaminants are found at similar concentrations as odorants in salmonid habitats (Figure 1.10).

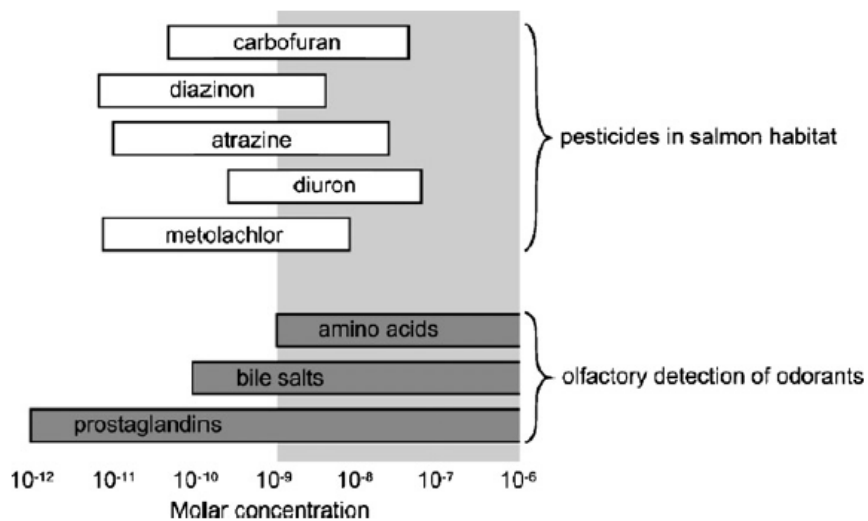


Figure 1.10. Concentrations of pesticides measured in the environment compared to concentrations of odorants needed to cause a detectable olfactory response. The pesticide data comes from surface water monitoring by the United States Geological Survey (reviewed in Gilliom et al., 2006). Odorant concentration data is summarized in Hara, 1992. Figure from Tierney et al., 2010.

Teleosts, including salmonids, have a paired olfactory organ with an olfactory cavity that interacts with the external environment through specific openings (Figure 1.11). While swimming, a flow of water is directed through these openings and the olfactory organ. A pair of olfactory rosettes, each located within a chamber on either side of the rostrum, makes up the peripheral portion of the olfactory system. A sensory olfactory epithelium (OE) containing olfactory sensory neurons (OSNs) covers the surface of each olfactory rosette (Yamamoto, 1982). Once an odorant is taken into the olfactory chamber, the odorant molecule interacts with an OSN in the OE. Individual OSNs have differentially expressed receptor proteins to which odorants bind.

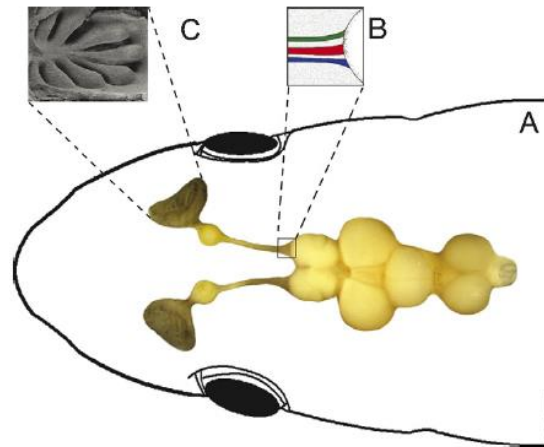


Figure 1.11. Overview of the fish brain with emphasis on the olfactory system. (A) Dorsal view of the brain and olfactory system of a crucian carp. (B) Schematic drawing of the olfactory tract entering the telencephalon. Three distinct bundles are shown. (C) A scanning micrograph image of an olfactory rosette. Figure from Hamdani and Døving, 2007.

These receptors are G-protein coupled receptors (GPCR) and have been classified into subfamilies, odorant receptor type (e.g. OR), vomeronasal receptor type (e.g. V2R), and G-protein coupled receptor family type (e.g. GFB). These subfamilies correspond to morphologically distinct OSNs anatomically classified as ciliated, microvillus, and crypt, respectively. Ciliated cells have cilia extending from a knob, while microvillus cells have larger unciliated protuberances, and crypt cells have an apically focused ciliary grouping (Zielinski and Hara, 2001; Schmachtenberg, 2006). OSN groups respond differently to odorant classes and are dispersed across the OE. OSNs which share a common odorant binding receptor extend their axons via the olfactory nerve to the olfactory bulb at separate sub-regions containing one or more glomeruli (Friedrich and Korsching, 1998).

OSNs express one of two types of heterotrimeric GPCRs (Figure 1.12). The first type stimulates phospholipase C which produces inositol triphosphate. The second type

stimulates adenylyl cyclase which produces cAMP (Sorensen and Sato, 2005). Both types lead to second messenger cascades which cause the opening of cation channels. The resulting influx of calcium activates calcium-gated chloride channels (Zhainazarov and Ache, 1995). This can then cause action potential propagation in the OSN axons projecting to the olfactory regions of the brain. Once information is passed to the olfactory bulb, it is then relayed from the glomeruli by mitral cells to networks in other brain centers. This information is then processed and results in physiological and or behavioral responses (reviewed in Tierney et al., 2010). Impairment results in harm to peripheral olfactory function and impacts olfactory mediated behaviors that are crucial for survival, growth and reproduction (reviewed in Laberge and Hara, 2001).

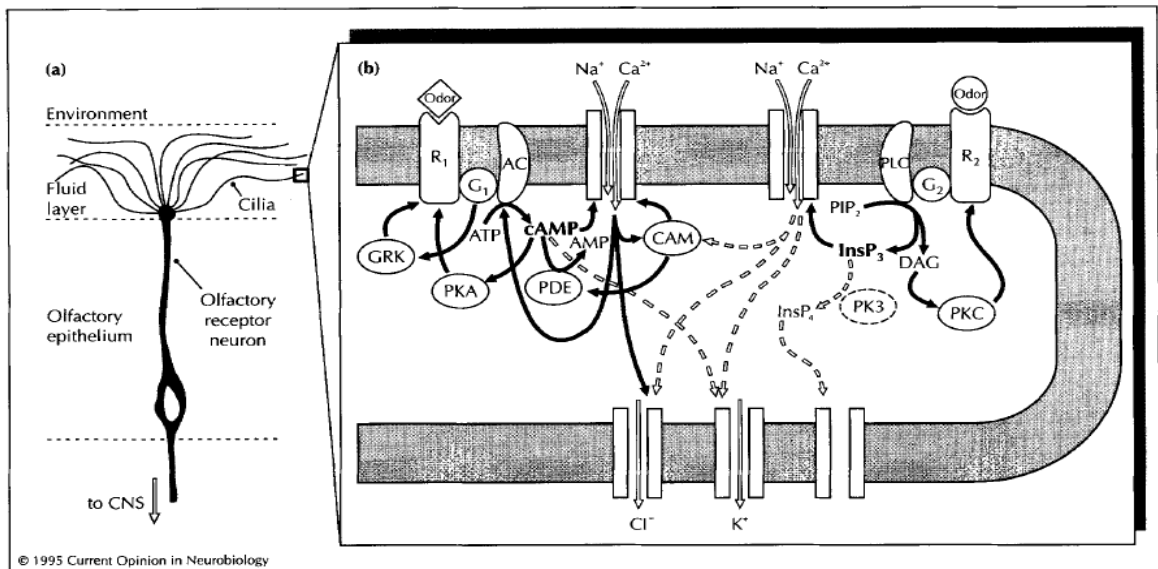


Figure 1.12. Diagram of the dual second messenger pathways in olfactory signal transduction. Figure from Ache and Zhainazarov, 1995.

Mechanisms of how dissolved contaminants impair olfaction are complex. Compounds can modify odorant perception, mimic molecules, act directly on other targets of the nervous system, and or impact physiological responses not directly through olfaction (Figure 1.13). The resulting olfactory toxicity falls into three categories: anosmia, the inability to smell; hyposmia, decreased ability to smell; or dysosmia, olfactory information is incorrectly processed. Hyposmia is the most common outcome from contaminant exposure, which at high doses can become functional anosmia (reviewed in Tierney et al., 2010).

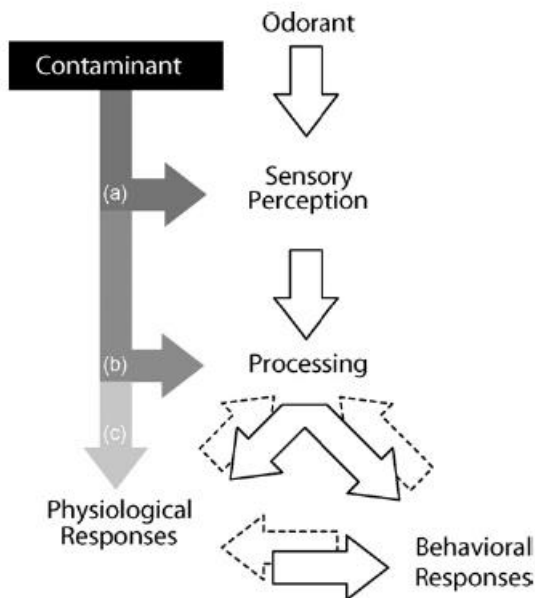


Figure 1.13. The olfactory pathway for processing of odorants or contaminants which can be impacted at different steps causing different responses. Contaminants can act on the olfactory pathway in three main areas. Contaminants can act as odorants or modify odorant perception (a); and or act on the nervous system through other pathways (b); and or alter other physiological responses (c). Figure from Tierney et al., 2010.

Chlorpyrifos has been shown to impair olfaction. Sandhal et al., (2004) exposed juvenile coho salmon to chlorpyrifos for seven days at environmentally relevant

concentrations of 0.625 – 2.5 $\mu\text{g/L}$. Impacts on olfaction were determined by examining odor-evoked field potentials from the sensory epithelium, using electroolfactograms (EOGs), and olfactory forebrain, using electroencephalograms (EEGs), with the odorants L-serine and taurocholic acid (TCA). Chlorpyrifos exposure decreased the amplitude of the epithelial and bulbar response to both odorants in a concentration-dependent manner (Figure 1.14). This study also investigated AChE inhibition in the exposed fish, but did not find a correlation with olfactory impairment. It was hypothesized that chlorpyrifos may act on other targets that are necessary for olfactory signal transduction, such as adenylyl cyclase (Sandhal et al., 2004). As chlorpyrifos individually has been shown to impair olfaction, understanding how hypersaline acclimation may impact the effect is crucial, as well as how olfactory impairment may influence olfactory mediated behaviors that are important for salmonid survival and migration.

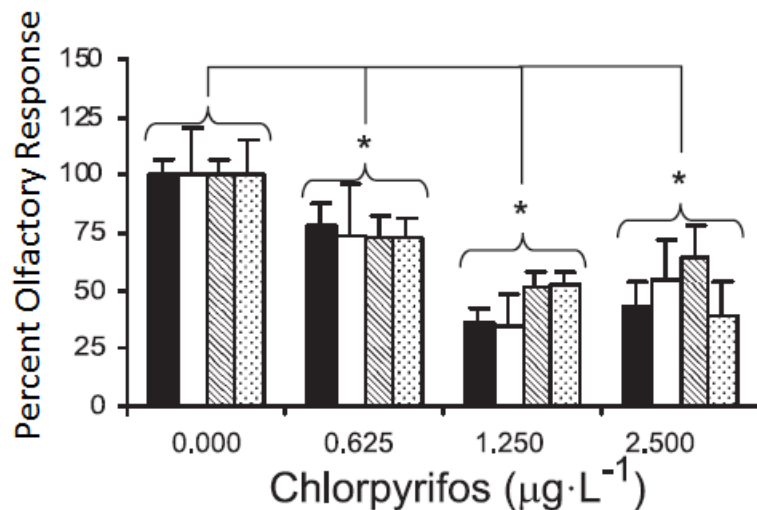


Figure 1.14. Percent olfactory response as compared to controls for juvenile coho salmon exposed to three concentrations of chlorpyrifos. * indicates a significant difference from controls. Solid bars, EOG responses to TCA; open bars, EEG responses to TCA; hatched bars, EOG responses to L-serine; stippled bars; EEG responses to L-serine. Figure from Sandhal et al., 2004.

Behavior

Behavioral endpoints can provide valuable information about environmental pollutants because they can link physiological and ecological processes. Although understanding impacts on the physiological level can provide vital information, it can be challenging to link the impacts to endpoints of ecological importance, such as growth, survival, and reproduction. Numerous interspecific and intraspecific interactions are closely associated with life histories of fish and these interactions depend on the performance of appropriate behaviors. Changes in behaviors can impact populations and potentially lead to ecological collapse, typically at much lower concentrations than those causing physiological impairment. Even if a contaminant does not overtly harm an organism, it may cause them to be unable to function in an ecological context if their normal behavior is altered (reviewed in Scott and Sloman, 2004).

External stimuli act through neural networks to trigger specific physiological sequences causing organisms to perform normal behaviors (Figure 1.15). Contaminants can cause disruption of these sequences before they are completed and lead to harmful alterations in behavior. Contaminants can impact the initiation of these sequences as well with detrimental results. Overall, contaminants of aquatic environments can cause organisms to have inappropriate behavioral responses which can have severe implications on survival. Alterations may occur in behaviors associated with feeding, predator avoidance, and reproduction (reviewed in Scott and Sloman, 2004).

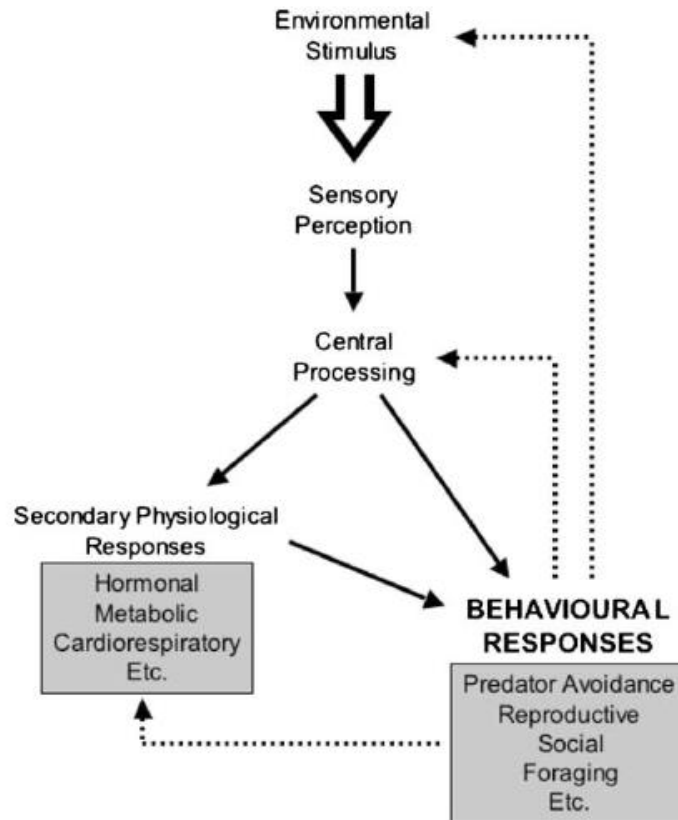


Figure 1.15. Sequence of events leading to behavioral responses. Figure from Scott and Sloman, 2004.

Foraging behavior is crucial to allow fish to feed and is primarily mediated by olfaction. The most common result from impaired feeding behavior after toxicant exposure is reduced food consumption and or cessation of feeding (Sandheinrich and Atchison, 1990). This change in feeding behavior can then impact growth and survival.

Examples of quantifiable feeding behaviors include striking at prey and successful prey capture. Little et al., (1990) examined the impact of six different contaminants on two types of feeding behavior, frequency of strikes at daphnid prey and prey capture, on juvenile rainbow trout. The six contaminants included four distinct chemical groups used

in agriculture: carbamates, organochlorines, organophosphates, and phenolics. It was found that all six contaminants significantly impaired strike frequency and prey capture. Higher concentration exposures appeared to cause an inhibition in motivation to feed. Lower concentrations exposures reduced feeding efficiency and strike frequency (Little et al., 1990).

To avoid predation fish employ a complex set of behaviors. Sublethal exposure to certain toxicants can alter predator avoidance and cause reduced survival (reviewed in Scott and Sloman, 2004). Contaminants can interfere with these behaviors in various ways. Sensory systems and motivation to respond can be disrupted which can alter behavioral responses to early warning signs of predation risk. Responses during later stages of predation can also be impacted. Contaminants can alter the escaping ability of individual fish or the schooling behavior of groups. Alterations in normal predator avoidance behavior may impact aquatic relations between predator and prey in ecosystems. This could impact community structure by increasing the predation susceptibility of prey fish populations (reviewed in Scott and Sloman, 2004).

An example of a specific behavioral mechanism to avoid predation is the response to chemical signals of predation threat. Chemical signals can be released from epidermal cells of prey fish skin when a predatory attack causes sufficient damage. This chemical signal acts as an alarm for other prey fish, which detect this signal through olfaction and then carry out behaviors to minimize further predation. For individual fish this involves decreased swimming and feeding potentially leading to increased schooling. Contaminants can impact the detection of and or proper response to chemical signals of

alarm which would impact susceptibility of prey fish to predation (reviewed in Scott and Sloman, 2004). For example, Scholz et al., (2000) exposed juvenile Chinook salmon to diazinon, an organophosphate, for two hours at 1 and 10 µg/L. They found that these exposures eliminated the behavioral responses of individual fish to alarm substance made of conspecific skin extract (Scholz et al., 2000).

Several different behaviors are involved in fish reproduction. Contaminants can potentially alter the performance of these various behaviors and impact reproductive success. Fish reproductive behaviors include spawning site selection, territorial defense of spawning site, nest building, courtship and spawning, and post-fertilization investment. Post-fertilization investment includes nest cleaning, guarding, and fanning behaviors. Alterations can impact mate selection, fertilization success, and survival of offspring (reviewed in Scott and Sloman, 2004).

Reproductive behavior may be impacted by toxicant exposure altering spawning site selection and natal homing. Many fish, including salmonids, return to their natal stream to spawn. Salmonids use olfaction to guide them back to their natal streams. Exposure to 10 µg/L diazinon for 24 hour hours disrupted the homing ability of Chinook salmon, causing a 60% decrease in return to their natal hatchery (Scholz et al., 2000). Disruption of this reproductive behavior by contaminant exposure could have detrimental effects, especially on salmonid populations that are threatened or endangered.

Chlorpyrifos is of particular concern when it comes to behavior alteration because sublethal exposure has previously been shown to alter behavior in various fish species. Levin et al., (2004) examined the effects of chlorpyrifos on the swimming activity of

newly hatched zebrafish (*Danio rerio*). Exposure to 100 µg/L chlorpyrifos for five days post fertilization significantly slowed swimming activity on days six and nine post fertilization. Additionally, Kienle et al., (2009) found that behavior was a more sensitive endpoint than morphological abnormalities or mortality for zebrafish larvae exposed to chlorpyrifos. Rice et al., (1997) found that chlorpyrifos caused an under reactive startle response and overall hypoactivity in juvenile Japanese medaka. Another study showed similar impacts of chlorpyrifos on startle response in juvenile medaka indicating that medaka generally appeared to be more susceptible to predation after exposure (Carlson et al., 1998).

Studies focusing on behavioral impacts of chlorpyrifos have also been performed on salmonids. Sandahl et al., (2005) compared the impacts of chlorpyrifos on AChE activity and behavior in juvenile coho salmon. Spontaneous swimming and feeding behavior were assessed using a computer-assisted, three dimensional video imaging system. After exposure for 96 hours to 0 – 2.5 µg/L tissue AChE activity and behaviors were inhibited in a dose-dependent manner (Table 1.1; Figure 1.16). Furthermore, brain AChE inhibition and decreases in both behaviors were significantly correlated (Sandahl et al., 2005). Swimming performance and rapid acceleration swimming in coho salmon were likewise impacted by chlorpyrifos exposure in another study (Tierney et al., 2007).

Table 1.1. Behavioral measures and acetylcholinesterase activity of juvenile coho salmon after exposure to chlorpyrifos. Table from Sandahl et al., 2005.

Chlorpyrifos ^a	Brain AChE ^b	Muscle AChE ^b	Spontaneous swimming rate ^c	Feeding swimming rate ^c	First strike ^d	Total strikes ^e
0	23.2 ± 0.5	38.9 ± 1.6	4.9 ± 0.3	4.6 ± 0.3	2.9 ± 0.6	19.6 ± 0.6
0.6	17.9 ± 0.3*	34.3 ± 1.1*	3.6 ± 0.2*	4.4 ± 0.3	3.1 ± 0.5	17.7 ± 1.1
1.2	11.3 ± 0.3*	20.7 ± 0.9*	2.8 ± 0.2*	3.8 ± 0.2*	5.1 ± 1.2	14.5 ± 1.1*
1.8	10.3 ± 0.4*	15.7 ± 0.8*	2.3 ± 0.3*	3.5 ± 0.2*	6.1 ± 2.0	12.7 ± 1.2*
2.5	8.3 ± 0.2*	12.9 ± 0.9*	1.1 ± 0.2*	2.0 ± 0.2*	16.1 ± 3.0*	5.4 ± 0.6*

^a Nominal chlorpyrifos concentrations are in µg/L.

^b Acetylcholinesterase activities are in µmol/min/g tissue.

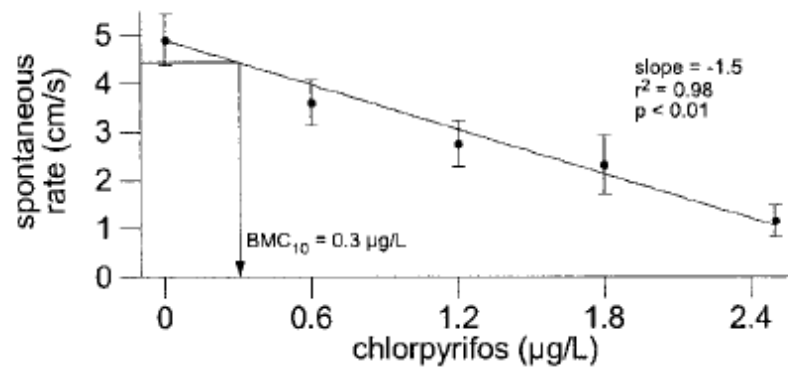
^c Swimming rates are in cm/s.

^d First strikes are in s.

^e Total strikes at brine shrimp are after a 1-min interval.

* Asterisks denote statistically significant difference from controls (analysis of variance, Dunnett's test, $p < 0.05$).

A)



B)

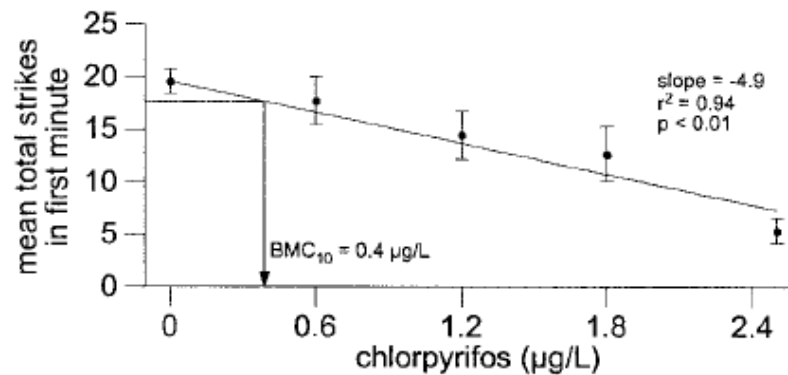


Figure 1.16. Behavioral measures, (A) spontaneous rate and (B) mean total strikes in first minute, of juvenile coho salmon after exposure to chlorpyrifos. Figure from Sandahl et al., 2005.

In addition to individual impacts on physiology and behavior, sublethal exposure to chlorpyrifos has the potential to impact salmonid populations. Baldwin et al., (2009) created a model to assess how episodic or continual sublethal exposure to organophosphate or carbamate pesticides at the individual level may impact populations of ocean type Chinook salmon (*Oncorhynchus tshawytscha*). The model was based on the idea that organophosphates and carbamates inhibit AChE which can impact feeding behavior, which in turn affects food uptake, ultimately influencing daily somatic growth rate. Size has been shown to be a determining factor in survival for ocean type Chinook. Overall the model predicted that organophosphates would have a more severe impact on populations than carbamates, as organophosphates bind AChE irreversibly and carbamates bind reversibly. For organophosphates, a four day exposure to sublethal concentrations was predicted to result in outmigrating salmonids that were around 10% shorter in length than unexposed fish. Furthermore, when extended for 20 years, the model predicted that seasonal exposure of salmonids to four days of sublethal organophosphate would reduce spawner abundance by 73% as compared to unexposed fish (Baldwin et al., 2009).

Macneale et al. (2014) expanded this modeling approach for predicting population level effects from individual exposures to organophosphates and carbamates by including impacts of pesticide exposure on salmonid food webs. The model predicted that for organophosphates that are long acting and highly toxic to salmonids and invertebrates, such as chlorpyrifos, the impacts at the population level would be driven by reductions in

salmonids population growth from impacted feeding behavior and that reductions in prey abundance would have minimal additional impact (Macneale et al., 2014).

Hypotheses

While previous studies have examined the impacts of hypersaline acclimation on the acute toxicity of certain thioether organophosphate and carbamate pesticides on salmonids, few studies have explored these impacts on sublethal endpoints. Although acute toxicity data can provide useful overall assessments of compounds, it can lack environmental relevance as concentrations required for lethality are not typically environmentally realistic. The purpose of this study is to assess the possible impacts on hypersaline acclimation on both the acute and sublethal toxicity of the organophosphate chlorpyrifos on salmonids. The following hypotheses will be addressed:

- a. Evaluation of differential gene expression after hypersaline acclimation will elucidate mechanisms responsible for differences in pesticide toxicity as well as provide general knowledge of pathways affected by hypersalinity.
- b. Hypersaline acclimation will enhance the acute toxicity of chlorpyrifos to salmonids.
- c. Differences in acute toxicity of salmonids to chlorpyrifos after hypersaline acclimation will be associated with differences in metabolism, acetylcholinesterase inhibition, bioavailability of chlorpyrifos, or regulation of signal transduction related genes.
- d. Sublethal impacts of acclimation to hypersaline conditions with concurrent exposure to chlorpyrifos will include impairment of olfactory responses to odorant stimuli.

e. Impacts of olfactory impairment will be linked between the molecular, physiological, and behavioral levels of biological organization.

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Chapter 2: Differential Gene Expression in Liver, Gill and Olfactory Rosettes of Coho Salmon (*Oncorhynchus kisutch*) after Acclimation to Salinity

Abstract

Most Pacific salmonids undergo smoltification and transition from freshwater to saltwater. Saltwater acclimation requires salmonids to make various adjustments in color, shape, size, metabolism, catabolism, and osmotic and ion regulation. The molecular mechanisms underlying this transition are largely unknown. In the present study, we acclimated coho salmon (*Oncorhynchus kisutch*) to four different salinities (<0.5, 8, 16, and 32 ppt) and assessed gene expression through microarray analysis of gill, liver and olfactory rosettes. Gills are involved in osmotic regulation, liver plays a role in energetics, and olfactory rosettes are involved in behavior. Between all salinity treatments, liver had the highest number of differentially expressed genes at 1,616. By contrast, the gills had 1,074 differentially expressed genes and there were 924 differentially expressed genes in the olfactory rosettes. The difference in the number of differentially expressed genes may be due to the higher responsiveness of liver to metabolic changes after salinity acclimation to provide energy to fuel other metabolic and osmoregulatory tissues such as the gills. Differentially expressed genes were tissue- and salinity- dependent. There were no genes differentially expressed that were common to all salinity treatments and all tissues. Five genes were targeted for microarray confirmation by qPCR and included CCAAT/enhancer binding protein β (CEBPB), calpain 1 (CAPN1), proto-oncogene, serine/threonine kinase (Pim1), aldolase B, fructose-bisphosphate (aldob), and complement component 3 (c3). qPCR expression

profiles of these genes were in good agreement with those obtained by microarray analysis. Gene ontology term analysis revealed biological processes, molecular functions, and cellular components that were significantly affected by salinity, as well as being tissue-dependent. For liver, oxygen binding and transport terms were highlighted, suggesting possible impacts on metabolism. For gills, muscle and cytoskeleton related terms predominated and for olfactory rosettes, immune response-related genes were accentuated. Interaction networks were examined in combination with GO terms and determined similarities between tissues for potential osmosensors and signal transduction cascades. In conclusion, our study suggests that Pacific salmon share many salinity acclimation molecular mechanisms with other species, with a few new genes identified, and that although the three tissues shared certain underlying mechanism, many of the differentially expressed genes were tissue-specific.

Introduction

Pacific salmonids are economically and ecologically critical species in the northwestern United States. Most of these species are anadromous, hatching in freshwater, migrating to the ocean for growth and maturation, and migrating back to their natal streams for reproduction (Quinn, 2005). The process of transitioning from freshwater to saltwater, smoltification, involves various changes in color, shape, size, metabolism, catabolism, and osmotic and ion regulation (reviewed in Hoar, 1988). However, there are still many aspects of Pacific salmonid smoltification that are currently unknown, particularly the regulation of smoltification at the molecular level.

Seear et al., (2010) conducted a transcriptomic study of Atlantic salmon (*Salmo salar*) smoltification in brain, gill, and kidney tissues to try to elucidate some of the molecular mechanisms involved in smoltification regulation. These tissues were selected based upon their key roles in smoltification. The gills had the highest number of differentially expressed genes and the differentially expressed genes in the three tissues were mostly involved in growth, metabolism, oxygen transport, or osmoregulation. Many of the genes that were upregulated complimented previous findings from other studies on salmonid smoltification physiology and biochemistry (D'cotta et al., 1996, Chakravarti et al., 1998, McCormick et al., 1989, Vanstone et al., 1964, Gallagher et al., 2008). Pacific salmon have also been the subject of a transcriptomics study of migration, although the focus was on the migration from the ocean to freshwater (Evans et al., 2011). Additionally, Gallagher et al. (2007) assessed differences in gene expression between smolts and adult coho salmon (*Oncorhynchus kisutch*) liver and found that differentially

expressed genes were mainly involved in cellular processes related to protein biosynthesis and degradation, ion transport, transcription, cell structure, and cellular energetics. There is still a need to better understand the molecular responses of Pacific salmon transitioning from freshwater to saltwater.

In addition to salmon, transcriptomic studies of other euryhaline fish undergoing salinity acclimation have also been conducted. For example, Evans and Somero (2008) performed a time course acclimation of goby, *Gillichthys mirabilis*, to hyper- and hypo osmotic stress in gills. The authors examined changes in gene expression between 0, 1, 2, 4, and 12 hours of acclimation and showed that cell signaling genes were among the most predominant groups of differentially expressed genes. Many of the genes they found to be differentially regulated had no previous reported role in osmotic stress adaptation, highlighting the value of the transcriptomic approach. Fiol et al., (2006) also examined changes in gene expression after short term acclimations. Tilapia (*Oreochromis mossambicus*) were transferred from freshwater to saltwater and 20 novel genes involved in immediate hyperosmotic stress were identified after four hours in gill epithelial cells. Pathway analysis revealed that more than half of the identified genes interact within a cellular stress response signaling network, with other important pathways comprising stress response signal transduction, compatible organic osmolyte accumulation, energy metabolism, lipid transport and cell membrane protection, actin-based cytoskeleton dynamics, and protein and mRNA stability. These and other novel genes may also be important in Pacific salmonid salinity acclimation, which could be determined through a transcriptomics approach.

The goal of the current study was to assess how salinity acclimation of coho salmon (*Oncorhynchus kisutch*) impacted gene expression in gills, liver, and olfactory rosettes. Gills were chosen because they play a vital role in osmoregulation. Liver was selected based on its role in energetics and olfactory rosettes were chosen for their role in behavior and homing to natal streams. To our knowledge, ours is the first transcriptomics study to examine changes in the olfactory rosettes from salinity acclimation. Establishing the underlying molecular responses of Pacific salmonids to changes in salinity will help in understanding the natural process of smoltification.

Methods

Organisms

Juvenile coho salmon (*Oncorhynchus kisutch*) (21.6 ± 1.7 cm length and 100.7 ± 28.7 g weight) were obtained from the Nimbus Hatchery (Gold River, CA), collected and maintained in freshwater. Organisms were maintained in a flow-through living-stream system at the University of California, Riverside, with dechlorinated carbon-filtered municipal water at 11-13°C and acclimated for a minimum of two months before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT).

Hypersalinity acclimation

Fish were transferred and sequentially acclimated to hypersaline water up to concentrations of <0.5 (control), 8 (low), 16 (medium), and 32 ppt (high) saline

concentrations, with an increase of 4 ppt every two days (CrystalSea Marine Mix, Marine Enterprises International, Baltimore, MA) in 24 L tanks. Previous studies in salmonids have indicated direct relationships with plasma osmolality, stress responses and survival of individuals using this method (El-Alfy et al., 2002). All fish were left for one week at the specific salinity. After acclimation to each salinity regime, animals were weighed, measured and euthanized using tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) with subsequent removal of the liver, gills and olfactory rosettes, which were frozen in liquid nitrogen and stored at -80°C. There were 4 biological replicates per tissue and condition. All experiments were done in accordance with IACUC guidelines at The University of California, Riverside.

RNA Extraction

Total RNA was isolated from salmon tissue using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the vendor's defined method, and stored at minus 70 degrees C. The quantity (ng/uL) of RNA was determined measuring the OD₂₆₀ with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the RNA purity was assessed measuring OD_{260/280} and OD_{260/230} ratios. RNA integrity was characterized using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only total RNA samples with appropriate size distribution, quantity, and OD_{260/280} as well as OD_{260/230} ratios of 1.8–2.1 were used for microarray-based analysis.

RNA sample processing for microarray analysis

The RNA samples were labeled and prepared for hybridization onto Agilent 4X44K salmon arrays (Cat.# G2519F-020938; Agilent Technologies, Inc. Santa Clara, CA) using the manufacturer's established protocols. Hybridization and washing of these arrays was accomplished using HS 400 Pro hybridization and wash stations (Tecan Systems, Inc., San Jose, CA) and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc. Santa Clara, CA) using previously established methods.

Microarray data analysis

Expression level data from the Agilent Microarray Scanner and Feature Extraction Software were also normalized using a multiple-*loess* algorithm (Sasik et al., 2004). Probes whose expression level exceeds a threshold value in at least one sample were called detected. The threshold value was found by inspection from the distribution plots of (log) expression levels. Detected probes are sorted according to their q -value, which is the smallest false discovery rate (FDR) at which the gene is called significant. FDR is the expected fraction of false positive tests among significant tests (Benjamini and Hochberg, 1995). We evaluate FDR using Significance Analysis of Microarrays (SAM) and its implementation in the official statistical package *samr* (Tusher et al., 2001). In order to not be unduly impressed by accidentally small variances, we set the percentile of standard deviation values used for the exchangeability factor in the test statistic to 75.

Each gene ontology term or a pathway was treated simply as a set of genes. The probe list, sorted by q -value in ascending order, is translated into Entrez gene ID's and parsed so that whenever several different probes represent the same gene, only the highest-ranking probe is kept for further analysis. The sorted list of genes is subjected to a non-parametric variant of the Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), in which the p -value of a gene set of size n is defined as follows: Let us denote the k -th highest rank in gene set as r_k , and define p_k as the probability that out of n randomly chosen ranks (without replacement) the k -th highest is not smaller than r_k . The p -value of the gene set is defined as $\min_k [P_k]$. It is designed to detect overrepresented gene sets at the top of the list. Unlike the Kolmogorov-Smirnov statistic used in GSEA, it will not detect underrepresented or other, pathologically distributed, gene sets. Finding the p -value of a gene set of size n requires calculation of n rank-order values p_k , however, there is no need to adjust the p -values for multiple testing as the rank-order tests are highly statistically dependent. A Bonferroni adjustment of gene set p -values for the number of gene sets tested was performed, even though there are often several gene sets with overlapping gene content (and therefore are statistically dependent), which is partly due to the design of the gene ontology database and partly because genes tend to be involved in multiple processes. We report only gene sets with adjusted p -values ≤ 0.01 .

qPCR validation

Primers were designed using Primer3 software for CCAAT/enhancer binding protein β (CEBPB), calpain 1 (CAPN1), proto-oncogene, serine/threonine kinase (Pim1),

aldolase B, fructose-bisphosphate (aldob), and complement component 3 (c3). CEBPB was selected based on its potential function as a transcription factor in response to salinity acclimation. C3 was chosen for its role in immune response and differential regulation in two tissues. CAPN1, Pim1, and aldob were chosen as representatives for the different tissues based on similar patterns of differential regulation (all up or all down regulated) in all three salinity treatments. β -actin was used as the housekeeping gene. Primer sequences are listed in table 2.1. Primers were optimized based on annealing temperature, template concentration, and primer concentration. qPCR was run for each gene comparing freshwater acclimated fish with fish acclimated to the high salinity (32 ppt; n = 6 - 8) using the iScript One-step RT-PCR kit with SYBR Green from Bio-Rad. 250 nM of each primer was added to 25 μ L PCR reactions containing SYBR Green RT-PCR Reaction Mix, and 100 ng of cDNA sample. Thermocycling parameters were as follows: 5 mins at 95°C; 40 cycles of 10 sec at 95°C and 30 sec at 57°C. At the end of each cycle fluorescence data was collected. A melting curve analysis was run between 60°C and 95°C following the amplification reaction. The C_t was selected to be in the linear phase of amplification. The reactions were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) and data analysis was done using IG5 (Bio-Rad).

Table 2.1 Primer sequences used to qPCR validation.

Target	Primer sequence (5'-3')	Reference
CCAAT/enhancer binding protein β (CEBPB)	AGAATGGACAACGGTTCAAC CGTGCATTTACCTGACAGTG	
Calpain 1 (CAPN1)	GCCAAAACATTGCCTGTTATCTTAG ATAGGAGGCCGTATCAAAATTCC	
Proto-oncogene, serine/threonine kinase (Pim1)	TGCTGGACTGGTTTGAGGTG CATCCCTATGCACGATCCCC	
Aldolase B, fructose-bisphosphate (aldob)	TGACTACCCAGTTCCCATCC CTGTGCCTTTGTCCACCTTA	
Complement component 3 (c3)	AAGACCCCATCATTGTGCGT CTCTTACCCCGTCATTGCT	
β -actin	GTCCTTCATGATTCTCTGCTGA ACTCGGGTTCATTTGCATAAACA	Lavado et al., 2009

Results

Using a 1.5-fold cutoff at $p < 0.05$, we observed 119, 101, and 81 differentially expressed genes in all three salinity treatments (low, medium, and high) as compared to the freshwater controls in liver, olfactory rosettes, and gills respectively (Figure 2.1). Among the salinity treatments, liver had the highest number of differentially expressed genes (1,616 differentially expressed genes). Gills had 1,074 differentially expressed genes and olfactory rosettes had 924. The high salinity treatment had the highest number of differentially expressed genes for liver with 556, whereas in the gills and olfactory rosettes the medium salinity treatment caused the highest number of differentially expressed genes with 440 and 361, respectively. The number of differentially expressed genes in each individual salinity treatment was greater than the shared genes between treatments for all salinities and all tissues.

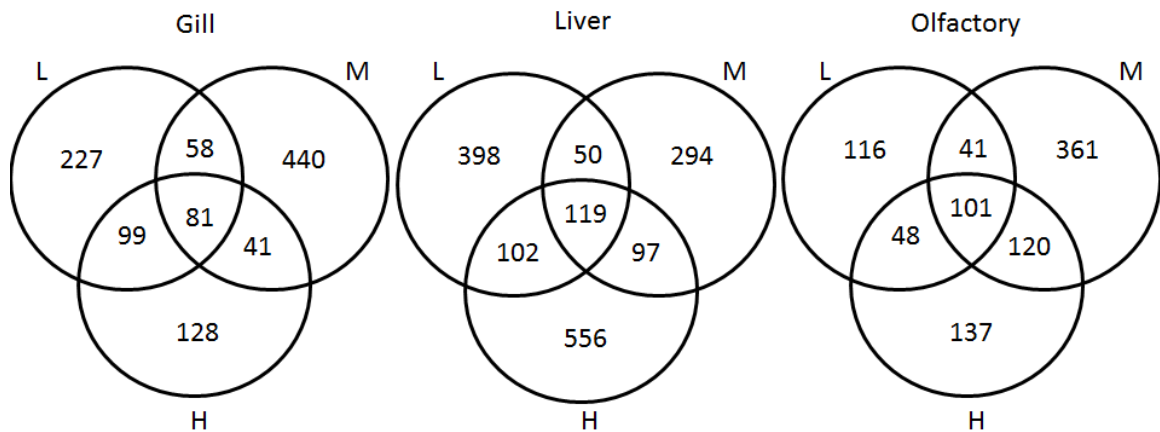


Figure 2.1.

Venn diagrams for differentially expressed genes in gills, liver, and olfactory rosettes after acclimation to low, medium, and high salinity as compared to freshwater controls. A 1.5 fold cutoff was used.

There were no known differentially expressed genes that were common and inclusive to all salinity treatments and all three tissues. Five genes were differentially expressed in all salinity treatments and shared between two tissues (Table 2.2). Sodium/potassium-transporting ATPase subunit alpha-1 precursor (*atp1a1*) was differentially expressed in gills and olfactory rosettes, although this gene was upregulated in gills and down regulated in olfactory rosettes. Sodium-coupled neutral amino acid transporter 2 (*SNAT2*) was upregulated in both gills and liver. Gastrulation-specific protein G12 (*g12*), complement C1q-like protein 2 precursor (*c1ql2*), and complement C3-1 (*C3*) were all differentially expressed in both liver and olfactory rosettes relative to freshwater controls. G12 was down regulated in liver and upregulated in olfactory

rosettes, while c1ql2 was upregulated in liver and down regulated in olfactory rosettes. C3 was down regulated in both liver and olfactory rosettes relative to freshwater controls.

Table 2.2 Genes differentially expressed in all salinities in two tissues.

Gene	Tissues		Expression	
	Gill	Olfactory	Up	Down
Na/K-transporting ATPase subunit alpha-1 precursor	Gill	Olfactory	Up	Down
Na-coupled neutral amino acid transporter 2	Gill	Liver	Up	Up
Gastrulation-specific protein G12	Liver	Olfactory	Down	Up
Complement C1q-like protein 2 precursor	Liver	Olfactory	Up	Down
Complement C3-1	Liver	Olfactory	Down	Down

Gene ontology (GO) term analysis revealed that most significant terms were tissue specific. For liver, 13 GO terms were significant: 3 biological processes, 5 molecular functions, and 5 cellular components (Table 2.3; Bonferroni $p < 0.05$). For gills, 17 GO terms were significant: 6 biological processes, 4 molecular functions, and 7 cellular components (Table 2.4; Bonferroni $p < 0.05$). For olfactory rosettes 26 GO terms were significant: 14 biological processes, 10 molecular functions, and 2 cellular components (Table 2.5; Bonferroni $p < 0.05$). No GO terms were significant for all three tissues. Liver did not share any significant terms with either gills or olfactory rosettes. Gills and olfactory rosettes shared three significant terms: the biological process of the classical pathway of complement activation, the molecular function of serine-type endopeptidase inhibitor activity, and the cellular component for extracellular space.

Table 2.3. Significant gene ontology terms for liver.

GO Term	GO Term Name	Bonferroni p
Biological Process		
GO:0055114	oxidation reduction	3.51E-05
GO:0042632	cholesterol homeostasis	2.26E-02
GO:0042254	ribosome biogenesis	3.85E-02
Molecular Function		
GO:0005344	oxygen transporter activity	5.75E-07
GO:0020037	heme binding	6.68E-07
GO:0019825	oxygen binding	1.72E-05
GO:0016491	oxidoreductase activity	1.87E-03
GO:0009055	electron carrier activity	3.34E-02
Cellular Component		
GO:0005833	hemoglobin complex	8.75E-06
GO:0005789	endoplasmic reticulum membrane	4.57E-04
GO:0005783	endoplasmic reticulum	2.43E-03
GO:0005788	endoplasmic reticulum lumen	6.75E-03
GO:0005792	Microsome	1.23E-02

Table 2.4. Significant gene ontology terms for gills.

GO Term	GO Term Name	Bonferroni p
Biological Process		
GO:0030049	muscle filament sliding	1.32E-05
GO:0006096	Glycolysis	1.72E-04
GO:0045214	sarcomere organization	1.91E-04
GO:0048739	cardiac muscle fiber development	7.14E-03
GO:0055010	ventricular cardiac muscle morphogenesis	2.21E-02
GO:0006958	complement activation, classical pathway	4.22E-02
Molecular Function		
GO:0008307	structural constituent of muscle	8.03E-05
GO:0005509	calcium ion binding	1.34E-04
GO:0003779	actin binding	2.23E-03
GO:0004867	serine-type endopeptidase inhibitor activity	2.46E-02
Cellular Component		
GO:0005615	extracellular space	3.75E-06
GO:0005861	troponin complex	1.89E-03
GO:0005581	Collagen	3.30E-03
GO:0005743	mitochondrial inner membrane	4.73E-03
GO:0005859	muscle myosin complex	6.29E-03
GO:0030016	Myofibril	1.25E-02
GO:0030018	z disc	2.60E-02

Table 2.5. Significant gene ontology terms for olfactory rosettes.

GO Term	GO Term Name	Bonferroni p
Biological Process		
GO:0006958	complement activation, classical pathway	3.12E-10
GO:0006956	complement activation	1.58E-05
GO:0006957	complement activation, alternative pathway	4.36E-05
GO:0007596	blood coagulation	5.04E-05
GO:0006879	cellular iron ion homeostasis	1.87E-04
GO:0050766	positive regulation of phagocytosis	4.92E-04
GO:0001798	positive regulation of type IIa hypersensitivity	8.28E-04
GO:0006953	acute-phase response	7.50E-03
GO:0007597	blood coagulation, intrinsic pathway	1.56E-02
GO:0006642	triglyceride mobilization	1.94E-02
GO:0016525	negative regulation of angiogenesis	2.21E-02
GO:0006916	anti-apoptosis	2.59E-02
GO:0006810	Transport	3.16E-02
GO:0008380	RNA splicing	4.47E-02
Molecular Function		
GO:0003823	antigen binding	8.97E-08
GO:0004867	serine-type endopeptidase inhibitor activity	7.97E-06
GO:0030414	peptidase inhibitor activity	1.10E-03
GO:0008289	lipid binding	1.53E-03
GO:0032052	bile acid binding	1.98E-03
GO:0002020	protease binding	5.00E-03
GO:0004252	serine-type endopeptidase activity	8.83E-03
GO:0043499	eukaryotic cell surface binding	1.00E-02
GO:0005529	sugar binding	3.23E-02
GO:0003705	RNA polymerase II transcription factor activity	4.12E-02
Cellular Component		
GO:0005615	extracellular space	3.14E-15
GO:0000502	proteasome complex	2.11E-02

The MetaCore™ knowledge base was used to create interaction networks for each tissue comparing freshwater acclimated fish and fish acclimated to the high salinity to depict how differentially expressed genes interact (Bugrim et al., 2004). Different fold

changes were used for each tissue to create pathways that gave an overall picture of the main changes occurring, without being too convoluted. As liver had the highest number of differentially expressed genes, a stricter cutoff of 1.75 fold change was used. For gills, only genes with 1.5 fold change or greater were included. For olfactory rosettes, which had the least genes differentially expressed, a 1.25 fold change was used.

For the liver, the interaction network revealed one main pathway with four major nodes, as well as seven pairs of interacting genes and one small network of three related genes (Supplemental Figure 1). The four nodes on the major pathway were complement component 3 (C3), CCAAT/enhancer binding protein δ (CEBPD), CCAAT/enhancer binding protein α (CEBPA), and jun proto-oncogene (JUN). The total number of genes included in the liver pathway was 37. Confirmation of hepatic C3 down regulation was achieved by qPCR analyses in animals from the high salinity treatments (Figure 2.2). To confirm upregulation of hepatic transcripts, qPCR was performed on CAPN1 in the liver where similar increases of mRNA were consistent with that of the array. The interaction network for gills, which had a lower fold-cutoff, was more complex than observed for liver (Supplemental Figure 2). For example, there was one major pathway with multiple nodes identified in the gills, as well as three separate interactions with two genes each. Sixty two genes were included in the gills pathway. For gills, both CEBPB and Pim1 were confirmed to be upregulated through qPCR (Figure 2.2). The olfactory rosettes network, which had the least strict fold-cutoff, was the most complex of the three tissues studied (Supplemental Figure 3). Specifically, the olfactory rosettes network contained one main network with multiple nodes and short pathways with four genes or less. A total

of 108 genes were included in the pathway. In olfactory rosettes, transcripts of ALDOB and C3 were both down regulated and consistent with array data in the high salinity treatment (Figure 2.2).

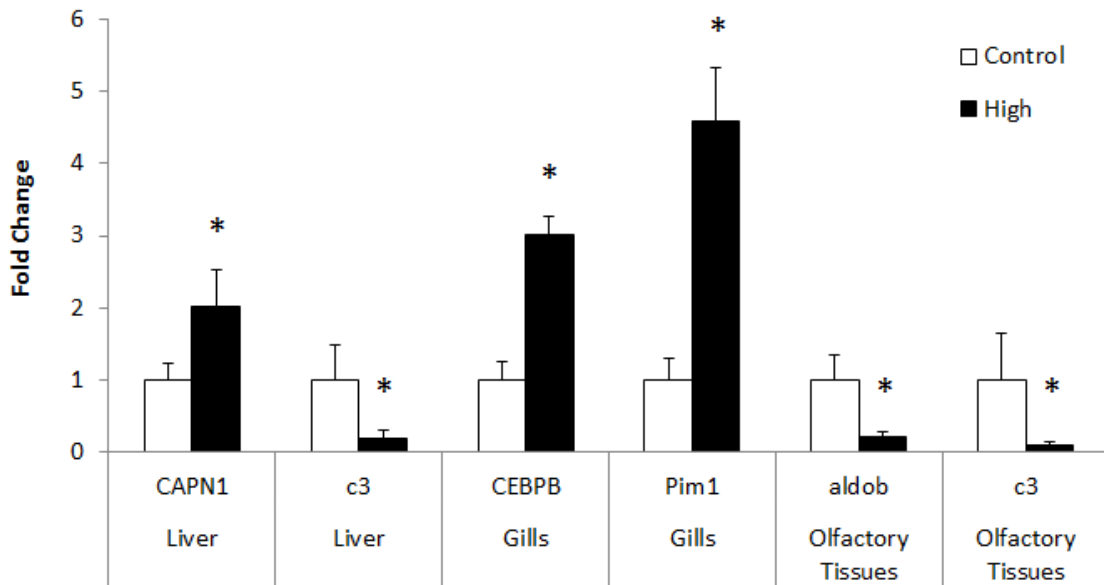


Figure 2.2. Relative gene expression for coho salmon tissues (liver, gills, and olfactory rosettes) acclimated to freshwater or high (32 ppt) salinity. Data are presented as fold change and mean \pm standard error ($n = 6 - 8$). * indicates significant differences from freshwater acclimated fish ($p < 0.05$, Student's T-test). β -actin was used as the housekeeping gene. CAPN1 = calpain 1; c3 = complement component 3; CEBPB = CCAAT/enhancer binding protein β ; Pim1 = proto-oncogene, serine/threonine kinase; aldob = aldolase B, fructose-bisphosphate.

A systems view network of interacting genes and gene products identified similarities and differences in how each tissue responded to the high salinity acclimation. For example, CCAAT/enhancer binding protein α (CEBPA) was upregulated and a main node in the pathways for all three tissues. The interaction this gene had with other

differentially regulated genes was similar between tissues. Another similarity between the three pathways was upregulation of growth arrest and DNA-damage-inducible genes (GADD45). For liver, GADD45G was upregulated. For gills, GADD45B was upregulated and for olfactory rosettes, both GADD45B and GADD45A were upregulated. Insulin-like growth factor binding protein (IGFBP) genes were also present in each pathway; however, the specific gene and direction of change differed by tissue. IGFBP1 mRNA levels were upregulated in liver, and down regulated in olfactory rosettes compared to freshwater controls. For gills, IGFBP5 mRNA levels were down regulated compared to freshwater controls. A few genes were involved in pathways for two of the three tissues. Jun proto-oncogenes (JUN) were also observed in both the liver and gills pathways, with JUN being upregulated in liver and JUNB being upregulated in gills. JUN genes also shared a connection with CEBPA in both pathways.

Although there was some overlap in genes between the different pathways, there were many salinity-modulated genes that were specific to each tissue. For liver, the four main nodes were shared with other tissues, although some of the genes branching from CEBPA were not shared, including genes involved in metabolic processes, (e.g. cytochrome P450 3A, which for salmonids would likely be CYP3A27) (Lee et al., 1998). None of the smaller two and three gene interactions separate from the main pathway were shared between tissues, but included genes involved in blood coagulation, such as coagulation factor XIII, B polypeptide (F13B), and protein folding, such as heat shock 70kDa protein 8 (HSPA8).

For the gills, some of the major nodes in the pathway were not shared with other tissues. Avian myelocytomatosis viral oncogene homolog (MYC) was upregulated and linked to 19 different genes. Transforming growth factor, β 1 (TGFB1), which was linked to 20 genes, not shared, and down regulated on high salinity acclimation. Another major node that was specific for gills was insulin (INS), which was upregulated by high salinity and connected to 19 genes. The gills pathway also included various genes that impact the cytoskeleton and cell growth, such as collagen, type I, alpha 1 (COL1A1), which was down regulated, and actin gamma 1 (ACTG1), which was upregulated. Many genes involved in immune response were also included in the pathway, such as lectin galactoside-binding soluble 3 (LGALS3), which was down regulated and chemokine (C-C motif) ligand 7 (CCL7), which was upregulated by high salinity.

Unshared nodes in the olfactory pathway included signal transducer and activator of transcription 1 (STAT1), which was upregulated and linked to 13 genes. Caspase 8 (CASP8) was also upregulated and interacted with 8 genes. Ubiquilin 4 (UBQLN4) was down regulated and connected to 9 genes. Genes involved in immune response were also present in the olfactory pathway, such as major histocompatibility complex, class I-related (MR1) and SLC46A2 solute carrier family 46, member 2 (TSCOT), which were both down regulated. Genes involved in growth and impacts of the cytoskeleton were also part of the pathway and included talin 1 (TLN1), which was upregulated by high salinity acclimation, and plasminogen activator, tissue (PLAT), which was down regulated.

Discussion

An interesting finding from our study was that gene expression induced by salinity was highly tissue-dependent, as we did not observe changes in gene expression that were common to salinity and all three tissues analyzed. Similarly, Seear et al. (2010) reported only one gene upregulated more than two fold in the three tissues in their study (gills, brain, and kidney) in Atlantic salmon after smoltification. It has previously been proposed that the different cell types within an organism may be able to detect and respond in a unique fashion to different ranges of osmolality (reviewed in Fiol and Kültz, 2007). As some of the tissues of euryhaline fish, such as gills and olfactory rosettes, are exposed directly to the aquatic environment, response to changes in salinity would be different compared to most of the other tissues, such as the liver which are in a more homeostatic environment.

Despite not being in direct contact with the external environment, we found it interesting that the liver had the highest number of differentially expressed genes following salinity treatments compared to the gills and the olfactory rosettes. As previously stated, the three tissues were chosen based on their different biological roles. Gills were evaluated because the tissue is directly exposed to the aqueous environment and involved in osmotic regulation, liver was selected as it plays a role in energetics needed for osmoregulation, and olfactory rosettes were picked based on the proximity of the tissue to the environment, but also its role in behavior. In previous studies, salinity acclimation caused tissue-specific reorganization of energy metabolism in sea bream (*Sparus aurata*) liver, brain, kidney, and gills (Sangiao-Alvarellos et al., 2003).

Interestingly, the nonosmoregulating organs, liver and brain, were more metabolically responsive to changes in salinity than the gills and kidney, which have established roles as osmoregulatory organs. Salinity acclimation may cause an increase in liver metabolism as liver is the main region for glycogen and glucose turnover in fish and could provide energy to fuel other metabolic and osmoregulatory tissues like gills (Sangiao-Alvarellos et al., 2003). Support for this hypothesis comes from a previous report showing enhanced liver metabolism in rainbow trout after salinity acclimation (Soengas et al., 1995). This difference in metabolism may impact the number of differentially expressed genes observed for each tissue in the current study.

Differential gene expression was also salinity dependent as the number of treatment specific genes was greater than the shared genes between treatments for all salinities and all tissues. Also, there was no discernable salinity concentration dependent pattern in the number of differentially expressed genes. In liver, the high salinity caused the greatest number of differentially expressed genes, followed by the low concentration, with the medium concentration causing the least number of differentially expressed genes. The medium salinity concentration caused the highest number of differentially expressed genes in gills and olfactory rosettes. However, for gills, the low concentration resulted in a greater number of differentially expressed genes than the high concentration, while for olfactory rosettes, the high concentration caused a greater number of differentially expressed genes than the low salinity treatment. Evans and Somero (2008) analyzed gene expression of goby gills at different times points after acclimation to hyper and hypo osmotic stress and found that many genes were only significantly altered at

specific time points. Similarly, salinity only alters certain genes at specific concentrations.

Five genes were differentially expressed in all salinity treatments and shared between two tissues (Table 2.1). Sodium/potassium-transporting ATPase subunit alpha-1 precursor (*atp1a1*) was upregulated in gills and down regulated in olfactory rosettes. Sodium/potassium ATPases are membrane proteins which establish and maintain electrochemical gradients that are necessary for osmoregulation, as well as for sodium-coupled transport of a variety of organic and inorganic molecules, and for electrical excitability of nerve and muscle. Sodium/potassium ATPases consist of two subunits: alpha, the larger catalytic subunit and beta, the smaller glycoprotein subunit. *Atp1a1* encodes an alpha 1 subunit; the alpha subunit is encoded by multiple genes (reviewed in Kaplan, 2002). Upregulation of *atp1a1* in the gill is consistent with previous studies as an increase in activity of gill sodium/potassium ATPases has been used in previous studies as a molecular indicator of smoltification (D'cotta et al., 1996, Yada et al., 2008, Riar et al., 2013). Evan and Somero (2008) reported a 1.6-fold increase in the alpha subunit of Na^+/K^+ ATPase mRNA during 12 hours of hyperosmotic stress of *Gillichthys mirabilis*. Additionally, Seear et al. (2010) found that Na^+/K^+ ATPase alpha subunit isoform 1b was upregulated 2.1-fold in gill tissue of Atlantic salmon smolts. The difference in expression of *atp1a1* in gills and olfactory rosettes may be due to tissue-specific distributions of alpha isoforms. Richards et al., (2003) demonstrated the expression of alpha 1c and 3 in several tissues of rainbow trout (*Oncorhynchus mykiss*), however olfactory rosettes were not included in the study. Other isoforms (alpha 1a, 1b, and 2) had tissue-specific

distributions that were altered after transfer to seawater, suggesting that the different isoforms play different roles in salinity acclimation (Richards et al., 2003). It is possible that the gills and olfactory rosettes have different distributions of subunit isoforms with unique roles in salinity acclimation.

Sodium-coupled neutral amino acid transporter 2 (*SNAT2*) was upregulated in both gills and liver. *SNAT2* is member of System A which transports small, non-branched amino acids (Jones et al., 2006) and couples the transport of amino acids against the inward movement of sodium down its electrochemical gradient (Baird et al., 2009). In humans, *SNAT2* has ubiquitous tissue distribution (Glover et al., 2005) and is involved in the adaptive regulation of System A to nutritional challenge (Franchi-Gazzola et al., 1999). Previous studies in a human placental cell line showed that cortisol upregulates *SNAT2* expression, which may ensure sufficient amino acid supply to the developing fetus (Jones et al., 2006). Salinity acclimation increases cortisol in euryhaline fish (Assem and Hanke, 1981, Borski et al., 1991, McLean et al., 1997), which could result in the upregulation of *SNAT2*. *SNAT2* has also been shown to be involved in cell volume control after hypertonic exposure as amino acids make up a large fraction of organic osmolytes within the cytosol. Neutral amino acids may act as compatible osmolytes in hypertonically stressed cells and *SNAT2* can help regulate cell volume through transport (reviewed in Franchi-Gazzola et al., 2006).

Expression of gastrulation-specific protein G12 (*g12*) was down regulated in liver and upregulated in olfactory rosettes in salinity acclimated salmon. Although *g12* was expressed in the enveloping layer of zebrafish during different developmental stages,

little is known about its function (Conway, 1995). This gene shares identity to mammalian genes, rat spot 14 and human MID1 interacting protein 1 (MID1). Rat spot 14 is purported to be involved in lipogenesis (Grillasca et al., 1997), whereas MID1 may function in maintaining microtubule dynamics (Schweiger et al., 1999). Evans and Somero (2008) found that MID1 interacting protein was down regulated in hypersaline acclimated goby gills. Other environmental factors have also altered *g12* expression in fish. Castilho et al., (2009) found that a *g12-like* gene was differentially expressed in tissues of bluefin tuna (*Thunnus orientalis*) after thermal acclimation in the ventricle and white muscle.

Complement C1q-like protein 2 precursor (c1ql2) was upregulated in liver and down regulated in olfactory rosettes. C1ql2 belongs to the C1q family which is involved in immunological processes. The C1q family plays a role in the classical complement pathway, impacting innate and acquired immunity (reviewed in Ghai et al., 2007). Seear et al., (2010) found that C1q-like protein mRNA was upregulated 3.38 fold in kidney tissue of Atlantic salmon smolts after smoltification. The final shared gene, complement C3-1, is also involved in the complement pathway and was down regulated in both liver and olfactory rosettes. C3 is involved in immunological processes and helps the host defend against infection. C3 has been suggested to be the most versatile and multifunctional component of the complement system (Lambris, 1990). Hardiman et al. (1994) found through Northern blot analysis that C3 was induced modestly during smoltification in Atlantic salmon liver. The difference from the current study may be

from species difference or from enhanced specificity from the techniques used in the current study compared to Northern blot.

Salinity has been shown to exhibit complex effects on the immune system, and the results of our study revealed that many differentially regulated transcripts in all three tissues examined were associated with maintenance of immunity. Birrer et al. (2012) found that salinity changes caused an increase in activity and proliferation of immune cells in the broad-nosed pipefish (*Syngnathus typhle*), but gene expression of certain immune related components were down regulated after infection of *Vibrio* in lower salinities. The authors suggested that energy needed for osmoregulation may result in less resources available to combat infection (Birrer et al., 2012). The stress response, as would occur during salinity acclimation, is thought to cause immunosuppression through release of cortisol and activation of corticosteroid receptors; however, this hypothesis may be tissue specific. For some species of euryhaline fish, salinity acclimation has enhanced some immune functions (reviewed in Yada and Nakanishi, 2002). Yada et al., (2007) found that acute stress of rainbow trout decreases the number of antibody producing leucocytes and circulating level of immunoglobulin, but, in gills expression of glucocorticoid receptor 1 and 2 was unaltered. In contrast, saltwater acclimation in steelhead trout increased mRNA of corticosteroid receptor genes in gill and body kidney, while head kidney and spleen were unaltered, and leucocytes and peripheral blood showed decreases (Yada et al., 2008).

In evaluating the complement component of immune function, gill and olfactory rosettes shared the biological process GO term of classical pathway of complement

activation; however, this term was not significant for liver. In the gills of Atlantic salmon, Seear et al. (2010) found down regulation of *CC chemokine SCYA112*. Chemokines play a role in innate immune response and are involved in responding to sites of injury or infection (Moser and Loetscher, 2001). Evans et al. (2011) also observed significant enrichment of ontologies associated with immune response, including the complement system, in gills from salmon from different migrating routes in the marine environment.

For olfactory rosettes, six of the top ten GO terms were related to immune response. Pathway analysis identified down regulated immune related genes in all three tissues. Although immune response in liver may not be one of most significant effects, as it did not appear in the GO terms, it is still affected, as seen by the interaction network. In the liver pathway, C3 was also adjoined to four other genes involved in immune response: complement component 5 (C5), complement component 7 (C7), clusterin (CLU), and complement component 8, beta polypeptide (C8B); all of which were down regulated. The current study is in agreement with previous studies mentioned above in that salinity acclimation can impact tissue immune response to differing extents, yet overall the current study observed immunosuppression in response to salinity acclimation for olfactory rosettes, gills, and liver.

A systems level analysis of interacting genes and gene products also helped to identify the differential regulation of cell cycle related genes, such as growth arrest and DNA-damage-inducible genes (GADD45) in the three tissues. Cell proliferation, resulting from hormones and growth factors, may play a role in osmoregulation (Evans and Somero, 2008). The expression of GADD45G genes was upregulated in all three

tissues. GADD45 increases following stressful growth arrest conditions and treatment with DNA-damaging agents and mediates activation of the p38/JNK pathway via MTK1/MEKK4 kinase (reviewed in Johnson and Lapadat, 2002). Evans and Somero (2008) found that GADD45 was down regulated after hyposmotic stress, supporting the role of GADD45 in salinity acclimation. DNA damage sensors have been suggested as possible osmosensors, recognizing osmotic stress and initiating signal transduction pathways (reviewed in Fiol and Kültz, 2007). As GADD45 genes were upregulated in all three tissues, it is possible that these genes are working as osmosensors.

Another gene identified by interaction pathways that appears to be regulated by salinity acclimation was insulin-like growth factor binding protein 1 (IGFBP1). It was linked to CCAAT/enhancer binding protein α (CEPBA), upregulated in liver, and down regulated in olfactory rosettes. IGFBP5 was down regulated in gills, but did not link to similar genes in liver or olfactory rosettes. Evans and Somero (2008) saw an increase in expression of IGFBP in goby gills after hypersaline acclimation. IGFBPs have been hypothesized to prevent proteolytic degradation of insulin-like growth factors (IGFs) by mediating the efflux of IGFs from the vascular space to the cell surface, and thus modulating interactions between ligands and receptors (Wood et al., 2005). IGFs couple with prolactin and cortisol to aid in osmoregulation (McCormick, 2001). Application of exogenous IGF-1 increases salinity tolerance of certain euryhaline fish (Mancera and McCormick, 1998).

Signal transduction pathways were also impacted by salinity. Knowledge on osmosensing and osmotic stress signal transduction is limited in euryhaline fish;

however, mitogen-activated protein kinase (MAPK) signaling has been identified in gills (reviewed in Fiol and Kültz, 2007). In gills of coho salmon, pathway analysis showed that MAPK14 was upregulated and interacted with 5 genes in the current study. MAPKs receive signals from osmosensors that are then integrated and amplified to activate downstream targets. MAPKs are important in regulating cell cycle, cell growth, differentiation, cell death, and pathogen defense systems (Treisman, 1996). Kültz and Avila, (2001) observed altered activity and phosphorylation of the three main euryhaline MAPKs in the gills of killifish (*Fundulus heteroclitus*) after osmotic stress leading to the conclusion that MAPKs are important parts of salinity acclimation osmosensory pathways. Similarly, jun proto-oncogenes (JUN and JUNB) were upregulated in liver and gills. JUN is activated by the JNK pathway, part of a MAPK cascade, which can be regulated by cellular and environmental stress. Marshall et al., (2005) saw an upregulation of JNK in gills of killifish (*F. heteroclitus*) after hypersaline acclimation.

In olfactory rosettes another kinase signaling pathway, protein kinase N1 (PKN1) was also upregulated. PKN1 is part of the protein kinase C (PKC) superfamily and is activated by Rho family small G proteins. PKC is involved in osmotic stress signaling and promotes volume regulation after hypo-osmotic stress (Ollivier et al., 2006). During hyperosmotic stress, PKC has been shown to activate Na⁺/K⁺/Cl⁻ co-transporter (Lionetto et al., 2002).

Fiol et al. (2006) found one of the main processes of novel immediate hyperosmotic stress response genes for tilapia four hours after transfer from freshwater to saltwater was actin-based cytoskeleton dynamics. The current study also observed

differential regulation of cytoskeletal related genes, which is apparent in the GO terms and interaction networks for the gills. One of the significant cellular component GO terms in the current study was collagen. Collagen is involved in growth or reorganization. Seear et al. (2010) found that multiple collagen related genes were upregulated in gills and brain. In the gill of coho salmon, actin, gamma 1 (ACTG1) was upregulated. ACTG1 is involved in cell motility and maintenance of the cytoskeleton. Evans and Somero (2008) observed differential regulation of a variety of cytoskeleton related genes in goby gills after hyperosmotic stress. Additionally, a majority of the significant GO terms for gills were related to muscle organization and movement. Seear et al. (2010) also saw upregulation of a gene, *myomesin 1*, involved in muscle contraction. Evans and Somero (2008) observed upregulation of myosin light chain 1, which is involved in cell contractile events after hypoosmotic stress in goby gills.

Transcription factors can also play a vital role in combating osmotic stress by responding to osmotic signal transduction through activating effector mechanisms (reviewed in Fiol and Kültz, 2007). Interaction network analysis identified CCAAT/enhancer binding protein α (CEBPA) as a main component that was upregulated in the pathways for all three tissues. CEBPA is a transcription factor that can regulate the expression of genes involved in cell cycle regulation and body weight homeostasis (Lekstrom-Himes and Xanthopoulos, 1998). CEBP was shown to be down regulated in gill of two types of mussel, *Mytilus galloprovincialis* and *Mytilus trossulus*, after transfer from saltwater to low salinity, supporting a role for CEBPs in saltwater acclimation.

Although the four main nodes of the liver pathway were shared with other tissues, some of the genes branching from CEBPA, were not shared, including genes involved in metabolic processes, such as CYP3A27, which was down regulated. Down regulation of CYP3A27 may impact metabolism of endogenous and exogenous compounds. None of the smaller two and three gene interactions separate from the main pathway were shared with gills or olfactory rosettes and included genes involved in blood coagulation, such as coagulation factor XIII, B polypeptide (F13B), and protein folding, such as heat shock 70kDa protein 8 (HSPA8). Salinity has previously been shown to impact blood characteristics, including coagulation, in redbelly tilapia (*Tilapia zilli*) (Farghaly et al., 1973). Protein unfolding can influence chaperones and trigger osmosensors to activate osmoregulatory signal transduction networks (reviewed in Fiol and Kültz, 2007). Other significant GO terms in the liver were related to oxygen binding and transport. One significant cellular component was the hemoglobin complex. During smoltification, the hemoglobin system of salmon becomes increasingly more complex, for example, increasing from three to ten hemoglobin forms from fry to juvenile and adult salmon (Vanstone et al., 1964, Giles and Vanstone, 1976, Sullivan et al., 1985). Seear et al. (2010) found that alpha and beta hemoglobin genes were upregulated in gills and brain of smolts, but liver was not included in their study.

Glycolysis was one of the significant biological processes identified in the array for gills. Seear et al. (2010) found that two genes involved in glycolysis were upregulated in gills. Evans et al. (2011) who studied the migration of salmonids from saltwater to freshwater also found that ontologies relating to glucose metabolism were significant in

gills of sockeye salmon (*Oncorhynchus nerka*) sampled in the marine environment from different migration routes. For the gills, some of the major nodes in the pathway were not shared with other tissues. Avian myelocytomatosis viral oncogene homolog (MYC) was upregulated and connected to 19 different genes. MYC is involved in cell cycle progression, apoptosis, and cellular transformation and is a transcription factor. MYC was down regulated in gills of two types of mussel, *M. galloprovincialis* and *M. trossulus*, after transfer from saltwater to low salinity, suggesting a role for MYC in saltwater acclimation. Also, transforming growth factor, beta 1 (TGFB1), which was linked to 20 genes, was down regulated. TGFB1 is a cytokine that regulates proliferation, differentiation, adhesion, migration, and the MAPK cascade. Evans and Somero (2008) saw differential regulation of genes that inhibited cytokines after hypersaline acclimation in goby gills.

Olfactory-specific nodes included signal transducer and activator of transcription 1 (STAT1), which was upregulated and connected to 13 genes. STAT genes are transcription activators that act in response to cytokines and growth factors. Caspase 8 (CASP8) was also upregulated and connected to 8 genes. CASP8 is involved in cell apoptosis. Cells may undergo apoptosis in response to salinity stress if they are no longer able to compensate and the amount of damage is too large (Schwartz and Osborne, 1993, Katsuhara, 1997). Programed cell death allows for the elimination of malfunctioning and potentially harmful cells from the organism (reviewed in Kültz and Burg, 1998).

In summary, the majority of differentially expressed genes were tissue and salinity dependent. Significant GO terms were also mostly tissue dependent. For liver,

terms involving oxygen binding and transport were significant, potentially impacting metabolism. For gills, terms involving muscle and cytoskeleton were enriched and for olfactory rosettes, immune response related terms were highlighted. When interaction networks were examined in addition to GO terms, similarities between tissues were identified. For example, GADD45 genes were upregulated in all three tissues and may function as potential osmosensors. Signal transduction cascades, which may integrate and amplify signals from osmosensors, were also differentially expressed in each tissue and deserve further study since the specific cascades also showed some tissue differences. CEPBA, which is a transcription factor and may play a role in activating salinity acclimation effector mechanisms, was also upregulated in all three tissues.

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Chapter 3: Impacts of Hypersaline Acclimation on the Acute Toxicity of the Organophosphate Chlorpyrifos to Salmonids

Abstract

Acclimation to hypersaline conditions enhances the acute toxicity of certain thioether organophosphate and carbamate pesticides in some species of euryhaline fish. As the organophosphate chlorpyrifos is commonly detected in salmonid waterways, the impacts of hypersaline conditions on its toxicity were examined. In contrast to other previously examined pesticides, time to death by chlorpyrifos was more rapid in freshwater than in hypersaline water (16 ppt). The median lethal time (LT_{50}) after 100 $\mu\text{g/L}$ chlorpyrifos exposure was 49 hr (95% CI: 31 – 78) and 120 hr (95% CI: 89 – 162) for rainbow trout (*Oncorhynchus mykiss*) in freshwater and those acclimated to hypersaline conditions, respectively. Previous studies with hypersaline acclimated fish indicated induction of xenobiotic metabolizing enzymes that may detoxify chlorpyrifos. In the current study, chlorpyrifos metabolism was unaltered in liver and gill microsomes of freshwater and hypersaline acclimated fish. Acetylcholinesterase inhibition in brain and bioavailability of chlorpyrifos from the aqueous exposure media were also unchanged. In contrast, mRNA expression of neurological targets: calcium calmodulin dependent protein kinase II delta, chloride intracellular channel 4, and G protein alpha i1 were upregulated in saltwater acclimated fish, consistent with diminished neuronal signaling which may protect animals from cholinergic overload associated with acetylcholinesterase inhibition. These results indicate targets other than

acetylcholinesterase may contribute to the altered toxicity of chlorpyrifos in salmonids under hypersaline conditions.

Introduction

Salmonids are economically and ecologically critical species in the northwestern United States and exhibit unique life histories. Many species are anadromous and move between freshwater and saltwater at different life stages eventually homing to discrete native areas to spawn. The unique life cycle of salmonids makes them especially sensitive to natural and anthropogenic alterations of their habitats. On the west coast of the United States, formerly abundant salmonid species have experienced dramatic declines in population during the past several decades leading to many salmon populations being listed under the Endangered Species Act for protection (Quinn, 2005).

Habitat degradation can play a role in salmonid population declines and habitats can be impacted in several ways. Migration pathways often go through urban or agricultural areas near estuaries. Saltwater intrusion due to irrigation and rising of groundwater tables has been especially problematic in Western arid and semi-arid agricultural regions where crop production consumes large quantities of water. In these areas salt concentrates and soil water becomes more saline as crops only absorb a fraction of the salt of the irrigation water. Salts may then be leached out through runoff and enter rivers (reviewed in Cañedo-Argüelles et al., 2013).

Climate change is another anthropogenic factor that can contribute to hypersaline conditions in certain areas. Increased temperatures can heighten the freeze line which

impacts alpine snow volume. Reductions in alpine snow volume result in decreased freshwater input into various waterways. Consequently climate models for the San Francisco Bay Delta predict continued increase of saline water (Knowles and Cayan, 2002, Knowles and Cayan, 2004, Howat and Tulaczyk, 2005).

Salmonid habitat has also been impacted through agricultural insult, which can co-occur with hypersalinity. This is particularly an issue in areas such as the San Francisco Bay Delta which receives agricultural runoff from the Central Valley of California. In the United States, pesticide input and salinization both rank among the top 15 causes of stream impairment (EPA, 2012b). Of particular interest is the organophosphate insecticide, chlorpyrifos. Chlorpyrifos, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is a broad spectrum insecticide approved for use to control foliage and soil borne insects on numerous crops including fruits, vegetables, nuts, and grains. It is also used on non-food sites such as golf course turf, nurseries, sod farms, greenhouses, wood products, and industrial sites (EPA, 2012a). In 2007, chlorpyrifos was the fourteenth most commonly used conventional active ingredient in the agricultural pesticide market sector with between seven and nine million pounds used (Grube et al., 2011).

Chlorpyrifos, along with other organophosphates, is acutely toxic with acetylcholinesterase (AChE) inhibition as the primary mode of action. Chlorpyrifos inhibits AChE by phosphorylating the enzyme at its esteratic site. During neurotransmission, acetylcholine (ACh) is released into the synaptic cleft from the nerve. ACh receptors on the post synaptic membrane bind ACh which relays the signal from the

nerve to another neuron, smooth muscle, or other effectors. After transmitting the signal, AChE hydrolyzes and breaks down ACh, effectively terminating synaptic transmission. If AChE is inhibited, ACh accumulates at synaptic junctions leading to over stimulation (Fukuto, 1990).

Although organophosphate usage has decreased in recent years, chlorpyrifos is still commonly detected in surface waters which provide habitat for numerous aquatic species. For example, chlorpyrifos concentrations frequently exceeded water quality criteria in recent monitoring studies in California (Phillips et al., 2007, Corbin et al., 2009, Ensminger et al., 2011). Between 2006 and 2010, chlorpyrifos was detected in 17.7% of samples, with 9.9% exceeding water quality criteria. The highest detected concentration was 3.7 µg/L in 2007 in the San Joaquin region (Xuyang et al., 2012). A 2009 to 2011 monitoring study in Washington which specifically focused on salmon-bearing streams, found that chlorpyrifos was among fourteen compounds that did not meet an assessment criterion or water quality standard (Sargeant et al., 2013). At the Spring Creek location, chlorpyrifos concentrations exceeded criteria on seven different instances during the three year period (Sargeant et al., 2013).

As salmonids may encounter hypersaline conditions and chlorpyrifos concurrently, understanding how hypersaline acclimation may impact chlorpyrifos toxicity may be important. Previous studies of certain thioether organophosphate and carbamate pesticides have shown enhanced acute toxicity after hypersaline acclimation (Schlenk and Lavado, 2011). It was hypothesized that hypersaline acclimation would follow the same pattern with chlorpyrifos and cause enhanced acute toxicity. The

enhancement in toxicity in previous studies was attributed to upregulation of phase I xenobiotic metabolizing enzymes, such as flavin-containing monooxygenases (FMOs) and cytochrome P450s (CYPs), which increased formation of more potent anticholinesterase metabolites. The current study evaluated this hypothesis with chlorpyrifos to determine if a consistent mode of action relationship occurs with all organophosphates. In addition, other potential mechanisms evaluating the impacts of acclimation to hypersaline conditions were also explored. AChE inhibition was examined as it is the primary target for organophosphates. Bioavailability was considered as hypersaline conditions may impact the ability of the compound to reach its target. Lastly, expression of signal transduction related genes was explored as chlorpyrifos has been shown to impact the adenylyl cyclase signaling cascade in other species (Song et al., 1997).

Methods

Chemicals

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl-phosphorothioate; CAS 2921-88-2) was purchased from Chem Service (West Chester, PA). Methanol and acetonitrile were analytic grade (Fisher, Pittsburg, PA). Ethanol and potassium chloride was also purchased from Fisher (Pittsburg, PA). Phosphoric acid was purchased from LabChem, Inc. (Pittsburg, PA). The Coomassie Blue commercial kit was purchased from ThermoFisher Scientific (San Jose, CA). The commercial alkaline phosphatase conjugation kit and the iScript One-step RT-PCR kit with SYBR Green were purchased

from BioRad (Hercules, CA). SV Total RNA Isolation System kits and Reverse Transcription System kits were purchased from Promega (Madison, WI). CrystalSea Marine Mix was purchased from Marine Enterprises International (Baltimore, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Organisms

Juvenile rainbow trout (*Oncorhynchus mykiss*) (age approximately 4 months, 10.23 ± 2.60 cm fork length, 16.04 ± 13.70 g) were obtained from the Jess Ranch Fish Hatchery (Apple Valley, CA). Fish were maintained on a 14 hour light – 10 hour dark cycle at constant temperature, 11 – 13 °C. Organisms were housed in a flow through living-stream system with dechlorinated carbon filtered municipal water. Fish were fed commercial pellets (Silver Cup, Murray, UT). Fish were allowed to acclimatize to the laboratory for at least two weeks prior to experimental use.

Salinity acclimations

After the acclimation period, fish were transferred to 20 L glass aquaria, five fish per aquarium, and maintained on the same light cycle and at the same temperature as previously stated. Fish were acclimated to one of two salinity treatments, freshwater (< 0.5 ppt) or hypersalinity (16 ppt). Sixteen ppt represents a typical brackish water environment where smoltification occurs throughout the west costal of the United States and comparable salinities have previously been used for combined exposure studies with euryhaline fish (Saiki et al., 1992, Bawardi et al., 2007). Fish were acclimated to salinity

in a stepwise manner with an increase in 4 ppt every two days as previously described (Lavado et al., 2009). Fish were allowed to adjust to salinity regimes for one week after reaching the final salinity with 48 hour complete water changes. Salinity treatments were created by reconstituting carbon filtered municipal water with CrystalSea Marine Mix.

LT₅₀

After the week long adjustment period fish were exposed to chlorpyrifos at one of five nominal treatments of 20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L, or 150 µg/L (57 nM, 114 nM, 228 nM, 285 nM, and 428 nM) for 7 days, three replicates per treatment (n = 3). Ethanol was used as the carrier solvent. Since complete water changes with retreatment were conducted every 48 hours and bioavailability was assessed below, measured values were not recorded. Survival was evaluated every two to four hours during the exposure period.

Metabolism

After the week adjustment period to salinity, fish were killed by cervical dislocation according to IACUC guidelines at The University of California, Riverside. Tissues were collected and immediately frozen at -80°C until assays could be conducted. Tissues collected included liver, gill, and brain.

In vitro metabolism of chlorpyrifos was assessed in liver and gill microsomes (liver: n = 16 for hypersalinity and 17 for freshwater; gill: n = 17 for hypersalinity and 18 for freshwater). Microsomes were obtained through subcellular fractionation according to

(Lavado and Schlenk, 2011). Briefly, tissues were rinsed in ice-cold 1.15% KCl and homogenized in a 1:5 w/v of cold 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing 100 mM KCl and 1 mM ethylenediametetraacetic acid (EDTA). Liver and gill tissues were analyzed from individual animals. Homogenates were centrifuged at 12,000g for 20 minutes. The supernatant was then collected and centrifuged at 100,000g for 60 minutes, producing the microsomal fraction. The pellet was resuspended in 200 μl of 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing 100 mM KCl, 20 % (w/v) glycerol, and 1 mM EDTA. The Coomassie Blue method using a commercial kit and bovine serum albumin as a standard was used to determine protein concentrations.

Chlorpyrifos biotransformation was evaluated as described by (Lavado and Schlenk, 2011). Formation of metabolites was assessed after microsomal incubations. Liver and gill microsomal fractions were incubated in 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer at pH 8.4 with 5 mg/mL (14.0 mM) chlorpyrifos, as well as with and without 15 mg/mL NADPH. The addition of the substrate initiated the reaction and the total incubation time was 120 minutes at 25°C. At 30 and 60 minutes the NADPH was refreshed. The reaction was stopped by the addition of an equal volume of ice cold acetonitrile. An internal standard of 1 mg/mL *R*-methyl-*p*-tolyl sulfoxide (MTSO) was added to each sample after the incubation. Negative controls consisted of identical additives except boiled protein was substituted for the microsomal protein.

Analysis of metabolites was conducted using reverse phase HPLC on a SCL-10AVP Shimadzu HPLC system equipped with a 250 x 4.6 mm Hypersil ODS C18 (5 μm) reverse-phase column (ThermoFisher Scientific Inc., Waltham, MA). Separation of

chlorpyrifos and metabolites employed an HPLC gradient system elution at a flow rate of 1 mL/min with mobile phases composed of (A) 89% water, 10% acetonitrile, and 1% phosphoric acid and (B) 99% acetonitrile and 1% phosphoric acid. The run consisted of a 15 min linear gradient from 100% A to 100% B, 5 min 100% B, 10 min linear gradient from 100% B to 80% A, and 10 min linear gradient to 100% A. Chromatographic peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 300 nm up to 10 min, 230 nm from 10 to 19 min, and 300 nm until the end of the run. Peaks were quantified by integrating the area under the peaks and identified by elution of authentic standards (the retention time observed for chlorpyrifos was 18.4 mins, chlorpyrifos-oxon was 14.8 mins, and 3,5,6-trichloro-2-pyridinol (TCP) was 12.3 mins).

Western blots were also performed on liver microsomes from freshwater (< 0.5 ppth) and hypersaline (16 ppth) acclimated fish to assess CYP3A27 protein levels following the methods of (Lavado et al., 2004). Briefly, microsomes were heated at 95°C for 5 minutes in SDS-PAGE buffer and 10 µg of protein was separated by electrophoresis using 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Samples were probed using a 1:1000 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP3A27. Membranes were incubated overnight at 25°C. Afterwards, blots were rinsed three times with Tris-buffered saline containing 0.2% Tween 20 (v/v) and 0.5% gelatin (w/v). Membranes were then incubated for 1 hour with alkaline phosphatase conjugated anti-rabbit IgG, the excess of secondary antibody was removed, and immunoreactive bands were visualized by incubation with the substrates p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate from a commercial

alkaline phosphatase conjugation kit. Bands were semiquantified by densitometry using Quantity One (BioRad) in a Molecular Imager Gel Doc XR System (BioRad) image analyzer and are presented as optical density units/mg protein.

Acetylcholinesterase Inhibition

The *in vitro* inhibition of AChE by chlorpyrifos oxon was evaluated using a subset of the freshwater and hypersaline acclimated rainbow trout brains from fish used in metabolism studies (n = 5). Brains from individual animals were homogenized in 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer at pH 7.4, containing 100 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA) and homogenates were centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was then collected and assayed following the methods of (Nillos et al., 2007) with minor modifications. Chlorpyrifos oxon solutions of various concentrations (25, 50, 100, 250, and 500 nM) were preincubated with the brain samples for 20 minutes at 25°C. Samples were then added to 96-well microtiter plates, followed by the addition of 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) solution. The AChE activity was determined using a SOFTmax Pro microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm for 2 minutes following the addition of acetylthiocholine (ATC). Protein concentrations of brain homogenates were determined using the Coomassie Blue method using a commercial kit and bovine serum albumin as a standard.

Bioavailability

Bioavailability of chlorpyrifos from the aqueous media was measured through time course experiments in freshwater (< 0.5 ppt) and hypersaline water (16 ppt). Twenty L tanks were maintained at 11 - 13°C under 14 hour light – 10 hour dark cycles and treated with 100 µg/L (285 nM) chlorpyrifos (n = 3). One L water samples were taken at 0, 12, 24, and 48 hours post chlorpyrifos addition. Solid phase extractions following the methods of Bogus et al., (1990) were conducted on water samples. Briefly, water samples were suction filtered through the activated C18-SPE columns with a vacuum manifold. One hundred mL of water was then passed through the column. The columns were eluted with 5 mL of methanol. After elution the extracts were spiked with an internal standard of 0.1 µg/uL of *R*-methyl-*p*-tolyl sulfoxide (MTSO).

Extractions were then analyzed also following the methods of Bogus et al., (1990) with slight modifications. Analysis of extracts was conducted using reverse phase HPLC on a SCL-10AVP Shimadzu HPLC system equipped with a 250 x 4.6 mm Hypersil ODS C18 (5 µm) reverse-phase column (ThermoFisher Scientific Inc., Waltham, MA). Separation of chlorpyrifos employed an HPLC isocratic system elution at a flow rate of 1 mL/min with mobile phase composed of 80% methanol and 20% water. The run time was 20 minutes. Chromatographic peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 230 nm. Peaks were quantified by integrating the area under the peaks and identified by elution of authentic standards (the retention time observed for chlorpyrifos was 9.9 mins).

qPCR of Signal Transduction Targets

Transcripts of genes were chosen based on upregulation in neurological tissues from a previous microarray study of coho salmon salinity acclimation (Maryoung et al., unpublished). Genes expressed during salinity acclimation were compared with gene expression profiles in zebrafish treated with chlorpyrifos (Tilton et al., 2011). Primers were designed using Primer3 software for calcium calmodulin dependent protein kinase II delta, chloride intracellular channel 4, and G protein alpha i1. β -actin was used as the housekeeping gene. Primer sequences are listed in Table 3.1. Primers were then optimized based on annealing temperature, template concentration, and primer concentration. After optimization, PCR products were run on DNA electrophoresis gels to confirm the size of the amplicon. After optimization and size confirmation, RNA was isolated from brain tissue from a subset of fish in the metabolism section using Promega SV Total RNA Isolation System kits (n = 6 for freshwater and hypersalinity). Total RNA was then converted to cDNA using Promega Reverse Transcription System kits. qPCR was run for each gene with β -Actin as the housekeeping gene using iScript One-step RT-PCR kit with SYBR Green from Bio-Rad. 250 nM of each primer was added to 25 μ L PCR reactions containing SYBR Green RT-PCR Reaction Mix, and 100 ng of cDNA sample. Thermocycling parameters were as follows: 5 mins at 95°C; 40 cycles of 10 sec at 95°C and 30 sec at 57°C. At the end of each cycle fluorescence data was collected. A melting curve analysis was run between 60°C and 95°C following the amplification reaction. The C(t) was selected to be in the linear phase of amplification. The reactions

were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) and data analysis was done using IG5 (Bio-Rad).

Table 3.1. Signal transduction target gene primers used in this study.

Target	Primer sequence (5'-3')	Reference
Chloride intracellular channel 4	GGATAAATGAGGGCAGGTTT	
	GGTCAGCATAAGGTCAAGGA	
Calcium calmodulin dependent protein kinase II delta	TCACCAGAACAAAAGCAAGC	
	TGGAGCATATTACCTGACATCTG	
G protein alpha i1	ACCTATATCCCGACCCAGCA	
	AAGCGCCACACAGAAGATGA	
β -actin	GTCCTTCATGATTCTCTGCTGA	Lavado, et al., 2009
	ACTCGGGTTCATTTGCATAAACA	

Statistical analyses

The median lethal time (LT₅₀) for freshwater and hypersaline acclimated fish exposed to five nominal concentrations of chlorpyrifos was determined by linear interpolation analysis using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, Ca). R (version 2.15.2) was used to perform Student's t-tests with unequal variance for comparison of LT₅₀ values and liver metabolism ratios between freshwater and hypersaline acclimated fish. R was used to perform Student's t-tests with equal variance for comparison of gill TCP production between freshwater and hypersaline acclimated fish. The median inhibitory concentration (IC₅₀) for AChE was determined by linear interpolation analysis using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). R was used to perform Student's t-tests

with equal variance for comparison of IC_{50} values between freshwater and hypersaline acclimated fish. R was used to perform Wilcoxon-Mann-Whitney Rank Sum Tests for comparison of bioavailability between freshwater and hypersaline acclimated fish. R was used to perform Student's t-tests with equal variance for comparison of gene expression between freshwater and hypersaline acclimated fish.

Results

LT₅₀

The median lethal time (LT_{50}) was significantly lower in freshwater compared to hypersaline acclimated fish at the two highest chlorpyrifos exposure concentrations, 100 and 150 $\mu\text{g/L}$ (285 and 428 nM) (Table 3.2). The LT_{50} after 100 $\mu\text{g/L}$ (285 nM) chlorpyrifos exposure was 49 hr (95% CI: 31 – 78) and 120 hr (95% CI: 89 – 162) for fish in freshwater and those acclimated to hypersaline conditions, respectively. The LT_{50} after 150 $\mu\text{g/L}$ (428 nM) chlorpyrifos exposure was 35 hr (95% CI: 19 – 63) and 105 hr (95% CI: 74 – 149) for fish in freshwater and those acclimated to hypersaline conditions, respectively.

Table 3.2. Median lethal time (LT₅₀) in hours for juvenile rainbow trout acclimated to either freshwater (< 0.5 ppth) or hypersalinity (16 ppth) and exposed to one of five nominal chlorpyrifos concentrations. Numbers in parenthesis are 95% confidence limits (n = 3). * Indicates a significant difference in time to death (p ≤ 0.05; Student's t-test with unequal variance).

Chlorpyrifos Concentration	LT ₅₀ (hours)	
	Hypersalinity (16 ppth)	Freshwater (<0.5 ppth)
20 ug/L (57 nM)	N/A	9434 (2837 - 31370)
40 ug/L (114 nM)	2733 (1392 - 5365)	694 (343 - 1404)
80 ug/L (228 nM)	1682 (1051 - 2691)	116 (83 - 162)
100 ug/L (285 nM)	120 (89 - 162)	49 (31 - 78)*
150 ug/L (428 nM)	105 (74 - 149)	35 (19 - 63)*

Metabolism

To compare metabolism between freshwater and hypersaline acclimated fish, a ratio of the rates of detoxified product (TCP) to activation product (chlorpyrifos-oxon) was calculated. The average *in vitro* ratio of detoxification to activation in rainbow trout livers was 1.43 ± 0.68 and 1.47 ± 0.40 for freshwater and hypersaline acclimated fish, respectively (Table 3.3). Significant differences in *in vitro* liver metabolism were not observed between hypersaline and freshwater acclimated fish. To calculate the average *in vitro* ratio of detoxification to activation for gills, half the limit of detection for chlorpyrifos-oxon was used as the amount of chlorpyrifos-oxon produced for all samples that were below the detection limit. The average *in vitro* ratio of detoxification to activation in rainbow trout gills was 0.64 ± 0.24 and 0.58 ± 0.18 for freshwater and hypersaline acclimated fish, respectively (Table 3.3). No significant difference in *in vitro* gill metabolism was observed between hypersaline and freshwater acclimated fish. Western blot analysis of CYP3A27 protein levels also failed to show a difference

between freshwater and hypersaline acclimated fish (data not shown). Correlations between CYP3A27 level and TCP production were not observed ($R^2 = 0.0026$).

Table 3.3. *In vitro* liver and gill chlorpyrifos metabolism detoxification ratios for juvenile rainbow trout, median *in vitro* AChE inhibition values for juvenile rainbow trout brains, and bioavailability of chlorpyrifos in aqueous media after 48 hours. Significant differences in metabolism ratios were not observed for liver or gill ($P = 0.8182$, Student's t-test with unequal variance, $n = 17$ for freshwater and $n = 16$ for hypersalinity for liver; $P = 0.2232$, Student's t-test with equal variance, $n = 18$ for freshwater and $n = 17$ for hypersalinity for gills). Significant differences were not observed in IC_{50} in fish from hypersaline or freshwater ($P = 0.4775$, Student's t-test with equal variance, $n = 5$). There was no statistical difference in chlorpyrifos concentration between freshwater and hypersaline conditions at any time point measured ($P = 1.0$ at 0 hours and $P = 0.7$ at 12, 24, and 48 hours, Wilcoxon-Mann-Whitney Rank Sum Test, $n = 3$).

	Metabolism		AChE IC_{50}	Bioavailability			
	Ratio of TCP to Oxon		nM	Chlorpyrifos concentration after			
	Liver	Gill		0 hr	12 hr	24 hr	48 hr
Freshwater	1.43 ± 0.68	0.64 ± 0.24	72.0 ± 19.2	88.5 ± 4.6	32.3 ± 10.5	19.2 ± 7.70	8.70 ± 2.30
Hypersalinity	1.47 ± 0.40	0.58 ± 0.18	84.5 ± 26.3	90.9 ± 10.9	26.8 ± 15.5	15.6 ± 10.1	7.60 ± 4.40

Acetylcholinesterase Inhibition

The median inhibitory concentration (IC_{50}) for AChE inhibition by chlorpyrifos-oxon was 72.01 ± 19.2 nM and 84.5 ± 26.3 nM in hypersaline and freshwater acclimated rainbow trout brain homogenates, respectively (Table 3.3). Significant differences were not observed in IC_{50} in fish from hypersaline or freshwater. Inhibition curves for hypersaline and freshwater acclimated fish show no difference at any chlorpyrifos concentration examined (Figure 3.1).

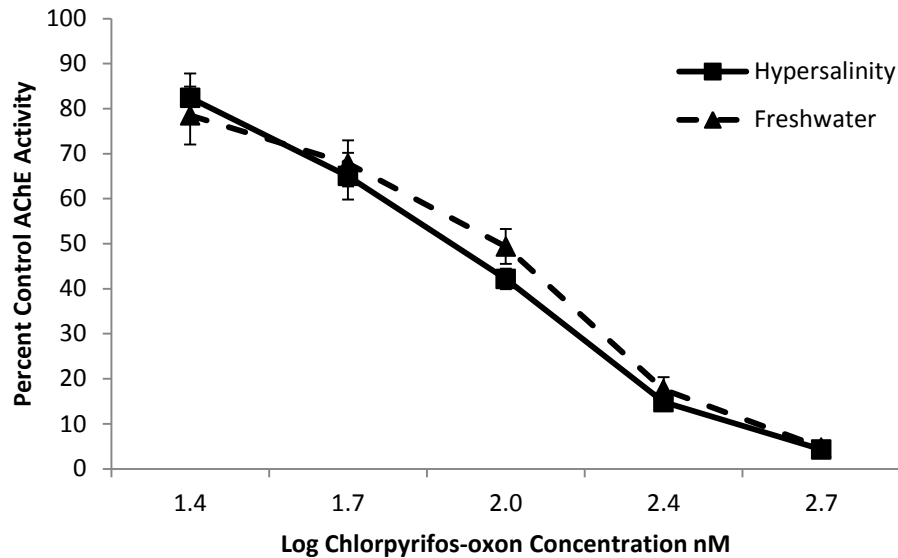


Figure 3.1. Inhibition curves for AChE inhibition in hypersaline and freshwater acclimated rainbow trout brain homogenates. Data are presented as mean \pm standard error ($n = 5$).

Bioavailability

The concentration of chlorpyrifos in exposure aquaria decreased over the 48 hour time period in a similar manner in freshwater and saltwater (Tables 3.3). There was no statistical difference in chlorpyrifos concentration between freshwater and hypersaline conditions at any time point measured.

Signal transduction targets

Calcium calmodulin dependent protein kinase II delta mRNA was upregulated 2-fold in hypersaline acclimated rainbow trout brain ($P = 0.055$; Student's T-test with equal variance; Figure 3.2). Similarly, chloride intracellular channel 4 was induced 3-fold by hypersaline acclimation ($P = 0.086$; Student's T-test with equal variance; Figure 3.2).

Additionally, G protein alpha i1 mRNA was upregulated 2-fold by hypersaline acclimation ($P = 0.079$; Student's T-test with equal variance; Figure 3.2).

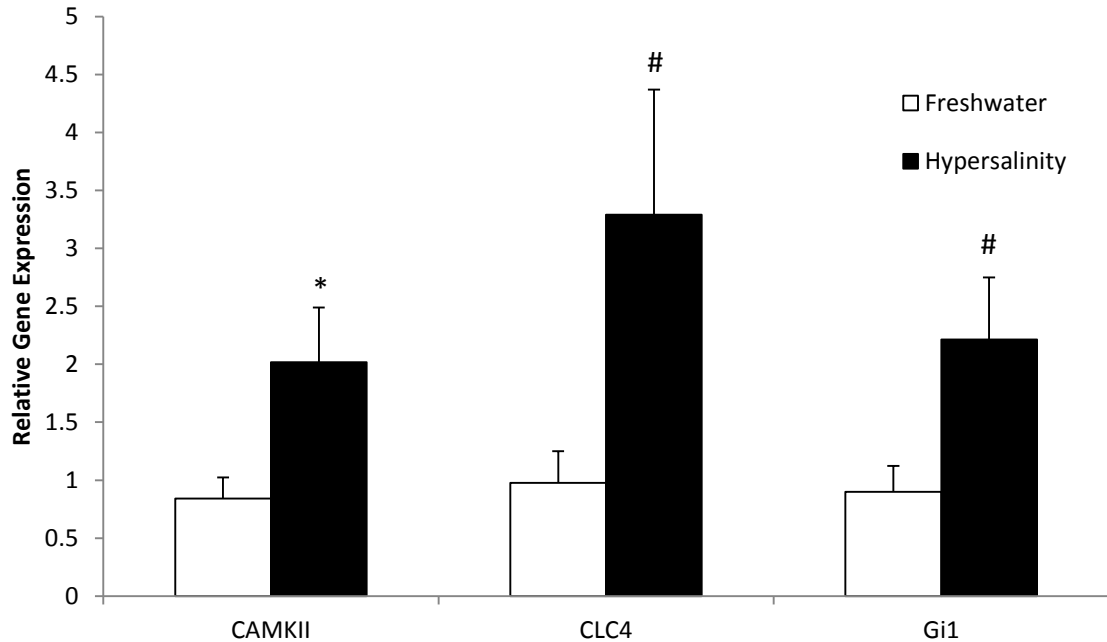


Figure 3.2. Relative gene expression of calcium calmodulin-dependent protein kinase II delta (CAMKII), chloride intracellular channel 4 (CLC4), and G protein alpha i1 (Gi1) in juvenile rainbow trout brains after acclimation to freshwater or hypersalinity (16 ppt). Data are presented as relative gene expression and mean \pm standard error ($n = 6$). * indicates significant differences ($p \leq 0.055$, Student's T-test with equal variance). # indicates significance difference ($p < 0.1$, Student's T-test with equal variance). β -actin was used as the housekeeping gene.

Discussion

Salmonid migration pathways go through urban or agricultural areas near estuaries allowing for habitat impairment by various factors. Salmonid habitats can be impacted through salinization in surface waters from arid and semi-arid zones causing alterations in lakes, rivers, and groundwater (Williams, 2001a, Williams, 2001b). Salinity

can be impacted by both natural and anthropogenic factors. In rivers and streams, salinity is influenced by weathering of the catchment, sea spray in coastal areas, and dissolved salts in rainwater resulting from evaporation of seawater. In semi-arid and arid regions with seasonally hot or dry climates developed for agriculture, dissolved ions can be concentrated by evaporation and transpiration. Thus, salinity in rivers and streams results from a complex combination of climate, geography of catchment, distance from the sea, topography, and vegetation (reviewed in Cañedo-Argüelles et al., 2013).

Along with hypersalinity, salmonid habitats can concurrently be impacted by agricultural chemicals and thus it is important to understand how this combined exposure may impact threaten or endangered species of salmonids. The two factors in this study, hypersalinity and chlorpyrifos, are of particular concern because previous studies have shown that acclimation to hypersaline conditions enhanced the acute toxicity of certain organophosphate and carbamate pesticides (reviewed in Schlenk and Lavado, 2011). In contrast to earlier studies on organophosphates, the time to death from chlorpyrifos exposure was more rapid in freshwater fish than in hypersaline acclimated fish. The mechanism of diminished toxicity in hypersaline conditions was thought to be consistent with an upregulation of cytochrome P450 3A27 (CYP3A27) as seen in previous studies of hypersaline acclimation and xenobiotic exposure (Lavado et al., 2009). CYP3A was involved in fenthion biotransformation and produced more potent anticholinesterase metabolites (Lavado et al., 2009).

In contrast to fenthion, where CYP3A stereoselectively activated the parent to more potent sulfoxide-oxon metabolites, CYP3A detoxified chlorpyrifos to 3,5,6-

trichloro-2-pyridinol (TCP) in mammals (Tang et al., 2001). Since CYP3A27 was induced by hypersaline conditions in previous studies with rainbow trout, it was hypothesized that upregulation of CYP by hypersaline conditions may detoxify chlorpyrifos and reduce toxicity (Lavado et al., 2009). However, *in vitro* studies in liver and gill showed no difference in chlorpyrifos metabolism between freshwater and hypersaline acclimated fish. There also was no relationship between TCP production and CYP3A27 protein level. In contrast to earlier studies, induction of CYP3A27 was not observed. It is possible that the size or age of the fish impacted the CYP3A27 levels, as fish in the current study were smaller than fish in the previous study (10.23 ± 2.60 cm fork length current study, 16 ± 3 cm fork length Lavado et al., 2009). CYP3A27 levels have been shown to differ by age in rainbow trout (reviewed in Buhler and Wang-Buhler, 1998). As differences in metabolism were not observed, other factors that may influence time to death, such as AChE inhibition, bioavailability, or interactions with other molecular targets were explored.

Hypersaline acclimation had no effect on the inhibition of brain AChE by chlorpyrifos-oxon. Chlorpyrifos, along with other organophosphates, is acutely toxic with AChE inhibition as the primary mode of action. Differences in toxicity could have resulted from differences in the target molecule, AChE, after hypersaline acclimation. Impacts of hypersaline conditions on AChE appear to be species, gender, tissue, and salinity dependent. Durieux et al., (2011) measured AChE activity in striped bass (*Morone saxatilis*) brain from different areas in the San Francisco estuary system and found no relationship with salinity. In contrast, El-Alfy et al., (2001) measured muscle

AChE inhibition by aldicarb in Japanese medaka and found differences based on gender and salinity. In channel catfish, a dramatic decrease in brain AChE inhibition accompanied aldicarb induced mortality; however muscle AChE inhibition also played a role (Perkins et al., 1999). Rainbow trout may be similar to striped bass in that no difference in brain AChE was observed after hypersaline acclimation; however, additional studies in muscle need to be conducted as it may have a more influential role on mortality than the brain enzyme.

Bioavailability of chlorpyrifos from water was not impacted by hypersaline conditions. Hypersaline conditions alter the ionic composition of water making it more ordered and compressible and the cavity volume available to accommodate neutral organic solutes is reduced. For neutral organic compounds an inverse relationship with ionic strength is typically observed (Ni et al., 2000). These changes in ionic composition can result in the “salting out” or precipitation of other compounds (Turner and Rawling, 2001). If a compound precipitates out of the water it is no longer available for uptake by aquatic organisms living in the water column. Chlorpyrifos concentrations were unaltered by hypersaline conditions. Since the log P for chlorpyrifos is relatively high, it may not adhere to compressed ionic characteristics of seawater and is not conducive to “salting out.”

Although AChE inhibition is known as the primary mode of action for the acute toxicity of chlorpyrifos, developmental studies have indicated other targets. Activity and expression of adenylyl cyclase, along with associated G-proteins, and neurotransmitter receptors involved in the signaling cascade showed deficits in neonatal rats after exposure

to chlorpyrifos (Song et al., 1997). Adenylyl cyclase plays a significant role in osmosensory and neuronal signal transduction in euryhaline fish (reviewed in Fiol and Kultz, 2007). The acute stress hormone cortisol has been shown to inhibit adenylyl cyclase in tilapia (*Oreochromis mossambicus*) (Borski et al., 2002). Tilapia acclimated to seawater showed transient increases in plasma cortisol levels during acclimation, which remained upregulated in fish acclimated to seawater for extended periods of time (Assem and Hanke, 1981, Borski et al., 1991). Cortisol has also been shown to be elevated in salmonids after acclimation to seawater (McLean et al., 1997). Additionally, rainbow trout acclimated to seawater showed a decrease in gill adenylyl cyclase levels by a factor of two as compared to freshwater acclimated fish (Guibbolini and Lahlou, 1987). It is possible that saltwater acclimation impacts adenylyl cyclase in other tissues, such as the central nervous system, of rainbow trout as well. Consequently, reduction of adenylyl cyclase could result from both chlorpyrifos and hypersaline acclimation, which could reduce overall signal transduction and may protect animals from cholinergic overload associated with AChE inhibition. Consistent with this hypothesis, transcripts of genes that down regulate adenylyl cyclase were induced in brains of fish acclimated to saltwater.

Adenylyl cyclase has been shown to be regulated by a number of kinases including calcium calmodulin-dependent protein kinase II delta (CAMKII). In the current study, CAMKII was upregulated in brains of hypersaline fish. CAMKII inhibited the activity of adenylyl cyclase III through phosphorylation (Wei et al., 1996, Cooper, 2003).

Adenylyl cyclase III is found in several tissues, including brain and olfactory tissue of vertebrates (Wei et al., 1996).

Expression of another gene involved the adenylyl cyclase signaling cascade, G protein alpha inhibitory 1 (Gi1), was also upregulated in brains of hypersaline acclimated fish compared to freshwater fish. This gene encodes for the alpha subunit of an inhibitory G protein complex of adenylyl cyclase. After a ligand binds to the G protein coupled receptor, a conformational change occurs and the attached intracellular heterotrimeric G protein complex then exchanges GDP for GTP, releasing the alpha and beta-gamma subunits. The alpha subunit subsequently binds to and inhibits adenylyl cyclase (reviewed in Birnbaumer, 2007). Previous studies have shown that inhibitory G proteins are involved in the inhibition of adenylyl cyclase in gills of rainbow trout after exposure to neurohypophyseal hormones (Guibbolini and Lahlou, 1992).

Chloride intracellular channel 4, which is also thought to be involved in neuronal signal transduction, was upregulated in hypersaline acclimated fish as compared to freshwater fish. In general, chloride channels function in regulation of cell volume, stabilizing cell membrane potential, intracellular organelle acidification, and transepithelial transport (Jentsch et al., 1999). Upregulation of these channels could result in enhanced cell depolarization and reduced signal transduction. CLC4 in humans has also been shown to interact with other genes involved in signal transduction. Interactions have been observed with YWHAZ, which belongs to a family of proteins that mediate signal transduction by binding to phosphoserine-containing proteins (Katanasaka et al., 2007). CLC4 interacts with HSP90AB1, which encodes a member of the heat shock

protein 90 family. HSP90s play a role in a number of signal transduction processes including nuclear receptors (Hannibal et al., 2011). Thus, changes in CLC4 could have both direct and indirect impacts on signal transduction.

In summary, median lethal time to death by chlorpyrifos was more rapid in freshwater than in hypersaline acclimated rainbow trout in contrast with previous studies which have found that hypersaline acclimation enhanced the acute toxicity of certain thioether organophosphates (Schlenk and Lavado, 2011). In previous studies, the proposed mode of action was upregulation of phase I xenobiotic metabolizing enzymes, such as FMOs and CYPs, which increased formation of more potent anticholinesterase metabolites. However, in the current study, liver and gill metabolism did not play a role in time to death. Other mechanisms were explored to explain the difference in time to death. AChE inhibition and bioavailability also did not play a role in time to death. However, expression of signal transduction related genes was altered in hypersaline acclimated fish. Upregulation of genes involved in diminishing neuronal signaling could in turn protect the animals from cholinergic overload typically seen with AChE inhibition and may explain the difference in acute toxicity. While this pathway may protect against acute cholinergic overload, it may have other chronic impacts at other neuronal targets such as olfactory components which may impair behavior or other apical endpoints. Studies exploring this possibility are currently underway.

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longirostris obsoletus), salt marsh harvest mouse (*Reithrodontomys raviventris*), Bay checkerspot butterfly (*Euphydryas editha bayensis*), valley elderberry longhorn beetle (*Desmocerus californicus dimorphus*), San Joaquin kit fox (*Vulpes macrotis mutica*), California freshwater shrimp (*Syncaris pacifica*), and Delta smelt (*Hypomesus transpacificus*). (Ed. O.o.P. Program).

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Chapter 4: Sublethal Toxicity of Chlorpyrifos to Salmonid Olfaction After Hypersaline Acclimation

Abstract

Salmonid habitats can be impacted by several environmental factors, such as salinization, which can also affect salmonid tolerance to anthropogenic stressors, such as pesticides. Previous studies have shown that hypersaline acclimation enhances the acute toxicity of certain organophosphate and carbamate pesticides to euryhaline fish; however, sublethal impacts have been far less studied. The current study aims to determine how hypersaline acclimation and exposure to the organophosphate chlorpyrifos (CPF) impact salmonid olfaction. Combined acclimation and exposure to CPF was shown to impact rainbow trout olfaction at the molecular, physiological, and behavioral levels. Concurrent exposure to hypersalinity and 0.5 µg/L CPF upregulated four genes (chloride intracellular channel 4, G protein *zgc:101761*, calcium calmodulin dependent protein kinase II delta, and adrenergic alpha 2C receptor) that inhibit olfactory signal transduction. At the physiological level, hypersalinity and chlorpyrifos caused a decrease in sensory response to the amino acid L-serine and the bile salt taurocholic acid. Combined acclimation and exposure also negatively impacted behavior and reduced the avoidance of a predator cue (L-serine). Thus acclimation to hypersaline conditions and exposure to environmentally relevant concentrations of chlorpyrifos caused an inhibition of olfactory signal transduction leading to a decreased response to odorants and impairment of olfactory mediated behaviors.

Introduction

As of 2012, five populations of salmonids have been listed as endangered and twenty-three as threatened in the Pacific Northwest by the National Oceanic and Atmospheric Administration (NOAA, 2012). Habitat degradation may contribute to population declines as migration pathways often go through urban or agricultural areas near estuaries. In addition, chemical and environmental stressors are likely to occur simultaneously in salmonid habitats and jointly contribute to overall effects (Sargeant et al., 2013).

Environmental stressors include temperature, pH, and salinity. Many salmonids are anadromous; naturally transitioning between freshwater and saltwater at different life stages, eventually homing to their native area for reproduction (Quinn, 2005). However, changes in normal salinity can impact salmonid habitats. Natural and anthropogenic factors can result in increased salinity in salmonid waterways. Salinity can be influenced naturally through weathering of catchment, dissolved salts in rainwater from seawater evaporation, and sea spray in coastal areas. A complex combination of climate, geography of catchment, distance from the sea, topography, and vegetation influences natural salinity in rivers and streams (reviewed in Cañedo-Argüelles et al., 2013).

As for anthropogenic routes, agricultural practices can impact salinity as crop production consumes large quantities of water. In Western arid and semi-arid regions near coastal embayments, saltwater intrusion can occur from freshwater export for irrigation and rising of groundwater tables. Crops take in only a portion of the solutes from irrigation water allowing for solutes to concentrate and soil water to become more

saline. Thus, solutes may enter rivers through runoff by leaching (reviewed in Cañedo-Argüelles et al., 2013).

Another anthropogenic route with the potential to impact salinity in certain areas is climate change. Temperature increases associated with climate change can influence freshwater inputs into certain water systems. For example, the San Francisco Bay Delta receives freshwater inputs from melting of alpine snow from the Sierra Nevada mountains (Cohen, 2000). Temperature increases would heighten the freeze line and decrease alpine snow volume, decreasing freshwater input into different waterways. Climate models predict continued increase of salinity for the San Francisco Bay Delta waterways (Knowles and Cayan, 2002, Knowles and Cayan, 2004, Howat and Tulaczyk, 2005).

Other stressors that may impact salmonid populations are contaminants, which can occur concurrently with hypersalinity. Pesticide input and salinization both rank in the top 15 causes of impairment to streams in the United States (EPA, 2012b). The organophosphate pesticide chlorpyrifos, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is widely used to control pests on crops as well as on non-food sites (EPA, 2012a). Chlorpyrifos was the fourteenth most commonly used conventional active ingredient in 2007 in the agricultural pesticide market sector (Grube et al., 2011).

Although usage has declined since 2009, chlorpyrifos is still frequently detected in surface waters which provide habitat for various aquatic species. Chlorpyrifos has been detected in salmonid habitats of Washington and California. From 2009 to 2011 samples from salmon-bearing waterways had chlorpyrifos concentrations exceeding water quality

criteria (Sargeant et al., 2013). For example, at the Spring Creek location, concentrations of chlorpyrifos were over the criteria guidelines on seven different occasions during the three year period (Sargeant et al., 2013). Additionally, in a recent monitoring study in California, chlorpyrifos was detected in 17.7% of samples, with 9.9% exceeding water quality criteria between 2006 and 2010, with the maximum detected concentration of 3.7 µg/L in 2007 in the San Joaquin region (Xuyang et al., 2012). Anderson et al., (2014) detected chlorpyrifos in all surface water samples analyzed from the Santa Maria estuary, which provides habitat for endangered steelhead trout. It was determined that elevated chlorpyrifos concentrations accounted for water-associated toxicity to *Ceriodaphnia dubia* and *Hyalella azteca* in the majority of samples from their study. Along with that study, Smalling et al., (2013) detected chlorpyrifos in 100% of fish tissue and water samples taken from the same estuary.

As salmonids may encounter hypersaline conditions and chlorpyrifos concurrently, understanding how hypersaline acclimation may impact chlorpyrifos toxicity is important. Previous studies of certain thioether organophosphate and carbamate pesticides have shown enhanced acute toxicity after hypersaline acclimation (reviewed in Schlenk and Lavado, 2011). However hypersaline acclimation was shown to be protective and decreased time to death by chlorpyrifos for rainbow trout (Maryoung et al., 2014). Previous studies on concurrent acclimation to salinity and exposure to pesticides have focused on acute effects. Although acute studies can provide vital mechanistic information, they typically lack environmental relevance. Studies on

sublethal effects of salinity acclimation and organophosphate exposure are limited even though they represent more environmentally realistic exposure scenarios.

One particular sublethal endpoint of importance for salmonids is olfactory impairment. Salmonids rely on olfaction to detect chemical cues that provide crucial information about food, predators, reproductive status of mates, environmental contamination, and natal streams of imprinting (reviewed in Laberge and Hara, 2001). Previous studies have shown that environmentally relevant concentrations of chlorpyrifos can cause decreased olfactory response of coho salmon (*Oncorhynchus kisutch*) to odorants (Sandahl et al., 2004). Since acetylcholinesterase (AChE) inhibition did not correlate with olfactory impairment; it was hypothesized that chlorpyrifos may act on other targets that are necessary for olfactory signal transduction, such as adenylyl cyclase (Sandahl et al., 2004). As chlorpyrifos individually has been shown to impair olfaction, understanding how hypersaline acclimation may impact olfaction is also crucial. In addition, the relationship of olfactory impairment to olfactory-mediated behaviors is important, particularly considering salmonid survival and migration.

Sublethal exposure of chlorpyrifos altered behavior in several fish species. Levin et al., (2004) examined the effects of chlorpyrifos on the swimming activity of newly hatched zebrafish (*Danio rerio*) and found that 100 µg/L significantly slowed swimming activity on days six and nine post fertilization following five days of exposure. Rice et al., (1997) found that chlorpyrifos caused an underreactive startle response and overall hypoactivity in juvenile Japanese medaka. Similar impacts of chlorpyrifos on startle

response in juvenile medaka also indicated that medaka generally appeared to be more susceptible to predation after exposure (Carlson et al., 1998).

Studies focusing on behavioral impacts of chlorpyrifos have also been performed on salmonids. Sandahl et al., (2005) compared the impacts of chlorpyrifos on AChE activity and behavior in juvenile coho salmon. Spontaneous swimming and feeding behavior were assessed using a computer-assisted, three dimensional video imaging system. After exposure for 96 hours to 0 – 2.5 µg/L, brain AChE activity and behaviors were inhibited in a concentration-dependent manner. Furthermore, brain AChE inhibition and decreases in both behaviors were significantly correlated (Sandahl et al., 2005). Swimming performance and rapid acceleration swimming in coho salmon were likewise impacted by chlorpyrifos exposure (Tierney et al., 2007a).

The current study aims to determine how hypersaline acclimation may affect the impacts of chlorpyrifos on salmonid olfaction utilizing an adverse outcome pathway for combined exposure to the two factors. Impacts were assessed at the molecular, physiological, and behavioral levels. It was hypothesized that hypersaline acclimation would augment olfactory impairment from chlorpyrifos by inhibiting olfactory signal transduction. Previous studies on the impacts of hypersaline acclimation on the acute toxicity of chlorpyrifos suggest that hypersaline acclimation decreases signal transduction by upregulating genes that inhibit adenylyl cyclase (Maryoung et al., 2014). Impaired olfactory signal transduction could disrupt olfactory function and associated behaviors.

Methods

Chemicals

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl-phosphorothioate; CAS 2921-88-2) was acquired from Chem Service (West Chester, PA). Methanol was analytic grade (Fisher, Pittsburg, PA). Ethanol and potassium chloride were also purchased from Fisher (Pittsburg, PA). The iScript One-step RT-PCR kit with SYBR Green was obtained from BioRad (Hercules, CA). SV Total RNA Isolation System kits and Reverse Transcription System kits were purchased from Promega (Madison, WI). Instant Ocean Marine Mix was purchased from PetSolutions (Beavercreek, OH). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Chemical Measurements

One liter water samples were collected immediately after dosing from randomly selected tanks (control = 3, low = 7, and high = 7) for analysis of chlorpyrifos concentration from the behavioral assay section. Analytical measurements of chlorpyrifos in water were conducted following the methods of Bogus et al., (1990) with slight modifications. Briefly, water samples were filtered through activated C18-solid phase extraction columns with a vacuum manifold. One hundred mL of water was then passed through the column, with subsequent elution with 5 mL of methanol. *R*-Methyl-*p*-tolyl sulfoxide was used as an internal standard.

Analysis of extracts was conducted using reverse phase HPLC on a SCL-10AVP Shimadzu HPLC system equipped with a 250 x 4.6 mm Hypersil ODS C18 (5 μ m)

reverse-phase column (ThermoFisher Scientific Inc., Waltham, MA). Separation of chlorpyrifos employed an HPLC isocratic system elution at a flow rate of 1 mL/min with mobile phase composed of 80% methanol and 20% water. The run time was 20 minutes. Chromatographic peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 230 nm. Peaks were quantified by integrating the area under the peaks and identified by elution of authentic standards (the retention time observed for chlorpyrifos was 10.3 mins).

Organisms

For electro-olfactograms, juvenile rainbow trout (*Oncorhynchus mykiss*) (5.72 ± 0.60 cm fork length, 2.57 ± 0.87 g) were sourced as eggs from Raven Brood Trout station (Caroline, AB, Canada) and grown at The University of Alberta (Edmonton, Alberta, Canada). Fish were maintained on a 12 hour light-dark cycle at constant temperature, 14 – 16 °C in 177 L tanks. Fish were maintained in dechlorinated carbon filtered municipal water and allowed to acclimate for at least two weeks prior to exposure. For behavioral assays, juvenile rainbow trout (7.61 ± 0.77 cm fork length, 6.51 ± 1.74 g) were obtained from the Jess Ranch Fish Hatchery (Apple Valley, CA). Fish were maintained on a 14 hour light – 10 hour dark cycle at constant temperature, 11 – 13 °C. Organisms were housed in a flow through living-stream system with dechlorinated carbon filtered municipal water. Fish were fed commercial pellets (Silver Cup, Murray, UT). Fish were allowed to acclimatize to the laboratory for at least two weeks prior to experimental use.

Salinity Acclimations

After the acclimation period, fish were transferred to 20 L glass aquaria, four fish per aquaria, and maintained on the same light cycle and at the same temperature as previously stated. Fish were acclimated to one of three salinity treatments, freshwater (< 0.5 ppt), hypersalinity (16 ppt) day seven, or hypersalinity (16ppt) day fourteen. The day, seven or fourteen, denotes when fish were exposed to chlorpyrifos. Fish were acclimated to salinity in a stepwise manner with an increase in 4 ppt every two days as previously described (Lavado et al., 2009). Salinity treatments were created by reconstituting carbon filtered municipal water with Instant Ocean Marine Mix. Ninety percent daily water changes were conducted.

Chlorpyrifos Exposures

Fish were exposed to chlorpyrifos for 96 hours after reaching the correct salinity (either freshwater, hypersalinity day seven, or hypersalinity day fourteen). Fish were exposed to nominal concentrations of ethanol solvent control, 0.5 µg/L, or 5.0 µg/L using static renewal.

Electroolfactograms

Electroolfactograms (EOGs), using the techniques described by Evans and Hara, (1985) and methods of Baldwin et al., (2003), were conducted to determine the impairment of neurophysiological responses of rainbow trout after acclimation to hypersalinity and exposure to chlorpyrifos. After the exposure period, EOGs were

conducted on each fish. To begin the EOG process, fish were anaesthetized with 150 mg/L tricaine methane sulfonate (MS-222; Syndell; Vancouver, BC Canada). Fish were put into a Plexiglas holder on a vibration isolation table and chilled, oxygenated water with 75 mg/L MS-222 was delivered through a mouthpiece to the gills. The skin that overlies the olfactory chamber, which contains the olfactory rosette, was then surgically removed. A perfusion tube was used to deliver a continuous flow of chilled source water to the exposed rosette. A computer controlled manifold controlled delivery of separated solutions of water only and water plus odorant.

Odorant stimulus solutions used consisted of the amino acid L-serine and the bile salt taurocholic acid (TCA). These odorants were chosen because they have previously been shown to evoke electrophysiological and behavioral responses in salmonids (Hara, 1992). In fish, amino acids and TCA act on nonoverlapping groups of olfactory receptor neurons (reviewed in Laberge and Hara, 2001). Odorant stock solutions were made weekly and dilutions were made daily.

To record odor-evoked EOGs, a pair of glass microelectrodes was used. A stereomicroscope mounted on a boom stand was used to aid in placing the recording electrode along the midline of the rosette. A reference electrode was placed in the water of the holding chamber. The signal obtained from the readings required amplification and filtration. This was completed with a direct current amplifier. The signal also needed to be digitized through use of a computerized data acquisition system. Before the actual testing procedure, fish were allowed to acclimate for ten minutes after electrode placement. Throughout the acclimation and the testing procedure, the rosette was

continuously perfused with water or test solution. The response of each fish to L-serine 10^{-4} M and TCA 10^{-4} M was tested by alternating pulse of the two odorants every two minutes. Between fish the order of which odorant was tested first was alternated. After three pulses of each odorant were conducted, the EOG trial was completed for that fish. Fish were then terminated by cervical dislocation and olfactory tissue was removed and immediately placed in RNAlater and frozen at -80°C for use in the molecular section of this study. For fish in the freshwater group, all odorant dilutions and flow water were freshwater. For fish in either hypersalinity group, all odorant dilutions and flow water were 16ppt.

Molecular Endpoints

Transcripts of genes to be measured were chosen based on upregulation in neurological tissues from a previous microarray study of coho salmon undergoing salinity acclimation (Maryoung et al., in review). Transcripts expressed during salinity acclimation were compared with gene expression profiles in zebrafish treated with chlorpyrifos (Tilton et al., 2011). Primers were designed using Primer3 software for G protein zgc:101761 (GNA11B) and adrenergic alpha 2C receptor (ADRA2C). Primers for calcium calmodulin-dependent protein kinase II delta (CAMKII) and chloride intracellular channel 4 (CLIC4) were previously designed using Primer3 (Maryoung et al., 2014). β -actin was used as the housekeeping gene. Primer sequences are listed in Table 4.1. Primers were then optimized based on annealing temperature, template concentration, and primer concentration. After optimization, RNA was isolated from

olfactory tissue using Promega SV Total RNA Isolation System kits. Total RNA was then converted to cDNA using Promega Reverse Transcription System kits. qPCR was run for each gene with β -Actin as the housekeeping gene using iScript One-step RT-PCR kit with SYBR Green from Bio-Rad. 250 nM of each primer was added to 25 μ L PCR reactions containing SYBR Green RT-PCR Reaction Mix, and 100 ng of cDNA sample. Thermocycling parameters were as follows: 5 mins at 95°C; 40 cycles of 10 sec at 95°C and 30 sec at 57°C. At the end of each cycle fluorescence data was collected. A melting curve analysis was run between 60°C and 95°C following the amplification reaction. The C(t) was selected to be in the linear phase of amplification. The reactions were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) and data analysis was done using IG5 (Bio-Rad). Gene expression was measured in select chlorpyrifos and salinity treatments based off results from the EOG section.

Table 4.1. Signal transduction target gene primers used in this study.

Target	Primer sequence (5'-3')	Reference
Chloride intracellular channel 4	GGATAAATGAGGGCAGGTTT GGTCAGCATAAGGTCAAGGA	Maryoung et al., 2014
G protein zgc:101761	ACAAAGCCAGGGATCACAGT CAGGGTCCACAACATTCCTC	
Calcium calmodulin dependent protein kinase II delta	TCACCAGAACAAAAGCAAGC TGGAGCATATTACCTGACATCTG	Maryoung et al., 2014
Adrenergic alpha 2C receptor	CAGCCTCAAAGTGACCCTTC GATTTGCAGGGAGAGCTGTT	
β -actin	GTCCTTCATGATTCTCTGCTGA ACTCGGGTTCATTTGCATAAACA	Lavado et al., 2009

Behavioral Trials

After the exposure period, fish were placed into a flow-through Y-maze behavioral trough for assessment of preference and avoidance. The Y-maze was constructed following the methods of Rehnberg et al., (1985). However the size was scaled for smaller fish. The Y-maze trough measured 1.22 meters in length. It was constructed out of plexiglass and caulked with an aquarium silicone sealant. The maze was broken into two main sections lengthwise. The first section measured 0.61 meters in length and was split into three sections widthwise. These three sections consisted of two arms of 0.14 meters in width and a separating channel (0.03 meters in width) between the two arms. Water flowed in at the head of the two Y-maze arms and the separating channel. Water was chilled to the correct temperature and adjusted to the correct salinity prior to entering the trough. The separating channel was used to help divide the flows from the two arms. This segregation was confirmed with dye tracers prior to experimental use. To prevent fish from entering the separating channel, a screen was placed over the opening. The flow averaged 3.1 L/min. The arms and separating channel were covered in black plastic to exclude light and encourage the fish to explore these areas. The arms also had an arm gate that could be rapidly raised and lowered without disturbing the fish. The second lengthwise section measured 0.61 meters and served as the fork area. Fish were initially placed in this area. This section was not covered in black plastic to encourage fish to move to other areas. The fork area housed the standpipe for the outflow, which

was screened to prevent fish from entering. Curtains were placed around the entire trough so the fish would be unable to see the observer.

Behavioral trials were conducted according to the methods of Stone and Schreck, (1994). For each trial, a single fish was placed into the fork area. The fish was allowed to acclimate for 5 minutes while both arm gates were lowered. The odorant was then added to the head tank of one of the arms and the fish remained in the fork area with the gates down for an additional 10 minutes. Afterwards, the gates were lifted and the fish was allowed 15 minutes to choose between entering the control arm, the odorant arm, or remaining in the fork area. After the 15 minute period, the arm gates were lowered and the location of the fish was recorded. At the end of the trial the fish were removed and the trough was thoroughly rinsed before resetting for the next trial. The arm receiving the odorant was alternated between trials. Control trials without odorant were conducted and did not differ from the expected random 1:1.

L-Serine at 10^{-7} M was chosen as the odorant and allows for comparisons between the olfactory EOG and behavior sections. L-serine is found in mammalian skin and has previously been shown to elicit a strong avoidance response in salmonids, has a well-established control response and allows for comparison with the EOG section (Idler et al., 1956, Rehnberg et al., 1985).

Statistical Analyses

Two-way ANOVAs were used to compare EOG responses to L-serine and TCA, separately, for all treatments with a Bonferroni multiple comparison post hoc test using GraphPad Prism version 5.00 for Windows (Graph-Pad Software, San Diego, CA). R was

used to perform a one-way ANOVA for relative gene expression between all treatments with a Tukey post hoc test. R was also used to perform a multinomial logistic regression analysis for Y-maze behavioral data using freshwater control results as the baseline comparison.

Results

Chemical Measurements

Average concentrations of chlorpyrifos determined in exposure tanks immediately after dosing were comparable to nominal concentrations, viz. 0.54 ± 0.06 and 5.01 ± 0.72 $\mu\text{g/L}$ (values are mean \pm standard deviation of seven different sampling days; table 4.2).

Chlorpyrifos was not detected in solvent control tanks (<5 ng/L; $n = 3$).

Table 4.2. Average chlorpyrifos concentration in exposure tank water immediately after dosing (values are mean \pm standard deviation; $n = 3$ for solvent control and $n = 7$ for both low and high chlorpyrifos concentrations).

<u>Treatment</u>	<u>Measured Concentration ($\mu\text{g/L}$)</u>
Solvent Control	b.d.l.
Low (0.5 $\mu\text{g/L}$)	0.54 ± 0.06
High (5.0 $\mu\text{g/L}$)	5.01 ± 0.72

Electroolfactograms

In freshwater acclimated fish, exposure to both low and high concentrations of chlorpyrifos caused a trend towards a decrease in olfactory response to L-serine, however it was not significant (Figure 4.1; $P > 0.05$, two-way ANOVA, Bonferroni post hoc test).

For fish acclimated to hypersalinity for seven days prior to chlorpyrifos exposure, in which fish were still in the acclimation process during exposure, no significant difference in olfactory response to L-serine was observed with either concentration of chlorpyrifos (Figure 4.1; $P > 0.05$, two-way ANOVA, Bonferroni post hoc test). For L-serine, hypersaline acclimation did not reduce olfactory response in solvent control fish (Figure 4.1; $P > 0.05$, two-way ANOVA, Bonferroni post hoc test). Fish acclimated to hypersaline conditions for fourteen days prior to chlorpyrifos exposure, in which fish would have had ample time to acclimate before exposure, showed a significant decrease in olfactory response to L-serine in the low concentration chlorpyrifos treatment group (Figure 4.1; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). Also for the low concentration exposure, there was a significant reduction in olfactory response to L-serine between freshwater fish and fish acclimated to hypersalinity for fourteen days prior to exposure (Figure 4.1; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). Thus hypersaline acclimation appears to be hindering the olfactory response when combined with chlorpyrifos.

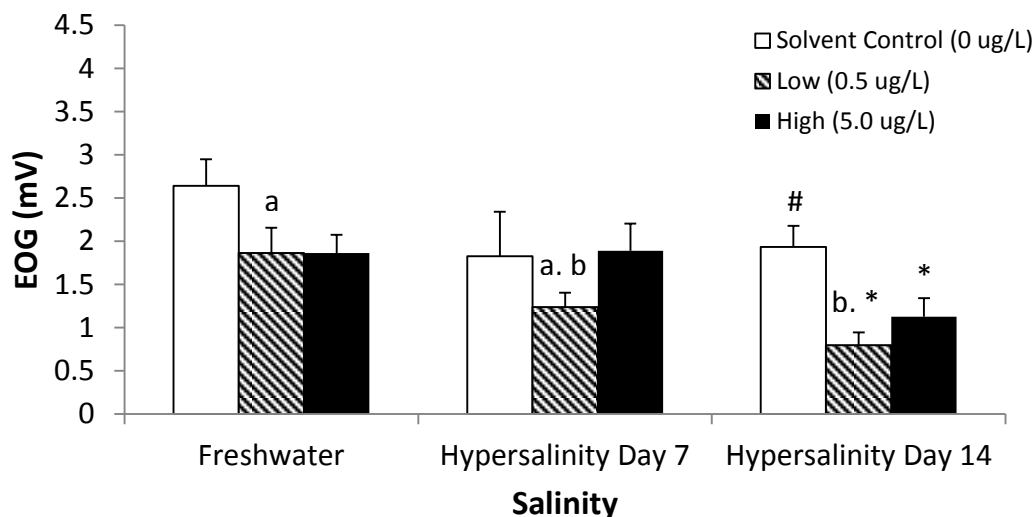


Figure 4.1. Olfactory response to L-serine (10^{-4} M) of rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos. Data are presented as mV and mean \pm standard error ($n = 7 - 11$). Different letter indicates significant differences between salinity treatments at the same concentration, while different symbols indicate significant differences between concentrations within the same salinity treatment ($p < 0.05$, two-way ANOVA, Bonferroni post hoc test).

In freshwater acclimated fish, exposure to the high concentration of chlorpyrifos ($5.0 \mu\text{g/L}$) significantly reduced the response to TCA as compared to solvent control fish (Figure 4.2; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). A significant decrease was not observed at the low concentration of chlorpyrifos ($0.5 \mu\text{g/L}$). For fish acclimated to hypersalinity for seven days prior to chlorpyrifos exposure, in which fish were still in the acclimation process during exposure, no significant difference in olfactory response to TCA was observed with either concentration of chlorpyrifos (Figure 4.2; $P > 0.05$, two-way ANOVA, Bonferroni post hoc test). Hypersaline acclimation for fourteen days prior to chlorpyrifos exposure was shown to significantly reduce the response to TCA in solvent control fish (Figure 4.2; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). Fish acclimated to hypersaline conditions for fourteen days

prior to chlorpyrifos exposure, in which fish would have had ample time to acclimate before exposure, showed a significant decrease in olfactory response to TCA in both the low and high concentrations (Figure 4.2; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). There was no significant difference in response to TCA between the low and high concentrations (Figure 4.2; $P > 0.05$, two-way ANOVA, Bonferroni post hoc test). At the low concentration of chlorpyrifos there was a significant decrease in olfactory response to TCA between freshwater acclimated fish and fish acclimated to hypersalinity for fourteen days (Figure 4.2; $P < 0.05$, two-way ANOVA, Bonferroni post hoc test). Hypersaline acclimation hindered the olfactory response when combined with chlorpyrifos exposure.

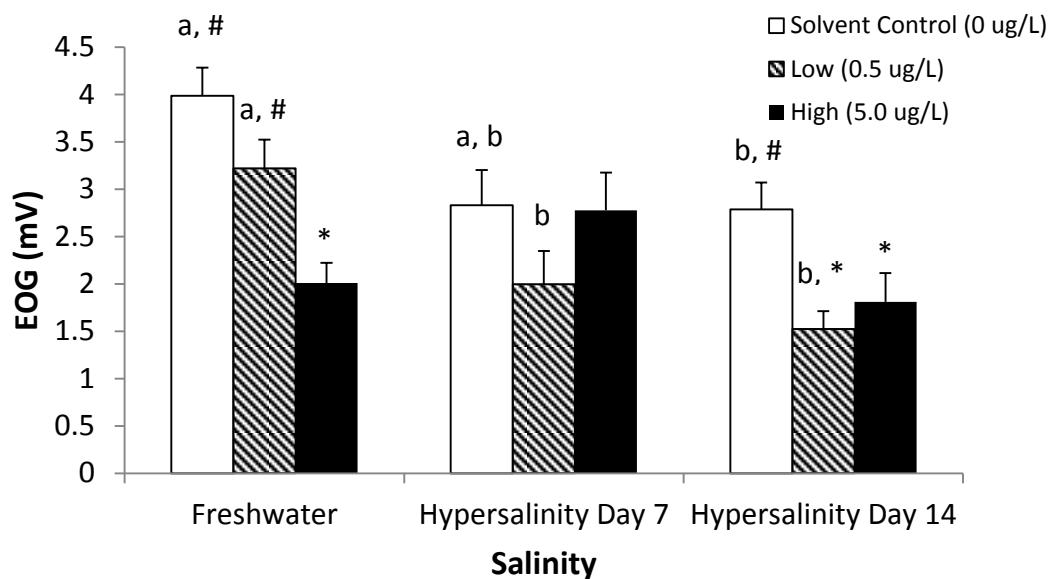


Figure 4.2. Olfactory response to TCA (10^{-4} M) of rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos. Data are presented as mV and mean \pm standard error ($n = 7 - 11$). Different letter indicates significant differences between salinity treatments at the same concentration, while different symbols indicate significant differences between concentrations within the same salinity treatment ($p < 0.05$, two-way ANOVA, Bonferroni post hoc test).

Molecular Endpoints

Gene expression was measured in olfactory tissue from freshwater control, hypersalinity day fourteen control, and hypersalinity day fourteen low chlorpyrifos treatment fish. These treatments were selected as they showed significant differences in response to odorants in the EOG section. The four target genes (CLIC4, GNA11B, CAMKII, and ADRA2C) were significantly upregulated in the hypersalinity day fourteen low chlorpyrifos treatment 33.6, 7.4, 18.5, and 12.3 fold, respectively, as compared to the freshwater control (Figure 4.3; $P < 0.05$, one-way ANOVA, Tukey's post hoc test). Only one gene, CLIC4, was also upregulated (12.3 fold) in the hypersalinity day fourteen control. All of the target genes followed a similar trend in differential regulation as was seen in Tilton et al., (2011) and microarray data mining (Maryoung et al., in review).

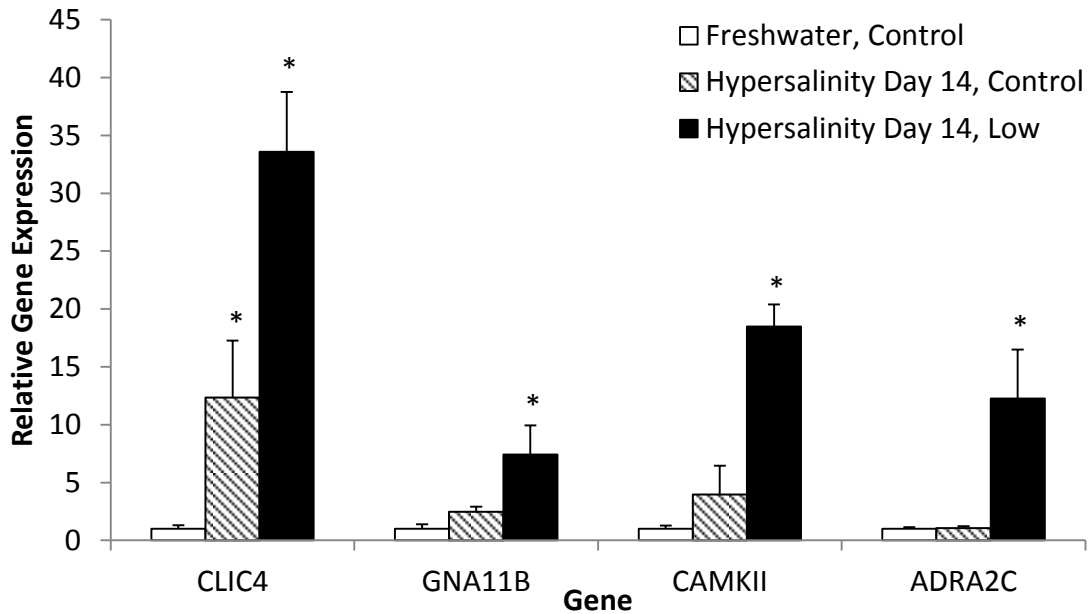


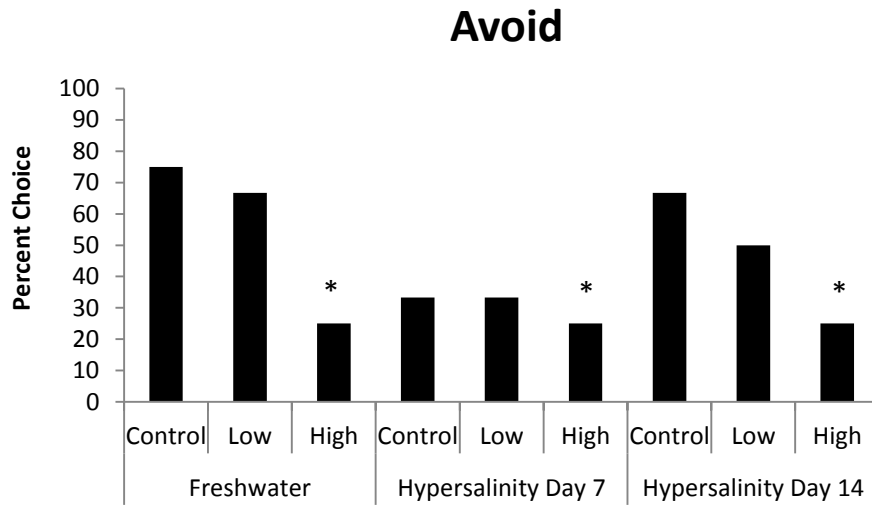
Figure 4.3. Relative gene expression of five signal transduction related genes in olfactory tissue of juvenile rainbow trout acclimated to freshwater or hypersalinity for fourteen days and exposed to chlorpyrifos. Data are presented as relative gene expression and mean \pm standard error ($n = 4 - 5$). * indicates significant differences ($p < 0.05$, one-way ANOVA, Tukey's post hoc test). B-actin was used as the housekeeping gene. CLIC4 = chloride intracellular channel 4; GNA11B = G protein (*zgc:101761*); CAMKII = calcium/calmodulin-dependent protein kinase II delta; ADRA2C = adrenergic (adrenergic, alpha-2C-, receptor).

Behavioral Trials

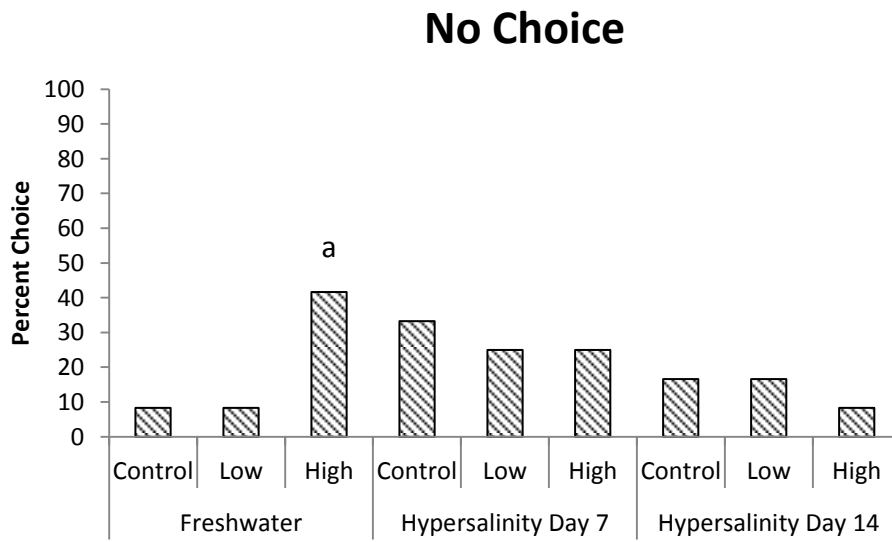
For y-maze behavior trials, freshwater solvent control fish avoided L-serine (10^{-7} M) seventy-five percent of the time. Avoidance of L-serine at this concentration is consistent with previous studies showing predator avoidance (Rehnberg et al., 1985). Response to L-serine was significantly different from freshwater solvent control fish in all high chlorpyrifos concentration fish. Fish acclimated to freshwater and exposed to the high concentration of chlorpyrifos had a significantly higher rate of making no choice than avoiding the odorant as compared to controls (Figure 4.4; $P = 0.023$, multinomial

logistic regression). Thus the high concentration of chlorpyrifos impacted the avoidance of the odorant. However, fish acclimated to salinity for seven days or fourteen days prior to exposure had a higher rate of preferring the odorant than avoiding it as compared to freshwater controls (Figure 4.4; $P = 0.037$ and $P = 0.016$, respectively, multinomial logistic regression). The salinity acclimation coupled with the high chlorpyrifos exposure resulted in a switching from avoidance to preference. Although there was a trend towards a concentration-dependent response in fish acclimated to salinity for fourteen days, there was no significant effect of the low concentration of chlorpyrifos on fish choice in any treatment.

a)



b)



c)

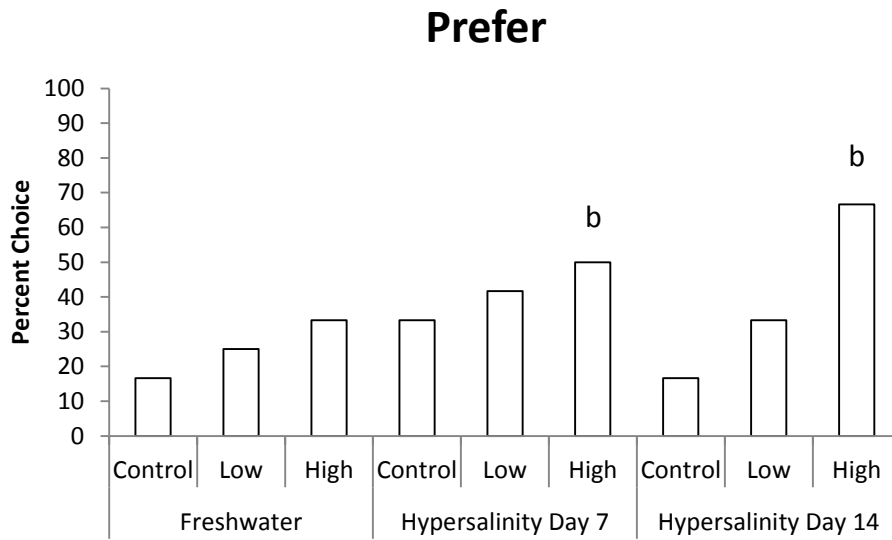


Figure 4.4. Behavioral response (a – avoid, b – no choice, c – no prefer) to L-serine (10^{-7} M) of juvenile rainbow trout after acclimation to freshwater or hypersalinity (seven or fourteen days) with co-exposure to chlorpyrifos. Data are presented as percent choice ($n = 12$). * indicate significant differences from freshwater solvent control fish avoidance response and letters indicate the specific response responsible for the difference ($p < 0.05$, multinomial logistic regression).

Discussion

Salmonid habitat can be impacted by hypersalinity which can influence salmonid tolerance to concurrent stressors, such as contaminants. Previous studies on euryhaline fish have shown that acclimation to hypersalinity enhanced acute toxicity to certain pesticides (reviewed in Schlenk and Lavado, 2011). However, freshwater acclimated rainbow trout showed a more rapid time to death by chlorpyrifos than did hypersaline acclimated fish (Maryoung et al., 2014). Furthermore, previous studies on impacts of salinity acclimation on pesticide toxicity have focused on acute toxicity, employing concentrations that are not typically environmental relevant. Determining impacts of combined acclimation and exposure on sublethal endpoints, such as olfactory responses, provides a more environmentally realistic scenario.

This study examined the physiological response to two odorants, the amino acid L-serine and the bile salt TCA. L-serine is thought to represent a predator scent as it is present in mammalian skin extract, while TCA is thought to represent a conspecific cue (Idler et al., 1956, Quinn and Hara, 1986). For L-serine, hypersaline acclimation did not impact olfactory response in control fish. In freshwater acclimated fish, exposure to both low and high concentrations of chlorpyrifos caused a trend towards a decrease in olfactory response to L-serine. Sandahl et al., (2004) exposed juvenile coho salmon to chlorpyrifos (0.625, 1.25, and 2.50 $\mu\text{g/L}$) for seven days and observed a concentration-dependent reduction in EOG response after seven days. Response to L-serine was shown to decrease in a concentration-dependent manner. Since the current study only exposed

animals for four days, the lack of significant differences in olfactory response may have been due to the shorter duration of exposure.

Fish acclimated to hypersalinity for seven days prior to chlorpyrifos exposure, showed no decrease in response to L-serine at either chlorpyrifos concentration. However, fish acclimated to hypersaline conditions for fourteen days prior to chlorpyrifos exposure, showed a significant decrease in olfactory response to L-serine after exposure to the low concentration of chlorpyrifos. Also for the low concentration exposure, there was a significant reduction in olfactory response to L-serine between freshwater fish and fish acclimated to hypersalinity for fourteen days prior to exposure. Thus hypersaline acclimation hindered the olfactory response when combined with chlorpyrifos. Decreasing the ability of fish to detect L-serine could impact survival as L-serine is thought to be a predator cue (Idler et al., 1956). Furthermore, amino acids are thought to be central to the migration of certain salmonids, hindering the ability to detect these cues could result in straying or ecological death (Shoji et al., 2000, Shoji et al., 2003).

In freshwater acclimated fish, exposure to the high concentration of chlorpyrifos significantly reduced the response to TCA as compared to control fish. A similar response was seen by Sandahl et al., (2004) in coho salmon. Sandahl et al., (2004) observed a concentration-dependent decrease in amplitude of olfactory response to TCA after a seven-day exposure to 0.625, 1.25, and 2.50 $\mu\text{g/L}$. In the current study a significant decrease was not observed at the low concentration of chlorpyrifos (0.5 $\mu\text{g/L}$), however, there was a concentration dependent trend toward diminished olfaction. Slight

differences in results between the current study and the Sandahl et al., (2004) study could be due to duration of exposure (see above) or concentration.

Acclimation to hypersalinity for seven days prior to chlorpyrifos exposure failed to affect olfactory response of fish to TCA at either chlorpyrifos concentration, but hypersaline acclimation for fourteen days prior to chlorpyrifos exposure was shown to significantly reduce the olfactory response to TCA. Fish acclimated to hypersaline conditions for fourteen days and then treated with chlorpyrifos, showed a significant decrease in olfactory response to TCA in both the low and high concentration. Decreasing the olfactory response to TCA could impact schooling, territorial aggression, and potentially mate selection as TCA is thought to represent conspecific cues and allow for sibling recognition (Quinn and Busack, 1985, Quinn and Hara, 1986).

We previously found that hypersaline acclimation resulted in protection from acute chlorpyrifos toxicity in rainbow trout (Maryoung et al., 2014). The proposed mechanism for this protection was diminished neuronal signaling in hypersaline acclimated fish from upregulation of genes inhibiting signal transduction. This mechanism was supported by previous studies in neonatal rats that showed that chlorpyrifos exposure also caused deficits in signaling cascades (Song et al., 1997). For acute exposures, diminished signal transduction would protect against acetylcholine overstimulation which occurs with AChE inhibition.

In the current study, the four target genes involved in reducing neuronal signal transduction (CLIC4, GNA11B, CAMKII, and ADRA2C) were all upregulated in the hypersalinity day fourteen low chlorpyrifos treatment as compared to the freshwater

control. Only one gene, CLIC4, was also upregulated in the hypersalinity day fourteen fish that were not exposed to chlorpyrifos.

CLIC4 is thought to play a role in neuronal signal transduction by regulating fundamental cellular processes such as cell membrane potential, transepithelial transport, maintenance of intracellular pH, and regulation of cell volume (reviewed in Jentsch et al., 1999). In humans, CLIC4 can interact with YWHAZ, which belongs to a family of proteins that mediate signal transduction by binding to phosphoserine-containing proteins (Katanasaka et al., 2007).

Information on GNA11B is limited, however for zebrafish it is reported to be involved in adenylyl cyclase modulating G-protein couple receptor signaling pathways (Oka and Korsching, 2011). We hypothesize that this G-protein may function as an inhibitory G-protein, resulting in inhibition of adenylyl cyclase. Another inhibitory G protein, G protein alpha i1, was shown to be upregulated in brains of rainbow trout acclimated to hypersalinity so it would be expected that hypersalinity would also upregulate inhibitory G proteins in the current study (Maryoung et al., 2014).

ADRA2C is a member of the G protein coupled receptor superfamily. Specific information on this gene in relation to olfactory signal transduction was not available. However this gene is involved in G alpha (i) signaling events. The classical signaling process for G alpha (i) also involved the inhibition of cAMP dependent pathways (Simonds et al., 1989). We hypothesize that this gene can also play this role in olfactory signal transduction, such that upregulation of this gene, as was seen in the current study, could also result in diminished signal transduction.

CAMKII inhibits adenylyl cyclase through phosphorylation and was also induced in rainbow trout brains after acclimation to hypersalinity (Cooper, 2003, Maryoung et al., 2014). In combination with inhibitory G-proteins, significant impairment of neuronal and or olfactory signal transduction would likely occur.

To better assess how hypersaline acclimation and chlorpyrifos exposure affect more apical endpoints, behavioral assays were conducted following treatments with each stressor. Freshwater acclimated fish avoided L-serine seventy-five percent of the time in behavioral assays, which is consistent with previous studies (Rehnberg et al., 1985) and its hypothesized role as a predator cue (Idler et al., 1956). Freshwater acclimated fish exposed to the high concentration of chlorpyrifos failed to avoid L-serine; making no choice indicating susceptibility to predation. The negative impact of the high concentration of chlorpyrifos on fish behavior is consistent with previous studies showing that chlorpyrifos negatively impacts fish behavior, such as spontaneous swimming and feeding activity, at concentrations comparable to those in the current study (Sandahl et al., 2005, Tierney et al., 2007a).

Impacts of xenobiotics on olfaction can be classified into three main groups. Contaminants can cause hyposmia, a reduced ability to smell odorants, anosmia, a complete inability to smell, or dysosmia, in which olfactory information is not processed correctly. The most common impact on olfaction is hyposmia, with higher concentrations causing anosmia (reviewed in Tierney et al., 2010). Based on previous studies, it would be predicted that chlorpyrifos exposure would cause hyposmia (Sandahl et al., 2004). From the EOG section of the current study we saw that there was a trend towards a

concentration-dependent decrease in olfactory response for L-serine. After exposure to the high concentration of chlorpyrifos, hyposmia was observed in freshwater acclimated fish.

Exposure to the high concentration of chlorpyrifos altered the behavior of rainbow trout in both salinity treatments. However, the alternative choice was different between freshwater and hypersaline acclimated fish. As mentioned previously, freshwater acclimated fish exposed to the high concentration of chlorpyrifos had no preference for L-serine or avoiding the odorant. Fish acclimated to hypersalinity either for seven or fourteen days actually preferred L-serine. Thus, hypersaline acclimation combined with chlorpyrifos exposure could cause fish to be at an even greater risk of predation as they prefer the odorant from predators instead of avoiding it or not recognizing it. Behavioral assays appear to be more sensitive for hypersaline day seven fish as EOGs were not different in this group. This is consistent with a previous study that found behavioral responses were generally more sensitive than EOGs in pesticide-exposed fish (Tierney et al., 2007b). For hypersaline day fourteen fish, behavioral assays and EOG experiments seem comparable as both assays found differences after acclimation and exposure to chlorpyrifos.

Several anthropogenic agents have been shown to cause dysosmia. Giattina et al., (1982) found that fish switched between avoidance and preference after treatment with copper or nickel. Similarly, Saglio and Trijasse, (1998) found that the herbicides diuron and atrazine were able to alter the chemical perception of skin extract solutions in goldfish. Exposure to 4-nonylphenol disrupted shoaling behavior of banded killifish

(*Fundulus diaphanous*) (Ward et al., 2008). In addition, environmental stressors can also cause dysosmia, specifically in marine environments. Munday et al., (2009) found that orange clownfish (*Amphiprion percula*) reared in conditions simulating CO₂-induced ocean acidification became attracted to odorants signaling habitat and settlement sites normally avoided. Orange clownfish larvae also exhibited attraction to predator cues instead of avoidance under the same environmental conditions (Dixson et al., 2010). Cripps et al., (2011) found that predators are impacted by this scenario as well and that they shifted from preference to avoidance of injured prey when exposed to simulated ocean acidification conditions. The mechanism causing the switching between preference and avoidance in larvae reared in acidified water was reversed by blocking neurotransmitter receptor function, specifically GABA-A receptor (Nilsson et al., 2012).

It is possible that the dysosmia in the current study in hypersaline acclimated fish exposed to chlorpyrifos is also related to neurotransmitter receptor function. GABA-A receptors initiate rapid inhibition through increases in chloride ion conductance, while GABA-B receptors mediate slow inhibition by interacting with G proteins to inhibit adenylyl cyclase, inactivate voltage-dependent calcium channels, or activate potassium channels (reviewed in Chebib and Johnston, 1999). As acclimation to hypersalinity and exposure to chlorpyrifos were shown to cause differential gene expression of a chloride channel, as well as members of the adenylyl cyclase signaling pathway, it is possible that GABA receptors are also impacted upstream. However, further research needs to be conducted to examine this possibility.

In summary, acclimation to hypersaline conditions and exposure to chlorpyrifos impacts rainbow trout olfaction at the molecular, physiological, and behavioral levels. Genes involved in inhibiting olfactory signal transduction were upregulated after acclimation and exposure. Physiologically, response to odorants decreased after acclimation and exposure. Furthermore, combined exposure caused impaired behavioral response to a predator odorant. Overall, acclimation to hypersaline environments caused fish to be more susceptible to olfactory impairment from environmentally relevant concentrations of a common organophosphate pesticide.

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Chapter 5: Summary and General Conclusions

Most Pacific salmonids undergo smoltification and transition from freshwater to saltwater as part of their unique life cycle. Smoltification involves adjustments in color, shape, size, metabolism, catabolism, and osmotic and ion regulation (reviewed in Hoar, 1988). The molecular mechanisms underlying this transition are largely unknown. Furthermore, salmonid habitats can be impacted by salinization, which can also affect salmonid tolerance to anthropogenic stressors. Increased salinity can result from natural processes, as well as anthropogenic factors, such as saline runoff from agriculture and reduced snow-based freshwater inputs from climate change (reviewed in Cañedo-Argüelles et al., 2013; Knowles and Cayan, 2004). Increased salinity can co-occur with chemical insult, such as pesticide runoff. In the United States, pesticides and salinization both rank among the top 15 causes of impairment to streams (U.S. EPA, 2012b). Acclimation to hypersaline conditions enhances the acute toxicity of certain thioether organophosphate and carbamate pesticides in some species of euryhaline fish. However, sublethal impacts have been far less studied (reviewed in Schlenk and Lavado, 2011). The organophosphate chlorpyrifos (CPF) is of particular interest as it is commonly detected in salmonid habitats in Washington and California (Sargeant et al., 2013; Xuyang et al., 2012). The main goals of the current study were to identify molecular mechanisms involved in Pacific salmonid salinity acclimation, as well as to determine how acclimation to hypersaline conditions impacts acute and sublethal toxicity of the common organophosphate CPF.

To understand the underlying molecular mechanisms, a transcriptomics approach was employed. Coho salmon (*Oncorhynchus kisutch*) were acclimated to four different salinities (<0.5, 8, 16, and 32 ppt) and gene expression was assessed through microarray analysis of gill, liver and olfactory tissues. Gills were selected because the tissue is directly exposed to the aqueous environment and involved in osmotic regulation, liver was chosen as it plays a role in energetics needed for osmoregulation, and olfactory tissues were evaluated based on the proximity of the tissue to the environment, as well as its role in behavior. Also, many previous studies on euryhaline fish salinity acclimation have focused on changes in the gills and to our knowledge; this is the first study to examine the olfactory tissues. Liver had the highest number of differentially expressed genes at 1,616 when considering all salinity treatments. Gills had 1,074 differentially expressed genes and olfactory tissue had 924. The difference in the number of differentially expressed genes may be due to the higher responsiveness of liver to metabolic changes after salinity acclimation to provide energy to fuel other metabolic and osmoregulatory tissues like gills (Sangiao-Alvarellos et al., 2003).

Differentially expressed genes were tissue and salinity treatment dependent. There were no genes differentially expressed in all salinity treatments and all three tissues. There was no discernable salinity concentration dependent pattern in the number of differentially expressed genes. Five genes were targeted for microarray confirmation by qPCR and included CCAAT/enhancer binding protein β (CEBPB), calpain 1 (CAPN1), proto-oncogene, serine/threonine kinase (Pim1), aldolase B, fructose-bisphosphate

(aldob), and complement component 3 (c3). qPCR expression profiles of these genes matched array outputs.

Gene ontology (GO) term analysis revealed biological processes, molecular functions, and cellular components that were significant. Most terms were tissue dependent. For liver, oxygen binding and transport terms were discovered, suggesting possible impacts on metabolism. For gills, muscle and cytoskeleton related terms were emphasized and for olfactory tissues, immune response related genes were highlighted. Interaction networks were examined in combination with GO terms and determined similarities between tissues for potential osmosensors and signal transduction cascades. Overall the transcriptomics portion of our study suggests that Pacific salmonids share many salinity acclimation molecular mechanisms with other species, with a few new genes identified, and that although the three tissues shared certain underlying mechanisms, many of the differentially expressed genes were tissue-specific.

After establishing molecular mechanisms involved in salinity acclimation, impacts of hypersaline acclimation on acute toxicity of CPF were examined. In contrast to other previously examined pesticides, time to death by CPF was more rapid in freshwater than in hypersaline water (16 ppt). The median lethal time (LT_{50}) after 100 $\mu\text{g/L}$ chlorpyrifos exposure was 49 hr (95% CI: 31 – 78) and 120 hr (95% CI: 89 – 162) for rainbow trout (*Oncorhynchus mykiss*) in freshwater and those acclimated to hypersaline conditions, respectively.

Hypersaline acclimated fish in previous studies showed induction of xenobiotic metabolizing enzymes, such as cytochrome P450 3A27, that may detoxify CPF (Lavado

et al., 2009). However, CPF metabolism was unaltered in liver and gill microsomes of freshwater and hypersaline acclimated fish in the current study. Acetylcholinesterase inhibition in brain was also unchanged, as was bioavailability of chlorpyrifos from the aqueous exposure media. In contrast, mRNA expression of neurological targets: calcium calmodulin dependent protein kinase II delta, chloride intracellular channel 4, and G protein alpha i1 were upregulated in saltwater acclimated fish, consistent with diminished neuronal signaling. Upregulation of these genes may protect animals from cholinergic overload associated with acetylcholinesterase inhibition and explain the difference in time to death. These results indicate targets other than acetylcholinesterase may contribute to the altered toxicity of chlorpyrifos in salmonids under hypersaline conditions.

Although acute toxicity studies can provide vital mechanistic information, they typically lack environmental relevance. To understand environmentally realistic impacts, sublethal toxicity studies of the impacts of hypersaline acclimation on salmonid olfaction were conducted. Combined acclimation and exposure to CPF was shown to impact rainbow trout olfaction at the molecular, physiological, and behavioral levels. Concurrent exposure to hypersalinity and 0.5 µg/L CPF upregulated four genes (chloride intracellular channel 4, G protein *zgc:101761*, calcium calmodulin dependent protein kinase II delta, and adrenergic alpha 2C receptor) that inhibit olfactory signal transduction. At the physiological level, hypersalinity and chlorpyrifos caused a decrease in sensory response to the amino acid L-serine and the bile salt taurocholic acid. Combined acclimation and exposure also negatively impacted behavior and reduced the avoidance of a predator cue

(L-serine). Thus acclimation to hypersaline conditions and exposure to environmentally relevant concentrations of chlorpyrifos caused an inhibition of olfactory signal transduction leading to a decreased response to odorants and impairment of olfactory mediated behaviors.

In summary, salinity acclimation causes molecular changes in Pacific salmonids which can impact the acute and sublethal toxicity of certain compounds. These results aid in understanding the underlying molecular mechanisms involved during salmonid salinity acclimation, as well as how acclimation to hypersaline environments, as could be seen from rising sea levels, may impact these mechanisms. Additionally, this study highlights the importance of conducting both acute and sublethal studies. At the acute level of exposure, a potential underlying mechanism was identified, however overall hypersaline acclimation had a protective effect at these concentrations. The sublethal studies which employed environmentally realistic concentrations determined that although a similar mechanism may be at work, the overall effect of hypersaline acclimation was a decrease in olfactory response. If only the acute studies had been conducted, the potential environmental impacts would likely have been missed. Also, many other studies on impacts of contaminants on olfaction have focused on one or two levels of effect. This study included molecular, physiological, and behavioral endpoints to identify an adverse outcome pathway for this type of combined salinity acclimation and contaminant exposure. Risk assessment strategies evaluating compounds of this nature in estuarine environments and freshwater environments that may be altered by hypersaline stress can benefit from this data.

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