AN ABSTRACT OF THE DISSERTATION OF

Jason Sandahl for the degree of Doctor of Philosophy in Toxicology presented on September 17, 2003.

Title: Biochemical and Physiological Indicators of Behavioral Impairment in Salmonids Exposed to Chlorpyrifos and Copper.

Abstract approved Redacted for privacy

Jeffrey J. Jenkins

The purpose of this work was to determine if environmentally-relevant concentrations of chlorpyrifos and copper, two commonly detected chemicals in Western surface waters, can negatively impact the biological health of salmonids. Both compounds are highly neurotoxic to fish, but each with distinct biological target sites and mechanisms of action. We used common biochemical and physiological indicators of toxicity, and correlated these effects with potentially significant behavioral alterations. For chlorpyrifos, the mechanism of toxic action is the inhibition of acetylcholinesterase (AChE) throughout the peripheral and central nervous system. Here, we conducted biochemical assays of AChE activity in brain and muscle tissues after exposing steelhead trout (*Oncorhynchus mykiss*) and coho salmon (*O. kisutch*) to chlorpyrifos for 96 hours. We then correlated the AChE inhibition with behavioral impairment in swimming and feeding activities. In juvenile steelhead and coho exposed to 0.6–2.5 μg/L chlorpyrifos, AChE activity was inhibited between ~10–65%. This biochemical indicator was significantly correlated with changes in behavioral patterns. Spontaneous swimming rates were reduced ~30–80% in the exposed fish, and strikes at food items (brine shrimp) were reduced ~10–70%. For copper and some other neurotoxicants, the olfactory nervous system is a sensitive target site in fish. The highly-developed olfactory system in salmonids is particularly susceptible to toxic insult by dissolved chemicals since receptor neurons are in direct contact with the aquatic environment. Here, we used electrophysiological techniques to record odor-

evoked responses from the sensory epithelium and the olfactory bulb as direct measures of olfactory function in juvenile coho salmon. In fish exposed to copper, chlorpyrifos, or esfenvalerate for 7 days, field potentials recorded from the sensory epithelium and the olfactory bulb showed reduced or obscured olfactory responses to two classes of odorants, which activate non-overlapping populations of receptor neurons. To determine if this reduced sensory input can subsequently alter or diminish olfactory-mediated predator avoidance behaviors, paired physiological and behavioral tests were conducted on juvenile coho exposed to copper. In fish exposed to 2–20 μg/L copper for 3 hours, olfactory sensitivity was reduced by ~50–90%. When these fish were presented with a predatory alarm cue (conspecific skin extract), fish with reduced olfactory function increasingly failed to exhibit antipredator behavior. In the following experiments, we show that chlorpyrifos and copper can impair the biochemical and physiological biology of salmonids at environmentally-relevant concentrations, and that these sublethal effects can alter potentially important behavioral patterns.

Biochemical and Physiological Indicators of Behavioral Impairment in Salmonids Exposed to Chlorpyrifos and Copper

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Jeffrey Jenkins provided oversight of all projects. Nathaniel Scholz was responsible for coordinating experiments, advising on experimental design, and contributing substantial editorial remarks to Chapters 3–5. David Baldwin provided technical expertise in the set-up of electro-physiological equipment, digital imaging instruments, and computerized data acquisition for Chapters 3–5. He also assisted in experimental designs, data analysis, and statistical interpretation of results.

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CHAPTER 1

GENERAL INTRODUCTION

Salmon and pesticides in the current public and regulatory scope

In recent years, the potential effects of pesticides on salmon have received a considerable amount of attention from the public, media, and government. Many salmon and steelhead (*Oncorhynchus* sp.) runs in the Pacific Northwest USA are at precariously low population levels, with 12 significant evolutionary units recently listed as threatened or endangered by the Federal government under the Endangered Species Act (National Marine Fisheries Service 2000). A report released by the General Accounting Organization in August, 2002, estimated that over 3.3 billion dollars have been spent over the past two decades (1.8 billion since 1997) to help restore degraded fish habitats, improve fish passage around dams, and develop strategies to protect the remaining viable populations of anadromous salmonids (U.S. General Accounting Office 2002). This report concluded, however, that expenditures have not helped to increase populations of at-risk species. According to the National Marine Fisheries Service, in addition to physical disturbances that have destroyed salmonid habitats (e.g., dam construction, channelization, sedimentation, removal of riparian cover), water pollution is a major unknown and possible limiting factor with respect to salmonid recovery efforts (National Marine Fisheries Service 1996, 1998). Contaminants such as current-use pesticides, heavy metals, fertilizers, and petroleum hydrocarbons are commonly discharged into rivers, lakes, and estuaries via urban and agricultural runoff (U.S. Geological Survey 1999a, Pew Oceans Commission 2001, 2003). The most serious water quality problems occur in regions with high rates of human population growth, such as the Pacific Northwest where the population is projected to increase by 50% over the next 25 years

(U.S. Census Bureau 2000). In these high growth geographic areas, there has been little or no progress in controlling non-point source contamination in surface waters over the past few decades (Pew Oceans Commission 2003). In a report to Congress, the U.S. Environmental Protection Agency concluded that non-point source pollution is the leading cause of degraded water quality in the United States (U.S. Environmental Protection Agency 2000).

In response to the continuing decline of salmonid populations in the Pacific Northwest, a few activist organizations have emerged to become influential forces in salmon protection issues (e.g., Washington Toxics Coalition, Northwest Coalition for Alternatives to Pesticides, Pacific Coast Federation of Fishermen's Associations, and Earthjustice). These organizations have combined their resources, and have obtained sufficient scientific and legal knowledge to challenge regulatory laws of the Local, State, and Federal government. At the foundation of their major challenges lie two Acts of Legislation; the Federal Insecticide, Fungicide and Rodenticide Act, and the Endangered Species Act.

As a requirement of the former Act, the U.S. Environmental Protection Agency must approve pesticides before they are sold or used in the United States. Part of this process involves estimating the potential for pesticides to move into the environment, based on modeled parameters that include use patterns, application rates, soil types, and the chemical properties of the pesticide, to name a few. These estimated exposure concentrations are then compared to toxicity values obtained from animal exposure tests, in order to determine the likelihood of risk, often expressed as a risk quotient. For aquatic species, toxicity values are obtained from a range of test organisms, which are intended to be representative of a broader distribution of species in the natural system. Based on the species toxicity distribution, sensitive organisms can be identified, and exposure standards established to protect similarly sensitive species.

For cold water fish species (which include salmon and steelhead), the representative species is often the rainbow trout (*O. mykiss*). Toxicological testing requirements do not typically include studies using special-status fish species, or testing for sublethal effects that may be relevant to specific biological requirements of fish. In considering potential

sublethal risks to endangered fish species in risk assessments, the U.S. Environmental Protection Agency has recommended to use the $1/20^{th}$ LC_{50} (acute lethality value) obtained from a representative species (U.S. Environmental Protection Agency 1986). If chemical exposures are below this level, it is "presumed that there will be minimal risk to endangered aquatic organisms." If exposures exceed this level, it "presumes there will be a risk to endangered species and, thus, a formal consultation with the [Office of Endangered Species] is initiated" (U.S. Environmental Protection Agency 1986; pg.8). Although this guideline document is nearly two decades old, the application of the $1/20^{th}$ LC_{50} for considering endangered species in risk assessments is still practiced (e.g. U.S. Environmental Protection Agency 1999a, Erickson and Turner 2003). However, activist organizations are challenging the U.S. Environmental Protection Agency's policies, citing a host of scientific literature suggesting that pesticides and other contaminants pose major risks to the sublethal ecological health of salmonids at much lower exposure levels (Ewing 1999).

Under the Endangered Species Act, federal agencies are required by law to ensure that any action they fund, authorize, or carry out will not risk the continued survival of a listed species or result in the adverse modification or destruction of its critical habitat (16 U.S.C. 1536 (a)(2)). If there is potential risk, the agency conducting the action must consult with a relevant expert agency on measures to eliminate or mitigate detrimental impacts. As argued by activist organizations, in the case of pesticide discharges into surface waters, the U.S. Environmental Protection Agency must consult with the National Marine Fisheries Service (newly named the National Oceanic and Atmospheric Administration Fisheries Service) to determine if current pesticide regulations "harm" endangered fish.

In issuing its salmon protection rules in June, 2000, the National Marine Fisheries Service concluded that, "concentrations of pesticides [in surface waters] may affect salmonid behavior and reproductive success. Current [Environmental Protection Agency] label requirements were developed in the absence of information about some of these subtle but real impacts on aquatic species such as salmonids" (National Marine Fisheries Service 2000). In July, 2002, a U.S. District Court in Seattle, WA, found the U.S.

Environmental Protection Agency was not in compliance with the Endangered Species Act with respect to pesticides, and ordered the timely evaluation of 54 current-use pesticides (many of which are organophosphate and carbamates, including chlorpyrifos), concerning their effects on the biological requirements of Pacific salmon (U.S. District Court 2002). These effects include sublethal alterations that can potentially affect the survival, migratory success, or reproductive success of special-status fish. More recently, in July, 2003, the U.S. District Court issued temporary restrictions on pesticide applications near salmon-bearing streams in Oregon and Washington states. These include 20 yard buffer zones for ground-based pesticide applications, and 100 yard buffers for aerial applications (amendment to U.S. District Court 2002). The U.S. Department of Agriculture estimated that the implementation of such buffers zones could result in more than 100 million dollars in lost agricultural crop revenues if this restriction includes all 54 named pesticides (Bernton 2003).

Contaminants detected in salmon-bearing surface waters

Over the past decade, the U.S. Geological Survey has assessed several major river basins in the United States for the occurrence and distribution of pesticides and trace metal contaminants. These documents are frequently cited in the current scientific literature, as they are the most thorough and comprehensive scientific data available describing the present state of the aquatic environment. Examples of surveyed water systems in the West include the Sacramento River Basin (Domagalski et al. 2000) and San Juaquin River Basin (Panshin et al. 1998) in California, the Willamette River Basin (Anderson et al. 1996, Anderson et al. 1997) in Oregon, and the Yakima River Basin (Ebbert and Embrey 2002, Hughes 2003) and Puget Sound Basins (U.S. Geological Survey 1999b, King County 2002) in Washington. The findings of these reports have concerned some environmentalists, ecological scientists, and government because of the frequent and widespread detections of highly toxic pesticides and heavy metals in the environment. However, concentrations of these chemicals are typically exceedingly low—near or below the detection capabilities of the analytical instruments (often 1–10 parts-per-trillion). On occasion, though, concentrations have exceeded state and federal

water quality standards that are intended to protect aquatic life, raising concerns with respect to potential sublethal risks to special-status fish species.

Of the detected pesticides, there is a primary concern for the organophosphate and carbamate insecticides, as these compounds are particularly neurotoxic to salmonids. Some examples of detected concentrations in river basins of California, Oregon, and Washington states are shown in Table 1.1. Some compounds included are azinphos-methyl, carbaryl, carbofuran, chlorpyrifos, diazinon, and malathion. These insecticides are primarily used to control crop pests in agricultural regions, and some are used by homeowners and commercial landscapers in urban areas (Anderson et al. 1996).

Table 1.1. Frequencies and concentrations of selected organophosphate and carbamate detections in some western United States river basins. Concentrations are in μg/L.

River Basin	chemical	detection frequency	90th percentile	maximum
San Juaquin Basin[a]	azinphos-methyl	9%	0.11	0.39
	carbaryl	23%	0.012	5.2
	carbofuran	13%	0.023	0.097
	chlorpyrifos	64%	0.048	0.26
	diazinon	76%	0.56	3.8
	fonofos	13%	0.062	0.26
	malathion	9%	0.044	0.39
Willamette Basin[b]	carbaryl	13%	0.027	0.11
	carbofuran	4%	<detection	0.084
	chlorpyrifos	14%	0.009	3.3
	diazinon	26%	0.031	0.31
	malathion	1%	<detection	0.03
Yakima Basin[c]	azinphos-methyl	65%	0.078	0.52
	carbaryl	67%	0.097	4.8
	chlorpyrifos	11%	0.004	0.007
	diazinon	16%	0.013	0.17
	malathion	27%	0.008	0.037
Puget Sound Basin[d]	carbaryl	69%		0.21
	carbofuran	31%		0.23
	diazinon	100%		0.57

[a] Panshin et al. 1998.
[b] Anderson et al. 1997.
[c] Ebbert and Embrey 2002.
[d] King County 2002.

In the following experiments of Chapters 2 and 3, chlorpyrifos was selected for investigation because it is one of the most widely-used insecticides throughout the United States, is one of the most frequently detected organophosphates in surface waters, is extremely toxic to fish, and the timing of its application in the Pacific Northwest on fruit crops coincides with the early life stages of salmon and steelhead.

Copper is also a contaminant of concern, although no lawsuits are pending on its use as a pesticide at this time. Copper is a major component of storm-water pollution, originating from both urban and agricultural landscapes. It is one of the most frequently detected heavy metals in surface waters in the western United States (U.S. Geological Survey 1999a, U.S. Environmental Protection Agency 2000). From these landscapes, copper leaches from various non-point sources including roofing and flashing materials, wood preservatives, and vehicle brake linings and clutch plates (Snohomish County 2002). It is also applied as an algaecide and fungicide on agricultural crops, and used to control algae in waterways (Snohomish County 2002). Examples of detected copper in Western surface waters are shown in Table 1.2.

Table 1.2. Levels of copper detected in some western United States surface waters. Concentrations are in μg/L.

surface water	mean[1] or median[2]	maximum
Willamette River Basin[a]	2.2[2]	21
Puget Sound Tributaries[b]	5.2.0–13.7[1]	70–160
King County Small Streams[c]	0.5–2.7[1]	
Yakima River Basin[d]	2.1[(75th percentile)]	9.1

[a] Anderson et al. 1996.
[b] Snohomish County 2002.
[c] King County 2002.
[d] Hughes 2003.

The presence of copper in surface waters is a prominent indicator of degraded environmental conditions (U.S. Environmental Protection Agency 2000), and dissolved copper is extremely toxic to salmonids (Marr et al. 1995, Hansen et al. 2002). Particularly, copper targets the olfactory nervous system of fish, a sensory function that underlies numerous ecologically-important behaviors in salmonids (reviewed in Klaprat et al. 1992). For these reasons, copper was also selected as a test compound in the following experiments within Chapters 4 and 5.

Toxicity of chlorpyrifos and copper to salmonids

Chlorpyrifos, as well as other organophosphate and carbamate insecticides, is a potent inhibitor of the acetylcholinesterase (AChE) enzyme (reviewed in Chambers 1992). Acetylcholinesterase is widely expressed throughout the central and peripheral nervous system of fish (Zinkl et al. 1991). Its primary role is to modulate electrical signaling at nerve terminals by rapidly metabolizing the neurotransmitter molecule acetylcholine after its release into the synaptic cleft (Massoulié et al. 1993). Inhibition of AChE prolongs the residence time of acetylcholine at cholinergic and muscarinic receptors—receptors that control neuro-muscular coordination, sensory systems, central processing, and autonomic homeostasis—resulting in excessive nervous stimulation. In skeletal muscle, persistent depolarization of the motor end-plate can result in muscular spasms, but eventually can develop into tetanus or paralysis (Taylor and Brown 1989). Toxicity within central networks can affect both autonomic and somatic systems, and responses can be excitatory or inhibitory, depending on the tissue. In fish, reported symptoms related to anti-cholinesterase exposures include twitching of pectoral fins and opercula, muscular rigidity, paralysis, altered social patterns, and loss of swimming control (reviewed in Zinkl 1991). For the rainbow trout, chlorpyrifos is acutely lethal (96-h LC_{50}) in the range of 6.0–9.4 µg/L (Macek et al. 1969, Holcombe et al. 1982).

For copper, acute toxicity in fish is primarily associated with cellular damage to the gills. Copper specifically binds to the sodium channels of gill membranes, disrupting ionic balance (Marr et al. 1995). Depending on the water chemistry, copper is acutely lethal (96-h LC_{50}) to juvenile salmonids in the range of 30-50 µg/L (Hansen et al. 2002).

However, more sensitive to toxic insult by dissolved copper in fish is the olfactory epithelium. The olfactory epithelium is directly exposed to the aquatic environment, and also contains protein structures that strongly complex with copper ions (Rhenberg and Schreck 1986, Klaprat et al. 1992). Examination of olfactory neurons exposed to copper have revealed a loss of receptor cilia and microvilli, ruptured plasma membranes, and swollen mitochondria (Moran et al. 1992, Juliard et al. 1996, Hansen et al. 1999a). At higher exposure concentrations or longer durations, this can eventually leads to gross necrosis of receptor cells, requiring synthesis of new neurons (Juliard et al. 1996).

Sublethal toxic effects of chlorpyrifos and copper to salmonids

Although concentrations of chlorpyrifos and copper rarely reach levels that are overtly toxic to salmon and steelhead in the environment, there is considerable scientific uncertainty as to whether these compounds can impact other important aspects of biology at relevant exposure levels. To estimate the potential risks of contaminants to organisms in the environment, biological markers or indicators are often used to evaluate toxic stress. These biological markers or indicators are typically measured at or below the scale of the individual (e.g., biochemistry, physiology), and are intended to be predictive of toxicological effects at higher scales of biological order (e.g., individual behavior, group organization, natural populations). However, a common (and justified) criticism of using such biochemical or physiological measures as an index of overall health is that they do not always have clear biological or ecological relevance (Little et al. 1990). In lay terms, these are the "so what?" questions. For example, what is the significance of a fish losing 10 or 20% AChE activity due to chlorpyrifos exposure? What does it mean for a fish to lose 10 or 20% olfactory sensitivity? Is it significant? These are worthwhile questions to pursue, especially in the context of the current political landscape regarding the sublethal impacts of pesticides on endangered salmon and steelhead.

A commonly used biological indicator of organophosphate and carbamate exposure in fish is the measurement of AChE activity in brain and muscle tissues (reviewed in Zinkl et al. 1991, also reviewed here in Chapter 2). Behavioral indicators of toxic stress have included alterations in swimming and feeding patterns (reviewed in Little and Finger

1990, also reviewed here in Chapter 3), but few studies have attempted to directly correlate AChE inhibitions to these impaired behaviors. In studies that have attempted to establish relationships, the levels of AChE inhibition required to alter behavioral patterns often exceeded 50% or more (e.g., Post and Leasure 1974, Cripe et al. 1984). Not many studies have attempted to correlate low-level (5–20%) AChE inhibition—effect levels more likely to occur in fish in natural systems—with behavioral effects that are potentially meaningful ecologically.

In a comparison of the relative toxicity of various organophosphate compounds on sublethal endpoints in fish, the effects on three aspects of biological health for salmonids are assessed below (Table 1.3). These three aspects, termed "*assessment endpoints*", reflect important life-history stages of Pacific salmon, and include survival, rearing and migration, and reproductive success (defined in; U.S. Environmental Protection Agency 1998, Washington State Pesticide/ESA Task Force 2001). Effects on these endpoints may be expected to reduce the viability or genetic integrity of natural salmonid populations. The attributes of the assessment endpoints are termed "*assessment measures*", which are defined by a "*quantifiable measure*". For example, reduced swimming speed (quantifiable measure) can increase susceptibility to predation (assessment measure) which can ultimately threaten survival (assessment endpoint). Since $1/20^{th}$ of the acute lethality (LC_{50}) value has been presumed to be protective of sublethal effects in endangered fish species, it has been included here as a sublethal "*survival*" endpoint. It is realized that the categories used here are somewhat broad, but regardless of the terms used, these are representative of the reported sublethal effects of organophosphate toxicity for fish. Almost no studies exist for the effects of chlorpyrifos on salmonid species. It should be noted that similar approaches to sublethal risk evaluation are currently being used by the National Oceanic and Atmospheric Administration Fisheries Service in issuing biological opinions for proposed State and Federal pesticide application projects in Oregon and Washington states.

Table 1.3. Comparative sublethal effects of organophosphates in fish.

Assessment Endpoints	Assessment Measures	Species	Chemical	LOEC[a] (µg/L)	Reference
Survival					
mortality	1/20 LC_{50}[b]	rainbow trout	chlorpyrifos	0.3	Holcombe et al. 1982
mortality	1/20 LC_{50}[b]	rainbow trout	chlorpyrifos	0.46	Macek et al. 1969
mortality	1/20 LC_{50}[b]	rainbow trout cutthroat trout lake trout	diazinon	4.5 85 40	Johnson and Finley 1980
mortality	1/20 LC_{50}[b]	brook trout	diazinon	37.5	Allison and Hermanutz 1977
mortality	1/20 LC_{50}[b]	rainbow trout	dimethoate	310	EPA 1999b
predation	olfactory anti-predatory response (food strikes, activity)	chinook salmon	diazinon	1.0	Scholz et al. 2000
predation	swimming (distance, speed, turning rate, tortuosity of path)	rainbow trout	diazinon	250	Beauvais et al. 2000
predation	swimming (erratic pattern)	European eel	diazinon	160	Ferrando et al. 1991
predation	swimming (erratic pattern)	European eel	chlorpyrifos	1290	Ferrando et al. 1991
predation	survival (predation by Large mouth bass)	rainbow trout	methyl parathion	10	Little et al. 1990
growth	foraging (prey ingestion)	Atlantic salmon	fenitrothion	6.0	Morgan and Kiceniuk 1991
Rearing and Migration					
holding	spontaneous swimming	rainbow trout	methyl-parathion	10	Little et al. 1990
holding	position holding (swimming stamina)	brook trout rainbow trout coho salmon	malathion	90 112 200	Post and Leasure 1974
upstream return	homing (number returning to hatchery)	chinook salmon	diazinon	10	Scholz et al. 2000
holding	territory defense (location)	Atlantic salmon	fenitrothion	100	Symons 1973
holding	territory defense (agonistic behaviors)	coho salmon	fenitrothion	100	Bull and McInerney 1974
Reproduction					
mating	detection of mate (electrophysiology)	Atlantic salmon	diazinon	1.0	Moore and Waring 1996
physiology	biological stimulation (hormone production, expressible milt)	Atlantic salmon	diazinon	0.3	Moore and Waring 1996

[a] Lowest-observed-effect-concentration. Exposure times vary between studies.
[b] Adjusted from the original reported value.
[c] EPA; U.S. Environmental Protection Agency.

For evaluating the sublethal impacts of copper on fish, electrophysiological measures obtained from the olfactory nervous system provide a reliable indication of neurotoxicity (reviewed here in Chapter 4). A well-established technique used to measure the impacts of contaminants on fish olfaction is the electro-olfactogram, or EOG (Hara 1992). The EOG records the active properties of receptor neurons, using an electrode positioned above the surface of the sensory epithelium in the olfactory chamber. The magnitude of the EOG response reflects the summated electrical generator potential of receptor neurons after they bind odorant molecules, providing a direct physiological measure of olfactory function in intact fish (Hara 1982). The electro-encephalogram, or EEG, is another electrophysiological technique, which measures oscillatory field potentials from the surface of the olfactory bulb in the forebrain. Previous work has demonstrated that copper and other neurological toxicants can diminish the sensitivity of peripheral olfactory neurons (Waring and Moore 1997), and presumably reduce odor-evoked electrical signals reaching the olfactory bulb (e.g., Hara et al. 1976, Moore and Waring 1996, Hansen et al. 1999a). Other studies have indicated that copper can also disrupt olfactory-mediated behaviors (Rhenberg and Schreck 1986, Hansen et al. 1999b, also reviewed here in Chapter 5). To our knowledge, no studies have attempted to directly link peripheral reductions in odor-evoked EOG responses with reductions in signals reaching the central networks, as measured by the EEG. In addition, no studies have attempted to directly correlate specific degrees of olfactory impairment to deficiencies in potentially important ecological behaviors.

Overall aim of current research

The overall aim of this research was to determine if chlorpyrifos and copper, at environmentally-realistic exposure concentrations, can alter behaviors that may be ecologically significant for survival in salmonids. Importantly, we attempted to correlate these behavioral effects with laboratory-based measures that could potentially be predictive indicators of impaired higher-order processes. The following are four experiments that 1) establish brain AChE as a sensitive biochemical indicator of chlorpyrifos exposure, 2) correlate inhibitions of brain AChE activity to altered

swimming and feeding behaviors, 3) determine the relative thresholds of contaminant-induced olfactory toxicity for three chemicals (copper, chlorpyrifos, esfenvalerate), using the EOG and EEG as direct measures of olfactory function, and 4) demonstrate that copper-induced olfactory impairment correlates with a failure to initiate olfactory-mediated antipredator behaviors.

CHAPTER 2

Pacific Steelhead (*Oncorhynchus mykiss*) Exposed to Chlorpyrifos: Benchmark Concentration Estimates for Acetylcholinesterase Inhibition.

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ABSTRACT

Steelhead trout (*Oncorhynchus mykiss*) were exposed for 96 h to the organophosphate chlorpyrifos to establish benchmark concentration (BMC) values in the low-effect range of brain acetylcholinesterase (AChE) inhibition. The U.S. Environmental Protection Agency (U.S. EPA) Benchmark Dose Software was used to model the data. BMCs were determined for a range of inhibition levels at 5%, 10%, 20%; 1 and 2 control standard deviations (SD); and at an experimental limit-of-detection level of 2.5%. One contributing difficulty in establishing precise inhibition BMCs in the lower-effect region is the variability associated with the AChE analytical method. To minimize this variability, the Ellman method was modified specifically for analysis of *O. mykiss* brain tissue. Laboratory-established BMCs for chlorpyrifos were then compared to the U.S. EPA 96-h water quality criteria and to the concentration levels detected in Northwest surface waters that are home to threatened steelhead trout. The U.S. EPA 96-h water quality criteria of 0.083 μg/L is below the $BMC_{02.5}$, the limit-of-detection value for this study. The average chlorpyrifos concentration detected during a two-week period in one monitored stream was 0.127 μg/L, which approaches the $BMC_{02.5}$. The peak chlorpyrifos concentration detected at 0.482 μg/L is near the BMC_{1SD} estimate.

INTRODUCTION

Certain pesticides have been detected in Northwest surface waters that provide migratory, spawning, and rearing habitat for wild salmonids (*Oncorhynchus* sp.) (Wentz et al. 1998). There is concern that sublethal exposures to these insecticides may harm endangered or threatened fish species. Organophosphorous (OP) insecticides are of particular concern due to their high exposure potential (based on quantity used and timing of application) and their high toxicity to salmonids.

The Hood River Basin is located in northern Oregon, USA between Mt Hood and the Columbia River. This area contains numerous pear and apple orchards, and growers apply chlorpyrifos extensively for scale and leaf roller control during March and April. An Oregon Department of Environmental Quality (ODEQ) study detected chlorpyrifos in the Hood River main channels and three of its tributaries (Foster, E.P., ODEQ 1999, unpublished report), which are habitat to threatened steelhead trout. Steelhead are the anadromous form of rainbow trout (*O. mykiss*), belonging to the family *Salmonidae*, and are similar to some Pacific salmon in their life cycle and ecological requirements. They are born in fresh water streams where they spend one to three years before migrating to the ocean. After one to four growing seasons in the ocean, steelhead return to their native stream to spawn.

In the spring of 1999, average chlorpyrifos concentrations at monitoring sites on six Hood River tributaries ranged from 0–0.127 µg/L, with one peak occurring at 0.482 µg/L. The U.S. Environmental Protection Agency 96-h water quality criterion for chlorpyrifos is 0.041 µg/L, and the 96-h chlorpyrifos median lethal concentration (LC_{50}) for *O. mykiss* ranges from 6.0–9.4 µg/L (Macek et al. 1969, Holcombe et al. 1982). It is uncertain to what degree environmental levels of chlorpyrifos affect acetylcholinesterase (AChE) activity of the steelhead brain, and ultimately whether this AChE loss affects the health of the fish.

Measurement of brain AChE activity is commonly used as an indicator of OP exposures for fish (Zinkl et al. 1991). The critical and common effect of most OP pesticides is the inhibition of AChE (reviewed in Chambers 1992). The function of AChE

is to hydrolyze the neurotransmitter acetylcholine (ACh) at synaptic junctions, terminating nervous stimulation. Inhibition of AChE is followed by accumulation of ACh and results in toxicity due to continuous stimulation of cholinergic receptors throughout the central and peripheral nervous system. Defining an abnormal AChE depression after exposure is difficult, regardless of species (Hill 1988, Grue et al. 1991). Individual variation of enzyme levels within a population, natural biological fluctuation, and the fact that some fish may tolerate enzyme depression with no apparent signs of toxic effect contribute to the difficulty in establishing a critical level of AChE depression (Christenson et al. 1994). As a result, there has been little consistency in the way AChE activity is evaluated in the literature. Relative departures for reported adverse AChE inhibition have included 10%, 20%, 1 SD, or 2 SD deviations from control mean activity, as well as no-observable-effect-levels (NOELs) and lowest-observable-effect-levels (LOELs). A need to standardize data reporting has been emphasized (Mineau and Peakall 1987, Hill 1988, Fleming et al. 1992). Grue et al. (1991) address some of the obstacles associated with defining abnormal AChE depression and provide recommendations for future study designs and data presentation.

Accurately reporting low-level (0–20%) AChE inhibition is challenging. Fulton and Key (2001) note that given the variability of individual AChE levels, a depression of >13% is often necessary to be indicative of OP exposure. More commonly in the literature, >20% is usually necessary to distinguish a depression of enzymatic activity with statistical confidence. When assessing cholinesterase activity, it is important to carefully consider methodological issues that may affect the accuracy and variability of the data (U.S. Environmental Protection Agency 2001). Sources of variability are well documented in the literature. These include age and size of the animal (Zinkl et al. 1987, Sturm et al. 1999), environmental conditions, stress, type and region of tissue sampled (Rattner and Fairbrother 1991, Sturm et al. 1999), individual and species variations (Hill 1988, Rattner and Fairbrother 1991), sample collection and storage (Zinkl et al. 1987), tissue preparation, and laboratory methodology (Fleming et al. 1992, Christenson et al. 1994, Wilson et al. 1996). Several methods are used to measure AChE activity including titrimetric, electrometric, radiometric, gasometric, fluorimetric, immunochemical, and

colormetric assays. The colorimetric method is the most commonly used procedure and is based on the Ellman reaction (Ellman et al. 1961).

The benchmark concentration (BMC) approach is one way to provide comparative data in the lower region of a dose-response curve for standardized reporting (U.S. Environmental Protection Agency 1995, 2001). The BMC is the estimated concentration that produces a specific change in response relative to controls. The corresponding lower-limit of a 95% confidence interval on the BMC is referred to as the BMCL. For example, a BMC_{10} could be the central concentration estimate corresponding to a 10% AChE inhibition, and the $BMCL_{10}$ the lower limit of a 95% confidence interval of the data fit.

The BMC approach has several advantages over the traditional NOEL/LOEL approach (Crump 1984, Crump 1995, U.S. Environmental Protection Agency 1995 and 1996, Faustman 1996, Clewell et al. 1997). These include the fact that the NOEL is limited to being one of the exposure levels of the study and is dependent on exposure selection and spacing. The NOEL does not account for data variability, and, therefore, no confidence interval can be associated with the estimate. A study with fewer animals is likely to have a higher NOEL than a study with more animals. In addition, continuum responses, such as enzymatic activity, may have no threshold (or NOEL) value. When using the NOEL/LOEL approach, the slope and shape of the dose-response curve is ignored, losing valuable toxicity information. A regression-based approach is an alternative for estimating low-range toxic effects (Crump 1995, Moore and Caux 1997). The BMC involves fitting the data to a model equation, then interpolating or extrapolating to the effect level of interest. The current U.S. Environmental Protection Agency benchmark dose software (BMDS) offers four different models that are appropriate for the analysis of continuous data: linear, polynomial, power, and Hill.

The objectives of this study were to optimize the Ellman method specific to *O. mykiss* brain tissue to reduce variability associated with the AChE assay, determine the natural distribution of AChE activity in non-exposed juvenile steelhead trout, and determine if chlorpyrifos concentrations found in Hood River surface waters are likely to affect brain AChE activity in resident juvenile steelhead, using BMC estimates based on laboratory exposures.

MATERIALS AND METHODS

Test system

Analytical grade chlorpyrifos (99.2% purity) was purchased from Chem Service, Inc. (West Chester, PA, USA). Test solutions were prepared in 5 ml acetone with acetone blanks for control groups. Exposures were static and conducted in fiberglass tanks filled with 1500 L hatchery spring water. Water temperature (± standard deviation) was maintained at 12 ± 1°C, pH 7.9 ± 0.1, dissolved oxygen 9.9 ± 0.3 mg/L, and hardness 44.5 ± 0.5 mg/L as $CaCO_3$.

Samples were collected from tanks at the beginning of each exposure and analyzed to confirm initial nominal chlorpyrifos values. Due to the large volume of water used, little chlorpyrifos loss was anticipated over the test period. Chlorpyrifos analyses were conducted using a Hewlett-Packard 6890 gas chromatograph (Avondale, PA, USA) coupled with a 5972A mass selective detector (GC/MS). Chemical extraction followed a modified in-vial elution protocol (Runes et al. 1999). Samples were collected on 25-mm C_8 bonded-phase silica Empore disks (Varian, Sugarland, TX, USA) by vacuum manifold, air dried, and placed directly into the GC/MS sample vial. Ethyl acetate was then added in-vial to elute the chlorpyrifos and allowed 5 h to equilibrate before analysis. Initial GC oven temperature was held for 1 min at 90°C, increased 15°C/min to a final temperature of 240°C, and held 3 min. The MS operated under selective ion monitoring (*m/z* 197, 199, 314) at 280°C. Laboratory extraction recoveries ranged from 86–100% and field recoveries 74–89% of nominal value. Nominal values were used in the BMC modeling.

Tests were conducted with steelhead trout at Oak Springs Hatchery (Maupin, OR, USA), source of the Hood River's stocked fish. Fish size (± standard deviation) averaged 7.6 ± 0.8 cm, and 3.8 ± 1.3 g. Feeding was stopped two days prior to tests. Chlorpyrifos solutions were mixed thoroughly in tanks before introducing fish. The test consisted of five evenly spaced exposure levels of 0, 0.625, 1.25, 1.875, and 2.50 µg/L replicated in triplicate (n = 60 total fish per exposure group), with the goal of producing AChE inhibition levels in the range of 0–20%. After the 96-h exposures, fish were anesthetized

in MS-222 and immediately frozen whole at -80°C until brain extraction and AChE analysis.

AChE assay optimization

Development of the *O. mykiss* brain AChE assay followed the methods of Ellman et al. (1961) and optimization procedures of Habig et al. (1988). Reagent preparation, protocol adaptation to the 96-well plate, and AChE activity calculations followed the recommendations of Padilla et al. (1998). Measurements were performed on a SpectraMax Plus Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA), and all reagents purchased from Sigma Chemical Co. (St. Louis, MO, USA). Samples were run in triplicate micro-plate wells, and included tissue and substrate blanks. Change in absorbance at 412 nm was measured at 12-s intervals for 10 min and measured as µmol substrate hydrolyzed/min/g tissue. All tests were replicated in triplicate, using five pooled fish brains per replicate. The method development investigated the effects of buffer type, pH, reagent concentration, temperature, and specific cholinesterase inhibitors. The conditions and ranges tested were selected from methods reported throughout the literature for fish AChE analysis. Testing was performed in the following order:

Buffer type and pH: Four buffer types, 0.025 and 0.05 M Tris buffer, 0.01 and 0.1 M sodium phosphate buffer, were compared over the pH range 6.5–9.0. Trizma hydrochloride (7.88 g/L ddH_20) and Trizma base (6.01 g/L ddH_20) stocks were mixed to prepare 0.05 M Tris buffers to appropriate pH, and 0.025 M Tris buffers were made by a 1:2 dilution. Monobasic (27.6 g/L ddH_20) and dibasic (28.4 g/L ddH_20) sodium phosphate stocks were mixed to prepare 0.1 M sodium phosphate buffers to appropriate pH, and 0.01 M sodium phosphate buffers were made by a 1:10 dilution. All solutions were adjusted to pH just prior to testing. Assay constants in reaction well: 25°C, 1 mM AtChI (acetylthiocholine iodide), 0.7 mM DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)], 0.7 mg/ml tissue.

Substrate: Acetylthiocholine iodide was tested over the range 0.1–20 mM. Solutions were prepared from stock 100 mM AtChI (29 mg/ml ddH_20). Assay constants in reaction well: 25°C, 0.1 M sodium phosphate buffer (pH 8.0), 0.7 mM DTNB, 1 mg/ml tissue.

Chromogen: 5,5'-dithio-bis(2-nitrobenzoic acid) was tested over the range 0.1–1.0 mM. Solutions were prepared from stock 5 mM DTNB (1.96 mg/ml in 0.1 M sodium phosphate buffer, pH 8.0). Assay constants in reaction well: 25°C, 0.1 M sodium phosphate buffer (pH 8.0), 3 mM AtChI, 1 mg/ml tissue.

Temperature: Temperature was tested over the range 20–30°C. Assay constants in reaction well: 0.1 M sodium phosphate buffer (pH 8.0), 3 mM AtChI, 0.25 mM DTNB, 1 mg/ml tissue.

Specific inhibitors: Inhibitors BW284c51 [BW1,5-bis-(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide] and iso-OMPA (tetramono*iso*propyl-pyrophosphortetramide) (specific AChE and butyryl-cholinesterase inhibitors, respectively), and eserine [1'methylpyrrolidino (2':3':2:3)1,3-dimethylindolin-5-yl *N*-methylcarbamate] (total cholinesterase inhibitor) were tested over the range of 10^{-8}–10^{-4} M to specify cholinesterase types present in brain tissue. Test solutions were prepared from 10^{-3} M stocks: BW284c51 (5.7 mg/10 ml ddH_20), iso-OMPA (3.4 mg/10 ml ddH_20), and eserine (2.8 mg/10 ml ddH_20). Brain homogenates were incubated 15 min at 30°C with the inhibitors before the addition of substrate. Enzymatic activities were compared relative to controls (no inhibitor). Assay constants in reaction well: 25°C, 0.1 M sodium phosphate buffer (pH 8.0), 3 mM AtChI, 0.25 mM DTNB, 1 mg/ml tissue.

AChE analysis of test fish

Fish were partially thawed, and the brains were excised and individually homogenized at 25 mg/ml in 0.1 M sodium phosphate buffer (pH 8.0) with 0.1% TritonX-100. Samples were processed in a randomized order. Homogenates were centrifuged at low speed (1000 rpm for 10 min) to remove large particles. For each sample, 50 µl of the homogenate was transferred to a 1.5 ml Eppendorf tube. Added were 900 µl of 0.1 M sodium phosphate buffer (pH 8.0) and 50 µl DTNB (6 mM). The

solution was thoroughly mixed, incubated at room temperature for 10 min to allow non-enzymatic activity to stabilize, and then 200 µl was transferred to plate wells in triplicate. The reaction was initiated by addition of 50 µl AtChI (15 mM), and change of absorbance (412 nm) was measured at 12-s intervals for 10 min at 25°C. Blanks for tissue and substrate were included for each sample. Final well concentrations were as follows: 3 mM AtChI, 0.25 mM DTNB, 1 mg/ml brain tissue. All activities were normalized to protein content by the method of Bradford (1976), using Coomassie Plus-200 Protein Assay Reagent and bovine serum albumin standard (Pierce, Rockford, IL, USA).

AChE activity calculation

The AChE activities were calculated as µmol/min/g brain or as nmol/min/mg protein using the method described by Padilla et al. (1998). The geometry of the plate well does not allow a simple determination of the solution path length, which is dependent on the sample volume inside the well. In order to account for this, a different method using glutathione as the supplier of sulfhydryl groups was used. First, a 0.1 mM solution of glutathione was prepared; a 0.1 mM solution contains 1 nmol of glutathione per 10 µl. A standard curve of 1–10 nmol glutathione was created by adding 10–100 µl of the glutathione solution into the plate well, along with the appropriate volume of buffer and DTNB, to equal the total volume and DTNB concentration used for the assay (250 µl). The glutathione and DTNB reacted immediately, and the plate was read at 412 nm. The slope of the glutathione curve equaled 0.041 optical density (OD) units per 1 nmol glutathione, so dividing the AChE assay reaction rate by 0.041 equaled the nmols of AtChI hydrolyzed per minute in the well. Converting the reaction volume to 1 ml and multiplying by the tissue dilution factor gave nmol/min/g of brain. The equation is summarized as follows;

$$\text{AChE activity} = \frac{\text{OD units/min}}{\text{0.041 OD units/nmol/250 µl}} \times \frac{\text{1 ml}}{\text{250 µl}} \times \text{tissue dilution.}$$

Finally, dividing by the protein content per gram of tissue produced nmol/min/mg protein.

Statistical analyses

Prior to BMC analyses, replicate order and fish weights were tested as possible covariates to AChE activity. Analysis-of-variance (ANOVA) followed by Tukey's multiple comparisons was performed to test replicate reproducibility (GraphPad Prism 3.02). To determine the statistical departure of AChE inhibition (U.S. Environmental Protection Agency 1995, U.S. Environmental Protection Agency 1996) for this experiment, a power test was conducted from a two-sample t-test with a power level of 50%, group size of 54, standard deviation of 8.2% (pooled standard deviation estimate), and a one-sided test with Type I error of 0.05 (S-Plus 2000, MathSoft, Inc.). The statistical departure is an estimate of the lowest detectable response under the experimental design, and is more generally defined as the 95% lower-confidence interval from the control mean.

The BMDS Version 1.3 statistical program was downloaded from the U.S. Environmental Protection Agency website (http://www.epa.gov/nceawww1/bmds.htm). The data were fit by a simple linear regression model with 95% confidence intervals and constant variance. The BMCs were determined at 2.5%, 5%, 10%, 20%, 1 SD and 2 SD responses. Confidence limits were calculated by BMDS using likelihood theory, based on the asymptotic distribution of the likelihood statistic.

RESULTS

Sodium phosphate buffers yielded higher enzymatic activity and were stable over a broader pH range than Tris buffers (Fig. 2.1A). The 0.025 and 0.05 M Tris buffer pH curves were indistinguishable from each other and are represented together in Figure 2.1A as one curve. Activities with 0.1 M sodium phosphate buffer were consistently higher than with 0.01 M sodium phosphate by approximately 10% and followed a similar curve. For clarity, only the 0.1 M sodium phosphate curve is shown in Figure 2.1A. Sodium phosphate buffers showed peak AChE activity over the pH range 7.5–8.5. Non-

enzymatic reaction became apparent at pH 8.5–9.0 with all buffers: sodium phosphate buffers about 5% and Tris buffers up to 25% of total activity.

The AtChI substrate curve shows a sharp rise in AChE activity from 0.1–2 mM, peaking from 2–5 mM and gradually decreasing out to 20 mM (Fig. 2.1B). A slight inhibition of activity at high substrate concentrations is typical of AChE.

Increasing concentrations of DTNB produced a slight downward trend from 0.1–1.0 mM (Fig. 2.1C). The DTNB in solution at these concentrations is acidic, so increased DTNB concentrations could have shifted the pH from its optimal range.

Increasing temperature from 20–30°C caused a steady rise in AChE activity (Fig. 2.1D). At 28 and 30°C non-enzymatic hydrolysis became apparent, accounting for approximately 2 and 5% of total activity, respectively.

Incubation with iso-OMPA in the range of 10^{-8}–10^{-4} M had no effect on brain enzymatic activity (Fig. 2.1E). Eserine and BW284c51 produced similar inhibition curves with near total inhibition occurring at 10^{-5} M. This indicates that ezymatic activity is attributed to AChE, and not other pseudo-cholinesterases.

Addition of 0.1% TritonX-100 to the homogenizing buffer significantly increased sample activity by 25–30%. TritonX-100 at 1% also increased sample activity but caused problematic foaming during transfers. Some activity (about 5%) was lost after centrifugation at 1000 rpm, but this generally increased precision of well replicates (data not shown). Increased precision between sample and well replicates was obtained by altering reagent concentrations and transfer volumes from those traditionally presented in the literature. Pipetting larger volumes of more dilute solutions kept the replicate-well coefficient of variation mostly below 2%. Activity was stable over the duration of the reaction, which was sampled every 12 sec for 10 min (Fig. 2.1F). Normalization of AChE activities to protein content helped reduce inter-animal variability (pooled standard deviation of 8.2%), compared to activities determined by wet tissue weight (pooled standard deviation of 11%).

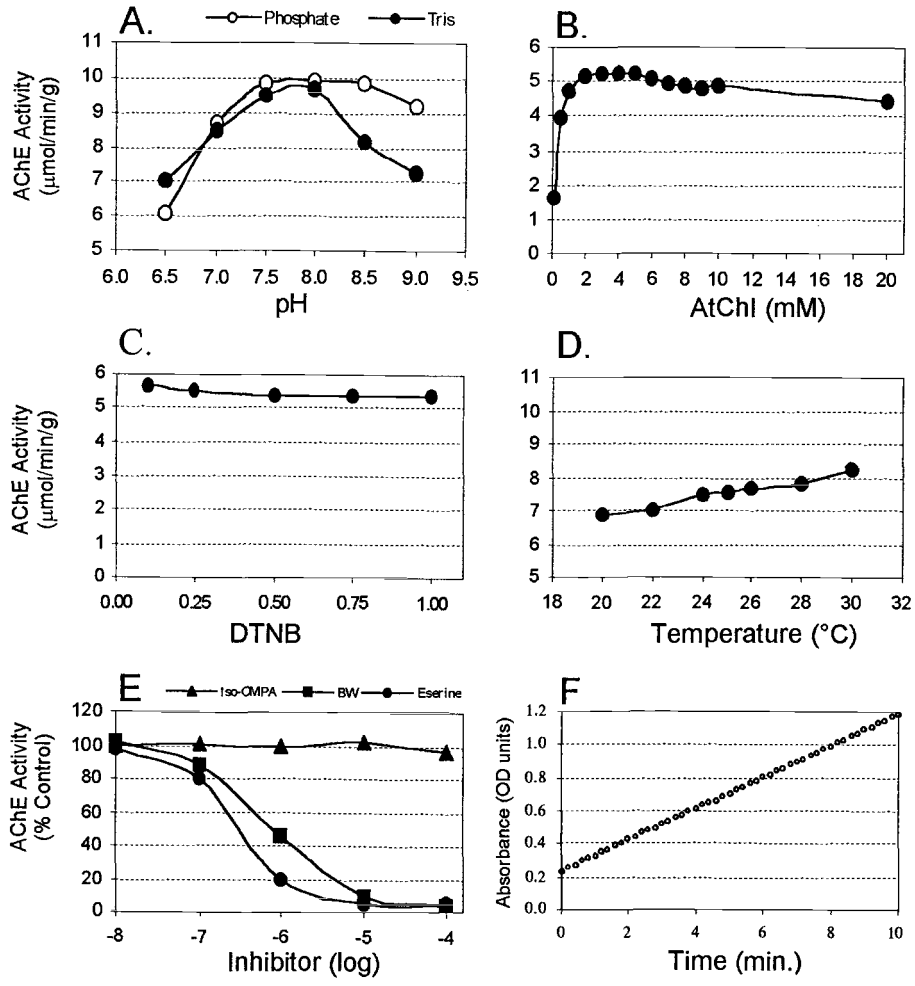

Figure 2.1. Acetylcholinesterase (AChE) assay profile for *Oncorhynchus mykiss* brain tissue. Each point is the mean of three replicate tests, with five pooled fish brains per test (except F). (A) pH-activity profiles for two buffer types, 0.1 M sodium phosphate and 0.05 M Tris-HCl. (B) Substrate-dependent activity rises sharply from 0.1 to 2 mM, peaks between 2 to 5 mM, and gradually decreases out to 20 mM. (C) Activity is lowered slightly as the chromogen (DTNB) concentration increases. (D) Activity increases with temperature. (E) Effects of specific cholinesterase inhibitors show that enzymatic activity is attributed to the presence of AChE. (F) Activity is very stable over the course of the assay; absorbance at 412 nm was measured in optical density (OD) units every 12 s for 10 min.

At the two highest exposure levels (1.875 and 2.50 μg/L), some individual fish showed erratic swimming behavior, loss of schooling pattern, aggression, and failure to maintain horizontal position in the water column approximately 72 h into the exposure.

No correlations were found between brain AChE activity and experiment replicate order ($p > 0.05$) or fish weight ($p > 0.05$). However, pilot experiments with younger fish weighing 2.5 ± 0.8 g did show a significant negative correlation between AChE activity and weight ($p < 0.01$). A weight-activity correlation with smaller fish, but not with larger fish, is consistent with data presented by Sturm et al. (1999). A Tukey's multiple comparison test showed only one test replicate (rep. 1, 1.25 μg/L) was outside its group distribution range ($p < 0.05$), indicating generally good reproducibility between experimental replicates. Figure 2.2 shows the distribution of individual AChE activities over the dose response. Table 2.1 provides the distribution parameters, including individual group totals, AChE activity means with standard error (SE), standard deviation (SD), and range values expressed as nmol/min/mg protein and as percent-control mean activity. The mean control activity expressed as wet weight was 15.3 μmol/min/g brain tissue. The statistical departure for brain AChE inhibition in this study was a 2.5% decrease from the control mean.

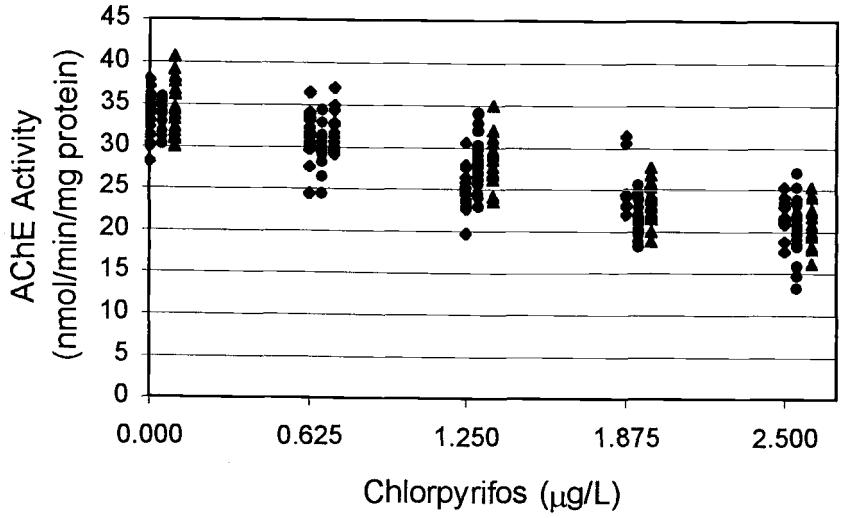

Figure 2.2. Dose response of *Oncorhynchus mykiss* brain acetylcholinesterase (AChE) activity after a 96-h exposure to chlorpyrifos. Each test concentration was replicated in triplicate, and each point represents an individual fish.

Table 2.1. Parameters of *Oncorhynchus mykiss* brain acetylcholinesterase (AChE) activity following a 96-h exposure to chlorpyrifos. Values describe the individual variation of AChE activity within the tested population.

chlorpyrifos[a]	n[b]	AChE activity[c]	SE[c,d]	SD[c,e]	range[c]
0	54	34.1 (100)	0.35 (1.0)	2.6 (7.6)	28-40 (83–118)
0.625	53	31.7 (93.0)	0.36 (1.1)	2.6 (7.6)	25-37 (72–109)
1.25	55	27.3 (80.0)	0.43 (1.3)	3.2 (9.4)	20-35 (58–103)
1.875	45	23.1 (67.7)	0.40 (1.2)	2.7 (7.9)	18-31 (54–92)
2.50	51	21.0 (61.7)	0.40 (1.2)	2.9 (8.4)	8.2-27 (39–80)

[a] Chlorpyrifos concentrations are expressed in µg/L.
[b] Total number of fish per exposure.
[c] Acetylcholinesterase (AChE) activities are expressed in nmol/min/mg protein and as percent control (in parenthesis).
[d] SE = standard error.
[e] SD = standard deviation.

As an example of the BMC approach, Figure 2.3 shows the BMC_{1SD} linear data fit. Error bars around points are 95% confidence intervals of observed activity means. The horizontal line from the y-axis is the 1 SD response (8.2% depression), the central concentration estimate associated with this response is the BMC, and the lower limit of the fit estimate is the BMCL. Table 2.2 shows the BMC response estimates at the 2.5%, 5%, 10%, 20%, 1 SD, and 2 SD brain AChE depression levels. The linear equation used to fit the data was

$$Y = \text{beta}(0) + \text{beta}(1)X$$

where Y equals AChE activity, beta(0) is the control AChE activity estimate, beta(1) is the slope of the dose-response, and X is chlorpyrifos concentration. The parameter estimates (± SE) for this data are beta(0) = 34.4 (0.3), and beta(1) = –5.56 (0.21). Homogeneous variance was determined to be appropriate for this modeling ($p < 0.001$) since activity variance did not change with dose. A goodness-of-fit test indicated only a moderate fit of the given data (Chi-Square probability $p < 0.05$), due to the slight curvature of the dose response.

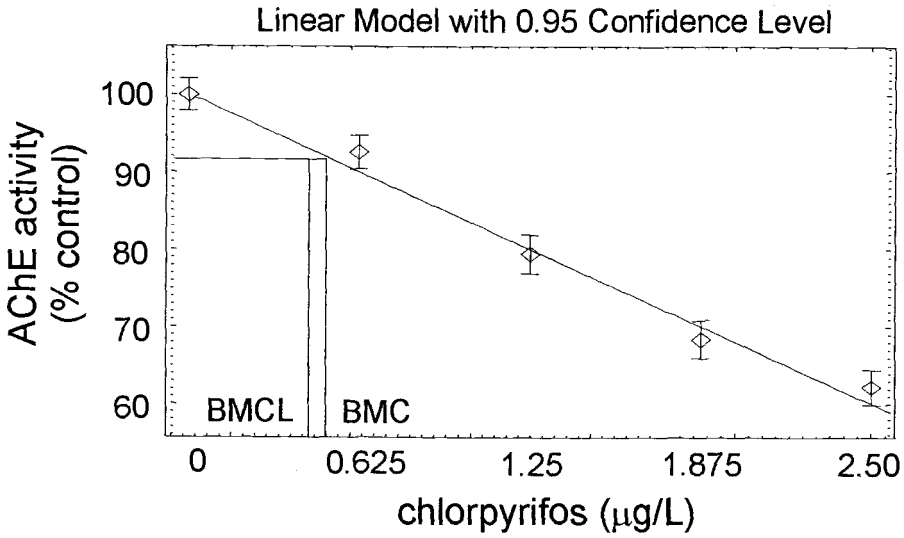

Figure 2.3. Benchmark concentration graphical output from a linear data fit. Points are group means of *Oncorhynchus mykiss* brain acetylcholinesterase (AChE) activity following a 96-h exposure to chlorpyrifos. The horizontal line indicates the 1 SD effect level (BMC_{1SD}), and the vertical lines represent the corresponding central estimate of the benchmark concentration (BMC) and the 95% lower limit confidence of the fit (BMCL). Error bars around points represent a 95% confidence interval of group means.

Table 2.2. Benchmark concentration estimates for *Oncorhynchus mykiss* brain acetylcholinesterase (AChE) activity following a 96-h exposure to chlorpyrifos, using a simple linear model to fit the dose response.

AChE inhibition	chlorpyrifos concentration (µg/L) BMC[a]	BMCL[b]
2.5%	0.16	0.15
5%	0.31	0.30
10%	0.62	0.59
20%	1.25	1.19
1 SD	0.51	0.47
2 SD	1.02	0.94

[a] Benchmark concentration estimate.
[b] 95% confidence lower limit confidence interval of the benchmark concentration estimate.

DISCUSSION

Variability associated with the AChE assay was minimized, which allowed for a more precise determination of chlorpyrifos-induced brain AChE inhibition in *O. mykiss*. For comparisons with future studies, there should be a common analytical protocol and a common expression and presentation of AChE activity. Properties of the AChE assay examined here included buffer type, pH, reagent concentrations, temperature and effects of specific cholinesterase inhibitors. Recommended conditions for *O. mykiss* AChE activity include using 0.1 M sodium phosphate buffer at pH 8.0, 3 mM AtChI, 0.25 mM DTNB, brain tissue at 1 mg/ml measured at 25°C for 10 minutes at 412 nm. The tissue should be homogenized at 20 to 50 mg/ml in sodium phosphate buffer, pH 8.0, with 0.1% TritonX-100. Correlating fish body weight and AChE activity should be considered, especially when analyzing small fish.

Several BMDS models were considered in evaluation of these data: linear, polynomial, power, and Hill. The cubic polynomial model fit the data well, but extrapolation above experimental doses resulted in a rapid increase of AChE activity. The Hill model provided a good sigmoidal fit, but created a y-axis asymptote near the 50% inhibition response when unconstrained. When constrained to converge at zero, it provided a poor goodness-of-fit. The exponent of the best-fit power model converged to 1, which is basically equivalent to the linear model. The simple linear model was chosen to model the data, although a logistic function might be expected to describe an enzymatic dose response more adequately. The linear model provided the best compromise between goodness-of-fit and biological plausibility. Extrapolation of the linear model, though not explained mechanistically, is consistent with reported LC_{50} values for *O. mykiss*. Brain AChE inhibitions above 80% can result in death, and reported 96-h chlorpyrifos LC_{50} values for *O. mykiss* range from 6.0–9.4 µg/L (Macek et al. 1969, Holcombe et al. 1982). Extrapolated BMC_{80} and BMC_{100} estimates are 4.3 µg/L and 6.2 µg/L, respectively, which are very similar to the reported LC_{50} values.

One of the goals in ecological risk assessment is to determine whether chemical concentrations in the environment reach or exceed a predicted no-effect level (Moore and

Caux 1997). One of the difficulties in assessing a continuous response (effects measured on a continuum, rather than discrete scale) is defining abnormality, or a critical effect level. In the case of AChE inhibition, abnormality could be based on an adverse biological departure (Grue et al. 1991). Relationships between AChE inhibition and biological function for fish have been investigated previously and include alterations in growth, reproduction, maturation, swimming, hyperactivity, and feeding. These biological correlations have been observed when brain AChE activity has been reduced 20–50% (Coppage 1972, Jarvinen et al. 1983, Lockhart et al. 1985, Zinkl et al. 1991, Beauvais et al. 2000). Mortality is an extreme effect, but one that is easily comparable and common to all species. Mortality for fish begins to occur when inhibitions exceed 70–80% (Coppage 1972, Coppage and Matthews 1974).

A relative departure for AChE abnormality could be based on more convenient, and comparable, values such as a 1%, 5%, 10%, 20%, 1 SD, 2 SD, or 3 SD cut-point (Ludke et al. 1975, Hill 1988, Gaylor and Slikker 1990, U.S. Environmental Protection Agency 1995, 1996, 2001). Relative departure values are useful in evaluating exposures to wildlife (Ludke et al. 1975, Mineau and Peakall 1987, Hill 1988) and in assessing human health risk (Crump 1984, Slikker et al. 1996, U.S. Environmental Protection Agency 2001). One limitation, however, is that any discrete value for AChE inhibition reflects an assumption about abnormality without considering any true biological meaning (Slikker et al. 1996).

Another approach is to assume that any statistical significance implies toxicological relevance (Christenson et al. 1994). A statistical departure, such as the lower end of a confidence interval, NOEL, or LOEL value can also be used determined (U.S. Environmental Protection Agency 1995).

Applying a regression-based approach to evaluate dose-response data, such as the BMC, can be used to report over a range of responses (Crump 1984, Slikker et al. 1996, Moore and Caux 1997). This approach provides convenient benchmarks for multiple comparisons, considers the entire dose-response relationship, is not constrained to be one of the experimental doses, and provides confidence limits for the estimate (U.S. Environmental Protection Agency 1995, 1996). When data are available for individual

animals, the BMC can be reported in relation to a natural distribution of the population, for example, 1 SD or 2 SD from a control mean (BMC_{SD}). If individual data are not available (i.e., several animals are combined during analysis) the BMC can be reported as a percent change relative to controls ($BMC_{\%}$). This method is also appropriate when comparing studies, tissues, or over a time course.

This study uses the BMC approach to report AChE inhibition data for *O. mykiss* exposed to chlorpyrifos at multiple inhibition intervals since no criteria for a single critical level exists. The U.S. Environmental Protection Agency 96-h water quality criteria for chlorpyrifos is 0.041 μg/L, a concentration value based on lethality tests across multiple species. A chlorpyrifos concentration at this level would result in approximately a 1% decrease in brain AChE activity. This effect is below the limit of detection for this study and is regarded only as a point of consideration. The limit of detection for this study is a 2.5% AChE depression. The $BMC_{2.5}$ and $BMCL_{2.5}$ model estimates are above the U.S. Environmental Protection Agency 96-h water quality criteria, but within the range of detected surface water concentrations.

In 1999, the ODEQ detected chlorpyrifos in one tributary of the Hood River at concentrations exceeding the U.S. Environmental Protection Agency water quality criteria (Foster, E.P., ODEQ 1999, unpublished report). The two-week average concentration (± standard error) was 0.127 ± 0.72 μg/L (n = 6), with a peak detection at 0.482 μg/L. The daily chlorpyrifos concentration profile in the Hood River and its tributaries is currently unknown, but resident steelhead may be exposed to concentrations up to 0.482 μg/L over a 96-h period. Results from this 96-h static exposure study suggest that the average detected chlorpyrifos concentration of 0.127 μg/L may not inhibit steelhead brain AChE activity to a statistically detectable level (2.5% inhibition) if exposures last for 96 h. The peak surface water concentration of 0.482 μg/L could inhibit AChE activity up to 1 SD of controls ($BMCL_{1SD}$ = 0.47 μg/L), but not likely more than 10% ($BMCL_{10}$ = 0.59 μg/L). Acetylcholinesterase inhibition by organophosphates is irreversible and the enzyme must be regenerated de-novo, requiring several weeks for full recovery. Therefore, short-exposure pulses of chlorpyrifos could have a cumulative effect.

Studies investigating the effects of diazinon on the Atlantic salmon (*Salmo salar*) olfactory system show that physiological impairment can occur after a 30 min exposure, when the pesticide is applied directly over the olfactory epithelium (Moore and Waring 1996). The salmonid olfactory organ is extremely sensitive and may be especially susceptible to aquatic toxicants because the olfactory epithelium, consisting of sensitive ordorant receptor neurons, is in direct contact with the surrounding environment (Klaprat et al. 1992). Salmon exposed to diazinon exhibited a reduced olfactory sensitivity to natural odorants, as measured by the electro-olfactogram, and decreased physiological responsiveness to reproductive pheromones. Similar diazinon concentrations eliminated a predator alarm response in Pacific chinook (*O. tshawytscha*; Scholz et al. 2000). These diazinon concentrations affecting the olfactory organ were >100-fold below the NOEL reported for brain AChE inhibition in rainbow trout (Beauvais et al. 2000). It is uncertain whether disruption to olfactory function was due to the inhibition of AChE within the olfactory epithelium, or by another unknown mechanism. Acetylcholinesterase is present in the olfactory epithelium, but its role is unclear since the first synaptic connections of the receptor neurons occur in the olfactory bulb located in the forebrain.

If other organophosphate compounds, such as chlorpyrifos, disrupt olfactory function as well, the electro-olfactogram or olfactory-mediated behaviors may be more sensitive measures of induced toxicity than brain AChE activity. Application of the BMC for other biochemical, as well as physiological and behavioral measures of toxicity, will be a useful approach for future studies in estimating potential toxic thresholds.

CHAPTER 3

Comparative Thresholds for Acetylcholinesterase Inhibition and Behavioral Impairment in Coho Salmon Exposed to Chlorpyrifos.

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ABSTRACT

Chlorpyrifos is an organophosphate insecticide that is frequently detected in salmonid habitats in the western United States. Concentrations rarely reach levels that are acutely lethal to fish, but occasionally reach levels that can affect some aspects of basic neurochemistry. The critical biological target of chlorpyrifos is acetylcholinesterase (AChE), an enzyme that modulates electrical impulses at nerve terminals. Measurement of brain or muscle AChE inhibition is a common biological indicator of exposure to organophosphate and carbamate compounds in fish, yet the biological or ecological significance of depressed AChE levels in salmonids is largely unknown. In this present study, we use paired measurements from individual fish to relate AChE inhibition to altered swimming and feeding behaviors in juvenile coho salmon. Two orthogonally-positioned digital cameras provided three-dimensional positional tracking of the fish as it swam within the tank, allowing for subtle changes in swimming movements to be measured. Both brain and muscle AChE were similarly inhibited by chlorpyrifos over a range of concentrations (0.6–2.5 μg/L, 96-h exposures). Spontaneous and feeding swimming rate, latency to strike, and total strikes were also reduced by chlorpyrifos exposures. Both brain AChE activity and spontaneous swimming rate were highly sensitive indicators of neurotoxicity, and were both significantly reduced at chlorpyrifos concentrations as low as 0.6 μg/L. Effect levels for AChE inhibition and behaviors were determined using the benchmark concentration method and were found to be similar (10% departures from control means were estimated to occur between 0.3–0.6 μg/L chlorpyrifos). Over the range of tested chlorpyrifos concentrations, brain AChE inhibition was highly correlated with reductions in spontaneous swimming speed and strike rate. Thus, measurements of brain AChE activity may be reasonably predictive of impairment to these behaviors.

INTRODUCTION

Organophosphate and carbamate insecticides are widely detected in salmon- and steelhead-bearing river systems in the western United States (Anderson et al. 1997, Panshin et al. 1998, Ebbert and Embrey 2002). Transport of these insecticides into surface waters typically occurs via storm-water runoff or drift from urban and agricultural landscapes (U.S. Geological Survey 1999, Pew Oceans Commission 2003). Although concentrations rarely reach levels that are acutely lethal to salmonids, there is considerable uncertainty as to whether these insecticides (individually or as mixtures) can impact other important aspects of biology at environmentally-relevant concentrations. To evaluate the potential effects of pollutants on organisms in the field, biological markers or indicators of toxicity are often assayed from non-target animals. However, using biochemical or physiological indicators as an index of overall fitness have sometimes been criticized because they often do not have clear biological or ecological relevance (Little and Finger 1990). Thus, it is important to establish links between measurable indicators of toxicity and higher-ordered processes that are indicative of overall health. This will allow for better estimates of potential risks that chemical contaminants may pose in the environment.

A common biological marker used to assess organophosphate and carbamate toxicity in fish is acetylcholinesterase (AChE), an enzyme that modulates neurotransmission throughout the nervous system. These classes of insecticides are potent inhibitors of AChE, and can cause systemic loss of neurological function in exposed animals (Ecobichon 1996). In skeletal muscle, persistent depolarization of the motor end-plate results in muscular spasms, but can eventually lead to paralysis or tetanus at higher concentrations (Taylor and Brown 1989). Toxicity within central networks can affect both autonomic and somatic systems, and responses can be excitatory or inhibitory, depending on the tissue (Taylor and Brown 1989). Thus, symptoms are wide-ranging and often non-specific, but can include gastrointestinal ailments, increased gland secretions, and lethargy in some vertebrate species (Ecobichon 1996). In fish, conditions such as hyperactivity, muscle twitching, and loss of balance preclude mortality, with near

complete AChE inhibition throughout the central and peripheral nervous system (reviewed in Zinkl et al. 1991). However, some fish species have been shown to tolerate greater than 80% brain AChE inhibition and still survive (e.g., Post and Leasure 1974, Cripe et al. 1984). Regardless, fish in a substandard or moribund condition are not likely to be in a positive state of physiological or behavioral health (Mesa et al. 1994). In the environment, fish are likely to encounter AChE inhibiting compounds at very low concentrations, but few studies have attempted to correlate low-level AChE inhibitions—levels most likely to result in degraded natural systems—with altered physiological or behavioral conditions.

Behavior is a sensitive indicator of biological stress in fish exposed to sublethal levels of neurological toxins (Little and Finger 1990). It is an integrative assessment of fitness that incorporates multiple layers of biology. Anti-cholinesterase compounds have been shown to disrupt a variety of ecologically important behaviors in fish including locomotory skills (Little et al. 1990, Beauvais et al. 2000) food acquisition (Bull and McInerney 1974, Morgan and Kiceniuk 1990), predator avoidance strategies (Hatfield and Anderson 1972, Scholz et al. 2000), and social interactions (Weis and Weis 1974, Saglio et al. 1996). The mechanistic causations of these behavioral deficits are uncertain, but are likely related to the combined effects of depressed AChE activity throughout sensory, central and neuro-muscular target sites.

This current study attempts to correlate low-level AChE inhibitions with altered behavioral patterns in juvenile coho salmon (*Oncorhynchus kisutch*) exposed to chlorpyrifos, an organophosphate insecticide that is commonly detected in Western surface waters. Paired biochemical and behavioral measures were obtained from individual fish, providing a direct comparison of neurotoxicity at two scales of biological order. Three-dimensional video analysis allowed for quantitative measures of swimming activities as the fish moved throughout the observation tank. Here we show that inhibited brain and muscle AChE activities are highly correlated with altered swimming and feeding behaviors in fish exposed to environmentally-relevant chlorpyrifos concentrations. Benchmark concentration methods were used to compare relative

reductions in brain and muscle AChE inhibition, spontaneous swimming speed, and strike rate.

MATERIALS AND METHODS

Animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA, USA) at the eyed egg stage and raised at the Northwest Fisheries Science Center's hatchery facility under natural photoperiod conditions. Coho fry were maintained in tanks supplied with filtered, dechlorinated municipal water (120 mg/L total hardness as $CaCO_3$, pH 6.6, dissolved oxygen 8.1 mg/L, temperature 11–13°C) on a single-pass flow system. Fish were raised on standard commercial salmon pellets, and then one month prior to experiments the diet was changed to frozen brine shrimp (Hikari, Hayward, CA, USA). Fish were age 4–5 months with an average size (± standard deviation) of 4.5 ± 0.3 cm, and 0.7 ± 0.2 g.

Chlorpyrifos exposure and analysis

Analytical grade chlorpyrifos (99.3 % purity; O,O-diethyl-O-(3,5,6-trichloro-2-pyridinol)-phosphorothionate) was purchased from Chem Service, Inc. (West Chester, PA, USA). Chlorpyrifos stocks were prepared in ethanol, and 100 µL additions of stocks to exposure tanks (25 L solution volume) produced nominal chlorpyrifos concentrations of 0, 0.6, 1.2, 1.8, and 2.5 µg/L. Fish were exposed for 96 h in aerated glass aquaria, using a static-renewal (12-h) regimen. Fish were exposed in groups of 5–6 individuals, and each exposure level was replicated in triplicate (n = 15–17 total fish per exposure level). No changes in water temperature, pH, or dissolved oxygen were observed over the exposure period. Six water samples from each chlorpyrifos level, 3 at the beginning (initial) and 3 at the end (12-h) of static-renewal periods, were analyzed to compare nominal and measured values, and to determine if chlorpyrifos concentrations decreased over the exposure period. Samples were collected in acid-washed, amber glass bottles fitted with teflon-coated lids and refrigerated at 4°C.

Extractions and analyses of water samples were done 3 weeks after collection. Analyses were performed by gas chromatograph-mass spectrometery (GC-MS), using previously established methods (Sandahl and Jenkins 2002). Hatchery water did not contain detectable amounts of chlorpyrifos (detection limit 0.02 μg/L). Initial recovered chlorpyrifos ranged between 50–68% of nominal values with no additional loss occurring over the 12-h exposure period (Table 3.1). Because chemical recoveries from the exposure solutions were lower than expected, the chlorpyrifos stocks were analyzed to determine the possible source of chemical loss. The stocks were determined to be (mean ± standard error) 98 ± 5% of their expected concentrations. Thus, the exposure solutions are expected to have been close to nominal values. Chemical loss may have occurred during sample handling, storage, or the extraction/analytical process. Here, exposures are reported as nominal concentrations.

Table 3.1. Nominal and measured chlorpyrifos concentrations (μg/L). Exposure solutions (n = 3 each) were sampled at the beginning (initial measured) and end (12-h measured) of static renewal periods.

	chlorpyrifos [a]	
nominal	initial measured	12-h measured
0	0 ± 0	0 ± 0
0.6	0.3 ± 0.1	0.3 ± 0.1
1.2	0.6 ± 0.1	0.8 ± 0.1
1.8	1.2 ± 0.1	1.1 ± 0.1
2.5	1.7 ± 0.3	1.6 ± 0.1

[a] Concentrations are mean ± one standard deviation.

Acetylcholinesterase analysis

In some species of fish, brain and muscle tissues can contain substantial amounts of both AChE and butyrylcholinesterase (BuChE) (Sturm et al. 2000). Together, AChE and BuChE are generally referred to as cholinesterase. Although the physiological role of

AChE is well established, there is no known biological role for BuChE in vertebrates (Massoulié 1993). Therefore, it is important to distinguish the two when reporting the inhibitory effects of anticholinesterase compounds in fish. Although salmonids are not believed to possess substantial amounts of BuChE in brain or muscle tissues (Lundin 1962, Sandahl and Jenkins 2002), to our knowledge no study has confirmed this in Pacific coho salmon.

To determine if AChE is the predominant cholinesterase type present in brain and muscle tissue of coho salmon, triplicate composite homogenates (n = 5–6 fish per composite) were treated with BW284c51 (1,5-bis(4-allyldimethylammoniumpheynl) pentan-3-one dibromide) and iso-OMPA (iso-tetramono-iso-propylpyrophosphorte-tramide), specific inhibitors of AChE and BuChE, respectively. The inhibitors BW284c51 and iso-OMPA were tested over a range of 10^{-9}–10^{-4} M and 10^{-8}–10^{-4} M, respectively. Control homogenates received a treatment of distilled water only. The treated and control homogenates (n = 3 each) were incubated at room temperature (20–25°C) for 30 min. Enzymatic activities were then determined using the same assay constants as described below.

Following behavioral trials, the chlorpyrifos-exposed fish were sacrificed by immersion in a lethal dose of MS-222 (tricaine methanesulfonate; Sigma Chemical Co., St. Louis, MO, USA), and immediately frozen at -80°C. Enzymatic activity analyses were conducted within 2 weeks upon collection. The rate of substrate hydrolysis by AChE was determined using the colormetric method of Ellman et al. (1961), as modified by Sandahl and Jenkins (2002). Measurements were performed on a SpectroMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and all reagents were purchased from Sigma Chemical. Acetylthiocholine iodide (AtChI) was used as the enzyme substrate and 5,5'-dithiobis-(2-nitro-benzoic acid) (DTNB) was used as the reactive chromogen. Final concentrations of tissue and reagents in the microplate wells used for the assay were 1 mg/ml brain or muscle, 3 mM AtChI, and 0.25 mM DTNB. Reactions were carried out in 0.1 M sodium phosphate buffer (pH 8.0) at 25°C. Activities of AChE are expressed in μmol/min/g tissue.

Quantification of swimming and feeding behaviors

Trials were conducted in a 30 L glass aquarium (observation tank) filled with 25 L hatchery water. The experimental area was enclosed with black plastic sheeting to shield the fish from outside disturbances. The observation tank had two adjacent clear glass walls (for front and left camera views), and two opaque walls (back and right side). The tank was lined with 1 cm of gravel substrate, and uniform overhead lighting was provided by wide-spectrum fluorescent lights. A small, in-flow water pump was positioned in the back-right corner of the tank which circulated water at a rate of approximately 100 ml per min. A short length of tygon tubing (50 cm) connected an outside injection port directly to the circulation system that was used to introduce brine shrimp into the observation tank.

Following exposures to chlorpyrifos, individual fish were transferred to the observation tank and allowed to acclimate for 30 min. After acclimation, the spontaneous swimming rate of the fish was measured over a 3 min interval. Following this interval, 30 adult brine shrimp (previously frozen) were injected into the circulation system (t = 0 s). The speed in which fish moved to feed on the brine shrimp (feeding swimming rate) was then measured for 1 min. A DV camcorder (digital video camera; Canon DR45) with a front view of the tank recorded the fish as they made strikes at brine shrimp, and the video was later analyzed to measure latency to first strike and the total strikes made.

Spatial movements of the fish were monitored by two orthogonally positioned Firewire digital cameras (Fire-i, Unibrain) connected to a laptop computer (iBook, Apple Computer, Cupertino, CA). One camera was positioned to view the front of the tank, while the second viewed the left side. Custom-written software displayed simultaneously acquired frames from the cameras at12 frames/second, recorded a pair of frames every 2 seconds, and continuously recorded keyboard input. Semi-automated computer video analysis of each pair of frames (custom-written scripts in VideoScript; Videoscript Inc., Corrales, NM) located the position of the fish in both two-dimensional views. Triangulation (performed in Excel, Microsoft, Redmond, WA) determined the three dimensional location of the fish. During the behavioral trial, pressing a key on the keyboard indicated the time of brine shrimp introduction.

Statistical analyses

The effects of chlorpyrifos on biochemical and behavioral measures were analyzed using either one-way analysis-of-variance (ANOVA) to test for statistical differences between groups (followed by a Dunnett's test for comparisons with controls), or regression analysis to test for concentration-dependent relationships. Benchmark concentration (BMC) analyses were performed using U.S. Environmental Protection Agency benchmark dose software (version 1.3.2; provided on the internet at http://cfpub.epa.gov/ncea/cfm).

The BMC is the estimated exposure concentration that results in a specific degree of adverse response (e.g., a 10% decrease in AChE activity or swimming rate), which is termed the benchmark response. The BMC method has several advantages in comparative toxicology and environmental risk assessment, which have previously been discussed by others (e.g., Crump 1995, U.S. Environmental Protection Agency 1995, Slikker et al. 1996). The BMC model is based on regression analysis, considers the entire dose-response relationship, and the BMC estimates are not constrained to be one of the experimental doses (as is the case in determining a lowest-observed-effect-concentration, or LOEC). Since it is not constrained by the actual administered treatments, and it incorporates control values in fitting the model, BMCs can be estimated for values between the control and the lowest experimental concentration, in carefully designed studies. Benchmark responses can be based on statistical departures or relative departures (Sandahl and Jenkins 2002). Statistical departures include the point at which groups can be statistically differentiated from controls, defined by the biological variability and sample size of the study (e.g., the 95% lower-confidence interval of the control mean). Relative departures are useful for effect comparisons between studies or between responses within a study (e.g., 5, 10 or 20% differences from the control mean). In this present study, BMCs were estimated for statistical departures (the level dependant upon the measure) and relative departures (10% effect level) for brain AChE inhibition, spontaneous swimming rate, and total strikes in fish exposed to chlorpyrifos.

RESULTS

Chlorpyrifos similarly inhibited AChE activity in brain and muscle tissues

Salmonids have previously been shown to be highly sensitive to the anti-cholinergic effects of chlorpyrifos (Macek et al. 1969, Holcombe et al. 1982, Sandahl and Jenkins 2002). Brain and muscle tissues contain high quantities of AChE at synaptic and neuromuscular junctions, and these tissues are critical targets of chlorpyrifos toxicity. In this present study, fish exposed to chlorpyrifos for 96 h showed significant AChE inhibition in both brain and muscle tissues.

Incubation of homogenates in BW284c51 (specific AChE inhibitor) and iso-OMPA (specific BuChE inhibitor) indicated that AChE is the primary cholinesterase type present in brain and muscle tissues of coho salmon (Fig. 3.1A). Incubation with iso-OMPA up to 10^{-4} M had no effect on enzymatic activity, indicating that BuChE is not present in substantial amounts in these tissues. However, BW284c51 inhibited relative enzymatic activity in both tissues (nearly identically) over the range of 10^{-9}–10^{-4} M, abolishing activity at the highest level. Thus, cholinergic activity can be attributed to the presence of AChE alone in these tissues.

Exposure to chlorpyrifos significantly inhibited AChE activities of brain and muscle tissues at all chlorpyrifos levels tested (ANOVA, Dunnett's test, $n = 5$ levels, $p < 0.05$; Table 3.2) and in a concentration-dependent manner (regression analysis, $p < 0.001$; Fig. 3.1B). For control fish, baseline AChE activity in the brain was lower than that of muscle tissue (mean ± SE, 23.2 ± 0.5 and 38.9 ±1.4 μmol/min/g, respectively). At the lowest chlorpyrifos concentration tested (0.6 μg/L), brain and muscle AChE activities were inhibited (mean ± SE) 23 ± 1% and 12 ± 3%, respectively, as compared to controls. Even though fish at the highest exposure concentration (2.5 μg/L) had mean brain and muscle AChE activity inhibited by 64% and 67%, respectively, no overt symptoms of toxicity were observed during the exposures. There was a high correlation between brain and muscle AChE activities within individual fish (linear regression, slope = 1.05, $r^2 = 0.85$, $p < 0.001$) as shown in Figure 3.1C, indicating that AChE in both tissues were similarly sensitive to chlorpyrifos over the range of concentrations tested.

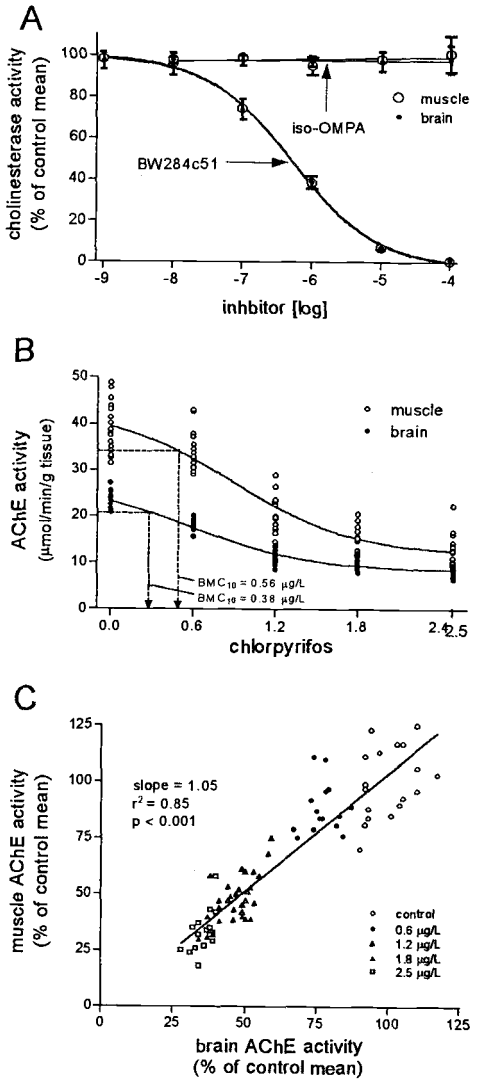

Figure 3.1. Acetylcholinesterase (AChE) is the primary acetylcholine-metabolizing enzyme present in brain and muscle tissue of coho fry, and both are sensitive to the inhibitory effects of chlorpyrifos. (A) The compounds BW284c51 and iso-OMPA are specific inhibitors of AChE and butyrylcholinesterase, respectively. Incubation of brain and muscle homogenates (n = 3; triplicate composites of n = 5–6 fish each) with the inhibitors indicate that AChE is the predominant cholinesterase type present in these tissues. (B) Chlorpyrifos inhibited the activity of brain and muscle AChE in fish (n = 15–17) over a range of concentrations. As an example of benchmark concentration analysis, the horizontal dashed line represents a 10% reduction in AChE activity. This corresponds to an estimated 0.38 µg/L and 0.58 µg/L chlorpyrifos exposure (BMC_{10}) for brain and muscle, respectively. (C) Brain and muscle AChE activities were compared in individual fish exposed to chlorpyrifos (each point represents AChE activities of single fish). Activities in both tissues were highly correlated ($r^2 = 0.85$), and shown to be similarly sensitive to the inhibiting effects of chlorpyrifos (slope = 1.05).

Both data sets were fit using a sigmoid logistic model with constant variance. For BMC estimates of brain and muscle AChE inhibition, the 95% lower-confidence interval of the respective control mean was used as the statistical departure value. For brain AChE activity, this departure corresponded to a 4% activity reduction (or -1.0 μmol/min/g). For muscle AChE activity, this departure corresponded to an 8% activity reduction (or -3.0 μmol/min/g). The relative departures for brain and muscle AChE inhibition were chosen to be a 10% reduction relative to the respective control means. An example of the BMC approach is shown in Figure 3.1B, and the calculated BMC estimates are shown in Table 3.3. In a direct comparison of brain and muscle AChE inhibitions, the BMC_{10} (relative departure) estimates were found to be similar (0.4 and 0.6 μg/L chlorpyrifos, respectively).

Chlorpyrifos altered swimming and feeding behaviors in exposed coho fry

In behavioral trials, spontaneous swimming rate, feeding swimming rate, latency to strike, and total strikes at brine shrimp were measured in juvenile coho exposed to chlorpyrifos. All behaviors were significantly altered, and this supports previous research showing that swimming and feeding behaviors are sensitive indicators of sublethal neurotoxicity in fish (Little and Finger 1990). An example of typical swimming charts obtained from individual control and chlorpyrifos-exposed (1.2 μg/L) fish are displayed in Figure 3.2, with $t = 0$ indicating the time brine shrimp were added to the observation tank. Control fish generally remained in motion throughout the entire trial, while chlorpyrifos-exposed fish tended to have periods of rest (< 1 cm movement per s), with the durations of rest increasing together with chlorpyrifos concentration. Group averaged swimming charts ($n = 15–17$ individual charts per exposure level) are displayed in Figure 3.3A.

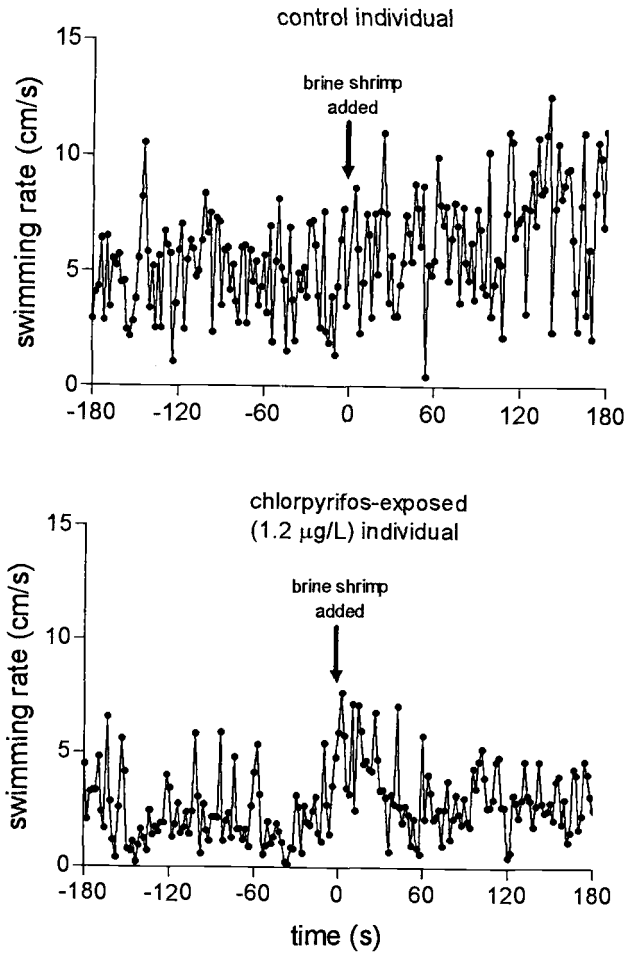

Figure 3.2. Shown are typical activity-grams obtained from individual control and chlorpyrifos-exposed (1.2 µg/L) fish. The three-dimensional position of individual fish was captured at 2-s intervals, providing a semi-continuous rate of movement as the fish swam throughout the tank. Spontaneous swimming rates were measured for 3 min (t = -180–0 s), and feeding swimming rates were measured for 1 min (t = 0–60 s) after the introduction of brine shrimp.

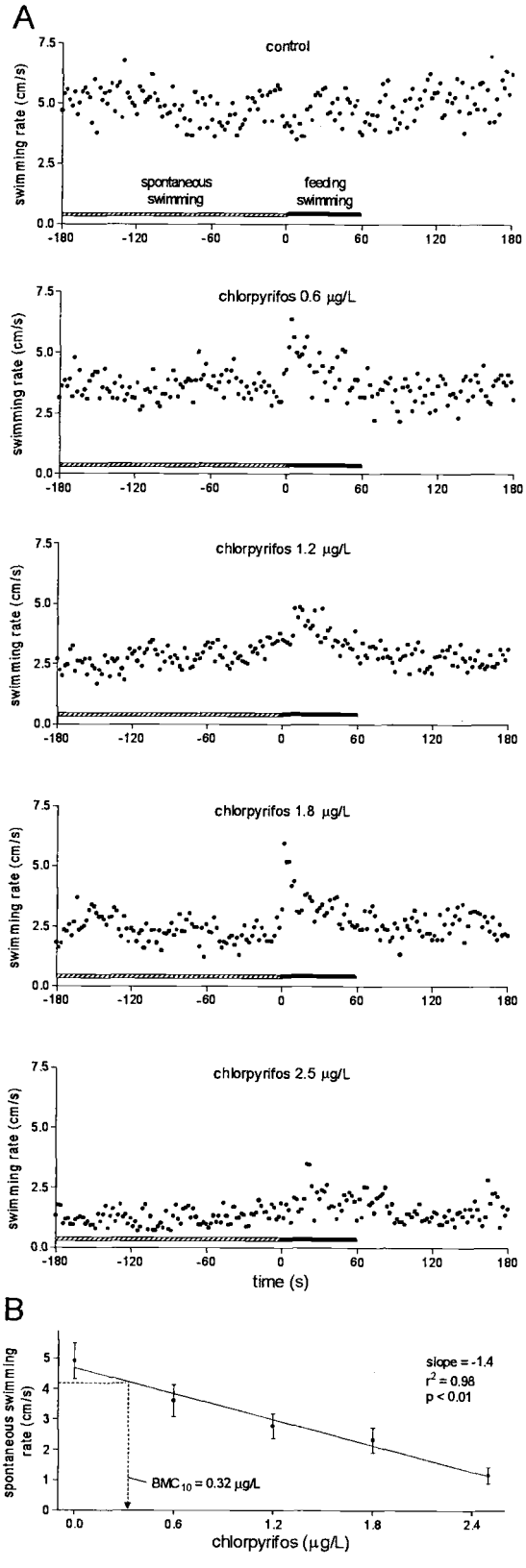

Figure 3.3. Spontaneous and feeding swimming rates were reduced after 96-h exposures to chlorpyrifos. (A) Data obtained from individual fish (n = 15-17) were combined to produce averaged locomotory rates (over 2-s intervals) at each chlorpyrifos exposure level. The crossed and solid bars at the bottom of graphs indicate the period in which spontaneous and feeding swimming rates were measured, respectively. (B) Mean spontaneous swimming rates of control and chlorpyrifos-exposed fish are plotted together and fit using linear regression. Spontaneous swimming rates were significantly reduced ($p < 0.01$) in fish exposed to chlorpyrifos. As an example of benchmark concentration analysis, the horizontal dashed line represents a 10% reduction in activity, which corresponds to an estimated 0.32 µg/L chlorpyrifos exposure (BMC_{10}).

Spontaneous swimming rates of fish were measured for the 3-min period before the introduction of brine shrimp (t = -180–0 s). Exposures to chlorpyrifos significantly reduced the spontaneous swimming rate at all chlorpyrifos levels tested (ANOVA, Dunnett's test, n = 5 levels, $p < 0.01$) and in a concentration-dependent manner (regression analysis, $p < 0.01$; Fig. 3.3B). For control fish, the spontaneous swimming rate averaged (mean ± SE) 4.9 ± 0.3 cm/s, which was reduced by 27 ± 5% (to 3.6 ± 0.2 cm/s) at the lowest chlorpyrifos exposure level (0.6 μg/L; Table 3.2). At 2.5 μg/L, the fish were severely immobilized, with movement averaging only 1.1 ± 0.2 cm/s.

Feeding swimming rate, latency to strike, and total strikes were also significantly affected by chlorpyrifos exposures (Table 3.2). When brine shrimp were introduced into the observation tank (t = 0) control fish averaged a first strike within 2.9 s, which took longer with increasing chlorpyrifos exposures (regression analysis, $p < 0.01$). During the observed feeding interval (t = 0–60 s), control fish maintained a consistent feeding swimming rate as they fed, which averaged 4.6 ± 0.3 cm/s (Fig. 3.3A). Chlorpyrifos-exposed fish tended to increase swimming activity to feed, but at rates slower than controls (ANOVA, Dunnett's test, $p < 0.05$, except 0.6 μg/L). Figure 3.4A visually displays the rate at which strikes were made by fitting the cumulative strikes by individual fish with an exponential decay curve. The mean total strikes made after 60 s were plotted together and fit using linear regression (Fig. 3.4B). On average, control fish made (mean ± SE) 23 ± 0.8 strikes at brine shrimp over the observation period, which was reduced with increasing chlorpyrifos concentrations (regression analysis, $p < 0.01$).

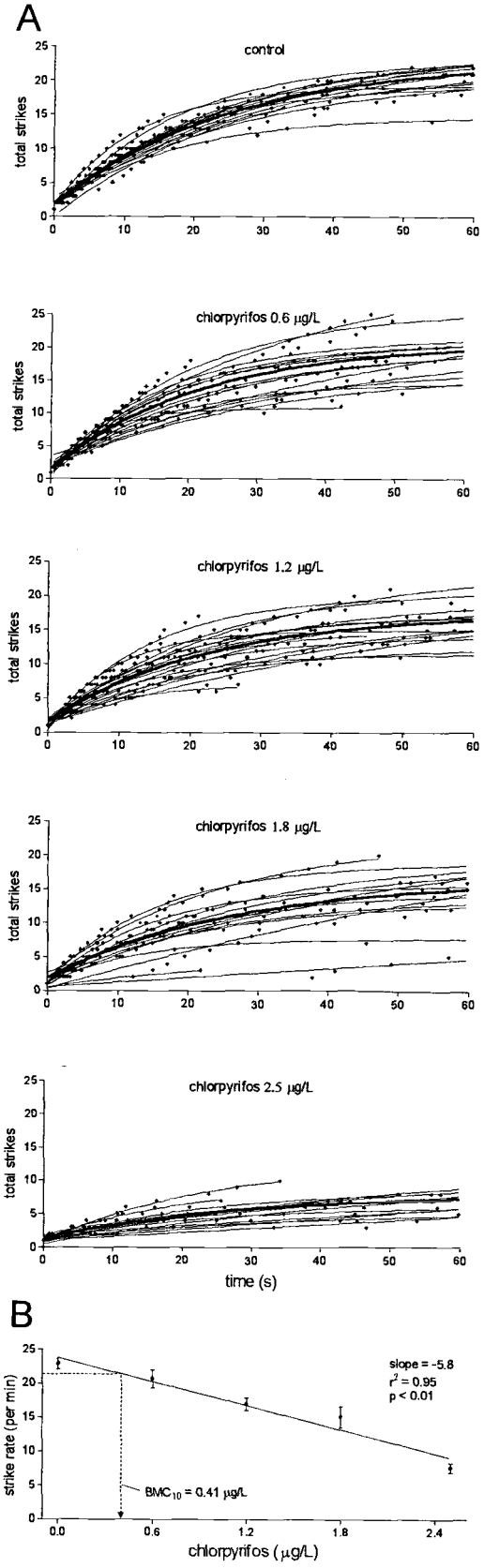

Figure 3.4. Strikes at brine shrimp were reduced in fish after 96-h exposures to chlorpyrifos. (A) Strikes were counted over a 60-s interval after introducing 30 brine shrimp into the observation tank. Each point indicates a strike made by an individual fish (n = 15-17 fish per exposure level). Strikes made by individual fish were traced with an exponential decay curve for visual affect. (B) Mean total strikes after 60 s of control and chlorpyrifos-exposed fish are plotted together and fit using linear regression. Total strikes were significantly reduced ($p < 0.01$) in fish exposed to chlorpyrifos. As an example of benchmark concentration analysis, the horizontal dashed line represents a 10% reduction in strike rate, which corresponds to an estimated 0.41 µg/L chlorpyrifos exposure (BMC_{10}).

Linear regression fits of data for spontaneous swimming rate (Fig. 3.3B) and total strikes (Fig. 3.4B) were used for BMC estimations. For spontaneous swimming rate and total strikes, the 95% lower-confidence interval of the respective control mean was used as the statistical departure value. For spontaneous swimming rate, this departure corresponded to an 11% reduction (or -0.5 cm/s). For total strikes, this departure corresponded to an 8% reduction (or -1.8 strikes). The relative departures for spontaneous swimming rate and total strikes were chosen to be a 10% reduction relative to the respective control means. Examples of the BMC approach are presented in Figures 3.3B and 3.4B, and the calculated BMC estimates are shown in Table 3.3. In a direct comparison with the BMC_{10} estimate for brain AChE inhibition (0.4 µg/L chlorpyrifos), the BMC_{10} estimates for spontaneous swimming rate and feeding were found to be similar (0.3 and 0.4 µg/L chlorpyrifos, respectively).

Table 3.2. Biochemical and behavioral measures from coho fry exposed 96 h to chlorpyrifos (n = 15–17 fish per exposure level, response indices are mean ± SE). Spontaneous swimming rate was measured for 3 min prior to the introduction of brine shrimp, and feeding swimming rate was measured for 1 min while the fish fed.

chlorpyrifos[a]	brain AChE[b]	muscle AChE[b]	spontaneous swimming rate	feeding swimming rate	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	22.9 ± 0.8
0.6	17.9 ± 0.3*	34.3 ± 1.1*	3.6 ± 0.2*	4.4 ± 0.3	3.1 ± 0.5	20.7 ± 1.2
1.2	11.3 ± 0.3*	20.7 ± 0.9*	2.8 ± 0.2*	3.8 ± 0.2*	5.1 ± 1.2	16.9 ± 1.0*
1.8	10.3 ± 0.4*	15.7 ± 0.8*	2.3 ± 0.3*	3.5 ± 0.2*	6.1 ± 2.0	15.1 ± 1.5*
2.5	8.3 ± 0.2*	12.9 ± 0.9*	1.1 ± 0.2*	2.0 ± 0.2*	16.1 ± 3.0*	7.7 ± 0.7*

[a] Nominal chlorpyrifos concentration in µg/L.
[b] Acetylcholinesterase (AChE) activity in µmol/min/g tissue.
[c] Swimming rates are in cm/s.
[d] First strike is in s.
[e] Total strikes at brine shrimp in 1 min.
* Asterisks denote statistical significance from controls (ANOVA, Dunnett's test, $p < 0.05$).

Table 3.3. Benchmark concentration (BMC) estimates for inhibitions in brain and muscle AChE activity, reductions in spontaneous swimming rate, and reductions in strike rate after chlorpyrifos exposures in coho fry. The benchmark response indicates the degree of adverse response for determination of the BMC estimate. Below, benchmark responses include the statistical departures and relative departures, as indicated in parentheses. For example, a benchmark concentration (BMC) for chlorpyrifos (0.4 μg/L) is estimated to cause a 10% inhibition (relative departure) in brain AChE activity of exposed fish.

	biochemical measures		behavioral measures	
benchmark response	brain AChE	muscle AChE	spontaneous swimming rate	total strikes
statistical departure[a]	(4% inhibition)	(8% inhibition)	(11% reduction)	(8% reduction)
BMC[c]	0.3	0.5	0.4	0.3
relative departure[b]	(10% inhibition)	(10% inhibition)	(10% reduction)	(10% reduction)
BMC[c]	0.4	0.6	0.3	0.4

[a] The statistical departure is the 95% lower-confidence interval of the control mean.
[b] The relative departure is a level used for comparative purposes.
[c] Chlorpyrifos benchmark concentration (BMC) estimates are expressed in μg/L.

Correlations between brain AChE inhibition and reductions in spontaneous swimming rate and total strikes

Coho fry exposed to chlorpyrifos showed significant concentration-dependent inhibitions of brain AChE activity (Fig. 3.1B), spontaneous swimming rate (Fig. 3.3B), and total strikes (Fig. 3.4B). The two behavioral measures were plotted as a function of brain AChE activity in Figure 3.5A and 3.5B, relative to respective control means (expressed as a percentage). Spontaneous swimming rate and total strikes were both highly correlated with changes in AChE activity (slope = 1.05, $r^2 = 0.93$, $p < 0.01$; and slope = 0.86, $r^2 = 0.79$, $p < 0.05$, respectively). Thus, brain AChE activity was a good biological indicator for reduced spontaneous swimming rate and total strikes in this study.

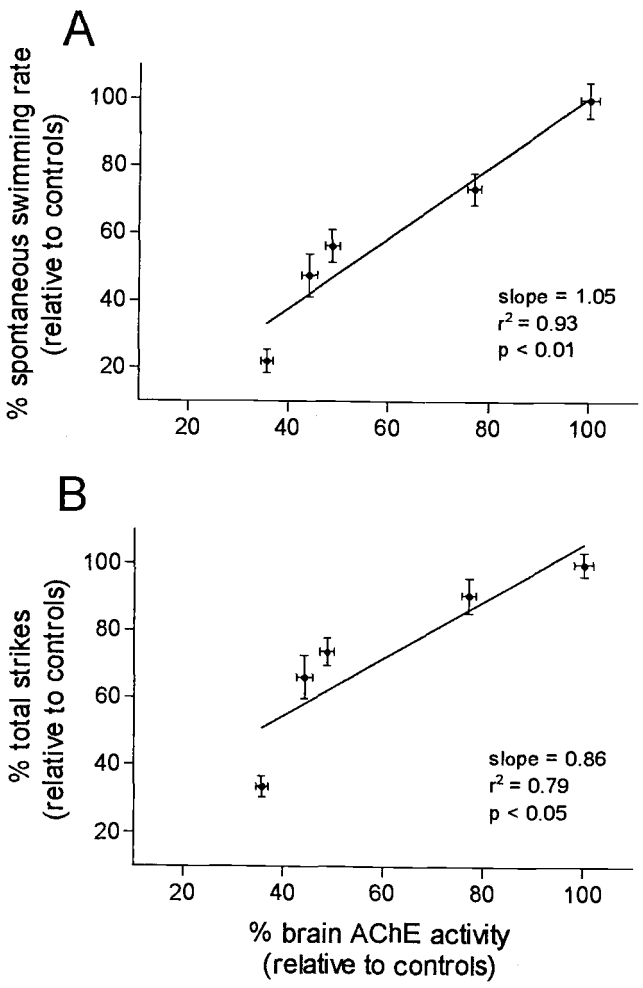

Figure 3.5. (A) Spontaneous swimming rate and (B) total strikes were highly correlated with changes in brain AChE activity ($p < 0.01$, and $p < 0.05$ respectively). Fish were exposed for 96 h to a range of chlorpyrifos concentrations between 0–2.5 μg/L ($n = 15$–17 per exposure level). Points indicate means ± one standard error and are expressed as percentage of respective control group means.

DISCUSSION

Estimating toxicological effects in the field typically requires the measurement of biological markers or indicators that can be indicative of overall biological health in wildlife. It is important to establish clear relationships between the two to adequately assess potential risks that contaminants may pose. This study showed that chlorpyrifos, an anti-cholinesterase insecticide, together inhibits AChE activity and alters potentially important behaviors in coho salmon fry. Biochemical measures of neurotoxicity proved to be highly reflective of altered behavior at exposure concentrations relevant to environmental conditions in urban and agricultural watersheds. Three-dimensional analysis of swimming movements allowed for quantitative measures of subtle changes in the fish behavior. The present data indicate that swimming and feeding behaviors in juvenile salmon are highly sensitive to the effects of chlorpyrifos. This lends support to previous evidence that behavioral endpoints are useful measures of neurological fitness in fish (reviewed in Little and Finger 1990), and that a common biological indicator (AChE activity) can be valuable in estimating potential sublethal impacts in fish.

AChE activity as a useful biological indicator of chlorpyrifos exposure in salmonids

Inhibition of AChE activity is a well-established biological indicator of organophosphate and carbamate toxicity in fish. Cold water species, including salmon and steelhead, are particularly sensitive to cholinesterase inhibiting compounds (Zinkl et al. 1991). Here, we show that AChE in both the central networks (brain) and peripheral networks (muscle) of juvenile coho salmon are similarly inhibited across a range of chlorpyrifos concentrations. Activity of AChE was inhibited by 23% in brain tissue and 12% in muscle tissue at the lowest chlorpyrifos concentration tested (0.6 μg/l). Regression-based analyses of the data suggest that the BMC_{10} (relative departure) for brain AChE inhibition is close to 0.4 μg/L chlorpyrifos. This is very similar to the estimated BMC_{10} for chlorpyrifos (0.6 μg/L) obtained in juvenile steelhead (*Oncorhynchus mykiss*) exposed for 96 h in an earlier study, under slightly different exposure conditions (Sandahl and Jenkins 2002). This finding is significant since it

implies that both coho salmon and steelhead trout share similar biochemical responses to chlorpyrifos exposures, and data may be interchangeable between the two species.

The relative importance of AChE inhibition in either the central or neuro-muscular systems in relation to swimming behaviors in fish is difficult determine, since neurotoxic symptoms may reflect targeted physiological impairment to skeletal muscle, or the general condition or motivational state of the fish. In skeletal muscle, AChE is primarily associated with the nicotinic receptors at the neuro-muscular junction of the end-plate (Taylor and Brown 1989). Depending on the degree of muscle AChE inhibition, effects can range from twitching of muscle fibers to fatigue, tetanus, or eventual paralysis in vertebrates (Ecobichon 1996). In the central nervous system, AChE is associated with both nicotinic and muscarinic receptors, with the latter activating a host of signal transduction proteins that control various sympathetic and parasympathetic functions (Taylor and Brown 1989). Depletion of AChE in the central nervous system can affect the cardiovascular system, sensory systems, secretory glands and the gastrointestinal tract, disrupting general physiological homeostasis. In this present study, we can not determine if the altered behaviors were the function of neuro-muscular impairment (e.g., fish were physiologically unable to maintain continuous swimming activity), or if it reflected the motivational state of the fish (e.g., fish were in a general state of lethargy).

Swimming and feeding behaviors as sensitive measures of sublethal neurotoxicity

Behavior is an important measure of sublethal toxicity, as it reflects impairment across multiple, integrated biological processes. In fish, swimming and feeding behaviors are recognized as being a particularly sensitive indicator of neurotoxicity (Little and Finger 1990), which is supported by the present results. Here, exposures to chlorpyrifos significantly altered behavioral patterns in coho fry at concentrations approximately an order of magnitude below concentrations that are acutely lethal to salmonids (6.0–9.4 µg/L, 96-h; Macek et al. 1969, Holcombe et al. 1982). Spontaneous swimming rate was the most sensitive, and robust, behavioral measure in this study. Because there is a general lack of behavioral data in the current literature, we can not quantitatively compare the behavioral effects measured in this present study to any previous research

with chlorpyrifos. However, qualitatively, fish exposed to other organophosphate and carbamate compounds have also shown reductions in swimming and feeding patterns (e.g., Little et al. 1990, Morgan and Kiceniuk 1990, Van Dolah et al. 1997, Beauvais et al. 2000).

Since swimming and feeding behaviors are known to be highly sensitive to several classes of neurological toxins (reviewed in Sandheinrich and Atchison 1990, and Little and Finger 1990), it is surprising that behavioral research involving anti-cholinesterase compounds is uncommon. One possibility for the disfavor is that some studies have not detected (or detected only slight) behavioral impacts in fish, even at high exposure concentrations and high AChE inhibitions (e.g., Post and Leasure 1974, Cripe et al. 1984). However, as was measured in those studies, swimming stamina and capacity are not sensitive indicators of sublethal neurotoxicity in fish (Little and Finger 1990). Swimming activities that require fine neuro-muscular coordination, such as frequency, duration and speed of movement, frequency and angle of turns, and swimming patterns have been shown to be more sensitive indicators of toxicity (Little and Finger 1990). For example, Beauvais et al. (2000) showed that rainbow trout with low-level inhibitions of brain AChE had behavioral deficits in swimming distance, speed, turns, and tortuosity. But in measurements of swimming stamina, greater than 50–70% depressions in brain AChE activity were required to impact behavior in three salmonid species (Post and Leasure 1974), and in the sheepshead minnow (Cripe et al. 1984).

Ecological implications for swimming and feeding behavioral impairment

Locomotory activities are instrumental for interacting with the dynamic physical, chemical, and biological challenges of the environment. Fish need to continually locate and defend territories, procure food, avoid predators, interact socially with conspecifics, and eventually mate and reproduce. Given that fish are behaviorally sensitive to chemical stressors, these complex ecological interactions are likely to be highly susceptible to the effects of toxicants that disrupt basic neurological function. In the context of the present study, swimming and feeding activities of coho salmon were impacted by chlorpyrifos exposures at environmentally-relevant concentrations. It is plausible that fish exposed to

chlorpyrifos in the wild could exhibit similar behavioral deficiencies. Fish with deviant swimming speed or patterns may become conspicuous to predators (Scholz et al. 2000), or may not be able to elude capture (Hatfield and Anderson 1972). Decreased food intake can also have negative consequences at a time when maximizing body size is essential for young fish. In coho salmon, high food intake and rapid growth are directly correlated with fitness (Dill et al. 1981). Larger body size often secures better habitat territory, which in turn provides better foraging opportunities for high-energy food items (Dill and Frasier 1984). Fish must be quick to capture a drifting prey, or they are likely to lose it to nearby territory holders. Fish that are hungry, or fish that are competing for resources, will take greater risks in the procurement of food even under the threat of a predator (Dill and Frasier 1984).

Chemical pollutants are not typically present in surface waters individually, but co-occur as complex mixtures of metals, petroleum hydrocarbons, and pesticides (U.S. Geological Survey 1999a). Some of these compounds share similar mechanisms of toxicity, such as the organophosphates and carbamates. Alone, chlorpyrifos may reach levels in the environment that cause AChE inhibitions and behavioral alterations in salmonids. Combined with other detected anti-cholinesterase compounds (e.g., azinphos-methyl, carbaryl, carbofuran, diazinon, and malathion), the effects are likely to be additive (U.S. Environmental Protection Agency 2001). This may be the case, as it has been shown that fish inhabiting waters contaminated with mixtures of organophosphates and/or carbamate insecticides can have significant depressions of brain AChE activity (Gruber and Munn 1998). Depending on the degree of enzymatic inhibition, AChE in fish may take several days or weeks to be replenished (Zinkl et al. 1991). Levels of functional AChE may remain depressed for substantial periods of time if exposures are periodic (pulsed discharges into surface waters from runoff) or become chronic.

Organophosphate and carbamate insecticides are widely used in the western United States, and assessing their potential risks to salmon and steelhead is a major priority of regulatory agencies. Although AChE inhibition has been a well-established biological indicator of exposure to anti-cholinesterase compounds, the behavioral and ecological

significance of AChE inhibition has not been adequately related. These present findings support the use of AChE activity as a sensitive biological indicator of toxicity in salmonids exposed to chlorpyrifos. Here were have demonstrated a strong correlation between changes in AChE activity and changes in potentially important behavioral patterns in juvenile coho salmon. If these laboratory-based behavioral effects are reflective of potential behavioral effects under natural conditions, exposures to chlorpyrifos could negatively impact the overall health of wild fish inhabiting contaminated surface waters. Since multiple organophosphate and carbamate insecticides can co-occur in fish habitat, and since they share a common mechanism of toxic action, an important area of future research will be to evaluate their combined effects on the biochemistry and behavior in salmon.

CHAPTER 4

Odor-evoked Field Potentials as Indicators of Sublethal Neurotoxicity in Juvenile Coho Salmon Exposed to Common Agricultural Pesticides.

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ABSTRACT

The sublethal effects of three different pesticides (a metal, organophosphate, and pyrethroid) on juvenile coho salmon (*Oncorhynchus kisutch*) were evaluated using paired electrophysiological recordings from the olfactory epithelium and the olfactory bulb. Animals were exposed to copper (5–20 μg/L), chlorpyrifos (0.625–2.5 μg/L) or esfenvalerate (0.05–0.20 μg/L) for seven days. The olfactory chamber was subsequently perfused with pulses of taurocholic acid or L-serine, two natural odorants that stimulate different classes of primary receptor neurons in salmonids. Sublethal neurotoxicity was measured as a reduction in the amplitude of odor-evoked field potentials in the sensory epithelium and in the olfactory forebrain. Copper and chlorpyrifos inhibited the olfactory response to both odorants in a concentration-dependent manner. Reduced sensitivity was evident in the epithelium and also in the bulb. Benchmark concentrations for a 20% loss of sensory function were 4.4 μg/L for copper and 0.72 μg/L for chlorpyrifos. Esfenvalerate did not affect the amplitude of odor-evoked field potentials. However, in the olfactory bulbs of coho exposed to 0.2 μg/L esfenvalerate, L-serine significantly evoked irregular bursts of postsynaptic activity, possibly indicating sublethal excitotoxicity to central networks. Collectively, these data show that three pesticides with different mechanisms of action, and at environmentally-relevant concentrations, can impair the normal function of the coho olfactory system—a sensory system critical for imprinting, kin recognition, predator avoidance, homing, and other important aspects of the life histories of Pacific salmon.

INTRODUCTION

Surface water monitoring studies in the Pacific Northwest of the United States (Anderson et al. 1996, Anderson et al. 1997, Rinella et al. 1999, U.S. Geological Survey 1999, Voss and Embrey 2000) have found a wide range of current-use pesticides and trace elements in watersheds that provide freshwater habitat for various species of Pacific salmon and steelhead (*Oncorhynchus* sp.). In general, pesticides occur in salmon habitats at levels far below thresholds for acute mortality. However, pesticides are still a concern for salmon, as these chemicals can cause sublethal effects that could ultimately lead to the ecological death of exposed animals (Kruzynski and Birtwell 1994).

Major efforts are currently underway to recover wild salmonid populations in the Pacific Northwest, including coho (*O. kisutch*), chinook (*O. tshawytscha*), steelhead (*O. mykiss*), and other freshwater teleost species that have been listed for protection under the U.S. Endangered Species Act. Much of this effort is focused on restoring the physical and chemical quality of degraded river systems and estuaries. Pesticides represent a key uncertainty for the recovery planning process, in part because of a lack of sublethal toxicity data that are specific to the biology and life histories of anadromous Pacific salmon. To date, very few studies have investigated the effects of pesticides over the range of concentrations that are actually detected in salmon habitats. In addition, few studies have focused on endpoints with clear significance for the viability of wild salmon populations—such as the survival, migration, or reproductive success of individual animals.

To address some of these data gaps, we have investigated the effects of copper, chlorpyrifos, and esfenvalerate on the sensory physiology of juvenile coho salmon. These pesticides are commonly associated with agricultural (and some urban) land-use activities in the Pacific Northwest. Copper, a metal, is used as a fungicide on various crops and as an algaecide in irrigation canals and other waterways. In urbanized watersheds, copper can originate from other, non-pesticide sources, such as wear from vehicle brake pads and clutch plates. Chlorpyrifos, an organophosphate insecticide, is widely used on fruit trees and other agricultural crops. For example, in the spring, chlorpyrifos is applied in

pear and apple orchards for scale and leaf roller control. Esfenvalerate is a pyrethroid insecticide that has been gaining use in Northwest agriculture in recent years, and this trend is expected to continue.

Copper, chlorpyrifos, and esfenvalerate are all known neurotoxicants in salmon and other vertebrates, and each has a different mechanism of toxic action. To assess the sublethal effects of these contaminants on the nervous system of juvenile coho, we used a paired neurophysiological recording method to monitor odor-evoked field potentials from the olfactory epithelium and the olfactory bulb in the forebrain. Coho were exposed to each pesticide for 7 days over a range of concentrations that approximate pesticide levels, and durations, that may be reached in salmon habitat. Sublethal neurotoxicity was measured as a reduction in the amplitude of field potentials that were evoked by two natural odorants; taurocholic acid (TCA) and L-serine. These odorants stimulate different populations of receptor neurons in the sensory epithelium (Hara 1982, Sveinsson and Hara 1990). We specifically focused on the functional properties of the olfactory system because olfaction plays a critical role in imprinting, kin recognition, predator avoidance, homing, and other important aspects of the life histories of Pacific salmon.

MATERIALS AND METHODS

Animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA, USA) at the eyed egg stage and raised at the Northwest Fisheries Science Center's hatchery facility under natural photoperiod conditions. Juvenile coho were maintained in tanks supplied with filtered, dechlorinated municipal water (hereafter referred to as hatchery water; 120 mg/L total hardness as $CaCO_3$, pH 7.1, dissolved oxygen 8.1 mg/L, temperature 11–13°C) on a single-pass flow system. A total of 120 fish were used for this study, with 8 fish per pesticide exposure group. Fish were age 10–12 months with an average size (± standard deviation) of 14 ± 0.8 cm, 30 ± 5.4 g.

Pesticide exposures

Fish were exposed to pesticide solutions for 7 days in aerated glass aquaria, using a static-renewal (12–h) dosing regimen. Nominal exposure solutions were prepared by diluting 0.5 mL of pesticide stock (or carrier alone for controls) into 25 L of source water. Three water samples from each pesticide exposure level were analyzed to compare nominal and measured values. Samples were collected in acid-washed, amber glass bottles fitted with teflon-coated lids and refrigerated at 4°C.

Copper chloride (99% purity; cupric chloride, dihydrate) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Copper stocks were made in distilled water and adjusted to pH 3.0 with HCl to maintain copper in ionic form. Exposure solutions were prepared at nominal concentrations of 0, 5, 10, and 20 μg/L copper. The solutions were analyzed for total dissolved copper by an outside laboratory (Frontier Geosciences, Seattle, WA, USA). Samples were filtered through a pre-cleaned 0.45 μm filter unit and preserved with concentrated HNO_3. The filtrate was subsequently analyzed by inductively coupled plasma-mass spectrometry (ICP-MS, Perkin-Elmer ELAN 6000, detection limit of 0.03 μg/L). The source water for the hatchery facility contained 0.4 μg/L total dissolved copper. Measured concentrations of copper in exposure tanks ranged from 70–72% of nominal values (Table 4.1).

Analytical grade chlorpyrifos (99.2% purity; O,O-diethyl-O-(3,5,6-trichloro-2-pyridinol)-phosphorothionate) and esfenvalerate (98% purity; (S)-alpha-cyano-3-phenoxybenzyl-(S)-2-(4-chloro-phenyl)-3-methylbutyrate) were purchased from Chem Service, Inc. (West Chester, PA, USA). The two pesticide stocks were made in ethanol. Nominal exposure solutions of chlorpyrifos were prepared at 0, 0.625, 1.25, and 2.5 μg/L, and were analyzed by gas chromatography-mass spectrometry (GC-MS), using established methods (Sandahl and Jenkins 2002). Nominal exposure solutions of esfenvalerate were prepared at 0, 0.05, 0.1, and 0.2 μg/L, and exposure solutions were analyzed following the methods of Fairchild et al. (1992) with slight modification, using a Hewlett-Packard 6890 gas chromatograph (Avondale, PA, USA) coupled with a 5972A mass selective detector (GC-MS). For esfenvalerate analysis, initial oven temperature was held at 70°C for 2 min, increased 15°C·min^{-1} to a final temperature of 290°C and

held for 7 min. The MS operated under selective ion monitoring (*m/z* 125, 225, 419). Measured chlorpyrifos and esfenvalerate ranged from 91–105%, and 85–116% of nominal values, respectively (Table 4.1).

Table 4.1. Nominal and measured pesticide concentrations from initial static-renewal (12-h) exposure solutions. Three samples were analyzed from each pesticide level.

	nominal (measured)[a]			
copper	0 (0.4)	5.0 (3.6)	10 (7.0)	20 (14.0)
chlorpyrifos	0 (0.0)	0.625 (0.57)	1.25 (1.31)	2.50 (2.59)
esfenvalerate	0 (0.0)	0.05 (0.06)	0.1 (0.09)	0.2 (0.23)

[a] Concentrations in μg/L.

Electrophysiological measurements

Prior to electrophysiological testing, fish were anesthetized by immersion for 15 min in 50 mg/L tricaine methanesulfonate (MS-222, Sigma) and then injected intramuscularly with the paralytic gallamine triethiodide (0.3 mg/kg body weight). Upon immobilization, fish were placed in a plexiglass holder with chilled (12°C), oxygenated source water containing MS-222 (25 mg/L) delivered to the gills at 120 mL/min (Fig. 4.1A). Skin overlying the right olfactory chamber was removed and the rosette (the supporting structure for the olfactory epithelium) was gently rinsed with 12°C hatchery water, pH 7.6. A section of the skull was then removed and the mesenchymal tissue cleared, exposing the olfactory bulbs, posterior olfactory nerve bundles, and anterior telencephalon. The rosette was subsequently perfused with hatchery water through a glass capillary tube at a rate of 4 mL/min. Fish were allowed to acclimate for 20 min before initiation of electrophysiological recordings.

Two classes of odorants were used to test the effects of pesticides on multiple populations of olfactory receptor neurons (ORNs). Taurocholic acid (a salmonid bile salt) and L-serine (an amino acid) were selected as the test odorants. Each odorant has previously been shown to activate separate populations of ORNs (Hara 1982, Sveinsson and Hara 1990). Stock solutions of TCA and L-serine (Sigma) were prepared in 1 mL aliquots of distilled water and stored at -20°C. Test solutions were prepared daily in acid-washed, amber glass bottles by diluting thawed aliquot stocks into source water. Test solutions of TCA and L-serine were prepared at 10^{-5} M and 10^{-4} M, respectively, as these concentrations elicit similar olfactory responses in juvenile coho (Baldwin et al. 2003).

Electro-olfactograms (EOGs) were obtained using the experimental method of Evans and Hara (1985) as modified by Baldwin et al. (2003). The glass recording micro-electrode was positioned along the midline of the rosette, just above the base of the large, posterior-most lamella and was held stationary during the recording phase of each experiment (Fig. 4.1B). The magnitude of each EOG response was measured as the negative phasic displacement (in mV) from pre-stimulus baseline to evoked peak.

Electro-encephalograms (EEGs) were recorded by pressing a blunt glass micro-electrode against the surface of the olfactory bulb. During the acclimation period, the location of the maximal EEG response to the two odorants was determined by positioning the micro-electrode across the olfactory bulb. Maximal responses to TCA were typically found at the central-medial region of the bulb, and responses to L-serine were maximal at the caudal-medial region (Fig. 4.1C). A third micro-electrode was placed on the head, mid-way between the olfactory bulb and rosette, serving as a reference for both the EOG and EEG recordings. Differential signals were acquired (10 k x gain) and filtered (1–100 Hz band pass) with an AC amplifier (A-M Systems, Carlsborg, WA). Recorded responses were measured in microvolts (μV), half-wave rectified, and integrated with a 3-s time constant. The magnitude of each EEG response was measured as the ratio of the integrated evoked peak over pre-stimulus basal activity.

The odorant delivery system has been previously described (Baldwin et al. 2003). Test EOG and EEG responses were measured simultaneously after brief (5-s) deliveries of each odorant to the rosette (Fig. 4.1D). Fish received 3 pulses of TCA followed by 3

pulses of L-serine with 3-min intervals between pulses. Triplicate responses to TCA and L-serine were averaged to produce a single response value for each odorant. A combination of four response measures (TCA-EOG, TCA-EEG, Ser-EOG and Ser-EEG) was obtained from each fish. In a few cases (8% of fish), surgeries were unsuccessful, and we were unable to obtain the EEG portion of the paired recording.

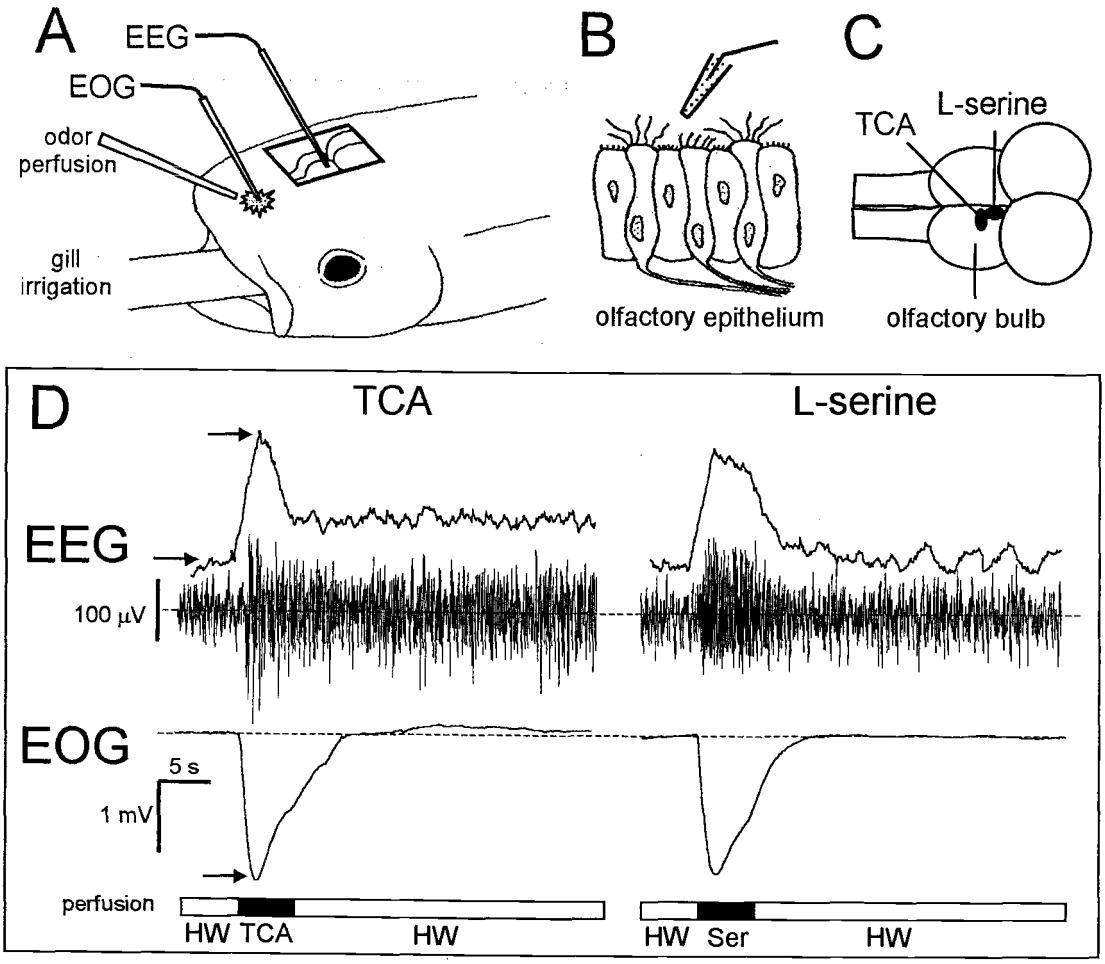

Figure 4.1. Paired electrophysiological recordings from the olfactory epithelium and olfactory bulb of juvenile coho salmon. (A) Positions of the gill irrigation, odorant perfusion, micro-electrode used to record odor-evoked field potentials from the sensory epithelium (electro-olfactogram, or EOG) in the olfactory chamber; micro-electrode pressed against the surface of the olfactory bulb to monitor odor-evoked field potential oscillations (electro-encephalogram, or EEG); reference micro-electrode is not shown. (B) The EOG recording electrode was positioned over the apical surface of the ciliated and microvillar receptor neurons in the sensory epithelium. (C) Locations for recording TCA and L-serine-evoked EEGs from the olfactory bulb. (D) Examples of EOGs and EEGs elicited by switching from a perfusion of hatchery water (HW) to TCA or L-serine (Ser) over the sensory epithelium. The EOG olfactory responses (lower traces) were measured as the negative phasic displacement from pre-stimulus baseline to evoked peak (arrow). The recorded EEG signals (middle traces) were half-wave rectified and integrated with a 3-s time constant (upper traces), and responses were measured as the ratio of the integrated evoked peak over pre-stimulus basal activity (arrows).

Acetylcholinesterase analysis

At the end of each experiment, the olfactory bulbs were removed and frozen at -20°C. The rate of substrate hydrolysis by AChE was analyzed using the colorimetric method of Ellman et al. (1961), as modified by Sandahl and Jenkins (2002). Sample protein content was determined by the method of Bradford (1976). Measurements were performed on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) and all reagents purchased from Sigma Chemical. Samples were analyzed in triplicate wells, including tissue and substrate blanks. Acetylthiocholine iodide was added as the enzyme substrate, and 5,5'-dithiobis-(2-nitro-benzoic acid) (DTNB) was used as a chromogen. The assay was run at 25°C, and change in absorbance (412 nm) was measured at 12-s intervals for 10 min. Acetylcholinesterase activities were normalized to total protein content and expressed as nmol of substrate hydrolyzed/min/mg protein.

Statistics

The effects of pesticides on the active properties of olfactory neurons in the sensory epithelium and the bulb were tested using two-way analysis-of-variance (ANOVA). Grouped measured responses were tested using one-way ANOVA followed by a Bonferroni multiple comparison (GraphPad Prism 3.02, San Diego, CA, USA). Individual and pooled response measures were fit by linear regression (y-intercept constrained to 100, non-constant variance), and the regression was used to calculate benchmark concentrations for copper and chlorpyrifos (U.S. Environmental Protection Agency Benchmark Dose Software, http://cfpub.epa.gov/ncea/cfm/bmds.cfm). Changes in activity patterns of bulbar neurons from esfenvalerate-exposed fish were evaluated using a Kruskal-Wallis test followed by Dunn's multiple comparison. Correlations between the two different odorants, and EOG and EEG recordings were evaluated by the Pearson correlation procedure. Relative AChE activity was compared using one-way ANOVA followed by a Dunnett's multiple comparison.

RESULTS

Odor-evoked field potential recordings from control fish

A brief delivery of odorants to the olfactory rosette evoked negative, or downward, shifts in the local field potential of the sensory epithelium. The waveform of the evoked potential consisted of both phasic and tonic components (Fig. 4.1D). When the rosette was rinsed with hatchery water, the field potential would return to the initial baseline typically within 10–20 s. Baseline bulbar activity, as recorded by the EEG, consisted of irregular waves of oscillation in the local field potential. These waves typically had amplitudes between 20–100 μV (peak to peak). Odor-evoked stimulation of the rosette induced a pattern of regular oscillations with a frequency typically between 8–12 Hz, and amplitudes between 50–200 μV (Fig. 4.1D).

The paired recording configuration allowed for comparisons of odor-evoked activity in two separate locations of the olfactory system. This includes the primary ORNs in the sensory epithelium and the post-synaptic neurons of the forebrain. The paired recordings generated by L-serine were similar in both relative amplitude and shape (phasic and tonic components) and were highly correlated temporally. The paired recordings elicited by TCA, however, were similar in amplitude but not in shape. The EEG response did not return to baseline immediately. Instead, the TCA-evoked potential had a sustained component that lasted 1–2 min after the sensory epithelium was rinsed (Fig. 4.1D).

For control fish ($n = 23$ individual animals), the mean (± standard deviation) EOG amplitudes elicited by TCA and L-serine were 2.1 ± 0.4 and 1.5 ± 0.6 mV, respectively, and the mean EEG response ratios were 1.9 ± 0.5 for TCA and 2.1 ± 0.5 for L-serine. Within fish, responses to the two odorants were highly correlated; i.e., fish that were relatively sensitive to TCA were also sensitive to L-serine. This was true of recordings from the sensory epithelium (Pearson correlation coefficient $r = 0.51$, $p < 0.02$) and also from the olfactory bulb ($r = 0.73$, $p < 0.001$). Moreover, the amplitude of the epithelial responses were highly correlated with bulbar responses for both odorants ($r = 0.41$, $p < 0.001$).

Copper and chlorpyrifos reduce the magnitude of the neurophysiological response to TCA and L-serine

Copper inhibited the olfactory responses to both TCA and L-serine. This included both the peripheral response as well as the bulbar response. An example of the inhibitory effects of copper (10 μg/L) on the active properties of the sensory epithelium and olfactory bulb is shown in Figure 4.2. The magnitude of the four measured responses (TCA-EOG, TCA-EEG, Ser-EOG, and Ser-EEG; n = 6–8 each) were not different within exposure groups ($p = 0.21$) and were together inhibited in a concentration-dependent manner ($p < 0.001$; Fig. 4.3A). The grouped responses at 10 and 20 μg/L were significantly different from controls, and were reduced by approximately 50 and 90%, respectively. Copper had no effect on AChE activity in the olfactory bulb (data not shown).

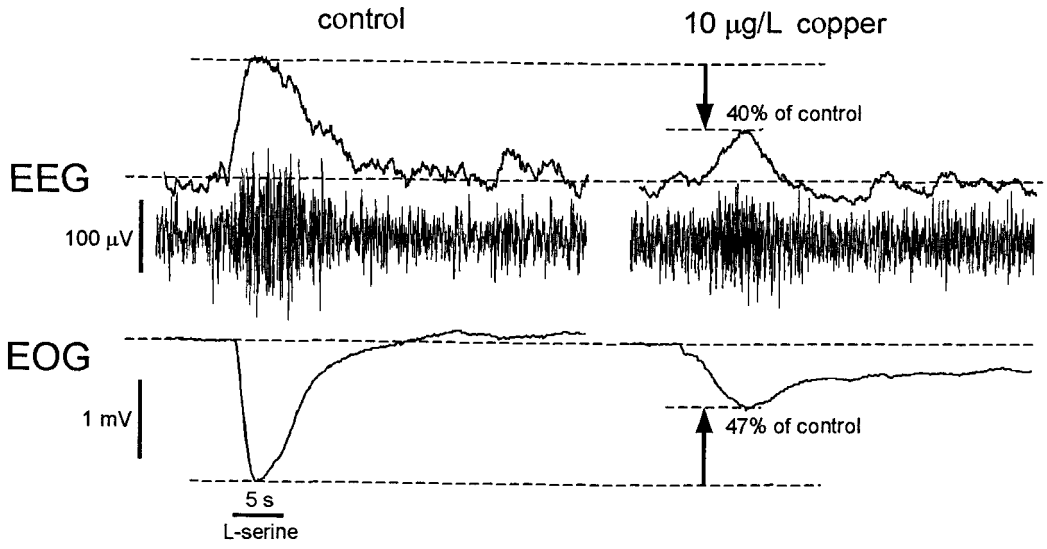

Figure 4.2. Paired EOG and EEG recordings from typical control and copper-exposed (10 μg/L) fish. Responses were elicited by a 5-s presentation of L-serine to the sensory epithelium in the olfactory chamber. In this example, the EOG and EEG responses were reduced to 47% and 40% of the control responses, respectively.

Fish exposed to the highest concentration of copper (20 μg/L) showed an atypical EOG response to TCA. This was a positive (upward) shift in the voltage potential, shown as a negative EOG amplitude relative to controls (Fig. 4.3A). It is uncertain if the paired EEG recordings reflected a specific TCA response or a non-specific response of damaged receptor neurons in the sensory epithelium.

Chlorpyrifos exposure also inhibited the olfactory response to both odorants in the sensory epithelium and in the bulb (Fig. 4.3B). The magnitude of the four measured responses ($n = 6–8$ each) were not different within exposure groups ($p = 0.79$), and were together inhibited in a concentration-dependent manner ($p < 0.001$). All grouped responses were significantly different from controls. At the 0.625 μg/L exposure level, the magnitude of the grouped response was reduced by approximately 25%. At the two highest concentrations tested (1.25 and 2.5 μg/L), the grouped responses were reduced by nearly 50%. Although chlorpyrifos significantly inhibited bulbar AChE activity at the highest exposure level (75% of control value; $p < 0.05$), there was no correlation between bulbar AChE activity and the magnitude of bulbar odor-evoked field potentials ($p > 0.8$).

Esfenvalerate treatment did not affect the magnitude of the measured odor-evoked responses ($n = 5–8$ each) at the olfactory epithelium or olfactory bulb ($p > 0.05$, Fig. 4.3C). There was also no effect on AChE activity in the olfactory bulb.

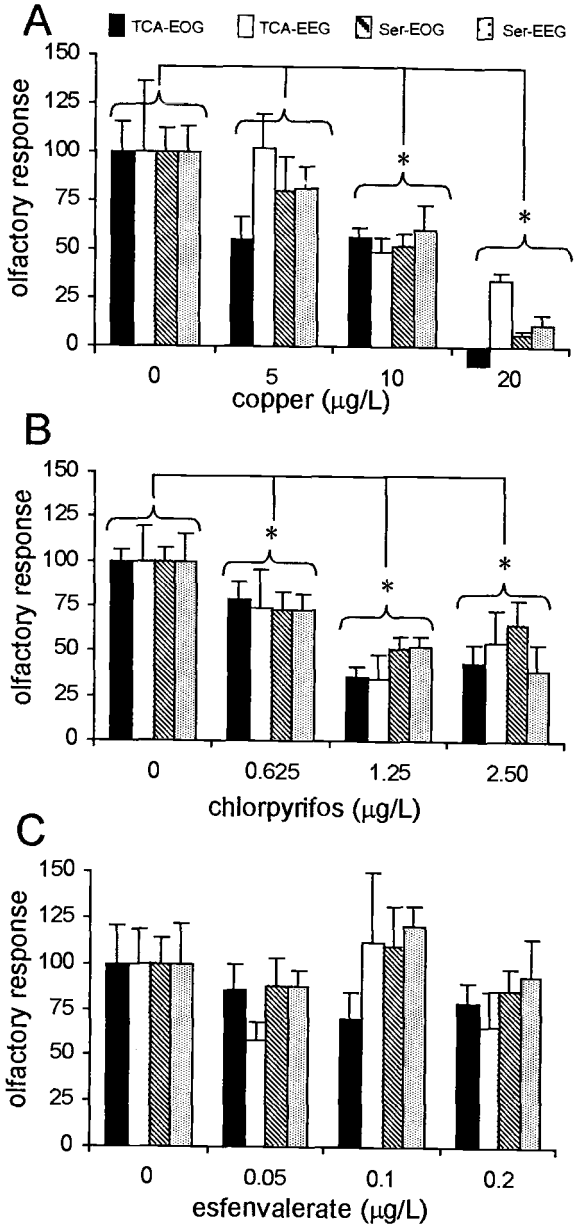

Figure 4.3. Increasing exposure concentrations of (A) copper and (B) chlorpyrifos reduced the magnitude of EOG and EEG amplitudes to taurocholic acid (TCA) and L-serine (two-way ANOVA, $p < 0.001$). The four response measurements ($n = 6–8$ each) were not different within exposures for both copper ($p = 0.21$) and chlorpyrifos ($p = 0.79$), and were therefore grouped for comparisons between controls and exposure levels. Exposures to (C) esfenvalerate showed no significant differences within the measured responses or across exposure concentrations ($p > 0.05$). Olfactory responses are expressed as a percentage of the control response means. Asterisks indicate a statistically significant difference from controls (grouped response measures; one-way ANOVA, Bonferroni multiple comparison, $p < 0.05$); error bars represent 1 SE.

Threshold determination of copper and chlorpyrifos olfactory neurotoxicity

The four normalized response measurements were separately fit by simple linear regression for both copper- and chlorpyrifos-exposed fish (Fig. 4.4). The individually derived slopes were not significantly different and were thus combined to produce a single dose-response fit for copper (pooled slope = -4.5, 95% C.I. -3.6 to -5.3) and chlorpyrifos data (pooled slope = -26.9, 95% C.I. -20.6 to -33.2). Benchmark concentration estimates were then calculated for 20% and 50% reductions in olfactory response (Table 4.2). The BMC_{20} values approximate reductions of two standard errors from copper (28%) and chlorpyrifos (15%) control means. The copper BMC_{20} and BMC_{50} estimates (and lower-bound 95% confidence limit) were determined to be 4.4 (4.3) and 11.1 (10.9) µg/L, respectively. The chlorpyrifos BMC_{20} and BMC_{50} estimates were determined to be 0.72 (0.56) and 1.81 (1.36) µg/L, respectively.

Table 4.2. Copper and chlorpyrifos benchmark concentration (BMC) estimates were determined for olfactory neurotoxicity in juvenile coho salmon. Responses to taurocholic acid (TCA) and L-serine (Ser) were each measured by simultaneous EOG and EEG recordings. Data from the four measured responses (TCA-EOG, TCA-EEG, Ser-EOG and Ser-EEG) were fit individually, and as a pooled response, by linear regression. Benchmark concentrations were determined for 20% and 50% reductions in the magnitude of the olfactory response to the two odorants.

		BMC_{20}[a]	BMC_{50}[a]
copper	TCA-EOG	3.6	8.9
	TCA-EEG	6.1	15.1
	Ser-EOG	4.3	10.7
	Ser-EEG	4.5	11.3
	pooled	**4.4**	**11.1**
chlorpyrifos	TCA-EOG	0.60	1.51
	TCA-EEG	0.78	1.96
	Ser-EOG	0.71	1.77
	Ser-EEG	0.69	1.71
	pooled	**0.74**	**1.81**

[a] Benchmark concentration estimates expressed in µg/L.

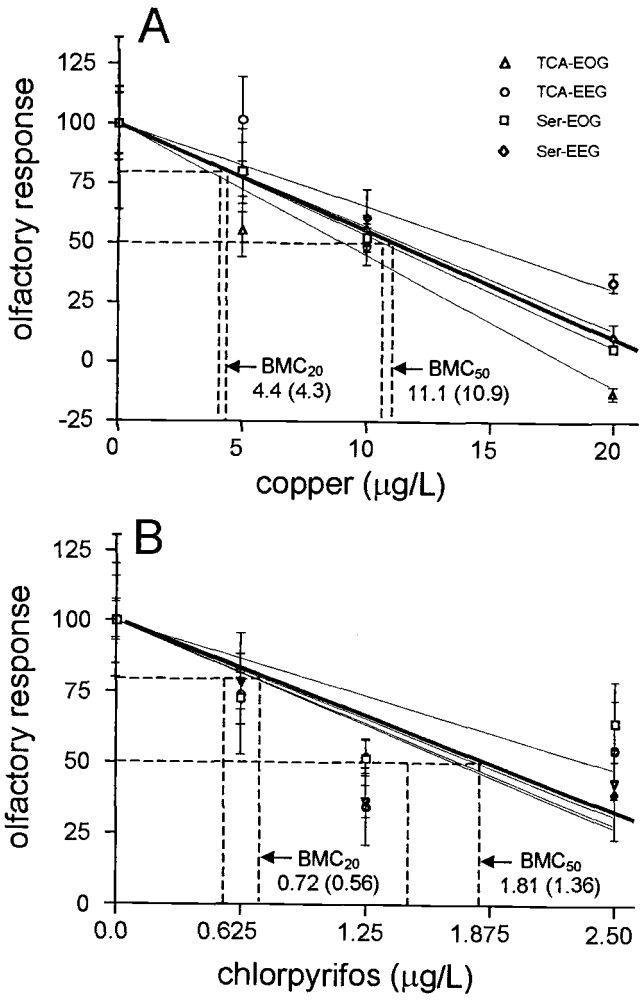

Figure 4.4. Benchmark concentration (BMC) response curves for the inhibitory effects of (A) copper and (B) chlorpyrifos on olfactory responsiveness to TCA and L-serine (Ser). Data from Fig. 4.3A and 4.3B are fit individually (fine lines), and as pooled responses (heavy lines), by linear regression. Horizontal dashed lines represent the 20% and 50% effect levels, while the vertical dashed lines represent central BMC and 95% lower-bound estimates (in parentheses). Esfenvalerate did not affect the magnitude of either the EOG or EEG responses (data not shown). Olfactory responses are expressed as a percentage of the control response means.

Esfenvalerate induces atypical postsynaptic burst activity in the olfactory bulb

Esfenvalerate-treated fish showed an atypical pattern of activity in the olfactory bulb after stimulation by L-serine. This consisted of a series of short (1–3 s), low-frequency

(0–10 Hz) bursts of activity (Fig. 4.5). This burst activity was not readily apparent following TCA stimulation. The excitatory postsynaptic activity may have been masked by the sustained component of the EEGs normally evoked by TCA. For the purpose of quantifying burst events in the bulb, postsynaptic activity was monitored for 2 min after stimulation by L-serine. To better distinguish burst events, the integration time constant of the recording signal was reduced to a 1-s window. A burst was defined as a 1–3-s event of low-frequency activity (0–10 Hz) that exceeded a threshold of three standard deviations from pre-stimulus activity. Using these criteria, 5 of the 7 control fish did not show any postsynaptic burst activity that exceeded the threshold. However, the number of bursts increased with esfenvalerate concentration, with the 0.2 μg/L exposure group significantly different than controls ($p < 0.05$, Table 4.3).

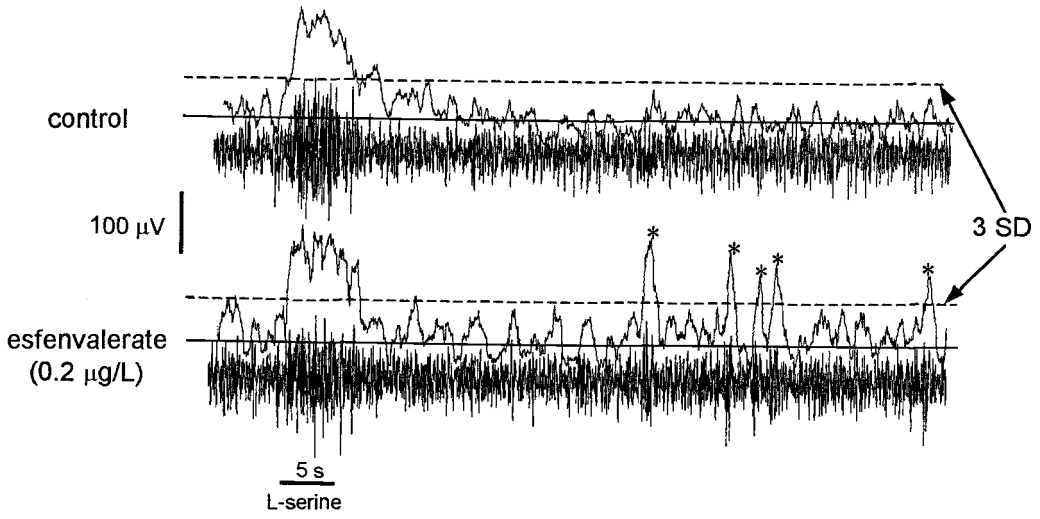

Figure 4.5. Example EEG recordings from control and esfenvalerate-exposed (0.2 μg/L) fish. Exposed fish showed bursts of low-frequency bulbar activity (0–10 Hz) after stimulation by L-serine (asterisks). Bursts were arbitrarily defined as 1–3-s events that exceeded a threshold integrated response (dashed line) of three standard deviations above pre-stimulus activity (solid line). The integration time constant used to generate the analyzed EEG signal was reduced to a 1-s window for better resolution of the burst events.

Table 4.3. Exposure to esfenvalerate increased spontaneous bursting in the coho olfactory bulb. Burst events were counted over a 2-min period beginning 30 s after stimulation of the rosette by L-serine. A burst was defined as a 1–3-s event of low-frequency activity (0–10 Hz) that exceeded a threshold integrated response (1-s time constant) of three standard deviations above pre-stimulus activity.

	esfenvalerate[a]			
burst events	0 (n = 7)	0.05 (n = 6)	0.1 (n = 8)	0.2 (n = 8)
mean -	0.4	1.7	1.4	3.7*
median -	0	1.5	1	3
range -	0-2	0-4	0-4	0-8

[a] Concentrations in μg/L.
* Means compared by a Kruskal-Wallis test followed by Dunn's multiple comparison, significant at $p < 0.05$.

DISCUSSION

It has been recognized for many years that the fish olfactory system is particularly vulnerable to the toxic effects of metals and other environmental contaminants (Klaprat et al. 1992, Sutterlin 1974). This is due, in part, to the direct physical contact between primary sensory neurons and dissolved toxicants in surface waters. Contaminants can also be transported to the central nervous system, where they have the potential to interfere with the processing of sensory information by various regions of the animal's brain (Tjalve et al. 1986). These two points in the olfactory pathway—signal transduction and transmission—can be monitored using paired electrophysiological recordings from the sensory epithelium and the olfactory bulb, respectively. This approach provides for direct, functional measures of sublethal neurotoxicity in salmon and other fish species.

In the present study, a bile salt (10^{-5} M TCA) and an amino acid (10^{-4} M L-serine) stimulated field potentials in the sensory epithelium of control fish that were similar in terms of waveform and amplitude. The bulbar responses to these two odorants, however,

were different. Whereas the response of forebrain networks to L-serine coincided with peripheral activation and termination, the bulbar response to TCA was sustained for a minute or more after the sensory epithelium was no longer activated. The significance of the sustained component is not known, but it does indicate a difference in the processing of the two odorants by the olfactory bulb.

Copper and chlorpyrifos exposures each reduced the responsiveness of the coho olfactory system to TCA and L-serine. The amplitudes of the evoked EOGs and EEGs (phasic peak) were diminished in a similar, concentration-dependent manner. Thus, like copper, chlorpyrifos appears to be a non-selective inhibitor of different classes of receptor neurons in the sensory epithelium. Seven-day exposures to 5, 10, and 20 μg/L copper reduced the response to TCA and L-serine by approximately 20, 50, and 90%, respectively. Baldwin et al. (2003) reported similar results after 30-min perfusions of copper directly to the olfactory rosette of juvenile coho. As the present data indicate, there does not appear to be a compensatory response to copper within a 7-day exposure period. Similar copper exposures lasting more than a few hours are known to trigger cell death among primary receptor neurons (Hara et al. 1976, Winberg et al. 1992, Hansen et al. 1999a). It is therefore likely that diminished EOG and paired EEG responses of copper-exposed animals were a consequence of peripheral degeneration and loss of receptor neurons.

Similarly, the diminished bulbar activity of chlorpyrifos-exposed fish is probably due to a smaller peripheral response to TCA and L-serine. A significant reduction in AChE activity in the olfactory bulbs from these animals indicates uptake and transport of chlorpyrifos to the forebrain. However, there was no significant correlation between reduced AChE activity in the bulb and reductions in odor-evoked EEGs. Rather, chlorpyrifos seems to be inhibiting the responsiveness of olfactory receptor neurons in the sensory epithelium, leading in turn to smaller EEGs. The mode of action for chlorpyrifos in the olfactory rosette has yet to been determined. Chlorpyrifos inhibits AChE, an enzyme that regulates neurotransmitter levels at cholinergic synapses in the vertebrate nervous system (Massoulié et al. 1993). Other anticholinesterase insecticides, including diazinon and carbofuran, also reduce odor-evoked EOGs in Atlantic salmon

(*Salmo salar*) (Moore and Waring 1996, Waring and Moore 1997). Although AChE is expressed in the olfactory rosette of Pacific salmon (chinook; N.L. Scholz, unpublished data), there are no known synapses in the sensory epithelium. Acetylcholinesterase may serve a non-synaptic function in the olfactory rosette. Alternatively, chlorpyrifos may target other cellular proteins such as adenylate cyclase (Song et al. 1997).

In contrast to the inhibitory actions of copper and chlorpyrifos, esfenvalerate is an excitatory neurotoxicant. More specifically, esfenvalerate binds to voltage-activated sodium channels and slows or blocks channel inactivation (Narahashi 1996). As a consequence, neurons are more likely to fire in response to synaptic input, and neurons that are actively firing trains or bursts of action potentials may be unable to terminate or repolarize. Positive ions passing into receptor neurons through cyclic nucleotide-gated cation channels, and not voltage-activated sodium channels, are the basis for odor-evoked, negative extracellular field potentials recorded from the olfactory epithelium (Hara 1992). Therefore, it is not surprising that esfenvalerate did not affect the amplitude of TCA- and L-serine-evoked EOGs. However, the effects of esfenvalerate on postsynaptic activity in the olfactory bulb are consistent with sublethal excitotoxicity. Esfenvalerate-exposed fish showed unusual bursts of activity for a minute or more after a brief 5-s stimulation with L-serine. Similarly, atypical burst patterns have been observed in auditory-evoked EEGs from rats exposed to the pyrethroid decamethrin (Ray 1980). The implication of this delayed burst activity for processing odorant signals from the periphery in the olfactory bulb is not known. However, these data show that esfenvalerate can trigger atypical bursting activity in the coho forebrain at concentrations in the low parts-per-trillion.

The ranges of pesticide exposures in this study were chosen to reflect actual environmental conditions in river systems from the Pacific Northwest of the United States. Copper is a common non-point source contaminant in urban and agricultural watersheds where copper compounds are used as fungicides and algicides. For example, in the Willamette River Basin, Oregon, copper was frequently detected in surface waters at concentrations ranging up to 21 μg/L (Anderson et al. 1996). In the Hood River Basin, Oregon, chlorpyrifos is applied intensively to orchards as a broad-spectrum insecticide

throughout the spring months, with detectable residues present in surface waters during this period. Concentrations of chlorpyrifos in streams from this drainage have reached near 0.5 μg/L (E. Foster, Oregon Department of Environmental Quality, personal communication). In other geographical regions, including the Willamette, Yakima, and Puget Sound Basins, chlorpyrifos co-occurs in streams with other anticholinesterase insecticides, including azinphos-methyl, diazinon, malathion, and carbaryl (Wentz et al. 1998, U.S. Geological Survey 1999b). This raises the possibility of cumulative effects on the sensory physiology of juvenile salmonids. These cumulative effects could occur within a contaminant class (e.g., between chlorpyrifos and other organophosphate insecticides) or across classes (e.g., between chlorpyrifos and copper). To our knowledge, environmental monitoring of esfenvalerate has not been conducted in Pacific Northwest streams, and therefore the concentrations of this insecticide in surface waters are not known, but likely to be below a microgram per liter. Collectively, our results indicate that non-point source contamination of surface waters with pesticides or mixtures of pesticides may interfere with the olfactory system of resident or migratory Pacific salmon.

Olfaction is an important sensory modality for salmonids. Accordingly, a loss of olfactory function could interfere with physiological processes or behaviors that are essential for survival, migration, or reproductive success. It is well established that Pacific salmon are unable to navigate back to their natal streams to spawn when olfactory function is lost (Wisby and Hasler 1954). Sublethal exposures to neurotoxic pesticides that inhibit olfaction could potentially increase rates of straying in wild salmon and hatchery fish (Scholz et al. 2000). Salmon rely on olfaction to avoid water pollution, and their risk of contaminant exposure is higher when their olfactory system is impaired (Hansen et al. 1999b). Olfactory neurotoxicity has been shown to disrupt electrophysiological responses to natural odorants, as well as pheromone-mediated reproductive priming in Atlantic salmon (Moore and Waring 1996, Waring and Moore 1997). Moreover, juvenile chinook salmon exposed to the organophosphate insecticide diazinon are significantly less responsive to chemical cues that signal a predation threat (Scholz et al. 2000). Similar results have been observed in Colorado pikeminnow

exposed to copper (Beyers and Farmer 2001). Presumably, a reduction or loss of olfactory sensitivity could interfere with imprinting, kin recognition, predator avoidance, homing, spawning, and other aspects of Pacific salmon biology that are reliant on olfaction. To explicitly address linkages between physiology and behavior, it will be necessary to relate sublethal thresholds for neurotoxicity, as measured with the electrophysiological recording techniques used in the present study, to individual behaviors that have clear significance at the scale of natural populations. This is an important focus for future research.

CHAPTER 5

Dissolved Copper Reduces the Sensitivity and Behavioral Responsiveness of Juvenile Coho Salmon to an Olfactory Predation Cue.

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ABSTRACT

In salmonids and other fish species, the olfactory system provides an important link between the organism and the dynamic conditions of the external environment. Olfactory receptor neurons are in direct contact with the water, making them highly susceptible to damage by dissolved toxicants. Contaminants that inhibit or alter chemosensory perception can modify the way in which fish respond to various ecological cues. Copper is an example of a pollutant that is commonly detected in salmonid habitats, and is known to be neurotoxic to fish. Here we show that copper, at environmentally-relevant concentrations, can inhibit olfactory sensitivity and diminish olfactory-mediated behaviors in juvenile coho salmon (*Oncorhynchus kisutch*). Short-term (3-h) exposures to copper (2–20 μg/L) reduced the olfactory responsiveness to conspecific skin extract and other natural odorants in a concentration-dependent manner, as measured by the electro-olfactogram. Fish with impaired olfactory capacity were subsequently less likely to initiate antipredator behavior when presented with skin extract solution. Copper significantly reduced both olfactory sensitivity and antipredator behavior at concentrations as low as 2–5 μg/L, although these exposures had no effect on background swimming activity. At 10–20 μg/L copper exposures, electrophysiological and behavioral responses were virtually eliminated in fish. These data suggest that brief pulses of copper into salmonid habitats can have potential negative consequences on the ability of salmon to detect and respond to predator alarm cues.

INTRODUCTION

Non-point source pollution is the leading cause of degraded surface water quality in the United States (U.S. Environmental Protection Agency 2000). Contaminants such as current-use pesticides, heavy metals, and petroleum hydrocarbons are commonly discharged into rivers, lakes, and estuaries via urban and agricultural runoff (U.S. Geological Survey 1999, Pew Oceans Commission 2003). The most serious water quality problems occur in regions with high rates of human population growth. In these geographic areas, there has been little or no progress in controlling non-point source contamination in surface waters over the past few decades (Pew Oceans Commission 2003). In the Pacific Northwest USA, the population is projected to grow by 50% over the next 25 years (U.S. Census Bureau 2000) and this can be expected to lead to an increase in the load of pollutants that are transported to aquatic and marine ecosystems. Consequently, non-point source pollution is an increasing concern for natural resource managers who are working to protect at-risk species throughout the region.

Wild stocks of Pacific salmon (*Oncorhynchus* sp.) are declining throughout much of northern California, Oregon, Washington, and Idaho (USA), with 12 distinct populations recently listed as threatened or endangered under the Endangered Species Act (National Marine Fisheries Service 2000). Of the remaining salmon and steelhead stocks, many are at very low numbers and some are expected to be included for regulatory protection in the foreseeable future (National Marine Fisheries Service 2000). In the past two decades, an estimated 3.3 billion dollars has been spent by the Federal government on salmon and steelhead recovery efforts in the western United States, with no evidence that the expenditures have helped rebuild the declining fish populations (U.S. General Accounting Office 2002). Clearly, resource managers have been faced with major challenges in restoring critical habitat for at-risk salmonids.

For salmon, the regional aim is to conserve healthy stocks and also to restore those runs that have been designated as threatened or endangered. The resource management objectives are therefore focused at the scale of natural populations. Success or failure in meeting these objectives is typically measured in terms of the number of fish that can be

counted at a particular life-history stage. In the context of water pollution, this approach to species conservation poses some important conceptual problems. The most significant difficulty is that the impacts of degraded water quality on salmon are almost always sublethal. Thus, non-point source pollution must be considered at biological scales below the level of the individual. This important disconnect between the disciplines of toxicology and conservation biology has been previously highlighted (Hansen and Johnson 1999a, 1999b). To effectively address the impacts of degraded water quality on salmon it will be necessary to establish the links between altered biological processes at the molecular or cellular level to higher-order processes, such as behavioral alterations, that can ultimately affect the viability of natural populations. Clearly, the most important sublethal impacts of pollutants are those that ultimately limit the survival, reproductive success, or distribution of salmonids. Therefore, from a conservation standpoint, the most informative toxicological measures are those that have clear significance for survival or similar endpoints at or below the scale of individual animals (Hansen and Johnson 1999a, 1999b).

Chemical contaminants can impact aquatic species in different ways, depending on their mode(s) of action. For example, endocrine disrupting chemicals can impair the reproductive biology of fish, and immuno-modulators can render fish more susceptible to disease. Still other classes of contaminants target the fish nervous system, and these pollutants have the potential to disrupt behaviors that are important for predator avoidance and survival (Scholz et al. 2000). For neuro-active contaminants, the olfactory system of salmonids has been a particularly useful experimental system for evaluating potentially important sublethal effects. As noted previously (Klaprat et al. 1992, Hansen et al. 1999b, Baldwin et al. 2003), the salmonid olfactory system is highly sensitive to dissolved pollutants. Moreover, chemical cues in salmon habitats convey important information about the surrounding environment. This includes the chemical profile of an animal's natal stream (for imprinting), the presence and location of food, conspecifics, and predators, the reproductive status of potential mates, and the quality of surface waters. Salmon rely on their sense of smell during home-stream migrations, and contaminants that interfere with olfactory function could potentially increase rates of

straying among wild and hatchery fish. Collectively then, there are many potential linkages between a loss of sensory capacity and the survival, migratory success, or reproductive success of wild Pacific salmon.

Copper is an example of an important contaminant that is frequently detected in salmonid habitats below levels that are acutely lethal to fish (Anderson et al. 1996, Hughes 2003), but at or near levels that may cause sublethal biological harm (Drummond et al. 1973, Juliard et al. 1996, Marr et al. 1996, Hansen et al. 1999a, Beyers and Farmer 2001, Baldwin et al. 2003). Copper is a major component of storm-water pollution that leaches from urban and agricultural landscapes. Within these environments, copper can originate from multiple non-point sources including copper roofing and flashing materials, wood preservatives, vehicle brake linings and clutch plates, and as algaecides and fungicides applied to agricultural crops (Anderson et al. 1996, Snohomish County 2002).

It has been well established that copper is neurotoxic to fish, and is especially damaging to the olfactory system (Brown et al. 1982, Klaprat et al. 1992). A direct electrophysiological recording that is commonly used to measure the impacts of degraded water quality on the salmonid nervous system is the electro-olfactogram (EOG). The EOG records the active properties of olfactory receptor neurons and is obtained with an electrode positioned above the surface of the olfactory sensory epithelium in the olfactory chamber. The amplitude of the EOG reflects the summated electrical generator potential of receptor neurons after they bind odorant molecules (Hara 1992). This technique provides a direct physiological measure of olfactory function in an intact fish. Using this approach, it has previously been shown that short-term copper exposures can impair the sensitivity of peripheral olfactory neurons in salmonids (e.g., Hara et al. 1976, Hansen et al. 1999a, Baldwin et al. 2003) at concentrations that are commonly found in some urban and agricultural watersheds.

While several studies have shown that dissolved copper damages the salmon olfactory nervous system, relatively few studies have evaluated the effects of copper on olfactory-mediated behaviors—and these have focused on avoidance of copper-contaminated surface waters (Rhenberg and Schreck 1986, Hansen et al. 1999b).

Moreover, the relative thresholds for physiological and behavioral toxicity have never been directly addressed. Therefore, the behavioral significance of sublethal neurotoxicity is largely unknown. From a conservation perspective, it is important to know if specific behavioral impairments (e.g., imprinting, homing, and reproductive synchronization) might be inferred from neurotoxicity studies induced under controlled laboratory conditions. The most direct way to address this uncertainty it to make a direct comparison of the thresholds for physiological and behavioral toxicity in animals exposed to copper over a range of environmentally-realistic concentrations.

To this end, we compared the sublethal effects of short-term (3-h) copper exposures on 1) odor-evoked responses from the olfactory epithelium, as measured via the EOG, and 2) odor-evoked predator avoidance behaviors, as measured using three-dimensional analyses of motor activity in juvenile coho salmon (*Oncorhynchus kisutch*). For behavioral measures, antipredator responses were evoked by exposing fish to different dilutions of a conspecific skin extract. In salmonids and other fish species, the physical disruption of specialized cells in the epidermis results in the release of a chemical alarm substance (reviewed in Smith 1992, and Chivers and Smith 1998). This substance triggers a stereotypical behavioral response in conspecifics that are able to detect this olfactory cue (Brown and Smith 1997, Berejikian et al. 1999, Schloz et al. 2000). Importantly, the antipredator response is robust and dependent on olfactory sensitivity. Here we show that the sensory system of copper-exposed coho is less responsive to conspecific skin extract, and that affected fish are less likely to respond to natural cues. Consequently, short-term exposures to copper may have important negative impacts on a wide range of olfactory mediated behaviors in salmon.

MATERIALS AND METHODS

Animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA, USA) at the eyed egg stage and raised at the Northwest Fisheries Science

Center's hatchery facility under natural photoperiod conditions. Coho fry were maintained in tanks supplied with filtered, dechlorinated municipal water (hereafter referred to as hatchery water; 120 mg/L total hardness as $CaCO_3$, pH 6.6, dissolved oxygen 8.1 mg/L, temperature 11–13°C) on a single-pass flow system. Fish were raised on standard commercial salmon pellets, and then one month prior to experiments the diet was changed to frozen brine shrimp (Hikari, Hayward, CA, USA). Fish were age 4–5 months with an average size (± standard deviation) of 4.6 ± 0.4 cm, and 0.9 ± 0.2 g.

Preparation of conspecific skin extract (alarm substance)

Many fish species exhibit predator avoidance behaviors when presented with the skin extract of a conspecific (reviewed by Smith 1992). Although conspecific skin extracts trigger predator avoidance behaviors in salmonids of the genera *Oncorhynchus* (Brown and Smith 1997, Berejikian et al. 1999, Scholz et al. 2000, Scott et al. 2003), the active alarm substance, believed to be contained in specialized epidermal club cells (Smith 1992, Brown et al. 2001), has not been identified. Therefore, we used skin homogenates from conspecific animals to trigger alarm (or predator avoidance behaviors) in the present study. A stock of skin extract was prepared by homogenizing approximately 600 cm^2 of skin collected from 16 juvenile coho in 50 mL dissolved water. The homogenate was filtered through polyester floss, and brought to a total volume of 6 L with additional distilled water. The stock skin extract was thoroughly mixed, aliquoted into 10 ml scintillation vials, and stored at -20°C (approx. 100 cm^2 skin/L). Just prior to electrophysiological and behavioral testing, stock skin extract was thawed, filtered through packed polyester floss to remove fine suspended particles and diluted 1:100 in hatchery water (approx. 1 cm^2 skin/L). Total protein content of this stimulus solution was determined to be 499 μg/mL, using a modified method of Bradford (1976) and Coomassie Plus-2000 Protein Assay Reagent (Pierce, Rockford, IL, USA). Although not a determination of the alarm substance itself (the actual substance is not likely a protein; Brown et al. 2001), this provides a proportional estimate of the total amount of skin present in the stimulus solution. Throughout this paper, concentrations of the skin extract stimulus solution will be expressed in μg protein of skin extract per liter of hatchery

water (μg protein/L, in final dilution within the observation tank), as described above. In this experiment, 10 μg protein corresponded to approximately 2 μm^2 skin.

Copper exposure and analysis

Copper chloride (99% purity; cupric chloride, dihydrate) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Copper stocks were prepared in distilled water. Additions of stocks (100 μL) to exposure tanks (25 L solution volume) produced nominal copper concentrations of 0, 2, 5, 10 and 20 μg/L. Prior to the introduction of fish, water samples (100 mL) from each exposure tank were collected in acid-washed, teflon bottles and refrigerated at 4°C. Fish (n = 8–12 per exposure concentration) were individually exposed for 3 h in aerated glass aquaria. No changes in water temperature, pH, or dissolved oxygen were observed over the exposure period.

Nominal exposure solutions were analyzed for total dissolved copper by an outside laboratory (Frontier Geosciences, Seattle, WA, USA). Samples were filtered through a pre-cleaned 0.45 μm filter unit and preserved with concentrated HNO_3. Individual samples per exposure concentration (n = 2–4) were then combined to produce a total of 3 composite samples per copper level. The composite filtrate was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS, Perkin-Elmer ELAN 6000, detection limit of 0.03 μg/L). Measured copper concentrations for the different nominal exposure groups are shown in Table 5.1. Hatchery water contained 0.3 μg/L total dissolved copper and recovered concentrations of copper in exposure tanks ranged between 84-102% of nominal values. Copper concentrations are expressed as nominal values throughout the text.

Odor-evoked recordings from the coho olfactory epithelium

Two sets of electrophysiological experiments were conducted, using the electro-olfactogram (EOG) as a measure of olfactory sensitivity. The aims were 1) to determine the detectable threshold for EOGs evoked by skin extract in control fish, and 2) to determine the impact of copper on the magnitude of EOGs evoked by skin extract and

other natural odorants in exposed fish. Although presented here first, electrophysiological experiments followed immediately after behavioral trials (below).

Table 5.1. Nominal and measured total dissolved copper (± standard deviation) used in exposure tests.

copper (μg/L)	
nominal	measured
0	0.3 ± 0.2
2	1.9 ± 0.4
5	4.7 ± 0.6
10	10.2 ± 1.6
20	16.8 ± 1.7

Odorant solutions were made daily from concentrated stocks of skin extract, L-serine (an amino acid) and taurocholic acid (TCA; a bile salt). For the threshold experiment, five skin extract solutions were prepared over the range of 10–1000 μg protein/L to generate a stimulus response curve using control fish (n = 8–9 fish per stimulus level). For experiments with copper-exposed fish (n = 9–12 fish per copper level), three different odorants were prepared at a single concentration each; skin extract (1000 μg protein/L), L-serine (10^{-5} M) and TCA (10^{-6} M). These concentrations were selected since they elicit similar, robust EOG amplitudes in coho fry. A second skin extract stimulus-response curve (40–4000 μg protein/L) was generated in a group of fish exposed to 2 μg/L copper (3–6 fish per stimulus level) to determine the shift in olfactory sensitivity over a range of stimulation.

Electro-olfactograms were obtained following the experimental method of Evans and Hara (1985) as modified by Baldwin et al. (2003). Prior to EOG recordings, fish were anesthetized by immersion for 15 min in 50 mg/L tricaine methanesulfonate (MS-222, Sigma) and placed in a plexiglass holder with chilled (12°C), oxygenated hatchery water

containing 50 mg/L MS-222 was delivered to the gills at 10 mL/min. The olfactory chamber was then perfused with 12°C hatchery water (1.2 mL/min) through a glass capillary tube, positioned at the opening of the incurrent nare. A glass recording micro-electrode was positioned through the opening of the excurrent nare and positioned at the midline of the olfactory rosette, just above the base of the large, posterior-most lamella. A reference micro-electrode was placed on the moist surface of the head.

Odorants were delivered to the sensory epithelium, and EOG responses were measured as previously described (Baldwin 2003). In brief, experiments began by bathing the olfactory chamber in background hatchery water until electrode positioning was completed and an electrical baseline was established. Odorants were then pulsed, in turn, over the olfactory epithelium, using computer-controlled manifolds (Neptune Research, West Caldwell, NJ, USA) that allowed rapid switching between solutions. Solutions were delivered at a rate of 1.5 ml/min, and the odorant pulses (5-s) were separated by at least 2 min. Each odorant was tested in triplicate and then averaged to produce a single EOG response value. The magnitude of the EOG response was measured as the negative phasic displacement (mV) of the pre-stimulus recording baseline to the evoked peak (Fig. 5.1A). The response to a blank pulse (hatchery water only) was subtracted from odor-evoked responses for each fish.

Quantification of the antipredator response

An initial set of experiments was conducted to determine the threshold skin extract stimulus required to elicit antipredator behavior in juvenile coho. Fish were presented with different concentrations of stimulus; 0, 4, 10, 40, and 100 μg protein/L. Each fish (n = 7–12 fish per stimulus level) was tested at only one stimulus concentration.

To determine the impact of copper on antipredator behavior, copper-exposed fish (n = 8–12 fish per copper level, no mortalities occurred in the study) were presented with a single concentration of skin extract at 10 μg protein/L. This concentration was determined from initial assays to be sufficient in eliciting stereotypical antipredator behavior in a most control fish.

Responses to the skin extract were measured using an experimental design modified from Berejikian et. al. (1999). Trials were conducted in a 30 L glass aquarium (observation tank) filled with 25 L hatchery water. The experimental area was enclosed with black plastic sheeting to shield the fish from outside disturbances. The observation tank had two adjacent clear glass walls (for front and left camera views), and two opaque walls (back and right side). The tank was lined with 1 cm of gravel substrate, and uniform overhead lighting was provided by wide-spectrum fluorescent lights. A small, in-flow water pump was positioned in the back-right corner of the tank which circulated water at a rate of approximately 100 ml per min. A short length of tygon tubing (50 cm) connected an outside injection port directly to the circulation system. Earlier tests indicated that dye injected into the circulation system dispersed evenly throughout the observation tank within 1 min.

Behavioral trials began by transferring individual fish to the observation tank and allowing them to acclimate for 30 min. At the end of the acclimation period, baseline swimming activity was measured for a period of 3 min. After this period, a solution containing skin extract (diluted to 0.5 mL total volume) was injected into the circulation system (t = 0 s), and then post-stimulus activity of the fish was measured for an additional 4 min. The antipredator response was quantified by comparing the change in swimming activity before (pre-stimulus baseline, t = -180–0 s) and after (post-stimulus, t = 45–75 s) the introduction of skin extract into the observation tank (Fig. 5.1B). A fish was determined to "freeze" if there was a 50% or greater reduction in activity over the post-stimulus interval. Other parameters that were measured, but not quantified during analysis, were latency and duration of the antipredator response.

Spatial movements of the fish were monitored by two orthogonally positioned Firewire digital cameras (Fire-i, Unibrain) connected to a laptop computer (iBook, Apple Computer, Cupertino, CA). One camera was positioned to view the front of the tank, while the second viewed the left side. Custom-written software displayed simultaneously acquired frames from the cameras at12 frames/second, recorded a pair of frames every 2 seconds, and continuously recorded keyboard input. Semi-automated computer video analysis of each pair of frames (custom-written scripts in VideoScript; Videoscript Inc.,

Corrales, NM) located the position of the fish in both two-dimensional views. Triangulation (performed in Excel, Microsoft, Redmond, WA) determined the three dimensional location of the fish. During the behavioral trial, pressing a key on the keyboard indicated the time of stimulus injection.

Statistical analysis

The effects of copper on electrophysiological and behavioral measures were analyzed using either one-way analysis-of-variance (ANOVA) to test for statistical differences between groups (followed by a Dunnett's test for comparisons with controls), or regression analysis to test for concentration-dependent relationships. Paired t-tests were used to determine differences in pre-stimulus baseline activity and post-stimulus activity for antipredator responses. Statistical analyses and graphing were performed with GraphPad Prism 4.0 (San Diego, CA, USA).

RESULTS

Neurophysiological and behavioral responses to conspecific skin extract over a range of stimulus concentrations.

In the aquatic environment, fish are likely to encounter olfactory predation cues that vary considerably in terms of stimulus concentrations. Presumably, there is a threshold below which a fish will not be able to detect and subsequently respond to the alarm substance released from an injured conspecific. We used two different methods to determine the response thresholds of coho fry to a conspecific skin extract over a range of stimulus dilutions. These include 1) electrophysiological recordings from the olfactory epithelium, and 2) video analysis of stereotypical, odor-evoked antipredator behaviors. It is important to note that a specific alarm pheromone has not been isolated for the family *Salmonidae*. However, data from other teleosts suggest that the pheromone is unlikely to be a protein (Brown et al. 2001). Given that the alarm substance in fish is contained within specialized club cells, and these are generally distributed throughout skin tissue,

the amount of alarm substance in the skin extract from this present study is expected to be proportional to the protein content, which we are expressing as μg protein/L. This improves the reproducibility of our current methods in terms of comparing relative concentrations of skin extract between studies, but it does not imply that the actual alarm pheromone is a protein.

Antipredator behavior typically consisted of a reduction in swimming activity 30–60 s after the introduction of skin extract to the observation tank (Fig. 5.1). This brief delay in response presumably reflects the time required for the skin extract to circulate throughout the tank, the position of the fish in the tank at the time of stimulus introduction, or inter-animal variability in terms of motivation to initiate antipredator response. Responses were characterized with an orientation "upstream" into the direction of water flow, and remaining motionless (freezing) via a rapid fanning motion of the pectoral fins. Fish that were positioned higher in the water column when the skin extract was introduced tended to slowly settle toward the bottom of the tank. Although the tendency towards motionlessness was highly stereotypical (fish typically moving less than 1.4 cm/s), the duration of the response was variable, with some fish freezing for tens of seconds and others freezing for four minutes or longer. Qualitatively, the antipredator behavior in coho salmon is very similar with the antipredator behaviors previously reported for other salmonid species, including rainbow trout (*O. mykiss*; Brown and Smith 1997, Scott et al. 2003), brook trout (*Salvelinus fontinalis*; Mirza and Chivers 2000), and chinook salmon (*O. tshawytscha*; Berejikian et al. 1999, Scholz et al. 2000). In this present study, swimming activity was the only parameter of the antipredator behavior monitored. Shelter use was not monitored since it has not been a consistent measure previously (Mirza and Chivers 2000, Scholz et al. 2000, Scott et al. 2003). Feeding activities were not used because, based on pilot tests, coho fry continued to feed on a variety of food items even in the presence of high skin extract concentrations.

Increasing concentrations of skin extract delivered to the sensory epithelium of the fish produced a corresponding increase in the amplitude of odor-evoked field potentials, or electro-olfactograms (EOGs). Skin extract concentrations used to generate the stimulus-response curve ranged from 10-1000 μg protein/L, with evoked EOG responses

ranging from 0.2–1.7 mV (Fig. 5.2). At concentrations of skin extract below 10 μg protein/L, the EOG responses were indistinguishable from responses to a blank solution (hatchery water only). Thus, 10 μg protein/L was the electrophysiological detection threshold for skin extract solution in this study.

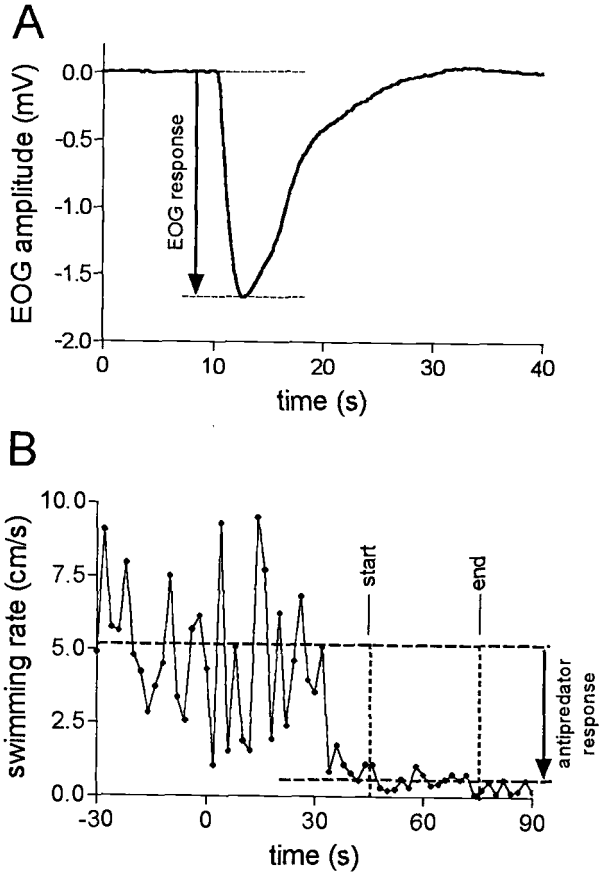

Figure 5.1. Paired physiological and behavioral measures were recorded for individual fish in response to skin extract. (A) Electro-olfactogram (EOG) responses to odorant solutions were recorded from the olfactory sensory epithelium of sedated fish. The magnitude of the EOG reflected the sensitivity of the epithelium to odor-evoked stimulation, measured as the negative phasic displacement from baseline to response peak. (B) Spatial positioning of the fish within the observation tank was determined at 2-sec intervals, using digital imaging and tracking software. This provided an estimation of continuous swimming rates pre- and post-stimulus introduction. The magnitude of the antipredator behavior was measured as the change in mean baseline activity (t = -180–0 s) and post-stimulus activity (t = 45–75 s), as indicated by the horizontal dashed lines in the figure. The vertical dashed lines indicate the interval in which the antipredator behavior was observed for measurement.

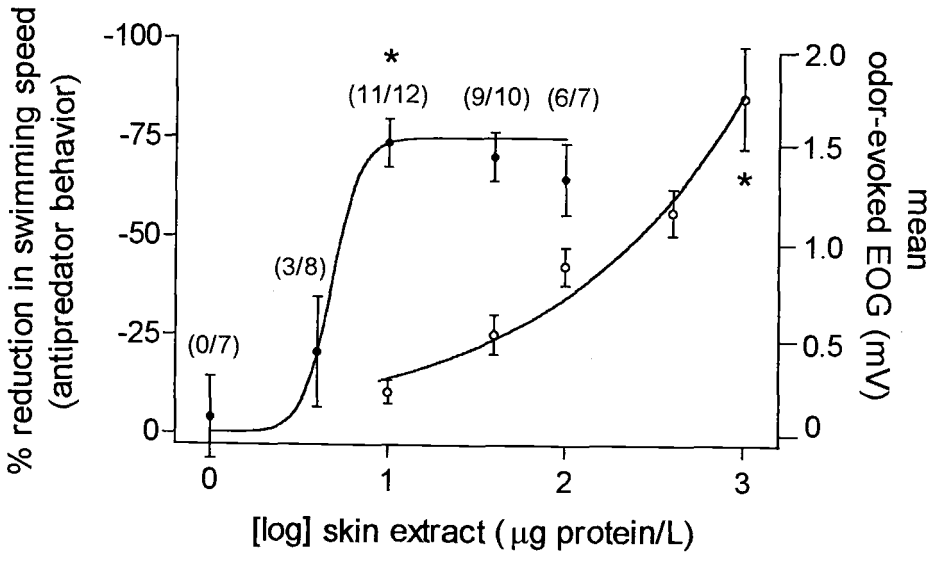

Figure 5.2. Curves were created for both electrophysiological (open circles) and behavioral responses (closed circles) to skin extract. Increasing the concentration of skin extract (10–1000 µg protein/L) delivered to the sensory epithelium resulted in increasing electro-olfactogram (EOG) response amplitudes. Fish presented with blank solution (filtered water only) did not elicit alarm behavior. Fish began to show antipredator behavior at 4 µg protein/L, which was measured as a decrease in swimming activity. Nearly all fish reacted to the 10 µg protein/L skin extract stimulus, and increases in stimulus above this point did not result in a further degree of antipredator behavior. Fractions within parentheses correspond to the number of fish eliciting a freeze response from the total number tested (> 50% reduction in activity).Error bars represent one standard error. Asterisks denote the skin extract concentration used in copper exposure experiments.

For behavioral trials, fish were presented with varying concentrations of conspecific skin extract, and the odor-evoked antipredator response was monitored using three-dimensional analysis of swimming behavior (Fig. 5.2). Initial pre-stimulus baseline activity was the same for all groups of fish (mean ± SE; 5.2 ± 0.2 cm/s, $p > 0.5$). Fish that received a blank stimulus (hatchery water only) showed no change in swimming activity over the pre- and post-stimulus observation period (paired t-test, $p > 0.5$), and no animals ($n = 0$ of 7) showed a freeze. As a group, fish presented with a 4 µg protein/L stimulus showed a slight antipredator response (mean ± SE; -21 ± 14% activity, paired t-test, p =

0.12), with 3 of 8 animals showing a freeze. At the next highest stimulus concentration (10 μg protein/L), there was a significant antipredator response (-74 ± 6% activity, paired t-test, $p < 0.001$), with 11 of 12 fish showing a freeze. Further increases in stimulus concentration (40–100 μg protein/L) still resulted in significant antipredator responses (paired t-test, $p < 0.001$), but did not further reduce activity compared to the 10 μg protein/L stimulus group, resulting in an apparent response plateau.

Relative thresholds for neurophysiological and behavioral toxicity among coho fry exposed to copper

Dissolved copper is known to impair the salmonid olfactory system, and has previously been shown to inhibit electrophysiological responses at the sensory epithelium and corresponding sensory input at the olfactory bulb (Baldwin et al. 2003, Hansen et al. 1999a, Sandahl et al.—see Chapter 4). To estimate the relative impacts of short-term (3-h) copper exposures (2–20 μg/L) on odor-evoked EOGs and odor-evoked antipredator behavior, we 1) recorded electrophysiological responses from the olfactory epithelium, using three different odorants to determine if neurotoxic effects impact multiple odorant receptor pathways, and 2) used video analysis to measure stereotypical antipredator responses in fish presented with 10 μg protein/L skin extract. From the behavioral stimulus-response experiment it was determined that this concentration approached the threshold for eliciting antipredator responses in most coho fry (see Fig. 5.2). The two data acquisition techniques were paired such that matched neurophysiological and behavioral data were collected from each animal. Examples of the paired electrophysiological and behavioral measurements from four control and four copper-exposed (10 μg/L) fish are shown in Figure 5.3.

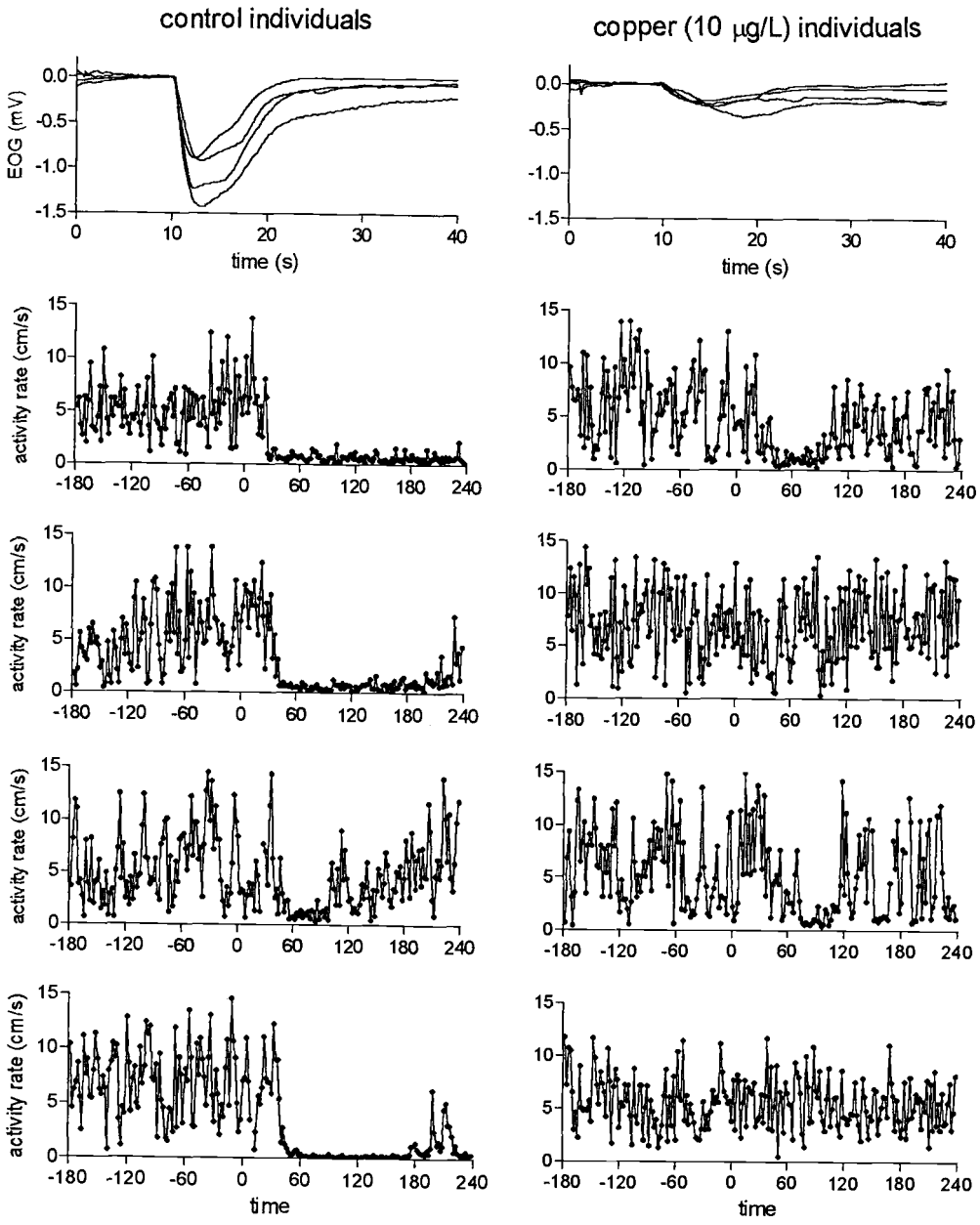

Figure 5.3. Representative examples of electrophysiological and behavioral responses to skin extract in control fish and fish exposed to 10 µg/L copper (4 each). The mean electro-olfactogram (EOG) response to skin extract was reduced approximately 85% in fish exposed to copper. Subsequently, fewer fish exposed to copper elicited antipredator behavior as compared to controls.

As measured by stimulus-evoked field potential recordings from the olfactory epithelium, copper inhibited responses to the three odorants (skin extract, L-serine, and TCA) in a concentration-dependent manner (ANOVA, $p < 0.001$). Data are presented in Figure 5.4A. For control fish, the mean EOG amplitudes to skin extract (1000 μg protein/L), L-serine (10^{-5} M) and TCA (10^{-6} M) were 1.2, 2.8 and 4.0 mV, respectively. The inhibitory effects of copper on the responsiveness to skin extract are presented in Table 5.2. At the lowest copper exposure concentration (2 μg/L), the mean skin extract-evoked EOG amplitude was 0.6 mV, which was significantly lower than the control mean (ANOVA, Dunnett's test, $p < 0.01$). The EOG responses to skin extract were nearly abolished at exposures to 10 and 20 μg/L copper (mean = 0.1 and 0.2 mV, respectively).

Table 5.2. Effects of copper on olfactory physiology and antipredator behavior.

copper [a]	EOG response [b]	baseline activity [c]	post-stimulus activity [c]	antipredator behavior [d]	freeze responses [e]
0	1.2 ± 0.2	5.6 ± 0.4	1.4 ± 0.3	-74%	11/12
2	0.6 ± 0.1*	6.0 ± 0.3	3.7 ± 0.7	-39%	6/12*
5	0.4 ± 0.04*	5.6 ± 0.3	4.8 ± 0.8	-15%*	3/12*
10	0.2 ± 0.04*	5.2 ± 0.5	4.1 ± 0.6	-13%*	2/12*
20	0.1 ± 0.03*	2.3 ± 0.4*	2.4 ± 0.6	+14%*	1/8*

[a] Nominal copper concentrations expressed in μg/L.
[b] EOG (electro-olfactogram) responses measured in mV (mean ± standard error).
[c] Activities measured in cm/s (± one standard error).
[d] Antipredator behavior expressed as the percent reduction in activity.
[e] A freeze respone was an individual fish with a 50% or greater reduction in activity; fractions represent the number of responders over the total number of fish tested.
* Asterisks represent statistical difference from controls ($p < 0.05$).

The effects of copper on antipredator behavior are graphically shown in Figure 5.4B, and quantified comparisons are presented in Table 5.2. In behavioral trials, baseline swimming activity was unaffected by copper concentrations up to 10 μg/L (Table 5.2). Fish exposed at 20 μg/L copper were lethargic, as indicated by a mean baseline activity

in these fish (mean ± SE; 2.3 ± 0.4 cm/s) that was significantly lower than the controls (5.6 ± 0.4 cm/s, ANOVA, $p < 0.05$) and the other groups (5.6 ± 0.2 cm/s). Upon presentation of the skin extract stimulus, control fish responded by significantly reducing mean swimming activity (mean ± SE, -74 ± 6% activity, paired t-test, $p < 0.001$), with all but one fish (11 of 12) showing a freeze (Fig. 5.4B, Table 5.2). In fish exposed to 2 μg/L copper, the mean anti-predator response (-39 ± 12% activity) was lower than controls but still significant (paired t-test, $p < 0.01$), with half of the fish (6 of 12) showing a freeze. At higher copper levels (5, 10 and 20 μg/L), antipredator responses were not significant (paired t-tests, $p > 0.1$), and the majority of fish failed to initiate a freeze. For copper-exposed fish that did respond to the stimulus, the duration of antipredator responses were generally shorter than those of controls. Overall, however, too few of the copper-exposed fish responded to allow for a statistical comparison of response duration.

Taken together, paired physiological and behavioral measures allowed for a direct comparison of the inhibitory effects of copper on the sensory biology and odor-evoked behavior of coho fry. In Figure 5.4C, EOG data are plotted together with antipredator behavioral data from control and copper-exposed fish. There was a strong correlation between inhibited olfactory sensitivity and the antipredator response ($r^2 = 0.94$, $p < 0.01$). From the slope of the correlated measures (linear regression, slope = -75 ± 11), a 25% decrease in olfactory sensitivity to skin extract (0.9 mV is the statistical threshold from the control mean in this study, based on sample size and variance of data) corresponded with a 29 ± 4% reduction in antipredator responsiveness.

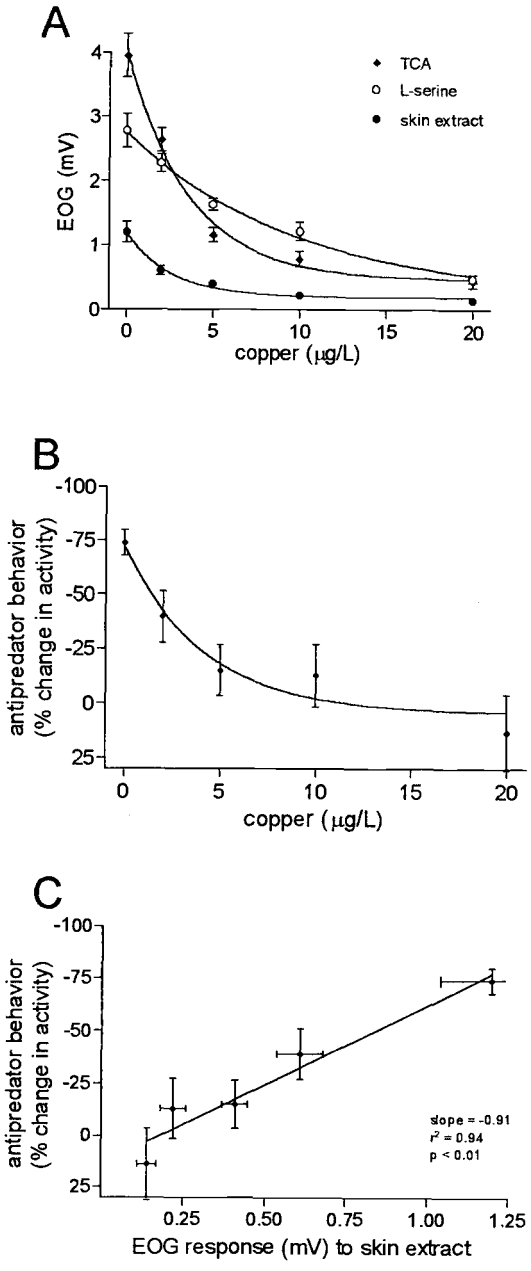

Figure 5.4. Copper diminished olfactory sensitivity and alarm behavior in coho fry. (A) Electro-olfactogram (EOG) responses to skin extract (1000 µg protein/L), L-serine (10^{-5} M), and taurocholic acid (TCA; 10^{-6} M) were each inhibited by increasing copper exposure. (B) Fish also failed to elicit antipredator behavior as copper levels increased. (C) The paired physiological and behavioral response means were highly correlated; i.e., fish with reduced olfactory sensitivity were less likely than controls to initiate antipredator behavior. Error bars in all graphs represent one standard error mean.

DISCUSSION

For salmon and steelhead, olfaction is a key sensory function that conveys important information about predator, prey, conspecifics, and the animal's surrounding environment. In the case of predation, the olfactory-mediated detection of an alarm pheromone is important for the detection and avoidance of potential bird or fish predators. As would be expected, fish that detect and respond to a conspecific alarm signal are more successful at avoiding predators than those that do not (Mathis and Smith 1999).

Here, we show that copper diminished the olfactory sensitivity of juvenile coho to skin extract (alarm substance), and that this loss of olfactory function led, in turn, to a failure to initiate predator avoidance response. Exposures to dissolved copper at nominal concentrations of 2 μg/L or higher were sufficient to significantly impair both olfactory physiology and behavior of coho. As indicated by the paired measurements, the amplitudes of electrophysiological responses to skin extract were significantly correlated with antipredator behavior in fish. Thus, the EOG was a relatively good predictive indicator of behavioral impairment.

Estimated effects of dissolved copper on olfactory-mediated behavioral thresholds

Experimental data (Fig. 5.2) indicate that the behavioral stimulus-response curve to skin extract is very steep, and that exposure to copper can shift the responsiveness of the olfactory system over a wide range of stimulus concentrations. As shown in Figure 5.5A, the principal effect of a short-term exposure to dissolved copper (2 μg/L) is to shift the stimulus-response curve, such that any given concentration of skin extract elicits a smaller electrophysiological response from peripheral olfactory neurons. For example, exposure to 2 μg/L copper for 3 h reduced the amplitude of EOG responses to skin extract over the entire stimulus range by approximately 40%, effectively shifting the stimulus-response curve to the right nearly a half log unit. This shift can be expected to increase with increasing concentrations of dissolved copper. This is shown in Figure

5.5B, where hypothetical stimulus-response curves were produced for fish exposed to copper at 2, 5, 10, and 20 μg/L.

For each concentration, a single measured value (from Table 5.2) was used to plot inhibitory effects at a single concentration of skin extract. The slope of the stimulus-response curve was then estimated by fitting a fixed exponential function to the points (the average of the two functions in Fig. 5.5A). As evident from Fig. 5.5B, copper exposures can cause a dose-dependent shift of the stimulus-response curves to the right. Thus, by rendering the olfactory system relatively insensitive to natural odorants, copper can be expected to mask or obscure the perception of environmental cues that would otherwise trigger avoidance behaviors.

We have previously shown that a copper-induced reduction in odor-evoked EOGs in the peripheral olfactory epithelium results in a corresponding reduction in the integrated response of the olfactory forebrain to chemical signals (see Chapter 4). Here we find that these electrophysiological measures also have direct behavioral correlations, and that a shift in the threshold for a neurophysiological response is reasonably predictive of a shift in the threshold for a behavioral response. This relationship is illustrated in Figure 5.5C. The bold lines represent control stimulus curves for EOG responsiveness to skin extract (labeled physiology), and the probability of a fish eliciting an antipredator response when presented with skin extract (labeled behavior). The control curves were generated from experimental data (Table 5.2), but are partially extrapolated beyond the range of data collected for demonstration purposes. The fine lines represent changes in physiology and behavior that may occur due to copper exposure (in this example, a copper exposure of 2 μg/L for 3 h). From experimental data shown in Fig. 5.2, a minimum physiological input of about 0.3 mV is required to elicit an antipredator response in most fish, and this is assumed to be the threshold EOG input in this model (labeled minimum EOG input). A skin extract concentration of 10 μg protein/L (2 μm^2 skin/L) was required to elicit this response. Since copper can shift the olfactory system's responsiveness to skin extract this can be expected to proportionately shift the concentration or intensity of the stimulus needed to trigger the anti-predator behavior. In the example presented in Figure 5.5C, a skin extract concentration of about 40 μg protein/L is required to generate the minimum

EOG input and subsequently elicit antipredator behavior in fish exposed at least to 2 μg/L copper—a four fold increase in necessary stimulus due to a 40% reduction in olfactory function.

A critical factor in the design of this current experiment was the selection of skin extract concentration to use in the behavioral trials. By using a concentration near response thresholds (10 μg protein/L), a small shift in olfactory sensitivity resulted in a significant shift in behavior; an effect that would be missed if using higher concentrations of skin extract. This was clearly demonstrated in preliminary trials when we presented fish with 4–10 times the above stimulus concentration, and nearly all fish elicited an alarm response, despite EOG data indicating that olfactory sensitivity had been inhibited by 70–90%. Although it is not currently possible to determine if the skin extract concentration used in the behavioral trials was reflective of real-world conditions, it may be a reasonable estimate. Reist et al. (1987) reported that up to 40% of surveyed whitefish possessed injury scars, presumably from attack encounters, and that damage and escapement may be common events in wild fish. This may also be true for juvenile salmon. As estimated from the behavioral threshold data in this present study, a skin tear of 0.5 x 4 mm (2 mm^2) contains enough alarm pheromone to active 1000 L (or 1 m^3) of water—or, 1 cm^2 of skin could activate a hypothetical stream 1 m deep, 2 m wide, and 25 m long (50 m^3). This is consistent with calculations made for fathead minnows (*Pimephales promelas*), where it was estimated that 1 cm^2 of skin can generate an active space of 58 m^3 (Lawrence and Smith 1989).

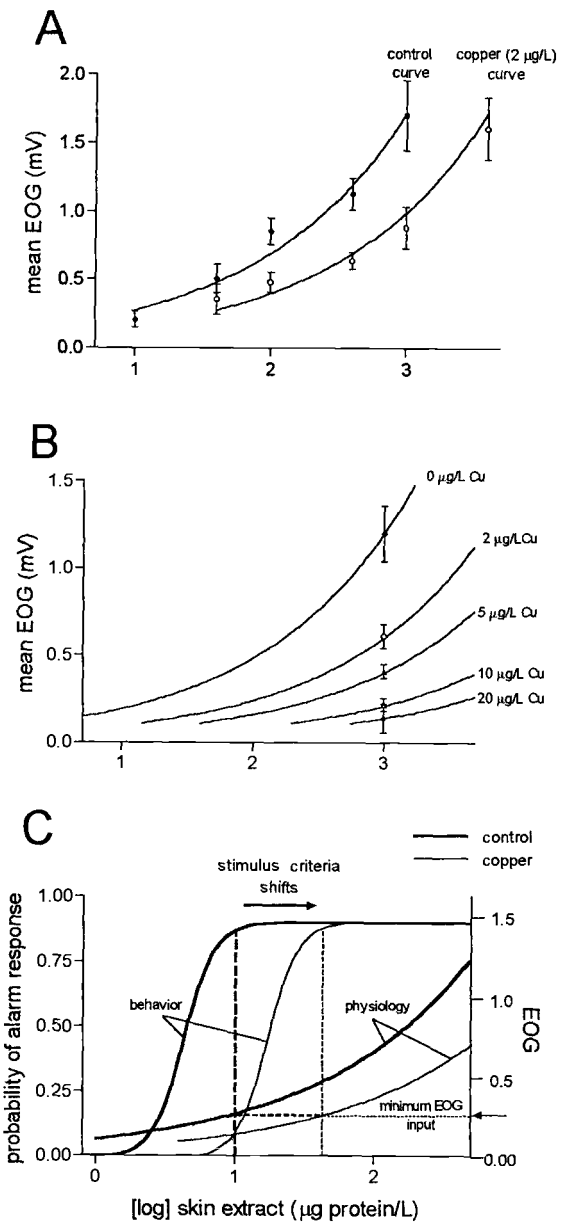

Figure 5.5. Theoretical response models indicate that shifts in olfactory sensitivity will result in corresponding shifts in antipredator behavior. (A) Experimental electro-olfactogram (EOG) data show that the skin extract stimulus-response curve is shifted to the right after copper exposure. (B) Hypothetical stimulus-response curves were produced for fish exposed to increasing concentrations of copper. Actual data points were used (from Table 5.2) to plot the effect of copper on a single concentration of skin extract, then the points were "fit" with a fixed exponential function. This shows how the stimulus-response curve may shift over a range of copper exposures. (C) A shift in olfactory physiology may also shift the stimulus requirements necessary to elicit antipredator behavior. In this theoretical model, a skin extract concentration of 10 µg protein/L is required to generate the minimum EOG input of 0.3 mV, and subsequently, elicit antipredator behavior in 90% of control fish. Exposure to copper (in this example 2 µg/L) shifts the requirement of skin extract concentration to about 40 µg protein/L. Error bars in graphs (A) and (B) represent one standard error mean.

Potential ecological relevance

There are two potential sublethal impacts of copper on juvenile coho with respect to antipredator behavior. First, animals at or near the edge of the active space of the alarm pheromone (i.e., at or near response threshold) may fail to respond to the predation cue.

Second, it may require, for example, a four-fold or higher stimulus concentration to evoke a response in fish exposed to copper at concentrations of 2 μg/L or more. The importance of olfactory acuity has been demonstrated in field tests with brook trout, showing that the recognition of alarm pheromones can increase survival time during encounters with a predator (Mirza and Chivers 2000). Olfactory impairment could put affected fish at an ecological disadvantage in the predator-prey interactions that exists in natural systems, since not all sensory systems are equivalently affected by toxicants. For example, previous research with juvenile salmon has shown that pesticide exposure can impair olfactory-mediated behavior, while leaving visual systems functioning properly (Scholz et al. 2000). To avoid detection by visually guided predators, such as birds and larger fish, color and patterning (bars and spots) help to render juvenile salmon cryptic against the gravel substrate when remaining motionless (Dill and Frasier 1984). Hatfield and Anderson (1972) noted that motionlessness was an important mechanism to enhance survival, since juvenile salmon that rapidly reduced movement in the presence of a predator suddenly became "invisible". Since juvenile salmon use olfactory cues as part of their defense strategy to avoid visually guided predators, the neurotoxic impact of copper could disproportionately affect the salmon. It is often the case that prey animals in substandard condition are more vulnerable to capture by a predator, even in situations where both animals are under the biological stress of a toxicant (reviewed in Mesa et al. 1994). In freshwater habitats receiving periodic inputs of copper, behavioral deficiencies could lead to the delayed ecological death of substandard fish (Kruzynski and Birtwell 1994).

Management implications

The presence of copper and other heavy metals in surface waters is a potential limiting factor to salmon and steelhead recovery efforts in the Pacific Northwest, USA (National Marine Fisheries Service 1996). In the Willamette Basin, Oregon, copper was detected in 19 of 23 surface water samples taken from tributaries of the Willamette River (Anderson et al. 1996). In that survey, the median concentration of copper was 2.2 μg/L, with a maximum detection at 21 μg/L. Agricultural and urban water systems in

Washington state have similarly contained dissolved copper, with mean detections ranging between 1-5 μg/L, and maximum levels reaching 9.1 μg/L (Hughes 2003) and 70-160 μg/L (Snohomish County 2002). Thus, the concentrations used in this present study (2-20 μg/L) reflect realistic conditions that fish are likely to encounter in degraded surface waters. In addition, our exposure duration of 3 h is relevant in terms of storm water events that can load streams with contaminated surface runoff. In this regard, the U.S. Environmental Protection Agency's water quality guidelines for copper may not be adequate for protecting salmon from sublethal neurotoxicity. For dissolved copper in freshwater systems (total water hardness of 100 mg/L), the U.S. Environmental Protection Agency's water quality guideline is 13 μg/L for a 1-h average maximum concentration. As shown in this present study, 2 μg/L copper is sufficient to significantly inhibit olfactory sensitivity to natural odorants, and 5 μg/L copper nearly eliminated antipredator behavior in exposed coho fry. Effects were further accentuated at higher levels of copper exposure. It is uncertain if coho fry can acclimate to sustained increases in background copper in the natural environment, but previous laboratory experiments with coho juveniles showed that odor-evoked electrophysiological responses remained inhibited in fish exposed (5–20 μg/L) for one week (Sandahl et al.—see Chapter 4).

In the Pacific Northwest of the United States, the current approach to evaluating the status and recovery of threatened or endangered salmonids is to measure the numbers and distribution of individual animals at a particular life-stage. Environmental monitoring efforts do not typically consider the health or performance of individual animals. As we have shown in this present research, degraded water quality in agricultural and urbanized watersheds may be limiting salmonid productivity in ways that would not be evident from field surveys. If this is true, watershed planners and natural resource managers throughout the region may be systematically underestimating the negative impacts of degraded water quality on at-risk species. The omission of sublethal toxicology data from the overall current habitat perspective stems from the traditional disconnect between the disciplines of toxicology and conservation biology (Hansen and Johnson 1999a, 1999b). To bridge this gap, it will be necessary to extend our current findings to future studies

using more environmentally realistic conditions and natural predators. From actual mortality data, it should be possible to estimate the relationship between water quality degradation and the ecological death of juvenile salmonids (e.g., Kruzynski and Birtwell 1994). This information could then be used to refine probabilistic estimates for stage-specific survival in an integrated life-cycle model. This is an important area for future research.

CHAPTER 6

CONCLUSIONS

Challenges in evaluating sublethal risks of environmental pesticide exposures

Surface water monitoring surveys have consistently shown that pesticides, including copper, are pervasive throughout the western United States (see Tables 1.1 and 1.2). It is also known that populations of Pacific salmon and steelhead have declined sharply over the past decades (National Marine Fisheries Service 1996, 1998). But is there a relationship? There is no doubt that these chemicals can be extremely toxic to fish at some concentration level, but the tenet of toxicological science is that toxicity is dependant on the dose. In this present case, contaminants typically occur in exceedingly low concentrations in the environment (low parts-per-trillion), often several orders of magnitude below levels that are known to be acutely harmful to fish. At times, however, spikes in chemical concentrations can occur due to surface-water runoff, spray drift, or accidental discharges. Are fish, then, exposed to pesticides or copper in substantial quantities and over a sufficient amount of time to cause toxic injury? If so, how can non-traditional sublethal effects, such as altered behavior, be quantified in ways that are ecologically significant? For risk assessments involving special-status species—as must be done with the 54 pesticides currently under evaluation with the Environmental Protection Agency—these questions pose a considerable conceptual challenge. In a region of the United States that relies on the economic viability of both agriculture and fishing industries, it is unlikely that pesticides will completely disappear from surface waters, and it is equally unlikely that lawsuits to protect salmon will cease to be utilized in the near future. It is certain, though, that science alone will not solve these highly politicized issues. Toxicological research (such as the work contained in this thesis) can help by reducing scientific uncertainties regarding the sublethal impacts of contaminants on salmonids, ultimately allowing for better management decisions to be made.

Toxicological linkages to conservation biology

A major challenge of resource management is linking basic toxicological science to the broader goals of population conservation (Hansen and Johnson 1999a, 1999b). The disconnect between the disciplines of environmental toxicology and conservation biology limits the long-term goals of both—to understand the mechanistic interactions between contaminants and biological systems, and to protect biological systems from the impacts of environmental stressors (Hansen and Johnson 1999a, 1999b). It is not difficult for resource managers to conceptually extrapolate acute lethality (LC_{50}) data at the individual level to probable effects on populations. However, contaminants in natural systems seldom reach levels that will directly kill organisms, such as fish, and toxic effects will almost always be sublethal. For toxicological research to be useful for managers, it is important to establish links between impacts that can be measured at the molecular or cellular level to higher-order processes, such as behavior, that can affect the viability of natural populations or result in delayed ecological death (Kruzynzki and Birtwell 1994).

For salmon and steelhead, two fundamental (and highly essential) aspects of biology that transcend to the scale of populations are locomotion and chemoreception. These behaviors are instrumental for fish in responding to the challenges of a dynamic physical, chemical, and biological environment. Young salmon are highly social, interacting with conspecifics via locomotory and olfactory-mediated behaviors to form social hierarchies, establish territories, and to coordinate schooling patterns. Since salmon rely on their sense of smell to develop olfactory memories and to return to their natal streams to reproduce (Hassler and Scholz 1988), the long-term genetic integrity of geographically distinct populations of fish depends on the olfactory acuity of individuals. Thus, pollutants that impair the locomotory or olfactory capabilities in individual salmon can potentially have negative consequences at the scale of natural populations.

Chemical contaminants may induce subtle changes to the biology of a fish that are not easily quantified or initially recognized as meaningful to long-term fitness. These subtle impacts may be overlooked by conservation biologists since they are not commonly (or easily) incorporated into population modeling (Hansen and Johnson

1999a, 1999b). Behavior is an important measure of sublethal toxicity in fish, as it reflects impairment across multiple, integrated biological processes (Little and Finger 1990). Locomotory and olfactory-mediated behaviors require the coordinated activities of sensory, central, and peripheral nervous system networks. Contaminants that interfere with basic neurological function in fish can therefore disrupt various physical, chemical, and biological interactions (Kleerekoper 1976).

Scope of the present work

We have demonstrated with this work that environmentally-relevant levels of chlorpyrifos and copper can impact salmonids at the biochemical and physiological scales of biology, and that this can translate to deficiencies in potentially ecologically-important behaviors.

Biochemical and behavioral indicators of sublethal toxicity. Brain acetyl-cholinesterase (AChE) activity was a sensitive biological indicator for assessing the inhibitory effects of chlorpyrifos in both coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*O. mykiss*). There were two substantial findings with the AChE studies presented here (Chapters 2 and 3). First, brain AChE activity is significantly inhibited in both fish species at environmentally-relevant exposures to chlorpyrifos. Benchmark concentration (BMC) analysis estimated that 10% reductions in AChE activity can result from chlorpyrifos exposures near 0.4 and 0.6 μg/L in salmon and steelhead, respectively. Statistical departures (95% lower-confidence interval of the control mean) of inhibition can occur at lower concentrations, approaching 0.2 and 0.3 μg/L chlorpyrifos, respectively. The fact that salmon and steelhead were similarly affected by chlorpyrifos suggests that data between the two species may be interchangeable.

Second, we showed a significant correlation between brain AChE inhibition and behavioral impairment in chlorpyrifos-exposed fish. The most sensitive swimming and feeding behaviors were found to be spontaneous swimming rate and total strikes at food items, which were both reduced by 10% at chlorpyrifos concentrations of 0.3 and 0.4

µg/L chlorpyrifos, respectively. Thus, brain AChE inhibition was highly reflective of altered behavior.

Comparing these experimental exposure concentrations to surface water detections (see Table 1.1), it is likely that salmonids occasionally encounter levels of chlorpyrifos in the environment that result in brain AChE inhibition, and possibly swimming and feeding behavioral impairment. In a broader context, fish are potentially exposed to a range of organophosphates in Western surface waters and their combined effects on sublethal endpoints are possibly additive (U.S. Environmental Protection Agency 2001). From previous literature investigating sublethal effects of chlorpyrifos in salmonids (Table 1.3), our present sublethal results can only be compared to the $1/20^{th}$ LC_{50} values obtained from Holcombe et al. (1982) and Macek et al. (1969). However, this makes for an interesting comparison since it has been presumed in some risk assessments that $1/20^{th}$ of the LC_{50} is protective of sublethal harm for endangered species (e.g., U.S. Environmental Protection Agency 1999a, Erickson and Turner 2003). The $1/20^{th}$ LC_{50} values for chlorpyrifos are 0.3 and 0.46 µg/L chlorpyrifos (adjusted from Holcombe et al. 1982, and Macek et al. 1969, respectively). These are very similar to the sublethal effect BMC_{10} estimates (0.3–0.6 µg/L) for chlorpyrifos determined in our present work. It does not appear that the $1/20^{th}$ LC_{50} assumption would provide adequate protection for threatened and endangered fish species in this case, if a 10% reduction in brain AChE activity, swimming ability, and feeding are considered ecologically detrimental. However, the $1/20^{th}$ LC_{50} value may provide a reasonable estimate of a sublethal lowest-observable-effect-concentration (LOEC).

Physiological and behavioral indicators of sublethal toxicity. For determining the neurotoxic effects of copper, chlorpyrifos, and esfenvalerate on the coho salmon olfactory system, electrophysiological techniques provided sensitive measurements of olfactory function. Experiments using paired recordings from the olfactory epithelium (electro-olfactogram, or EOG) and olfactory bulb (electro-encephalogram, or EEG) showed that three classes of pesticides can inhibit or obscure chemoreception of natural

odorants. Copper and chlorpyrifos were found to target the peripheral sensory system, while esfenvalerate targeted the olfactory nerve or bulb.

In this study (Chapter 4), 7-day exposures to copper and chlorpyrifos decreased the sensitivity of peripheral olfactory neurons by 20% (BMC_{20}) at estimated concentrations of 4.5 and 0.7 µg/L, respectively. The toxic mechanisms of copper- and chlorpyrifos-induced reductions in olfactory sensitivity are uncertain, but copper is known to bind various protein structures, block ion channels, and disrupt cellular membrane structures (Moran et al. 1992, Hansen et al. 1999a), which are likely to be contributing factors. For chlorpyrifos, its known mode of action is the inhibition of AChE activity. The olfactory epithelium of coho salmon contains low levels of AChE (data not shown), but it is uncertain what role AChE may have in olfaction. Esfenvalerate did not affect peripheral sensitivity, but EEG recordings indicated that signals reaching the olfactory bulb were affected at exposure concentrations in the range of 0.05–0.2 µg/L. Effects at the olfactory bulb consisted of irregular bursts of low-frequency (< 10 Hz) oscillations, which appeared after odor-evoked stimulation of the olfactory epithelium. These effects are consistent with the known mechanism for pyrethroid toxicity, which is the blocking of voltage-gated sodium channels along nerve axons.

For salmonids, olfaction is an essential chemosensory process that conveys important information about the surrounding environment. In the case of predation, salmon must quickly detect, and react to, chemical alarm signals to ensure survival. Chemical contaminants that impair olfactory function may render the fish more vulnerable to capture (Mathis and Smith 1993). In this study (Chapter 5), we show that copper diminished olfactory sensitivity to skin extract (alarm pheromone), and that this loss of olfactory function correlated with a failure to initiate predator avoidance behavior (antipredator response) in coho fry. It is important to note that a nominal exposure to 2 µg/L copper, a concentration commonly detected in degraded salmonid habits, was sufficient to impair both olfactory physiology and behavior in fish.

From some surveys of copper in Western surface waters (see Table 1.2), it has been shown that copper concentrations of 2 µg/L or higher are not uncommon. On occasion, detected levels of copper have exceeded 100 µg/L. In tributaries of the Puget Sound

(Washington, USA), the U.S. Environmental Protection Agency's water quality standards for dissolved copper are 10.5 μg/L for acute, and 7.3 μg/L for chronic exposures in wildlife (Snohomish County 2002). As a comparison to the effects shown in our study, after a 3-h exposure to a similar copper concentration (10 μg/L), olfactory sensitivities to natural odorants were reduced by an average of 70–84%, and antipredator behaviors were nearly abolished. It does not appear that the current water quality guidelines provide adequate protection for salmonids, if these laboratory-determined sublethal effects are reflective of potential effects in natural systems.

Recommendations for future research.

Anti-cholinesterase compounds. An important area of research that has received little or no attention is the effect of multiple chemical exposures on fish. As previously discussed, organophosphate and carbamate compounds typically co-exist in surface waters as diverse mixtures, usually at concentrations below levels known to inhibit AChE or impact behavior. Currently, risk evaluations must consider each compound individually, even though it is very likely that inhibitory effects would be additive. The U.S. Environmental Protection Agency has addressed this concern for human health purposes and is in the process of developing a "relative potency factor" approach to describe the relative toxicities of individual organophosphates to an index compound (U.S. Environmental Protection Agency 2001). The 10% AChE inhibition level will be used to compare the potencies of compounds using benchmark dose methodology. This will help estimate the cumulative effects of multiple anticholinesterase exposures.

A similar approach could be employed for ecological risk assessment using a representative fish species, such as the rainbow trout. But for this to be fully implemented would require a major undertaking. A smaller project using three of the most frequently detected organophosphates (azinphos-methyl, chlorpyrifos, and diazinon) could be possible in a shorter time frame. First, the BMC_{10} of each chemical could be determined for 24-h or 96-h exposure periods. Then, to test if AChE inhibitions are additive, the compounds could be applied in various combinations. For example, will mixtures of two or three compounds at the BMC_{10} level be equivalent to a total BMC_{20} or BMC_{30}? Understanding the cumulative effects of chemicals that share a common mechanism of

action would help to estimate risks in more realistic exposure scenarios that occur in the field.

Olfactory neurotoxicants. As shown in this current work, the olfactory nervous system is susceptible to toxic insult by various chemical groups (a metal, organophosphate, and pyrethroid). It is believed that copper and chlorpyrifos target the peripheral sensory system (as identified by EOG recordings), and that esfenvalerate targets the central networks of the olfactory nerve or bulb (as identified by EEG recordings). Chlorpyrifos also significantly inhibited AChE at the olfactory bulb, but the relevance of this finding is uncertain, since these did not appear to correlate with recorded EEG response amplitudes. Recently, a technique has been developed that functionally labels stimulated olfactory neurons in zebra fish (Michel et al. 1999) and salmon (Iwase et al. 2003), including neurons of the olfactory epithelium, the olfactory bulb and relay regions of the telencephalon. These techniques could be applied to toxicological studies to identify target sites, and to better understand the mechanisms of olfactory neurotoxicity throughout the entire olfactory system.

Functional labeling uses agmatine, a relatively small molecule that is able to permeate certain ion channels once they have been activated. This involves presenting the fish with an odorant (such as L-serine which activates the neurons) while simultaneously bathing the epithelium with agmatine. Labeling can be obtained in the olfactory bulb and telencephalon by perfusing agmatine directly over the forebrain while stimulating the epithelium with an odorant. This new technique allows direct visualization of regions throughout the olfactory pathway that are stimulated by odorants, and to determine if these regions are targeted by various toxins. Disruptions to olfaction may be inhibitory (less sensory input) or excitatory (multiple firing of neurons). By having the ability to label neurons along the entire olfactory pathway (sensory epithelium—olfactory nerve—olfactory bulb—telencephalon) a much greater understanding of how these toxicants fundamentally disrupt olfactory function can be obtained.

Closing remarks

For resource managers with the aim of improving habitat quality for threatened and endangered salmonids, pollution of water resources is a major, and possibly limiting, obstacle in population recovery efforts (National Marine Fisheries Service 1996, 1998). Since contaminants typically enter surface waters via diffuse non-point sources (U.S. Environmental Protection Agency 2000), concentrations seldom reach levels that are overtly toxic to salmon and steelhead. However, sublethal impacts by pollutants that limit survival, reproductive success, or the distribution of salmonids should be seriously considered. Thus, it is important for toxicological research that is conducted at or below the scale of the individual animal to have a clear ecological connection to higher orders of biology (Hansen and Johnson 1999a, 1999b). The impacts on biochemistry, physiology and behavior that we found in these studies resulted from concentrations of contaminants that are frequently detected in degraded Western surface waters. Clearly, there is a need to better understand the consequences of sublethal toxic effects on the ecological success of salmonids, and importantly, in the broader context of conservation management.

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