NEUROTOXICITY OF PESTICIDES TO SALMON: PHYSIOLOGY TO ETHOLOGY

by

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Abstract

Pesticides routinely found in the environment can adversely affect neurological systems of salmon. The major focus of the studies presented here is on the impairment of the relatively exposed olfactory sensory neurons (OSNs), since their functionality is critical to several indispensable behaviours. The responses of OSNs to various behaviourally-relevant odorants were impaired following exposure to several pesticide classes, including triazine (e.g. atrazine), carbamate (e.g. IPBC), organophosphorus (e.g. dimethoate), and phenylurea (e.g. linuron) pesticides, as well as a pesticide formulation (i.e. Roundup). In many cases, within minutes of exposure to environmentally realistic (part per billion) concentrations, impairments of greater than 50% in OSN responses were noted. Pesticides can selectively affect different OSN classes, since one pesticide (linuron) negatively affected amino acid but not bile salt responses. Lengthy (four-day) exposure to a mixture of pesticides modelled after that found in the Nicomekl River (British Columbia) also reduced OSN responses, confirming that pesticide mixtures found in salmon-producing waterways may be injurious. In fact, the ability to discern a change in odorant intensity was reduced by 25%. This may have partially been due to inadequate neuroprotection, as glutathione-S-transferase, an enzyme that protects against cellular damage, was not upregulated to any greater of an extent than it was in fish given a 10-fold lower exposure concentration.

To relate changes in OSN responses to behavioural responses, experiments were conducted on two olfactory-mediated behaviours: an amino acid-evoked searching behaviour, and an anti-predator alarm response. In the first case, exposure to three different pesticides disproportionately impacted searching behaviour with respect to neurological impairment, as the searching response was completely eliminated at greater than 60% impairment of neurological response. In the second case, the freezing behaviour salmon perform after detecting harm in a conspecific was progressively reduced with exposure to increasing concentrations of a carbamate. The work here endeavoured to assess how pesticides affect salmon neurophysiology, then relate the effects to altered behaviours, and finally place the findings within a context of environmental realism, regulations, and the status of salmon within Canada and the world.

Keywords: salmon, olfaction, swimming, behaviour, pesticides

To Brian, Lorna, and even Cecil

Wealth is not determined by how much you have,

but by how much you give.

- Peter Tatoosh, 2006

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Remember Van Wilder's credo: 'Don't be a fool, stay in school'.

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Glossary

ACh	Acetylcholine
AChE	Acetylcholinesterase
COSEWIC	Committee on the status of endangered wildlife in Canada
EEG	Electro-encephalogram
EOG	Electro-olfactogram
GPCR	G-protein coupled receptor
G-protein	Guanine nucleotide binding protein
GSH	Glutathione
GST	Glutathione-S-transferase
IC_{50}	The concentration of an agent causing 50% response inhibition
LC_{50}	The concentration of an agent causing death in 50% of test subjects in a fixed amount of time
mV	Millivolt
OC	Organochlorine
OSN	Olfactory sensory neuron
OP	Organophosphorus
TChA	Taurocholic acid (a bile salt)
R_R	(Response ratio) the relative change in time spent in an area due to introductiojn of an odorant

Chapter 1:
Neurotoxicity and thesis introduction

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Background neurotoxicology

All organisms experience exposure to xenobiotics, i.e. foreign substances. The overwhelming majority of these exposures do not result in lethality, but rather sublethal alterations in an organism's biology. For organisms with nervous systems, sublethal effects can be mediated through neurotoxicity, which is the modification of 'the neuron, the axon, the myelinating cell, or the neurotransmitter system' (Anthony et al. 2001). The nervous system exerts control over a diverse array of physiological and behavioural responses, and so neurotoxin exposure has the capacity to affect organismal fitness and cause 'ecological death' (Kruzynski and Birtwell 1994). For these reasons, neurotoxicology receives considerable attention.

For many animals, the neurons of the olfactory system are essential. Olfactory sensory neurons (OSNs) provide environmental information about food, predators, mates, siblings or environmental condition (e.g. CO_2 levels) (Hildebrand and Shepherd 1997). Salmon are an especially good example of the importance of olfaction, as OSNs enable the above behaviours plus the incredible return migration for which they are well known (Hasler and Scholz 1983). For salmon, as with other species, OSNs by necessity exist relatively exposed to the environment, unlike other neurons. This places them in harms way: the waterways of the world are contaminated with an abundance of neurotoxic pesticides (e.g. Verrin et al. 2004; ENKON Environmental 2005).

Across continents, the decline in natural salmon populations, in spite of their continued enhancement, promotes various avenues of research designed to understand and ameliorate the causes of the decline. Maintaining salmon abundance is critical for cultural, economic and food supply reasons, as humans and a variety of animal communities depend on salmon for sustenance and nutrients. Examples include the nutritional financing of inland watersheds by return migrating salmon and the large (>75%) dietary proportion that chinook salmon make up for B.C.'s endangered killer whales (Grant and Ross 2002).

One of the suspected contributors to their decline is the impact of pesticides on their olfactory abilities. The first set of studies into salmon olfaction also tested the effect of metal (mercury) and surfactant exposure, both of which are used in pesticides (Sutterlin and Sutterlin 1971; Sutterlin et al. 1971). These experiments measured responses at the olfactory bulb, which lies downstream and integrates signals from the olfactory epithelium. The activity of neurons in any tissue is routinely determined by measuring changes in the voltage across neuronal membranes. For olfactory neurons, this can be carried out both at the olfactory bulb and epithelium (i.e. OSNs in the nose). Voltage change occurs when odorants bind OSN receptors and initiate an intracellular cascade that leads to an influx of sodium ions and an efflux of potassium ions.

In the majority of OSN toxicity studies, functionality has been assessed using the olfactory epithelium preparation. The technique, referred to as electro-olfactogram (EOG), was pioneered on frogs in the 1950s (Ottoson 1956) and optimized for salmon in the 80s (Evans and Hara 1985). Studies continue to use

recordings from the bulb as well, and this technique is known as electro-encephalogram (EEG) (Baldwin et al. 2003). A decrease in either EOG or EEG may indicate an odorant will be perceived as being present at a lower concentration or perhaps not present at all. To date, EOG/EEG impairments have been found after exposure to surfactants (Sutterlin et al. 1971), metals (Hara et al. 1976), low pH (Moore 1994), and various classes of pesticides (e.g. Sandahl et al. 2004), several of which are investigated in this thesis (e.g. Tierney et al. 2006).

Both EOG and EEG can help determine the mechanistic underpinnings of observed pesticide OSN toxicity. For example, some odorants are specific to distinct OSN classes for which basic knowledge of protein receptors and signalling pathways are known (e.g. bile salt and amino acid OSNs) (Zielinski and Hara 2007). If pesticide exposure results in differential effects across OSN classes, then the mechanism of toxicity may be narrowed to certain cellular traits. Whether the mechanisms are known or not, decreased OSN responses can also be related to altered behaviours. Such relationships may facilitate predictive modelling of effects determined in-lab to those that may occur in-field.

Several odour-evoked behaviours are amenable to lab testing. One of the most common is the preference or avoidance salmon show to particular odours. Amino acids can be associated with both attractive (e.g. food (Hara 2006) or the 'olfactory bouquet' of the home stream (Shoji et al. 2003)) or repellent odours (e.g predator scent (Idler et al. 1956)). Because of this, amino acids have been used as odorants in many EOG and EEG studies. The use of the same odorants

in both tissue and behavioural level tests provides an opportunity to determine relationships between OSN and behavioural responses.

Behavioural response can be associated with physiological responses. For many species of fishes, the scent of a damaged conspecific, which might arise through predator attack, evokes an innate, sometimes complex response. For salmon, this consists of an activity decrease or 'freezing' (Berejikian et al. 2003). This 'alarm response' behaviour is designed to enhance survival over brief periods (i.e. enable rapid escape), and is often accompanied by a physiologic stress response. As with EEG and EOG, a decrease in the stress response in fish with olfactory impairment may serve as a correlate of olfactory-based toxicity.

Thesis objectives

There were many objectives of this work. The first was to determine whether several currently-used pesticides were able to interfere with the function of OSNs. To achieve this, EOG responses were used to screen a sample of the spate of pesticides found in salmon habitat (see Chapters 2 and 3). To help achieve this objective, collaborations with Fisheries and Oceans Canada (DFO) and Environment Canada (EC) enabled collection of, and access to, environmental monitoring data from Canadian salmon habitat. Next, given the diversity of pesticides and olfactory receptors (i.e. ~300 pesticides are registered for use in Canada (Verrin et al. 2004) and there are at least 100 olfactory receptor proteins in fishes (Zhang and Firestein 2002)), another goal specific to the EOG technique was to determine if pesticides affected specific classes of

OSNs (Chapter 3). Such information would be a further step towards a mechanistic understanding of pesticide OSN toxicity.

Another objective was to use laboratory techniques to simulate an environmentally-observed situation. The EOG technique has historically not been used to achieve this goal. The technique is one that involves keeping an animal stable on a lab bench, which greatly restricts the duration of any bench-top pesticide exposure. Although environmental pesticide pulses can be of short duration (i.e. minutes to hours) (Thompson et al. 1995), environmental exposures are typically considered to be of long term (i.e. chronic) duration. To better mimic such duration, tank exposures could conducted prior to bench testing. However, some of the more subtle effects of exposure (i.e. small EOG decreases) become lost within the large variation (e.g. 20-fold (Tierney et al. 2006)) observed in the bench-top EOG preparations. For this reason, a technique capable of using EOG to resolve olfactory changes following lengthy exposures was devised (Chapter 4).

The next objectives were concerned with determining how pesticide exposure affects the behaviours that OSNs support. Two behaviours were chosen for study, one using a behavioural response to an amino acid (Chapter 5), which facilitated comparison to EOG data where amino acids were often used to evoke responses, the second using an odorant prepared from the fish themselves (Chapter 6). For the first behavioural experiments, fish were exposed to various pesticides and changes in their responses to an amino acid were recorded. Additionally, the behavioural responses of salmon to pesticides

alone were tested. The next set of experiments focussed on the alarm response. Here the objective was to determine how pesticide exposure affected the ability of salmon to carry-out innate olfactory driven behaviours. Additionally, since this response is one that includes a stress component, these experiments included measurements of stress.

In an exposure, the uptake and distribution of pesticides and their metabolites has capacity to alter other neurological systems. A final objective was to determine if neurotoxic pesticide exposure impaired something which salmon behaviour depends upon – swimming performance. Clearly, the impairment of this system without question translates to a genuine survival challenge (Chapter 7).

Lastly, a review paper integrating much of the work in this thesis with other neurophysiological and neurotoxicological studies on fishes is included (Chapter 8). Here relationships between OSN and behavioural responses are considered, as are comparisons between olfactory-based measures of toxicity and others.

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Chapter 2:
Changes in juvenile coho salmon electro-olfactogram during and after short-term exposure to current-use pesticides

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Abstract

For anadromous salmonids, olfaction is a critical sense, enabling return migration. In recent years, several pesticides have been identified that interfere with salmonid olfaction at concentrations in the µg/L range, thus they may pose a risk to species longevity. In this paper, we investigate the acute effects of five agricultural pesticides on juvenile coho salmon (*Oncorhynchus kisutch*) olfaction using the electro-olfactogram (EOG), a measure of odorant-evoked field potentials. Electro-olfactograms responses to the odorant L-serine were measured during and following a 30-min exposure of the left olfactory rosette to chlorothalonil, endosulfan, glyphosate acid, iodocarb (IPBC), trifluralin and 2,4-D. With the relatively insoluble pesticides endosulfan and trifluralin, decreases in EOG amplitude were only apparent at relatively high concentrations (100 µg/L and 300 µg/L, respectively) following 20-min of exposure, and absent for chlorothalonil (1 mg/L). With the water soluble herbicide glyphosate, significant EOG reductions occurred within 10-min of exposure to 1 mg/L, and more rapidly with higher concentrations. Recovery of EOG post-glyphosate exposure was concentration-dependent, and complete recovery was not observed with some concentrations at 60-min post-exposure. 2,4-D only affected EOG at high concentration (100 mg/L), where it eliminated EOG within 2-min of exposure. With IPBC, EOG was decreased at 25-min of exposure to 1 µg/L; higher concentrations caused decreases to occur more rapidly. Excluding IPBC and glyphosate, all EOG reductions occurred at concentrations above current Canadian water quality guidelines and reported 96-h lethality values. Our results

show that olfactory neurons can be impaired rapidly by some current-use pesticides even at low µg/L range exposure concentrations.

Keywords

Salmon, Pesticide, Glyphosate, 3-iodo-2-propynyl-N-butyl carbamate, Dichlorophenoxyacetic acid

Introduction

The olfactory sense is important throughout the animal kingdom. For vertebrates, olfactory ability can be assessed by measuring the field potential of olfactory sensory neurons (OSNs) following odorant presentation. This measure, referred to as the electro-olfactogram (EOG), was developed by Ottoson for use in frogs a half-century ago [1], fine-tuned for use in fish by Evans and Hara [2], and applied in recent years to determine the effects of current-use pesticides on the olfactory ability of salmonids. For example, EOG has detected sublethal olfactory toxicity of carbamates [3], chlorpyrifos [4], copper (a constituent of pesticides such as chromated copper arsenate) [5], diazinon [6], and triazines [7]. The olfactory ability of salmon has received attention at least in part because olfaction is behaviorally indispensable, enabling behaviors such as imprinting and thus return migration.

Determining the olfactory toxicity of pesticides to coho salmon (*Oncorhynchus kisutch*) is of added relevance, as some stocks are endangered in Canada [8] and the US [9]. Ideally, water quality guidelines are in place to protect species from injurious chemical concentrations. However, guidelines are often based on available toxicological data, which may consist of acute and chronic toxicity data. This approach may be limited in that critical physiological systems, such as olfaction, can be impaired at concentrations lower than those that result in lethality. For example, simazine negatively affects the EOGs of Atlantic salmon parr (*Salmo salar*) at concentrations as low as 1 µg/L [7], which is 0.001% of the 96-h LC50 (the statistically estimated concentration causing death

in 50% of animals in 4 d) for another salmonid, rainbow trout (*O. mykiss*) [10], yet it is permitted at 10 µg/L in Canada (The Canadian Council of Ministers of the Environment (CCME) (www.ccme.ca) [11]). Similarly, the carbamate fungicide IPBC, which has a maximum allowed fresh water concentration of 1.9 µg/L in Canada, significantly impairs the EOGs of coho salmon parr at 0.1 µg/L [3] or 0.1% of LC50 [12]. These studies demonstrate that salmonid olfaction can be impaired at toxicant concentrations substantially less than LC50 values, and less than existing water quality guidelines. Since olfaction is tantamount to survival for anadromous salmonids, this sublethal toxicity endpoint would need to be considered in determining no observed adverse effect concentration (NOAEC). An olfactory NOAEC may be of regulatory use and serve to help preserve salmonid stocks, especially those at risk.

Here we examine five agricultural pesticides identified as of concern to the aquatic environment: chlorothalonil, endosulfan, glyphosate, trifluralin, and 2,4-D [13]. Chlorothalonil is a fungicide used in various applications from food (fruit and vegetable) to turf. Endosulfan is an insecticide used on food crops. Glyphosate is a widely used herbicide, especially by the forestry industry, with greater than 120,000 kg glyphosate (acid and isopropylamine forms) used in British Columbia (BC), Canada in 2003 ([14]; www.env.gov.bc.ca/epd/epdpa/ipmp/technical_reports/pesticide_survey2003/survey_2003.html). Trifluralin is a pre-emergent herbicide used to control broadleaf weeds and annual grasses. 2,4-D, like glyphosate, is used to control broadleaf and aquatic weeds. Since the selected pesticides are currently used in

agriculture, there is potential for their presence in coho bearing streams bordering land with agricultural activity. The fungicide iodocarb (IPBC) was included in our testing, despite being previously identified as an olfactory toxicant [3], since we used a new testing protocol. Iodocarb (IPBC) is used routinely in BC (>10,000 kg sold in BC in 2003; [14]) as an antisapstain for dimensional lumber. To assess acute olfactory toxicity, we exposed the OSNs of juvenile coho salmon to concentrations of these pesticides for a period of 30-min and monitored changes in electro-olfactogram during the exposure and a 60-min recovery period.

Materials and Methods

Animals

Coho salmon parr were obtained from the Fisheries and Oceans Canada Capilano Hatchery (North Vancouver, BC, Canada) (n = 108, mass 16.5 ± 0.86g, length 11.4 ± 0.25cm, condition factor [fork length] K_{fl} = 0.971 ± 0.021), and maintained in indoor 700 l tanks supplied with filtered, dechlorinated municipal water (background water; dissolved O_2 at >90% saturation, pH 6.8, hardness 6.12 mg/L $CaCO_3$) under a 12:12 h light:dark photoperiod with 30-min dawn:dusk transition, temperature (~10°C), and fed commercial salmon pellets (EWOS, Surrey, BC, Canada) ad libitum.

Chemicals

All chemicals were purchased from Sigma Aldrich (Oakville, ON, Canada). The pesticides and their given purities are chlorothalonil (2,4,5,6-tetrachloro-1,3-

dicyanobenzene; 99.1%), endosulfan (alpha,beta-1,2,3,4,7,7-hexachlorobicyclo(2.2.1)-2-heptene-5,6-bisoxymethylene sulfite; 99%), glyphosate acid (*N*-(phosphonomethyl) glycine; 99%), IPBC (3-iodo-2-propynyl-*N*-butyl carbamate; 97%), trifluralin (a,a,a-trifluro-2,6-dinitro-*N*,*N*-dipropyl-p-toluidine; 99.5%), and 2,4-D (dichlorophenoxyacetic acid; 98%). Two-phenoxyethanol was used for anesthesia; L-serine (serine; 99%) was used as an odorant.

Electrophysiology

Electro-olfactograms were recorded according to the procedure of Evans and Hara [2] using the apparatus described in Jarrard et al. [3]. Briefly, fish were anesthetized using 2-phenoxyethanol (0.4 ml/L induction, 0.2 ml/L maintenance), placed in a Plexiglas holder, and the gills continuously perfused with background water and anesthetic. The left naris covering was excised and the exposed olfactory rosette was irrigated with background water and pesticide/vehicle mixtures (flow rate ~1.5 ml/min). All solutions were maintained at holding tank temperature (~10°C) by a Lauda chiller (Brinkmann Instruments, Westbury, New York, USA). Fish heart rate was monitored by paired internal ventromedially-placed stainless steel electrodes, amplified 100×, and displayed on an oscilloscope. Tests where arrhythmia was observed were not included for analysis. An Ag/Ag-Cl electrode in 2% agar (1M NaCl) inside a pulled glass pipette (tip diameter ~150 µm) was advanced until it was observed depressing the mediocaudal area of the rosette raphe, and then backed off until no tissue

depression was seen. A similar indifferent electrode was placed on the top of the head.

To elicit EOGs, 2-s pulses of stimulus were delivered in the nasal background water flow using a computer-controlled solenoid valve system. Direct current EOGs were amplified 1000×, digitized at 200 Hz, and acquired on a computer (LabView, National Instruments, Austin, TX, USA). A serine concentration-response curve over the concentrations of 10^{-7} to 10^{0} M was established to guide selection of a submaximal stimulus concentration for EOGs. Electro-olfactogram response peaked with 10^{-2} M serine, approximately double that at 10^{-3} M (Fig. 2-1A; for example traces, see Fig. 2-1B). Two-s pulses of concentrations greater than 10^{-2} M required more than 2-min to restore baseline, indicating that sensory adaptation took longer than 2-min to decline with 10^{-1} and 10^{0} M serine. For subsequent tests, 10^{-3} M serine was used to evoke EOGs. This amino acid concentration, which has been used for electrophysiology work previously with salmonids (*O. kisutch* and *mykiss*; [15]), is greater than the 10^{-5} M used in some EOG studies (e.g., [3,7]), but lower than the 10^{-2} M used in another [16]. In this study, 10^{-3} M was used as it provided robust and repeatable peaks. Neither 10^{-3} nor 10^{-5} M realistically reflect reported background amino acid concentrations, which can be less than 10^{-7} [17], but they may have relevance in situations of high organic material (such as with carcass decay).

To limit EOG variation during exposure and post-exposure periods, fish were acclimated to the apparatus and pre-exposure EOGs were taken until there was ≤5% variation between two EOGs taken 5-min apart. Following acclimation,

the nasal background water feed was switched to either control (one of three: either background water alone, or one of two vehicle controls) or pesticide solutions.

Exposures

In the present study, one of two factors was used to establish a sublethal exposure concentration range for each pesticide. With water-soluble pesticides (glyphosate and 2,4-D), initial exposure concentrations were based on 50% LC50 values for salmonids (Table 2-1). To establish a concentration-response relationship, incrementally higher and lower concentrations were also tested. With glyphosate, since formulations generally have higher toxicity due to surfactants (such as polyethoxethyleneamine) [18], our initial exposure concentration should have been well below lethality. For the remaining pesticides of lower solubility, their maximum solubilities [19] were used to set the maximum exposure, except for chlorothalonil where we used slightly more (solubility 0.6 mg/L, exposure concentration 1 mg/L). If pesticides affected EOG at 100% of their water solubility value (Table 2-1), then they were tested at 10% of this concentration. Concentrations for IPBC (1, 10 and 100 µg/L) were within the range tested by Jarrard et al. [3].

For pesticide exposure solutions, glyphosate was added directly to background water, and although our goal was to test other pesticides at concentrations approaching the upper limit of their solubility, we had to first solubilize endosulfan, chlorothalonil, trifluralin and 2,4-D in acetone (10, 10, 10 and 100 mg/ml acetone, respectively). Similarly, IPBC was solubilized in

polyethylene glycol (10mg/ml). Both solubilizing agents were tested at their highest dilution concentration (i.e., 10 µl/L for polyethylene glycol and 1 ml/L for acetone; n = 6 for each group). Pesticides were not added to 2-s stimulant serine pulses. All solutions were prepared daily; all reported concentrations are nominal.

To determine whether the pesticides were capable of evoking EOGs, each pesticide was first tested at its maximum exposure concentration using the same protocol as serine. If a 2-s pesticide pulse caused an EOG, progressively lower concentrations were tested.

For the exposures, single serine-evoked EOGs were recorded at 2, 5, 10, 15, 20, 25 and 30-min during exposure, and 2, 5, 10, 15, 20, 30, 40, 50 and 60-min post-exposure. Line washout following exposure took 30-s. All background water, vehicle, and pesticide solutions were delivered in Teflon® tubing, which was replaced with each test group. Control fish were distributed throughout pesticide trials.

Statistics

For each fish, exposure and post-exposure EOG peak amplitude size (in mV) was considered as a proportion of the last pre-exposure value. Differences between control groups (background water, polyethylene glycol, and acetone; n = 6 per group) were tested using a two-way (time and treatment) repeated measures analysis of variance (RM ANOVA) followed by a Holm-Sidak post-hoc test. As no differences were found, control groups were pooled, and pesticide exposure groups were tested against control using the same method as above.

For glyphosate, the IC50 value (the concentration inhibiting response by 50%) was determined for each exposure time (2, 5, 10, 15, 20, 25 and 30-min) by fitting curves to EOG inhibition versus glyphosate concentration and interpolating 50% EOG reduction. For the curves, given the small number of points (i.e., average values for control, 0.1, 1, 10 and 100 mg/L at each time point), for simplicity, a three-parameter exponential decay model (inhibition = $y_0 + a \times e^{b \times [\text{glyphosate}]}$) was used since a polynomial model did not provide a better fit. To establish 95% confidence intervals (CIs), curves were also fitted to 2 × standard error (SE) above and below each point. To view interactions in IC50 and exposure duration, IC50 was then plotted against exposure time, and again, best-fit models were used. To model the time required for IPBC and glyphosate to cause an EOG decrease, the time at which a significant drop in EOG was plotted against pesticide concentration and curves were fitted. Additionally, EOG changes at the beginning and end of exposure and recovery periods were plotted for IPBC and glyphosate, and polynomials were fitted for visualization purposes.

Five percent was used as the limit of significance for all comparisons. All values are presented as mean ± SE, unless stated otherwise. SigmaStat 3.0 and SigmaPlot 8.0 (Systat Software, Point Richmond, CA, USA) were used for statistics and graphing.

Results

Heart rate was not different between exposure groups and did not vary over the 90-min testing procedure (pooled average = 59 ± 0.90 beats per min). Across all fish, EOG acclimation took 49.3 ± 2.2 min and pre-exposure EOGs

were 4.31 ± 0.19 mV (range 0.496 to 10.6 mV). The EOG acclimation time was positively correlated to fish mass (range 7.53 to 42.8 g) [(min) = 1.4 × (g) + 30; r^2 = 15.6%, p = 0.0015].

To test if the pesticides themselves evoked EOGs, 2-s pulses of each pesticide were delivered in the nasal background water feed. None of the pesticides evoked EOGs over the concentration ranges tested except for 2,4-D and glyphosate, which evoked EOGs in a concentration-dependent manner similar to L-serine (Fig. 2-1A, B).

With 30-min exposures, vehicle control EOG peak amplitude did not vary significantly over the 90-min testing procedure (Fig. 2-2A). For IPBC, 1 µg/L exposure caused a significant decrease in EOG 25-min into exposure. Significant decreases occurred more rapidly at higher concentrations (time to significant decrease, in min = 24.9 × [IPBC µg/L] $^{-0.375}$, r^2 = 99.7%, p = 0.03), and these decreases were concentration-dependent (Fig. 2-3). At the end of exposure, EOG responses differed from control for all concentrations, with 1, 10, and 100 µg/L reaching respectively 71.8 ± 16.5%, 54.1 ± 5.14% and 57.9 ± 13.6% of control. At 60-min post-exposure, all IPBC exposure groups remained significantly depressed from control except the lowest concentration, which returned to control level 30-min following exposure.

Chlorothalonil did not affect EOG amplitude within the testing protocol even at an exposure concentration greater than solubility (1 mg/L) (Fig. 2-2B), and so other concentrations were not explored. One-hundred µg/L endosulfan caused significant EOG decreases with 20 to 30-min of exposure, but EOG

returned to control levels 2-min post-exposure (Fig. 2-2C). Ten µg/L endosulfan did not cause any difference compared to controls. Similarly, with high-concentration (300 µg/L) trifluralin exposure, EOG was decreased at 30-min, but recovered within 2-min of exposure cessation (Fig. 2-2D). As with endosulfan, EOG effects were absent when trifluralin exposure was reduced by ten-fold (30 µg/L).

One and 10 mg/L 2,4-D exposure did not affect serine-evoked EOGs (Fig. 2-2E). However, in the presence of 100 mg/L 2,4-D the EOG response was atypical and in the opposite direction. Since a 2-s pulse of water in place of serine had the same effect we conclude that the apparently reversed EOG was simply a rapid washout effect (EOG recovery), as 2,4-D was not included in the stimulant pulse. For the purposes of this paper, reversed washout EOGs were considered as EOG loss and are considered as 0% of pre-exposure EOGs. Two-min following 100 mg/L exposure, EOG appeared to recover to 53.2 ± 9.4% and remained at a similar level up to 60-min post-exposure. A clear concentration–response relationship was not established with 2,4-D, as both 1 and 10 mg/L exposures did not alter EOG significantly from control values.

Glyphosate reduced EOG in a concentration-dependent manner (Fig. 2-2F, Fig. 2-3) and significant decreases occurred more rapidly with increasing concentration (time to significant decrease, in min = 10.0 × [glyphosate mg/L] $^{-0.325}$, r^2 = 99.6%, p = 0.04). One-hundred mg/L glyphosate exposure, like 100 mg/L 2,4-D exposure, caused small reversed EOGs due to washout. After 30-min exposure, EOGs recorded in response to exposure to 1, 10, and 100 mg/L

exposed fish were 66.1 ± 8.0%, 44.4 ± 13.9%, and 0 ± 0% of their pre-exposure level. Exposure at 0.1 mg/L did not affect EOG during or after exposure. Unlike the 100 mg/L 2,4-D exposure, EOG recovery post-100 mg/L was time-dependent (Fig. 2-2F). By 60-min post-exposure, 1 and 100 mg/L exposures remained significantly different from control at 74.5 ± 11.7% and 72.8 ± 11.0% of their pre-exposure EOG.

The point where EOG was 50% of pre-exposure value (IC50) was interpolated for glyphosate. At 2-min into exposure, IC50 was 10.9 mg/L, and at 5-min it dropped to 8.17 mg/L and remained at a similar level for the balance of the exposure (IC50 = $7.66 \times e^{[0.218/(\text{time}-1.39)]}$, r^2 = 88.4%, p = 0.01; upper CI = $9.76 + 11.5 \times e^{(-0.289 \times \text{time})} + 0.322 \times \text{time}$, r^2 = 96.3%, p = 0.01; lower CI = $4.91 + 28.5 \times e^{(-1.32 \times \text{time})} - 0.102 \cdot \text{time}$, r^2 = 95.8%, p = 0.01).

The IC50 values were not calculated for other pesticides since >50% inhibition was not reached, except for 2,4-D. However, IC50 was not calculated with this pesticide since a concentration–response curve was not reasonably established.

Discussion

Five of the six pesticides examined affected coho salmon EOG amplitude, with some pesticide concentrations doing so within 2 min of exposure. These effects were typically noted in the high µg/L to mg/L concentration range, except for IPBC, where effects were noted in the low µg/L concentration range. To relate these sublethal toxicity findings to lethality data and water quality regulations as they apply to salmonids, we present our findings in a three-part

comparison (summarized in Table 2-1). First, we asked if an acute 30-min pesticide pulse alters olfaction, setting our pulse concentrations according to either maximum solubility (since potentially there is little likelihood a salmon will encounter a pulse of concentration >100% solubility) or an estimate of sublethal exposure concentration (50% 96-h LC50). Second, we present our findings in comparison with existing lethality data, which answers whether olfactory impact (if found) represents a viable sublethal toxicity endpoint. Last, we ask whether water quality guidelines are at or below the concentration where EOG was negatively affected, acknowledging that these guidelines may or may not have bearing on environmental pesticide concentrations.

In the environment, negative EOG effects may alter survivorship since olfactory-mediated behaviors such as the alarm and avoidance reactions will be impaired at some level of EOG loss. For example, 1 µg/L of diazinon lowers EOG by 70% [6], and impairs alarm response [20]. Like Moore and Waring [6], we explored decreases in EOG response to stimulation by serine. This amino acid washes off mammalian skin [21] and has been shown to elicit an avoidance response in both juvenile [22] and adult salmonids [21], and so presumably functions as an anti-predator behavior [21]. Recently, it was demonstrated that amino acids can also function as 'home stream olfactory bouquet' constituents for chum salmon [17]. Thus, pesticides that reduce amino acid-elicited EOGs may also impair imprinting. However, as mentioned earlier, the amino acid concentrations of salmon EOG studies are likely greater than environmental amino acid concentrations, which hampers real-world extrapolation.

We found similar decreases in serine EOG amplitude with IPBC as Jarrard et al. [3]. These authors found a 50 and 70% decrease in EOG 30-min following a 1 and 10 µg/L exposure; we noted a 30 and 45% decrease for the same concentrations. The more conservative impairment estimate of our study may be attributable to a higher L-serine stimulus concentration (10^{-3} M this study; 10^{-5} M [3]) or differences in the exposure system. If the difference is due to the greater stimulus concentration, it suggests that EOG alteration, as assayed through EOG peak size, may become a less sensitive measure of pesticide impact with increasing stimulus concentration. Future studies may benefit from examining an additional measure of EOG, such as the maximum rate of EOG generation (the maximum of the curve-slope) after toxicant exposure. Iodocarb (IPBC) is clearly toxic to coho salmon olfaction, at concentrations below that which cause lethality (Table 2-1). The Canadian Water Quality Guidelines for the Protection of Aquatic Life (CWQG; [11]) set a limit at 1.9 µg/L. Based on this study and Jarrard et al. [3], the guidelines may not provide adequate safeguard for coho salmon olfaction.

In contrast to IPBC, chlorothalonil does not appear to affect EOG within 30-min, even at a concentration far greater than that which causes lethality and the CWQG guidelines of 0.18 µg/L (Table 2-1). Chlorothalonil was tested just above its theoretical maximum solubility (0.6 mg/L; [19]) a concentration well above the 96-h LC50 range of 14.3 to 17.1 µg/L [23,24], and so chronic effects or other sublethal effects are likely to develop prior to olfactory toxicity.

Endosulfan reduces EOG, but only at high concentrations (100 µg/L). Much lower concentrations (0.5 µg/L) of endosulfan impair pheromone-based behaviors in red-spotted newt (*Notophthalmus viridescens*) following 4-d exposure [25,26]. The fact that we only noted olfactory effects after exposure to 200-fold higher concentration suggests that either coho salmon olfaction is not as sensitive to this pesticide, or that lower, µg/L range concentrations act in a more chronic fashion, one missed with acute exposure. Although our findings support the latter notion, since the time to EOG impairment increased with decreasing concentration for both IPBC and glyphosate exposures, it is likely that acute and chronic endosulfan olfactory toxicity differ in mechanism of action. For example, the olfactory effects noted with newts and chronic exposures may be mediated through altered hormonal profiles. Any future EOG studies of endosulfan should consider extended exposures, such as those carried out over days in tanks. Unfortunately, variation between individual EOG preparations can be considerable: in our study, there was a greater than 20-fold range in EOG peak size prior to pesticide exposure. Thus, isolating subtle effects on EOG following a tank exposure might be challenging. In any case, as long as concentrations of this pesticide do not exceed the current CWQG guidelines (0.02 µg/L; see Table 2-1), acute sublethal olfactory effects would not be anticipated for newts or salmonids.

Like endosulfan, trifluralin only reduced EOG at a very high (300 µg/L) exposure concentration, and even then, only caused a small effect. Since lethality occurs at a much lower concentration (Table 2-1), and since CWQG

guidelines are much lower (0.2 µg/L), this pesticide likely does not pose an acute risk to salmonid olfaction.

Serine-evoked EOGs were not affected with low concentration (1 and 10 mg/L) 2,4-D exposure. However, 2,4-D at these exposure concentrations evoked EOGs similar to serine, suggesting that 2,4-D may be acting as a ligand to a set of OSNs different than serine. This could be tested by cross-adaptation experiments using 2,4-D and ligands that likewise include phenolic rings, such as aromatic amino acids (e.g., L-pheylalanine or L-DOPA). One of these, L-DOPA, has been shown to evoke EOGs in other teleost species (*Carassius auratus* [27]; *Solea senegalensis* [28]). At high concentration (100 mg/L), 2,4-D eliminated serine-evoked EOGs. This effect may be due to a nonselective effect on multiple OSNs, or from 2,4-D acting as a weak serine-receptor ligand (i.e., cross-adapting). This second explanation may not completely account for the effect given that the serine-evoked EOG did not recover after 100 mg/L 2,4-D exposure as it did with 100 mg/L glyphosate exposure. The approximately 50% recovery observed could be due to persistent effects or actual physical damage resulting from prolonged depolarization.

Folmar [29] found rainbow trout avoided 2,4-D concentrations of 1 and 10 but not 0.1 mg/L, suggesting that salmonids may behaviorally reduce 2,4-D exposure where possible. Our results suggest salmonids can detect 2,4-D through olfaction. Alternately or additionally, a sensorial mechanism other than OSNs in the olfactory rosette may be used for 2,4-D detection, as Ishida et al. [30] suggested with carp (*Cyprinus carpio*) avoidance of pesticides benthiocarb,

isoprothiolane, and fenitrothion. With our EOG findings, in view of the high concentration required for the observed effect and the fact that lethality occurs at a lower concentration, acute pulses of this pesticide likely do not pose a specific olfactory risk. Should existing guidelines be applied to stream habitat (Table 2-1), salmonids will be protected from any short-term olfactory alteration.

With the widely used herbicide glyphosate, our results show that exposure can reduce EOG responses rapidly, with a calculated IC50 value of 10.9 (upper and lower 95 CIs: 6.72, 16.8) mg/L, 2-min into exposure. However, glyphosate also evoked EOGs in concentration-dependent manner. This is not surprising given glyphosate's similarity to the amino acid glycine. Thus during exposure, the reduced serine EOG peak size may indicate that glyphosate was acting as an agonist, and consequently there may have been receptor adaptation. Several minutes after glyphosate washout, it was unlikely that there was significant residual receptor binding (i.e., antagonist or agonist action). However since the serine-evoked EOG did not return to control levels for all exposure concentrations (1 and 100 mg/L), there is evidence of either persisting adaptation, or toxic effects on OSNs or other cells of the olfactory rosette.

In our tests we used pure glyphosate acid, in spite of product formulations generally having elevated toxicity due to formulation constituents. However, the glyphosate formulation used for weed control in the aquatic environment (Rodeo®; Monsanto, St. Louis, MO, USA) does not include inert ingredients. In Willapa Bay, Washington, Rodeo® has been used repeatedly to control the invasive exotic smooth cordgrass (*Spartina alterniflora*) [31]. For this reason,

testing the effects of glyphosate in the absence of inerts or other formulation constituents is warranted. Furthermore, the method and sheer volume of glyphosate application over areas including or adjacent to salmon bearing streams, and its high solubility, make exploring its olfactory toxicity of interest, even though it tends not to persist unbound (reviewed by Giesy et al. [32]).

Our study noted that glyphosate has sublethal neurophysiological effects (i.e., salmonid olfaction would be impaired before lethality would be expected; see Table 2-1). Fish may be able to reduce any sublethal effects, as our data suggests fish can detect it via OSNs. However, a previous study suggested that may not be the case, as rainbow trout did not to avoid glyphosate over a concentration range of 0.1 to 10 mg/L [29]. Thus, our study with [29] suggest that salmonid OSNs detect both 2,4-D and glyphosate, but do not avoid glyphosate. Regardless, in view of the existing Canadian water quality guidelines for glyphosate (65 µg/L) and the relatively high (1 mg/L) concentration required to significantly decrease EOG, and the physico-chemical properties of glyphosate, it is unlikely that acute exposures of glyphosate are a routine risk to olfactory systems of anadromous salmonids. Nevertheless, should a salmon-bearing location receive concentrated pulses of this pesticide, olfaction and consequently ecological fitness may be impaired.

Most of the pesticides known to inhibit salmonid olfaction, such as the organophosphorus insecticide diazinon [6], atrazine [7], and the carbamate fungicide IPBC [3], have acetylcholinesterase (AChE) inhibiting properties. Acetylcholine (ACh) is of central importance since it acts as a neurotransmitter

for the peripheral nervous system, controlling skeletal muscle contraction, and by the autonomic, parasympathetic nervous system, inhibiting heart rate [33] and stimulating mucous secretion [34], among other functions. Because inhibition of acetylcholinesterase will cause elevated ACh, tetanus (sustained muscular contraction) and elevated heart rate or mucous secretion may result. Considering that elevated peripheral AChE may lead to increased mucous production, it is not unreasonable to hypothesize that olfaction could be diminished by an anticholinesterase-caused mucous plug, which is exactly what Jarrard et al. [3] hypothesized. In our tests, we did not observe any heart rate changes, so it is unlikely that AChE was systemically inhibited. Since our exposure system was designed to isolate pesticide exposure to the naris, even if a pesticide had AChE inhibiting-properties, altered heart rate would not have been expected.

In summary, pulses of the pesticides we tested can rapidly affect olfaction in coho salmon parr. The carbamate pesticide IPBC can impair olfaction within 25-min even at 1 µg/L. Runoff pulses of this semi-persistent pesticide should thus be considered hazardous to olfaction in migratory salmonids. The existing Canadian water quality guidelines would likely better protect salmonid olfaction if set at a NOAEC, which for the olfactory epithelium would be approximately ten-fold lower than at present. The highly soluble pesticide glyphosate impairs olfaction within 10-min at 1 mg/L, and the effects appear to persist. Overall, EOG can provide a useful measure for acutely toxic pesticides such as carbamates and some organophosphorus pesticides. Electro-olfactograms could be of

greater use and have better real-world applicability if new methods could be evolved to use it to detect any olfactory effects arising from longer-term exposures.

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Table

Table 2-1 Lethality data, water quality standards, and electro-olfactogram (EOG) effects (a measure of sublethal neurophysiological toxicity) of five current-use agricultural pesticides and a carbamate fungicide (iodocarb, IPBC). Highlighted values in the final two columns show where the EOG was a more sensitive measure than median lethal concentration (LC50), and if effects were noted at concentrations below existing water quality standards. CI = confidence interval

Pesticide	Test animal	Size (g)	Temp (°C)	LC50		95CI	Test length	[a]	Ref.	Solubility[b] Half-life		Water standards (Canadian)[c]		EOG (coho salmon) screen range		effect	EOG sensitivity < LC50	< stand.
Chlorothalonil	Rainbow trout	1.1-2.5	12-15	>8.2	µg/L	-	10 d	I	[35]	0.6	mg/L	0.18	µg/L	1	mg/L	no	no	no
	Rainbow trout	6-11	15	14.3	µg/L	-	96 h	I	[23]	30	d							
	Rainbow trout	6-12	15	17.1	µg/L	-	96 h	I	[24]									
Endosulfan	Bluegill sunfish	1	18	1.2	µg/L	0.9-1.7	96 h	T	[36]	100	µg/L	0.02	µg/L	10, 100	µg/L	100	no	no
	Rainbow trout	1.3	13	1.4	µg/L	1.2-1.6	96 h	T	[36]	59	d							
	Clarias batrachus	80-90	-	576	µg/L	-	96 h	T	[37]									
Glyphosate	*Cyprinus carpio*	4.0-5.5	20 ± 1	645	mg/L	632-655	48 h	I	[38]	12	µg/L	65	µg/L	0.1-100	mg/L	1	yes	no
	Cyprinus carpio	4.0-5.5	20 ± 1	620	mg/L	607-638	96 h	I	[38]	47	d							
	Rainbow trout	0.8	12	130	mg/L	108-156	96 h	T	[36]									
	Coho salmon	11.8	11 ± 1	22	mg/L	12-38	96 h	F	[39]									
	Chinook salmon	4.6	11 ± 1	20	mg/L	17-27	96 h	F	[39]									

Cont.

Pesticide	Test animal	Size (g)	Temp (°C)	LC50		95CI	Test length	[a]	Ref.	Solubility[b] Half-life		Water standards (Canadian)[c]		EOG (coho salmon) screen range		effect	EOG sensitivity < LC50	< stand.
Trifluralin	Rainbow trout	0.6-1.5	12.7	42	µg/L	38-46	96 h	U	[40]	300	µg/L	0.2	µg/L	30, 300	µg/L	300	no	No
	Rainbow trout	0.6-1.5	7.2	152	µg/L	132-175	96 h	U	[40]	60	d							
	Rainbow trout	0.6-1.5	1.6	210	µg/L	182-240	96 h	U	[40]									
	Rainbow trout	-	13	11	µg/L	-	48 h	U	[41]									
2,4-D	Perch; Roach	-	-	75	mg/L	-	48 h	U	[42]	890	mg/L	4.0	µg/L	1-100	mg/L	100	no	no
	Tinca tinca	200	-	800	mg/L	-	96 h	U	[43]	10	d							
	Pink salmon	Fry	10	21.1	mg/L	-	96 h	I	[44]									
IPBC	Coho salmon	10-mo	10-12	95	µg/L	86-100	96 h	F	[12]	156	mg/L	1.9	µg/L	1-100	µg/L	1	yes	yes
										139	d							

[a] I = pure material, T = technical material, F = formulation, U = unknown.
[b] From Wauchope et al. [19], except for IPBC information which was taken from Juegensen et al. [45].
[c] Canadian water quality guidelines for the protection of aquatic life, fresh water guidelines. In the United States, the Federal Insecticide, Fungicide and Rodenticide Act controls exposure through labelling restrictions on pesticide applications, based on modelled environmental concentrations.

Figures

Figure 2-1 (A) Electro-olfactogram (EOG) responses and (B) example EOG traces taken from the nasal epithelium of coho salmon in response to 2-s pulses of various concentrations of L-serine, glyphosate acid, and 2,4-D [(A) n = 6 per group; (B) serial EOG traces were taken 10-min apart from one fish]. Curve equations for (A): L-serine, EOG = 0.896 + 79.0 × 2.59 log[serine], r^2 = 99.3%, p = 0.0005; 2,4-D, 0.207 + 193 × 2.78 log[2,4-D] r^2 = 99.6%, p = 0.0037; glyphosate, EOG = 0.559 + 67300 × 12.7 log [glyphosate], r^2 = 99.9%, p = 0.0005).

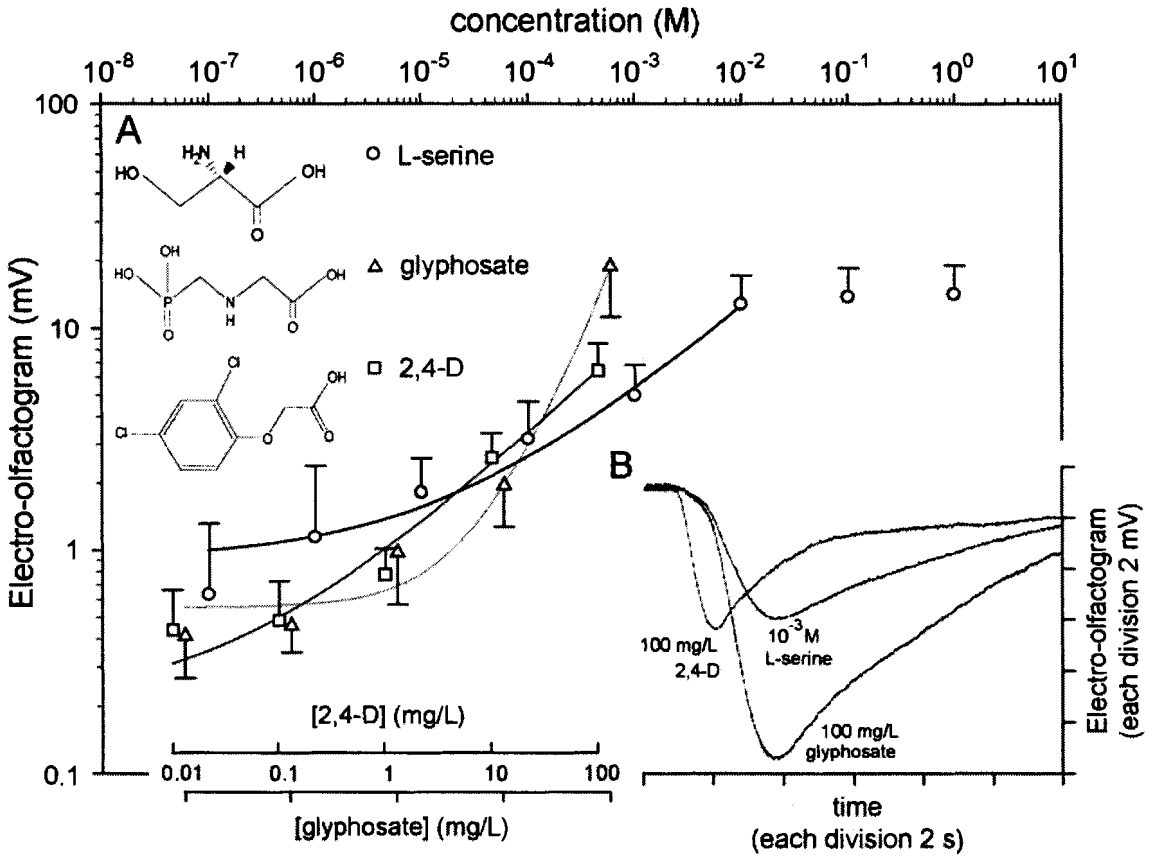


Figure 2-2 Changes in coho salmon electro-olfactogram (EOG) during 30-min exposure and 60-min recovery from (A) control (n = 18), the carbamate fungicide IPBC (iodocarb), (B) chlorothalonil, (C) endosulfan, (D) trifluralin, (E) 2,4-D, and (F) glyphosate (n = 6 per exposure concentration). Hatched area represents the exposure washout and transition period. For reference, the EOG of control fish are shown in panes (A) through (F) as a solid line. Line equation for 100 mg/L glyphosate recovery (32 – 90-min) is EOG = 0.875 × (min) – 5.40, r^2 = 94.3%, $p < 0.0001$. Significant differences from control are denoted with asterisks (two-way repeated measures analysis of variance, Holm-Sidak, $p < 0.05$).

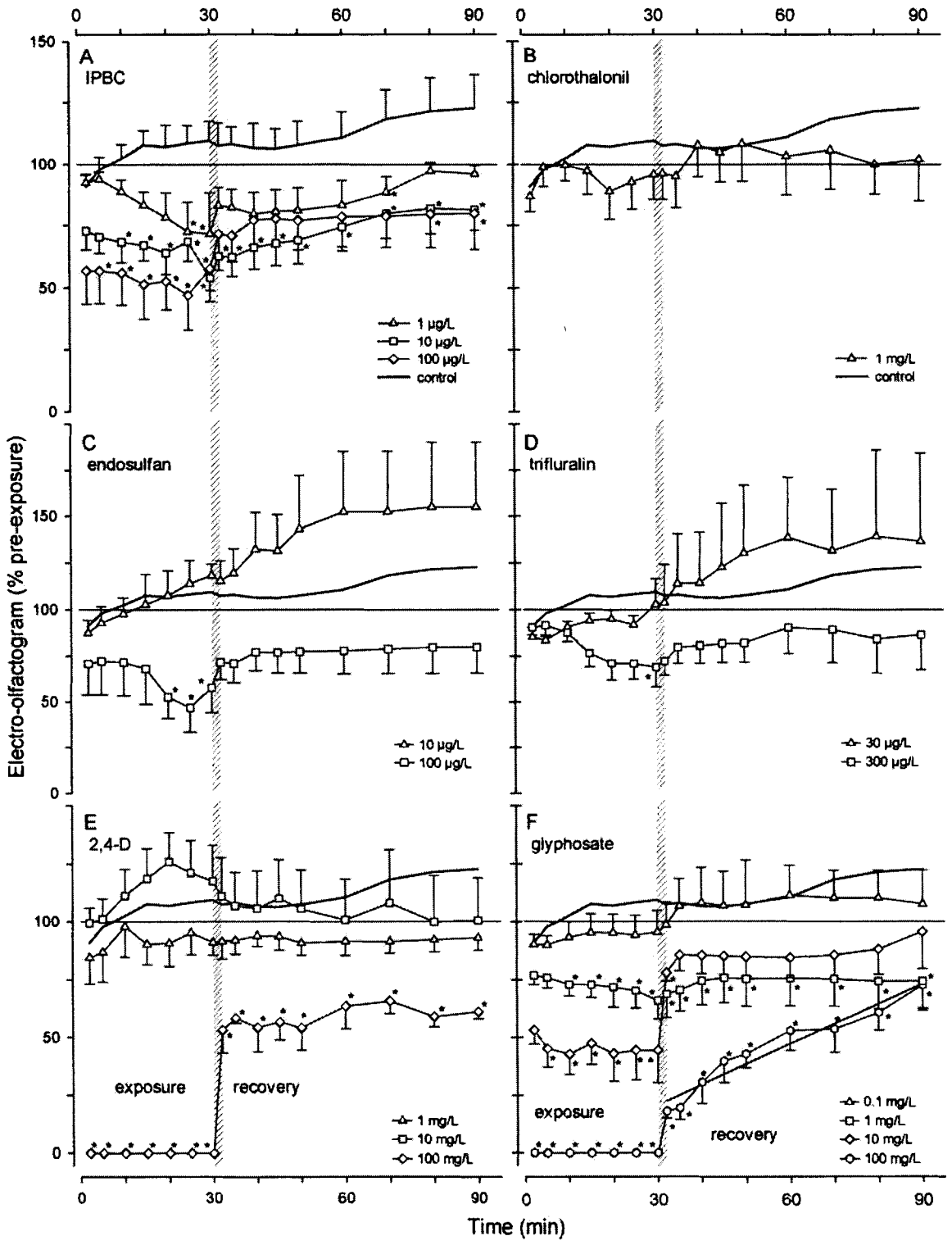
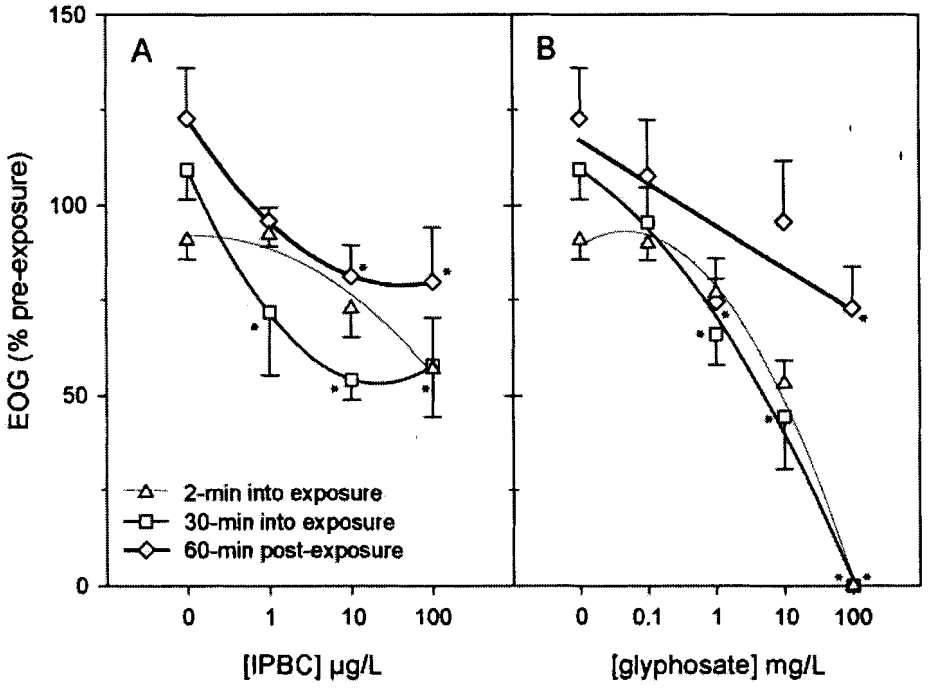

A
IPBC
1 µg/L
10 µg/L
100 µg/L
control
B
chlorothalonil
1 mg/L
control
C
endosulfan
10 µg/L
100 µg/L
D
trifluralin
30 µg/L
300 µg/L
E
2,4-D
exposure
recovery
1 mg/L
10 mg/L
100 mg/L
F
glyphosate
exposure
recovery
0.1 mg/L
1 mg/L
10 mg/L
100 mg/L
Electro-olfactogram (% pre-exposure)
Time (min)

Figure 2-3 Changes in electro-olfactogram (EOG) at 2 and 30-min into and 60-min post (A) IPBC (iodocarb) and (B) glyphosate exposure. Polynomial curves were fitted for visualization purposes only. Significant differences from control are denoted with asterisks (two-way repeated measures analysis of variance, Holm-Sidak



Chapter 3:
Linuron and carbaryl differentially impair baseline amino acid and bile salt olfactory responses in three salmonids

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Abstract

For salmon, amino acid and bile salt detection forms the basis for important behaviors including predator evasion and conspecific recognition, respectively. For this reason, decreases in olfactory sensory neuron responses to the amino acid L-serine and the bile salt taurocholic acid (TChA) have been used in studies as indicators of acute olfactory pesticide toxicity to environmental contaminants such as metals and pesticides. In this study, we first compare baseline responses to these two odorant classes across three salmonids, and then explore how two currently-used pesticides alter these responses. We found baseline differences in electro-olfactogram (EOG) responses and their sensitivity to pesticide exposure between rainbow trout, coho and sockeye salmon. For example, rainbow trout had lower baseline EOGs than either coho or sockeye (e.g. 10^{-5} M TChA EOGs of 1.34 ± 0.17 vs. 2.57 ± 0.46 and 2.72 ± 0.43 mV, respectively). At 15-min after exposure to 10 µg/L of the herbicide linuron, rainbow L-serine-evoked EOGs were 49.6% of control vs. 78.5 and 69.8% for sockeye and coho, indicating rainbow were more sensitive to linuron. In contrast, at 30-min of exposure to 100 µg/L carbaryl, L-serine-evoked EOGs of sockeye were 49.7% of control vs. 60.3 and 62.3% for rainbow and coho, suggesting sockeye were more sensitive to carbaryl. In all species the L-serine-evoked EOGs did not return to baseline by 15-min after 100 µg/L carbaryl exposure, suggesting persisting impairment of amino acid detection. The TChA-evoked EOGs were less affected by carbaryl exposure (i.e. EOGs were 83.3, 84.9 and 66.0% of control 15-min after exposure) and not affected at all by 100 µg/L

linuron exposure. Species-specific differences in pesticide sensitivity may limit extrapolation of toxicity across salmonids while the generally greater sensitivity of amino acid olfaction may lead to selective impairment of behaviors such as predator evasion.

Keywords: olfaction; EOG; coho; sockeye; salmon; rainbow trout; carbaryl; linuron

Introduction

Several studies demonstrate that current-use pesticides affect the baseline olfactory sensory neuron (OSN) responses of salmon through acute, sublethal exposures (Moore and Waring, 1996; Waring and Moore, 1997; Moore and Waring, 1998; Moore and Lower, 2001; Moore and Waring, 2001; Jarrard et al., 2004; Sandahl et al., 2004; Tierney et al, 2006). These studies have been conducted on different salmon species, using different odorants, sometimes at different concentrations, and after exposure to a variety of pesticides. This reflects the large number of species in the family Salmonidae, the number of laboratories involved, and the number of registered pesticides in current use (e.g. ~300 in Canada). The overarching justification for all of these studies remains the same: for salmonids, especially anadromous ones, the sense of smell enables crucial behaviors throughout the lifecycle, such that its loss represents a potentially important survival concern. This is exacerbated given the endangered status of some of these ecologically, economically and socially indispensable salmonid stocks (NIMFS, 1996; COSEWIC, 2002). There are presently no studies that have endeavored to compare the neurotoxic effects of pesticides across salmonid species. Such comparisons could facilitate the extrapolation of pesticide effects to untested salmonids and so strengthen our understanding of exposure risk.

Electrophysiological studies of pesticide effects have been largely limited to two salmonids, owing largely to the salmon species endemic to the regions in which the studies were performed. Not surprisingly, Atlantic salmon (*Salmo*

salar) were used in Atlantic (UK) studies while coho salmon (*Oncorhynchus kisutch*) were used in Pacific (Canada and the US) studies. However, rainbow trout baseline olfactory responses (Evans and Hara 1985; Hara and Zhang, 1996) and the effect of metals and pH on them have been tested (Klaprat et al., 1988). Furthermore, the effects of pesticides on olfactory-mediated behaviors have been studied in other salmon species, for example diazinon was found to impair return-migrating chinook salmon (*O. tshawytscha*) (Scholz et al. 2000).

In these toxicological and basic research studies of salmon olfaction, three classes of odorants, representing distinct OSN classes are routinely used: pheromone, amino acid and bile salt. These classes underpin distinct behavioral responses. For example, F-prostaglandins (PGFs) function as salmonid mating pheromones (Laberge and Hara, 2003), amino acids function in predator avoidance (L-serine washes of mammalian skin and salmon downstream of a plume of it avoid it; Brett and MacKinnon, 1954; Idler et al., 1956; Rehnberg and Schreck, 1987), feeding behavior (Hara, 2006) and for imprinting (Shoji et al., 2003), while bile salts can enable kin and conspecific recognition (Quinn and Hara, 1986). Morphologically, there are also three types of OSNs: ciliated, microvillar and crypt. For salmonids, ciliated OSNs are generalists that respond to all three odorant classes (amino acid, bile salt and pheromone) whereas microvillar OSNs are specialists that respond to amino acids (Sato and Suzuki, 2001). It is unknown what odorants correspond to crypt cells (Hansen and Zielinski, 2005), although sex pheromones have been hypothesized (Hamdani and Døving, 2006).

In this paper, we first measured baseline concentration-responses of coho salmon, sockeye salmon (*O. nerka*) and rainbow trout to the amino acid L-serine and the bile salt taurocholic acid (TChA) using electro-olfactogram (EOG), a measure of change in field potential in response to a stimulus (Ottoson, 1956). Next, olfactory rosettes were exposed to either linuron or carbaryl (Table 3-1) and EOG changes were noted during and following exposure. A reduction in L-serine-evoked EOG by either pesticide would suggest diminished predator avoidance, food location or imprinting abilities, while a reduction in TChA-evoked EOG would suggest altered schooling or sibling fidelity. These pesticides were chosen for two reasons: one, we had reason to suspect both pesticides would alter olfaction, and two, both pesticides are exposure risks to salmon as they are used in proximity to and have been detected in salmon producing waterways of British Columbia (BC) and have been listed as pesticides of concern (Verrin et al., 2004).

Linuron is a pre- and post-emergent herbicide that inhibits photosynthesis in broadleaf weeds. It is also known to have anti-androgenic activity, for example it has been shown to alter sexual differentiation in rats (Lambright et al., 2000). We suspected it may also alter olfaction in salmon since a structurally similar herbicide, diuron, impaired olfactory-mediated behaviors in goldfish (Saglio and Trijasse, 1998). As for exposure risk, linuron has been found in several streams in BC, one (Frew Cr.) at a concentration of 1.05 µg/L, which is not too surprising given sales in this region were 4585 kg in 2003 (ENKON, 2005).

The other pesticide we tested, carbaryl, is a carbamate insecticide used to inhibit acetylcholinesterase (AChE) in a diverse array of insects that infest crops and livestock. The omnipresence of AChE across the animal kingdom provides ample opportunity for non-target species effects. Furthermore, carbamates have previously been shown to alter both EOG responses (Jarrard et al., 2004; Tierney et al., 2006a) and olfactory-mediated behaviors in coho salmon (Tierney et al. 2006b). The method in which carbamates impair olfaction remains unknown, as AChE impairment does not obviously translate to impaired olfaction. Although, it has been hypothesized that since acetylcholine (ACh) stimulates mucous secretion, inhibition of AChE could lead to an overabundance of ACh and so a mucous plug (Jarrard et al., 2004). Like linuron, its sales and use patterns provide salmonid exposure risk. For example, its 2003 sales in the Georgia Basin were 1646 kg. Additionally, carbaryl is used for control of ghost shrimp by oyster farmers in Willipa Bay, Washington, and has been found in pore water from this area at 1.15 µg/L 60-d following application (Stonick, 1999).

In this study, coho were included to facilitate comparisons with other data on coho EOG-pesticide effects (e.g. Jarrard et al., 2004; Sandahl et al., 2004; Tierney et al. 2006a). However, coho may not be the best model for olfactory neurophysiologic research. Although bred for enhancement purposes, they are not typically raised to adulthood within captivity and can suffer from high mortality during the smoltification period. Furthermore, sacrificing animals that are bred for restorative purposes may be less than helpful. Therefore, we examined the potential use of the cultured, ubiquitous and frequently toxicity-screened rainbow

trout as a surrogate in pesticide–olfaction toxicity studies. We also used sockeye salmon, a species more closely related to coho. It was our expectation that any differences in olfaction or olfactory-sensitivity to pesticide exposure would be proportional to the relatedness of these species.

Methods

Fish

Coho salmon parr were obtained from the Fisheries and Oceans Canada (DFO) Capilano Hatchery (North Vancouver, BC) (N = 56, mass 16.1 ± 1.0g, length 11.5 ± 0.2cm, condition factor 1.02 ± 0.02), sockeye salmon parr were obtained from DFO Weaver Creek (Harrison, BC) (N = 68, mass 16.8 ± 0.8g, length 11.6 ± 0.2cm, condition factor 1.06 ± 0.02), and juvenile rainbow trout were obtained from Sun Valley Trout Farm (Mission, BC) (N = 56, mass 21.9 ± 1.1g, length 12.3 ± 0.1cm, condition factor 1.13 ± 0.02). All fish were maintained in indoor 170 l tanks supplied with filtered, dechlorinated municipal water (background water or BGW; dissolved O_2 at >90% saturation, pH 6.8, hardness 6.12 mg/l $CaCO_3$) under a 12:12 light:dark photoperiod with 30-min dawn:dusk transition, temperature (~10°C), and fed commercial salmon pellets (EWOS, Surrey, BC) *ad libitum*. Fish were held for at least 2 weeks prior to experiments and were tested under SFU animal care permit 716B.

Chemicals

All chemicals were purchased through Sigma Aldrich (Oakville, ON). The chemicals and their given purities were carbaryl (1-naphthy-N-methyl carbamate;

99.8%), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea; 99%), L-serine ((S)-2-amino-3-hydroxypropionic acid; 99%), taurocholic acid (TChA) (2-[(3α,7α,12α-trihydroxy-24-oxo-5β-cholan-24-yl)amino]ethanesulfonic acid; ≥95%), and 2-phenoxyethanol.

Experiment 1. Baseline L-serine and TChA olfactory concentration-response curves

The field generator responses of olfactory sensory neurons were measured with electro-olfactograms (EOGs), as detailed by Evans and Hara (1985) using the apparatus described in Jarrard et al. (2004). Briefly, fish were anesthetized using 0.5 and 0.25 ml/L (induction and maintenance) of 2-phenoxyethanol, placed in a Plexiglas holder and the left olfactory rosette was surgically exposed. The nasal cavity was continuously irrigated at ~1.5 ml/min with a Teflon feed in which we could supply background water, pesticide/vehicle mixtures or odorant pulses. Both the nasal feed and the anesthesia solutions were maintained at fish acclimation temperature by a Lauda chiller (Brinkmann Instruments, Westbury, NY). EOGs were taken as the change in potential difference across two Ag/Ag-Cl electrodes (2% agar in 1M NaCl), one placed just above the caudal area of the rosette raphe (tip diameter 580 μm) and the other on the top of the head (tip diameter ~1 mm). EOGs were amplified 1000×, digitized at 200 Hz, and acquired on a PC running LabView (National Instruments Inc., Austin, TX). All fish were acclimated to the EOG rig to provide a repeatable EOG response (i.e. < 5% variation in EOGs taken 10-min apart) before experimentation. Fish heart

rate was monitored using paired intraperitoneal stainless steel electrodes amplified 100× and displayed on an oscilloscope.

After the apparatus acclimation period, serial EOG responses were evoked using 2-sec pulses of 10^{-7}, 10^{-5} and 10^{-3} M L-serine delivered in the nasal feed by a computer-controlled solenoid valve system. Three responses were taken at each concentration, with 2-min given between 10^{-7} and 10^{-5} M pulses, and 3-min between 10^{-3} M pulses since EOGs for 10^{-3} M TChA or L-serine were only repeatable after 3-min. Five minutes after measuring the last L-serine response, the same procedure was repeated using TChA as an odorant. The average values of the three responses at each concentration were used in calculations for each fish (N = 10 per species).

Experiment 2. The effect of linuron and carbaryl on EOG

Pesticide exposure concentrations were selected as being at least 10× below reported 96-h LC50 values. With carbaryl, similar-sized coho and rainbow trout had 96-h LC50 values between ~1000 and 3000 µg/L; for example, juvenile coho had values of 2,700 and 1,150 µg/L (Mayer and Ellersieck, 1986), and juvenile rainbow trout had a value of 860 µg/L (Phipps and Holcombe, 1985). To our knowledge, sockeye have not been tested. However, given other species tend to be less susceptible to carbaryl, e.g. fathead minnow had a value of 14,600 µg/L (Johnson and Finley, 1980), we were confident that our exposure concentrations were sublethal. There aren't the same breadth of 96-h LC50 values for linuron, nevertheless, the values appear similar or greater than those of carbaryl. For example, rainbow trout fry had a value of 3,000 µg/L, whereas

an unspecified lifestage had a value of 16,400 µg/L (Office of Pesticide Programs, 2000). Therefore, 100 µg/L was chosen for the initial exposure concentrations of both carbaryl and linuron. However, since this concentration of linuron appeared to drastically reduce L-serine-evoked EOG, two lower range finding trials were performed with 1 and 10 µg/L with one of the species (sockeye), and the minimum concentration shown to significantly alter EOG (10 µg/L) was used for the remaining species.

Before testing the effects of pesticide exposure on EOG, it was first determined whether the pesticides themselves evoked EOGs. Fish were given 2-sec pulses of either pesticide, similar to *Experiment 1* but over a 100× concentration range (i.e. 1, 10 and 100 µg/L).

To determine the effects of linuron or carbaryl on amino acid or bile salt-evoked EOG during and after exposure, fish were first acclimated to the EOG apparatus as above and EOG responses to either 10^{-5} M L-serine or TChA were recorded. After reaching a stable EOG response (5-40-min), nasal water flow was then switched to vehicle control (100 µg/L acetone) or a pesticide solution and EOG responses to one of the odorants were taken at 0, 5, 10, 15, 20, 25 and 30-min during exposures. After this, the nasal water flow was returned to background water and EOGs were taken at 2, 5, 10 and 15-min after exposures. All solutions were delivered in Teflon tubing, which was replaced with each test group. Control fish were distributed throughout the experiments.

Statistics

For *Experiment 1*, differences between EOGs (in mV) at each L-serine and TChA concentration within each species were tested using a two-way (odorant and odorant concentration) repeated-measures (RM) analysis of variance (ANOVA) and a Holm-Sidak post-hoc test. The L-serine and TChA responses were then compared across species in a similar manner, except odorant concentration and species were used in the two-way RM ANOVA.

For *Experiment 2*, differences in linuron or carbaryl concentration-response curves were tested as above using an RM ANOVA. Differences in EOG during and after linuron or carbaryl exposure were tested in a different manner. First, exposure and post-exposure EOG peak sizes (in mV) were considered as a proportion of the stable pre-exposure value, then differences within a species over exposure concentrations were tested using a two-way (time and treatment) RM ANOVA followed by a Holm-Sidak post-hoc test. To determine whether L-serine or TChA evoked EOGs were more sensitive to pesticide exposure, the change in either L-serine or TChA-evoked EOGs from control at each exposure time was calculated (e.g. 2-min EOG value for control – 2-min value for exposed) and then these differences were tested against each other using a two-way (odorant and time) ANOVA. A similar procedure was used to determine differences in species pesticide sensitivity, except that the factors used in the ANOVA were species and time. To illustrate differences in sensitivity, the change from control values for each species were normalized (i.e. the difference at each time point for each species was divided by the sum of all differences at that time point) and plotted on a ternary graph. Any increased

sensitivity (i.e. greater EOG impairment) will be shown as increased apical clustering.

For sockeye exposed to three linuron concentrations, to estimate the concentration of linuron inhibiting L-serine EOG by 50% (IC_{50}) at 30-min of exposure, three-parameter exponential decay functions were fitted to average values of the relative EOG inhibition at each exposure concentration (inhibition = $Y_0 + a \times e^{-b[linuron]}$) and 50% inhibition was interpolated. Curves were also fitted to ± 2× S.E.M. of the averages and the same procedure was used to calculate 95% CIs. The fiducial limit of significance was set at 5% for all tests. Reported values are mean ± S.E.M. unless stated otherwise. SigmaStat 3 and SigmaPlot 9 (Systat Software, Inc., Point Richmond, CA) were used for statistics and graphing; Adobe Illustrator 10 (Adobe Systems Incorporated, San Jose, CA) was used to produce the figures.

Results

Experiment 1. L-serine and TChA olfactory concentration-response curves

In all species, over the 10,000× concentration range tested, L-serine-evoked EOGs (in mV) were best explained by exponential growth models while increases in TChA-evoked EOGs were best explained by linear models (Fig. 3-1). At the highest (10^{-3} M) odorant concentration, this difference translated to significantly greater L-serine- than TChA-evoked EOGs for sockeye and rainbow trout, but not for coho, which had lower L-serine evoked-EOGs.

Across species, the EOG responses of rainbow trout tended to be the lower than the other species at any given odorant concentration (Fig. 3-1c). With

L-serine responses, sockeye had significantly greater responses than either coho or rainbow ($p=0.000$) (Fig. 3-1b).

Experiment 2. The effect of linuron and carbaryl on EOG

Linuron and carbaryl concentration-response curves

Linuron did not evoke typical concentration-response curves (Fig. 3-2a). In fact, successive pulses of increasing concentrations evoked the same or lower EOG responses (Fig. 3-2c,d,e). The phasic portion of the EOG response linuron typically evoked resembled those of L-serine or TChA (see Fig. 3-3a,c), although in two cases repeated 2-s 100 µg/L pulses generated small (i.e. <0.5 mV) inverted responses. Furthermore, at this exposure concentration the tonic portion of the EOG was atypical and slow to return to baseline (Fig. 3-2d).

Carbaryl likewise evoked EOGs in a manner irrespective of concentration (Fig. 3-2e). This was especially clear for rainbow trout, where the response evoked by 100 µg/L was significantly lower than that of 10 µg/L. Unlike linuron, the phasic and not the tonic portion of the EOG responses were atypical (Fig. 3-2f,g,h). Like linuron, in one case repeated 100 µg/L pulses generated a small inverted EOG response.

EOG responses following linuron exposure

Linuron exposure caused decreases in L-serine-evoked EOGs (Fig. 3-3a,b) but had no effect on TChA-evoked EOGs (Fig. 3-3c,d). Comparatively, coho and sockeye were more sensitive to linuron at the beginning of exposure than rainbow (Fig. 3-4a,b,c), as evidenced by the immediate EOG decreases

during 10 µg/L exposure (p = 0.028 and 0.005, respectively) noted in these species vs. rainbow trout, where significant decreases occurred at 15-min (p = 0.039). However, only in rainbow trout did the effect of linuron exposure persist up to 15-min following exposure (p = 0.001) (Fig. 3-4c). Given a set of range finding exposures was performed for L-serine-evoked EOGs in linuron-exposed sockeye, the IC50 EOG response was determined at 30-min of exposure to be 12.7 (33.4, 1.75, upper and lower 95 CIs) µg/L.

EOG responses following carbaryl exposure

Carbaryl exposure reduced both L-serine and TChA-evoked EOGs (Fig. 3-3e,f,g,h). Significant decreases in L-serine-evoked EOGs of coho and sockeye were apparent immediately (p values of 0.017 and 0.009) (Fig. 3-5a,b), whereas differences for rainbow trout were apparent at 10-min of exposure (p=0.043) (Fig. 3-5c). In all species, EOG recovery was not noted within 15-min after exposure cessation. Comparing the relative L-serine-evoked EOG decreases across species, sockeye were more sensitive (i.e. had greater relative EOG decreases) than coho (p=0.003), but not rainbow (p=0.071); coho and rainbow had similar sensitivities (p=0.496) (Fig. 3-6).

Decreases in TChA-evoked EOGs from carbaryl followed a similar trend (Fig. 3-5d,e,f). Specifically, carbaryl exposure caused an immediate difference from control in coho (p = 0.025), and this difference continued to 2-min after exposure (Fig. 3-5d). In rainbow trout the difference occurred later (5-min into exposure) (p = 0.025) and it ended later (5-min after cessation) (Fig. 3-5f). Sockeye had similar numeric EOG decreases, although the difference over both

exposure and recovery was not statistically significant ($p = 0.055$) (Fig. 3-5e). Across species, rainbow trout were more sensitive than sockeye ($p = 0.027$), but not coho salmon ($p = 0.355$); coho and sockeye were similarly sensitive ($p = 0.449$) (Fig. 3-6).

The relative size of the EOG decreases caused by carbaryl exposure depended upon what odorant was used to evoke the responses. Overall, the L-serine-evoked EOGs were more sensitive to carbaryl exposure in all species (p-values of 0.002, 0.000 and 0.003 for coho, sockeye and rainbow). Specific differences in sensitivity tended to occur either late in or following carbaryl exposure. Explicitly, for coho, L-serine responses were affected more than TChA responses at 5 to 15-min after exposure (p-values of 0.019, 0.036 and 0.032), with sockeye from 25-min into exposure ($p = 0.042$) to 15-min after ($p = 0.003$), and with rainbow from 10 to 15-min post exposure (p values of 0.005 and 0.002).

Discussion

There were two purposes to this study: 1) to compare baseline olfactory sensory neuron responses in three juvenile salmonids using two odorant classes routinely used in toxicity testing and known to underlie distinct behavioral responses, and 2) to explore how two currently-used pesticides alter these responses. We report novel findings in both areas, as differences were noted in baseline amino acid and bile salt responses, and the pesticides caused both species- and olfactory sensory neuron-specific effects on these responses.

Baseline EOG responses and their comparability

Several studies have measured L-serine responses in salmonids (Table 3-2). The exponential relationship observed for L-serine evoked EOGs in all three species tested here has been observed previously in rainbow trout (Evans and Hara, 1985; Klaprat and Hara 1988). Furthermore, the 10^{-5} M L-serine-evoked EOGs of the coho tested here (1.56 ± 0.36 mV) resemble those of another study at the same concentration (i.e. 0.3 to 4 mV; Jarrard et al., 2004) and are slightly greater than another using 10^{-4} M (1.5 ± 0.6 mV; Sandahl et al. 2004) (Table 3-2). Of the three salmonids tested here, rainbow trout had the lowest responses. The difference is unlikely due to size since the rainbow were of larger mass, which positively correlates with EOG response magnitude (Tierney et al. 2006a). Across studies of other species, the L-serine responses of Atlantic salmon were the lowest. Given both rainbow trout and Atlantic salmon are cultured, the lower EOG responses of these species may indicate olfaction has become less sensitive. Since some of the behaviors supported by amino acid detection (e.g. predator evasion or imprinting) are likely unnecessary to survival in cultured salmonids, there would likely be no cost to reduced sensitivity. Differences in sensitivity with cultured fish could be tested by measures of 'wild' vs. cultured fish.

There is less data available on salmonid TChA responses (Table 3-2). Nevertheless, the TChA-evoked EOGs of our study (2.57 ± 0.46 mV) are similar to another (2.1 ± 0.4 mV) (Sandahl et al., 2004) at the same molarity (10^{-5} M). Again, rainbow trout had the lowest responses of the species tested. Comparing the magnitudes of TChA to L-serine responses, Quinn and Hara (1986) found

that 10^{-5} M TChA evoked EOG responses 182% that of 10^{-5} M L-serine in juvenile coho salmon (mV not given). Similarly, Hara and Zhang (1996) reported EOGs of ~4.2 mV for 10^{-5} M L-serine in rainbow trout, and ~3.2 mV for 10^{-6} M TChA. Correcting for the difference in TChA molarity using the equation from our rainbow trout work, this would indicate a 10^{-5} M TChA EOG response of ~4.3 mV and so a TChA:L-serine ratio of 135%. In the present study, the ratios for all species were similar at 124, 125 and 165% for rainbow, sockeye and coho, respectively. The difference in EOG values between TChA and L-serine is not likely attributable to electrode placement since the central raphe location used was shown to maximize both L-serine and TChA responses in rainbow trout (Hara and Zhang, 1996). The difference may be due to intrinsic differences in generator responses between these OSN classes.

Pesticide effects on baseline EOG

A priori, we expected similar sensitivities of amino acid and bile salt OSNs given both OSN classes were affected equally in coho exposed to copper (Baldwin et al., 2003). Nevertheless, it would be reasonable to predict that any differential sensitivity in these OSN classes to contaminants could reflect the importance of the specific survival-based behavior that each OSN class enables. For example, increased resistance in the L-serine chemoreception (ciliated and microvillar OSNs) vs. TChA chemoreception (ciliated OSNs) to contaminants could suggest predator evasion is more important than schooling behavior. Alternately, these OSN classes may have no historic contaminant analog and so have neuroprotective mechanisms that do not function properly (i.e. to protect the

more critical chemoreception) or at all. In any event, our results found that L-serine-evoked EOGs were more sensitive to both linuron and carbaryl exposure, with TChA-evoked EOG responses being unaffected by 100 µg/L linuron exposure. This suggests retention of social cues may be relatively more crucial than amino acid cues, and that salmonids exposed to certain currently-used pesticides may experience decreased predator evasion ability prior to altered kin and con-specific related behaviors.

Of the species tested, rainbow trout experienced a slower onset of linuron toxicity, but were unable to recover baseline responses as coho and sockeye did. This overall difference between rainbow trout cf. coho and sockeye salmon follows from our hypothesis that phylogenetic separation could help account for any differences. Nevertheless, with carbaryl exposure, sockeye were more sensitive, but only for L-serine-evoked EOGs. With TChA-evoked EOGs, rainbow trout were again more sensitive. A difference for all species was that L-serine-evoked EOG responses did not return to baseline levels within 15-min of carbaryl exposure cessation. Here again this indicates that amino acid responses may be more susceptible to contaminants, but it also suggest that these effects may persist past exposure.

In all salmon tested, the EOG effects occurred well below lethality but higher than Canadian Water Quality Guidelines for the Protection of Aquatic Life (CWQG). However, the interim CWQG for linuron of 7 µg/L (Caux et al., 1998) may still be injurious to salmon as we noted an L-serine-evoked EOG IC50 of 12.7 µg/L (33.4, 1.75). The CWQG for carbaryl is 0.2 µg/L, which is well below

any observed acute, negative olfactory effects and so these regulations if adhered to likely protect salmon olfaction.

Toxicity mechanism(s)

An earlier study suggested that the reduction in EOG from carbamate exposure may have been caused by a mucous plug (Jarrard et al. 2004), since carbamate pesticides inhibit acetylcholinesterase and so block the cleavage of acetylcholine, the neurotransmitter that stimulates mucous secretion (Inglis et al., 1997). This theory is substantiated by the finding that carbaryl inhibited 50% of chinook salmon AChE with an exposure to approximately 100 µg/L (Scholz et al., 2006), a concentration identical to our exposure groups where EOG was inhibited by 50% in some cases. Our results do not necessarily contradict this mucous theory as we noted similar decreases in both TChA and L-serine-evoked EOGs with carbaryl exposure, suggesting a non-specific effect. However, the effects of carbaryl occurred immediately with application (Fig. 3-5). This indicates that at least some of the EOG change may have occurred at the receptor or cell membrane level; a finding given credence since carbaryl evoked EOG responses.

In most carbaryl-exposed salmon of the present study, EOG returned to control levels within 15-min (Fig. 3-5a,b,c). This may indicate that carbaryl was only adapting (acting as an odorant to) both TChA and L-serine receptors. However, this is unlikely given the lack of OSN specificity and the atypical shape of the EOG responses (e.g. Fig. 3-2f). Furthermore, the mode of action of carbamates does not rule out a toxic mechanism of EOG impairment.

Specifically, carbamates can add a carbamoyl group (in the case of carbaryl, a methyl carbamoyl (O_2CNCH_3) group) to serine hydroxyl (SER-OH) residues, a site which is known to exist in other receptors and be likewise susceptible to attack (e.g. as in the estrogen receptor; Koffman et al., 1991). A bound SER-OH site may render the active site unavailable, as is the case with carbamates and acetylcholinesterase, or through steric hindrance. In any event, the forward and reverse reactions for addition/removal of a carbamoyl group to SER-OH can take just seconds (Lotti, 1995), which could explain both the rapid EOG reduction and restoration observed in this study during and after carbaryl-exposure. Organophosphorus exposure can likewise add a group to SER-OH (in this case a phosphate (PO_4) group), and so may reduce TChA and L-serine-evoked EOG responses in a similar manner (e.g. as is noted for chlorpyrifos; Sandahl et al., 2004). However, the forward and reverse rate for the phosphate group is much slower and so persisting effects would be more likely. Future studies could lend support to this theory by comparing the EOG recovery times of carbamate vs. organophosphorus pesticide-exposed fish.

The effects of linuron are in contrast to the general effects noted with carbaryl, and so alternate toxic mechanisms can be suggested. Given ciliated OSNs respond to both amino acids and bile salts whereas microvillar OSNs respond only to amino acids (Sato and Suzuki, 2001), it is conceivable that only microvillar OSNs were impacted by linuron. Furthermore, given the differential effect of carbaryl (i.e. TChA was less sensitive to exposure), it may be that ciliated OSNs are more resistant to contaminant exposure in general. An

alternate explanation may lie in differential sensitivity of the odorant receptor proteins expressed and/or the signal transduction machinery in ciliated vs. microvillar OSNs. Ciliated OSNs express OR-type proteins whereas microvillar OSNs express V2R-type proteins (Hansen and Zielinski, 2005). Each pesticide could have toxicity specific to one of these OR classes and so alter either bile salt or amino acid responses. With signal transduction machinery, ciliated and microvillar cells can express different G-protein-coupled receptors. Specifically, $G_{\alpha olf}$ exists in ciliated OSNs and stimulates adenylyl cyclase to catalyze cAMP production, while $G_{\alpha o}$ exists in microvillar OSNs and stimulates phospholipase C (PLC) to catalyze IP_3 production (Hansen and Zielinski, 2005). Conceivably the differential toxicity could be explained by increased sensitivity of the $G_{\alpha o}$ PLC pathway.

Conclusion

Baseline amino acid and bile salt olfactory responses differ across cultured and 'wild' salmonids. Furthermore, these salmon differ in sensitivity to the currently-used pesticides linuron and carbaryl. This difference has implications for the use of a cultured salmonid to adequately predict pesticide toxicity that may occur in a wild salmonid, and underscores the need for cautious inter-species extrapolation in fish toxicology. Furthermore, linuron and carbaryl appear able to differentially impair certain classes of odorant receptors. These effects typically occur immediately with exposure and can persist ≥15-min past the exposure period. Given that the responses of both OSN classes tested here facilitate distinct and life-supporting behavioral responses, these pesticides if

found in salmon supporting waterways represent a survival concern to salmon and the myriad species that depend upon them. With the importance of rainbow trout, coho and sockeye salmon, and the ongoing use of linuron and carbaryl, further research is warranted into the behavioral implications of exposure to these pesticides, especially longer-term exposures, and to the mechanisms by which they affect toxicity.

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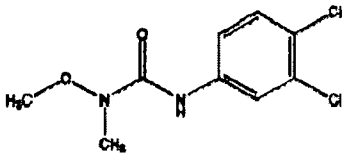
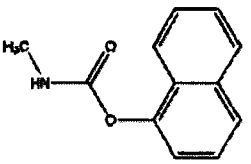
Tables

Table 3-1 The structure, names and physicochemical properties of linuron and carbaryl.

	linuron	**carbaryl**
structure		
pesticide class	phenylurea herbicide	carbamate insecticide
chemical name	3-(3,4-dichlorophenyl)-1 -methoxy-1-methylurea[a]	1-napthyl methylcarbamate[a]
CAS number	330-55-2	63-25-2
molecular weight	249.11	201.23
water slubility (mg/L)	81 @ 25 C[a]	40 @ 30 C[a]
melting point ©	93-94[a]	142 a
vapor pressure (mPa @ 25 C)	2[a]	<5.3[a]
partition coefficient	3.0043[b]	-
adsorption coefficient	400[c]	300[a]

[a]Kidd and James (1991)
[b]Weed Science Society of America (1994)
[c]Wauchope et al. (1992)

Table 3-2 Baseline olfactory responses of salmonids to various concentrations of amino acids (L-serine or L-histidine) and a bile salt (taurocholic acid; TChA).

	Species	Odorant concentration					Ref.
		10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M	
Amino acid							
L-serine	*S. salar*			0.90 ± 0.07			a
				0.86 ± 0.12			b
				0.75 ± 0.08			c
				0.72 ± 0.11			d
				0.68 ± 0.09			e
	O. kisutch	0.58	0.99	1.86			f
				0.3 to 4.2			g
					1.5 ± 0.6		h
						3.6 ± 0.5	i
		1.10 ± 0.20		1.56 ± 0.36		3.80 ± 0.69	j
	O. nerka	1.30 ± 0.26		2.18 ± 0.40		6.14 ± 0.71	j
	O. mykiss	0.48 ± 0.08		1.08 ± 0.11		3.78 ± 0.41	j
				4.17			k
L-histidine		0.90 ± 0.25		2.61 ± 0.83		7.10 ± 1.94	l
Bile salt							
TChA	*O. kisutch*	1.16	1.74	3.31			f
				2.1 ± 0.4			h
		1.16 ± 0.22		2.57 ± 0.46		4.37 ± 0.60	j
	O. nerka	1.27 ± 0.16		2.73 ± 0.43		3.74 ± 0.53	j
	O. mykiss	0.78 ± 0.09		1.35 ± 0.17		2.20 ± 0.27	j
			3.25			-	k

[a]Moore and Waring (1996); [b]Waring and Moore (1997); [c]Moore and Waring (1998); [d]Moore and Lower (2001); [e]Moore and Waring (2001); [f]Baldwin et al. (2003); [g]Jarrard et al. (2004); [h]Sandahl et al. (2004); [i]Tierney et al. (2006a); [j]present study; [k]Hara Zhang (1996); [l]Tierney et al. (2007). Values without error are estimates.

Figures

Figure 3-1 Serial electro-olfactogram responses evoked by increasing concentrations of the amino acid L-serine and the bile salt taurocholic acid (TChA) in three salmon species (N = 10 per species). Significant differences within species are indicated by dissimilar letters (p-values are given for differences at an odorant concentration); significant differences within an odorant concentration across species are indicated by dissimilar numbers (RM ANOVA, Holm Sidak). Curve equations for L-serine and TChA for coho: (mV) = 0.977 + 30.2 × $2.20^{(\log [SER])}$, perfect fit, and (mV) = 6.71 + 0.802 × (log [TChA]), R^2 = 99.5%, p = 0.0454; for sockeye: (mV) = 1.05 + 48.7 × $2.12^{(\log [SER])}$, perfect fit, and (mV) = 5.66 + 0.618 × (log [TChA]), R^2=98.9%, p= 0.0652; and for rainbow trout: (mV) = 0.312 + 33.1 × $2.12^{(\log [SER])}$, perfect fit, and (mV) = 3.22 + 0.356 × (log [TChA]), R^2 = 98.6%, p = 0.0755.

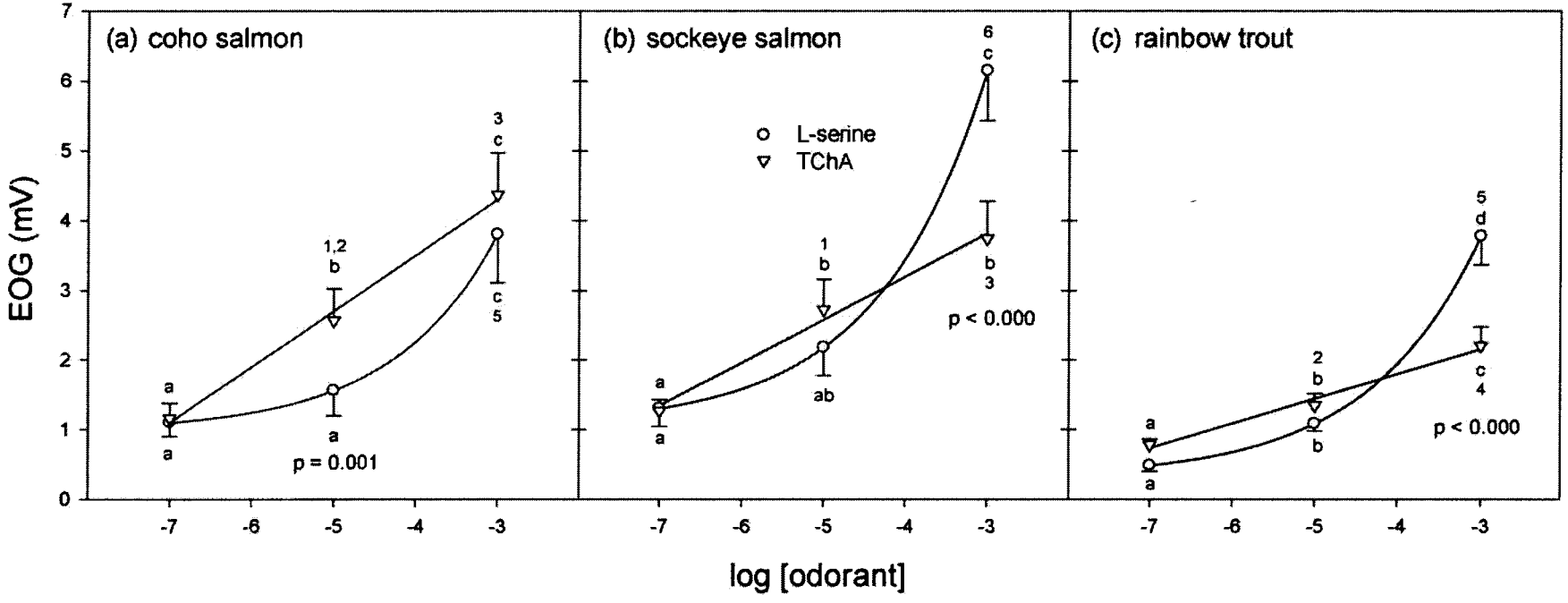


Figure 3-2 Serial electro-olfactogram responses evoked by increasing concentrations of the herbicide linuron and the insecticide carbaryl in three salmon species (N = 5 per pesticide per species). For each pesticide, significant difference within a species is shown by dissimilar letters, across species by an asterisk (RM ANOVA, Holm Sidak). The example EOG traces were taken from separate rainbow trout and were the third trace in a series of three for each concentration (1, 10 and 100 µg/L).

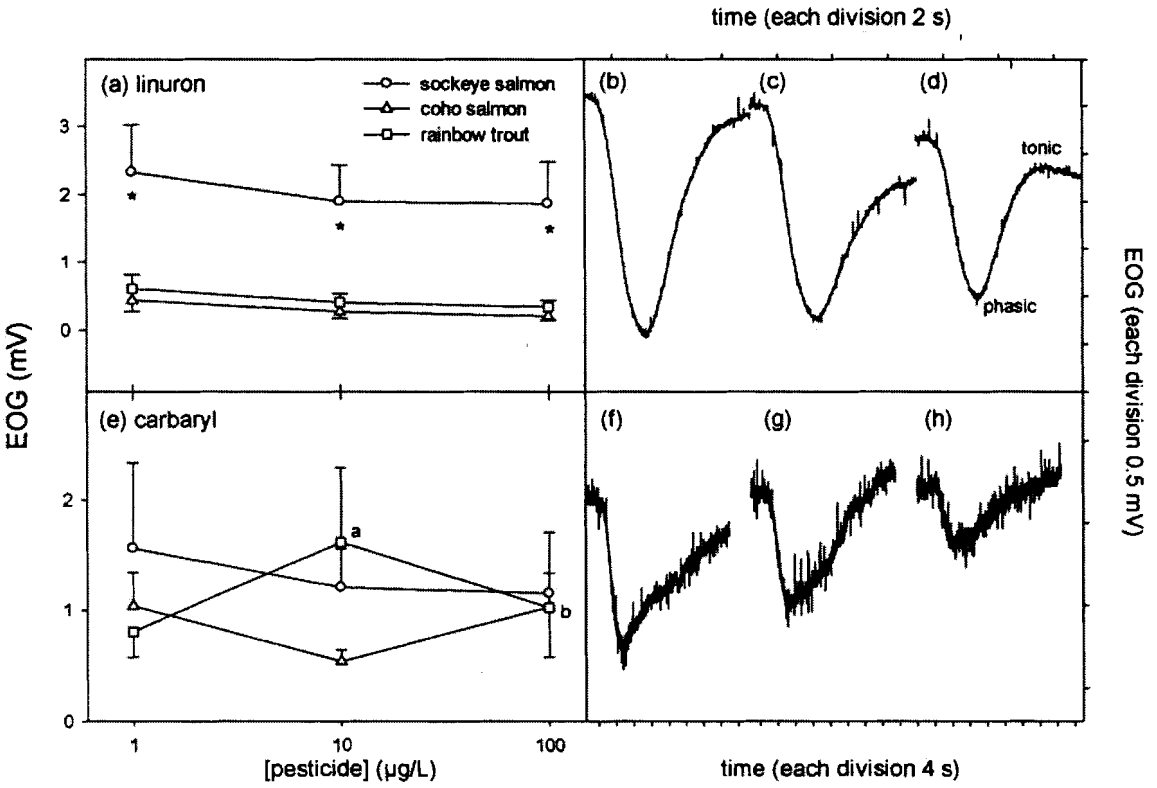


Figure 3-3 Electro-olfactogram responses evoked by either L-serine (a,e) or taurocholic acid (TChA) (c,g) in rainbow trout before and at 30-min of exposure to either linuron (a-d) or carbaryl (e-h).

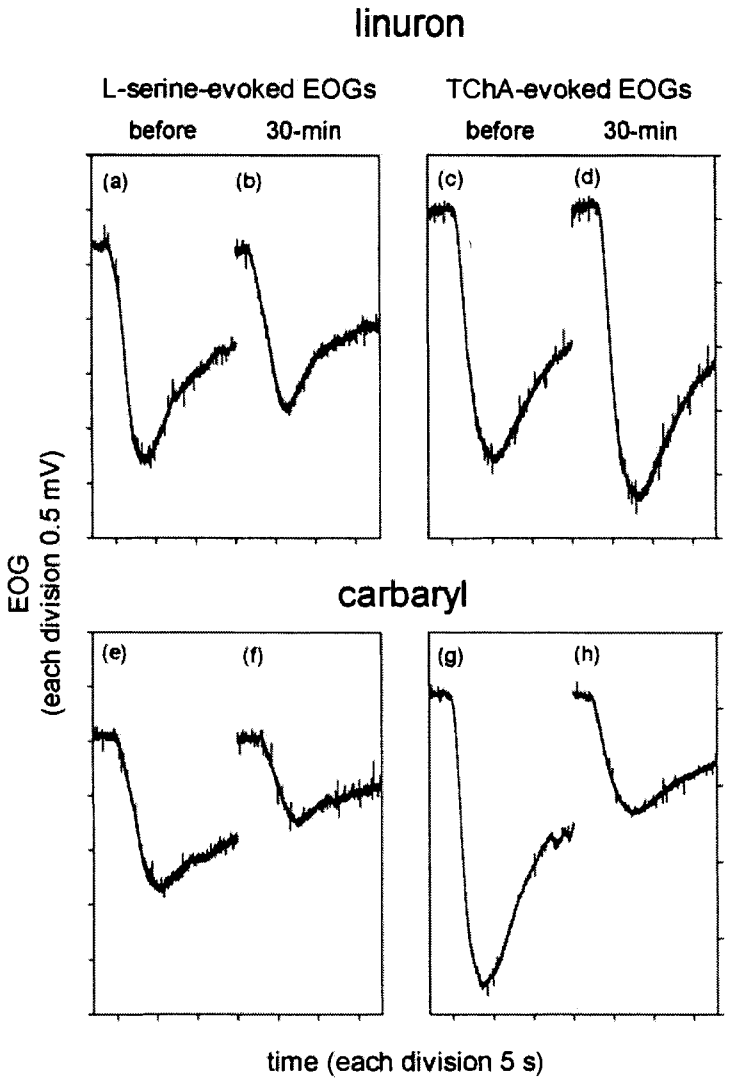


Figure 3-4 Changes in L-serine and taurocholic acid (TChA) -evoked electro-olfactogram responses of three salmon species during and following 30-min exposures to linuron (N = 6 per line). Values are considered as proportions of the last pre-exposure value. Significant difference from control within a species is shown by a '•' (two-way RM ANOVA, Holm-Sidak).

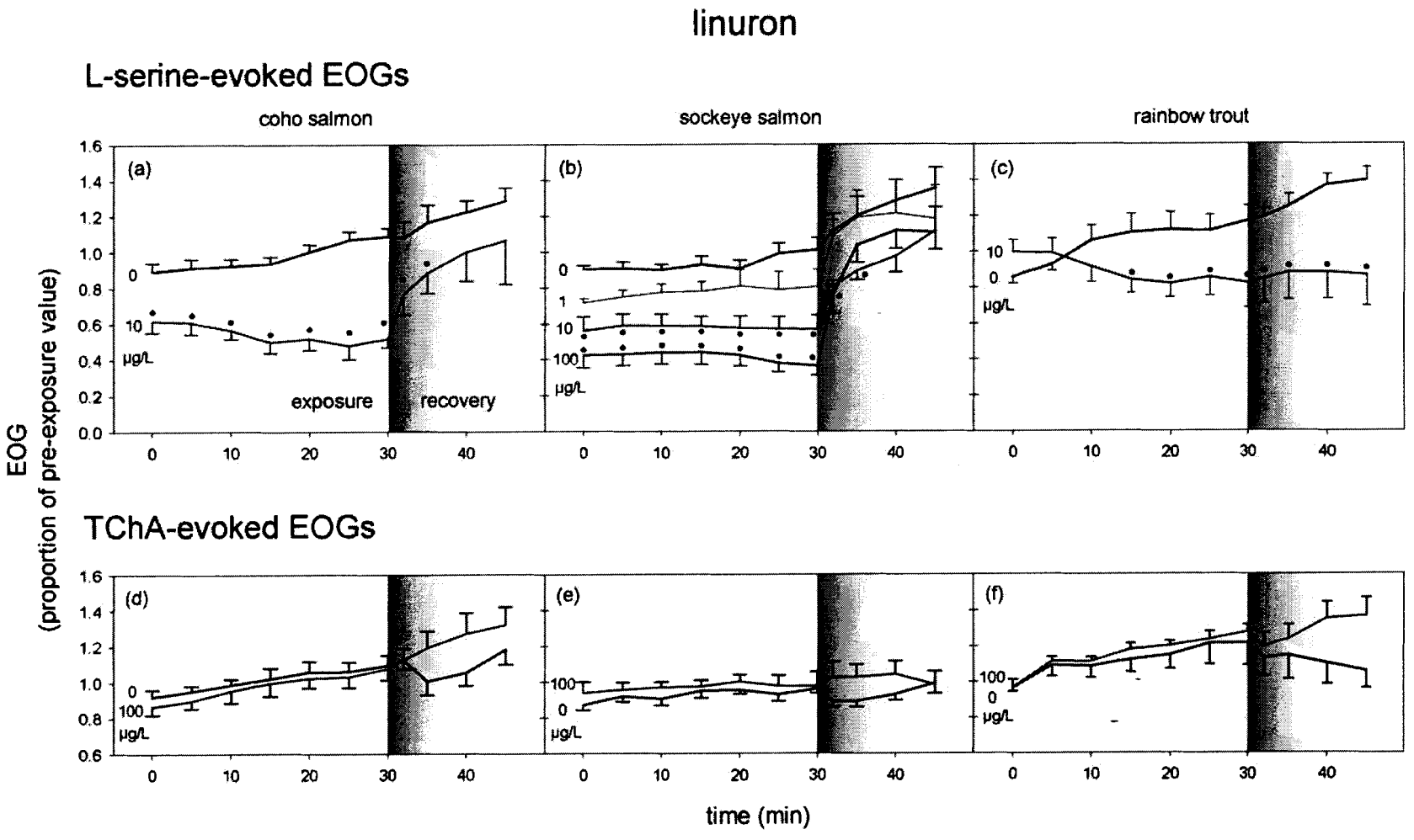


Figure 3-5 Changes in L-serine and taurocholic acid (TChA) -evoked electro-olfactogram responses of three salmon species during and following 30-min exposures to carbaryl (N = 6 per line). Values were considered as proportions of the last pre-exposure value. Significant difference from control within a species is shown by a '•' (two-way RM ANOVA, Holm-Sidak).

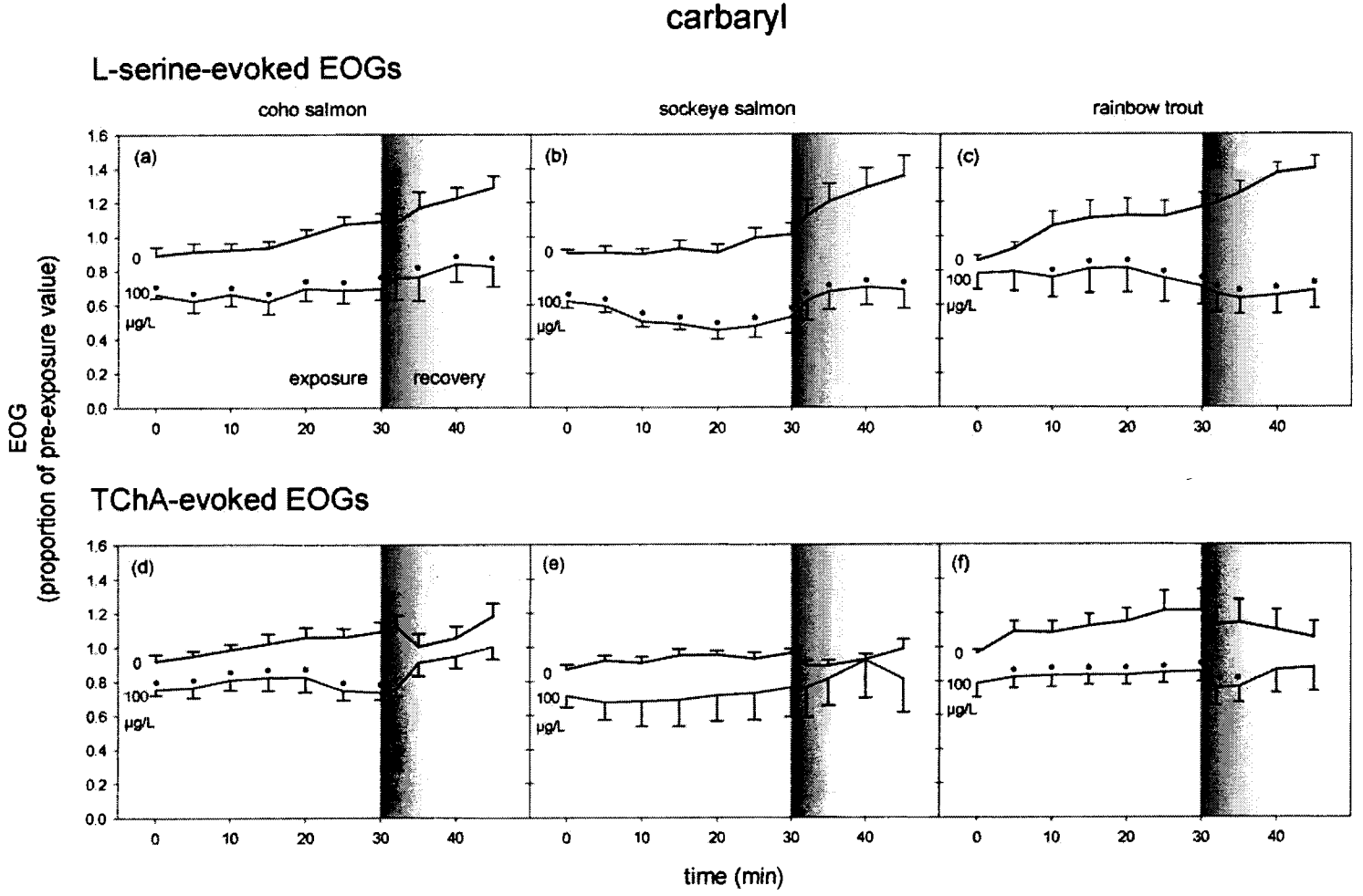
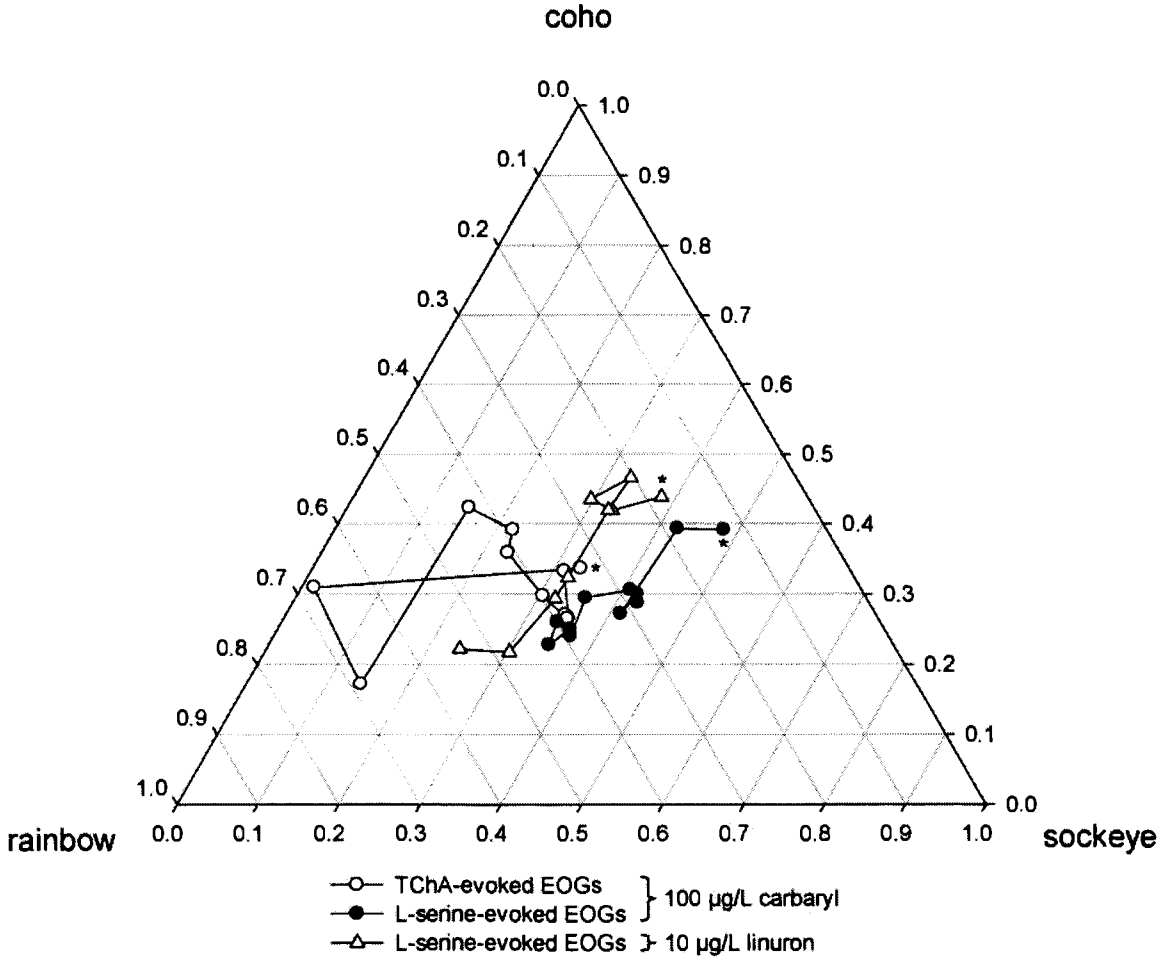


Figure 3-6 The relative reduction in electro-olfactogram of two different classes of olfactory sensory neurons during exposure to 10 µg/L of the herbicide linuron and 100 µg/L of the insecticide carbaryl in three salmon species (N = 36 per line). Points closer to any of the apices indicate increased species pesticide sensitivity. Asterisk indicates the beginning of the exposure and serial points are for 0, 5, 10, 15, 20, 25, 30-min during exposure and 2, 5, 10 and 15-min post-exposure, except for linuron where the series begins at 10-min of exposure. Note: linuron had no effect on TChA-evoked EOGs up to 100 µg/L and so data is not shown.



Chapter 4:
Does neuroprotection spare olfaction in rainbow trout exposed to an environmentally realistic pesticide mixture?

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Abstract

The salmon producing waterways of the world are polluted with pesticides, which is alarming since pesticide exposure can alter the function of the olfactory sensory neurons that guide many salmonid behaviors. To simulate a realistic exposure scenario, juvenile rainbow trout (*O. mykiss*) were exposed in-lab to 0, 0.1, 1 and 10× concentrations of a mixture consisting of the ten most frequently occurring pesticides found in British Columbia's Nicomekl River. This mixture consisted of organophosphorus, triazine, organochlorine, and phenylurea constituents. Following 96-hr exposure, the activity of an enzyme class that prevents cellular damage (glutathione-S-transferase), was increased in the olfactory epithelium of all mixture-exposed fish (32.4, 28.4 and 19.2% increase for 0.1, 1 and 10×, respectively). This upregulation did not appear to prevent damage to olfactory sensory neurons in fish given 1 and 10× exposure, since the ability of these cells to adapt to a change in background odorant concentration was impaired (by 42.3 and 53.3%, respectively). Exposure to a second, brief (10-min) and high (20×) mixture concentration reduced OSNs responses in all groups except the 10× fish. The impairments were greater than predicted by a model constructed using data from previous studies of salmon pesticide olfactory toxicity, which suggests there have been synergistic interactions between the pesticides. This study demonstrates that environmentally-observed pesticide mixtures can injure salmon olfactory tissue, which given the importance of olfaction to several salmon behaviors, suggests pesticide mixtures may be contributing to the threatened and endangered status of many salmon stocks.

Keywords: rainbow trout, steelhead, salmon, olfaction, neuroprotection, pesticide

Introduction

For migratory salmon such as steelhead (*Oncorhynchus mykiss*), olfaction enables the imprinting to the natal stream odors that years later will guide return migration. During the return trip, olfactory sensory neurons must be sensitive to changes in odorant intensity. The ability of sensory systems to discern an increment of intensity was proposed in the 1800s by Ernst Weber to remain constant. This was later described by Gustav Fechner as Weber's law, where the ratio between a change in stimulus intensity (ΔI) and the initial stimulus (I) remains constant ($k = \Delta I / I$) (Fechner 1860).

Since the 1970s, a series of studies have shown pesticides and some other waterborne contaminants alter the functioning of the olfactory system of salmonids (e.g. Sutterlin and Sutterlin (1971), Hara et al. (1976), Moore and Waring (1996a), Baldwin et al. (2003), Tierney et al. (2007a)). None of these studies have explored the ability to resolve odorant intensity. Additionally, these studies do not resemble an environmentally realistic scenario in two other ways: chronic low-concentration exposure to pesticide mixtures has not been tested, nor has the ability of fish to adjust their physiology to compensate for exposure. The former deficit exists for practical reasons: testing single pesticides allows for unequivocal determination of toxicity, and possibly toxicity mechanism. Toxicities and mechanisms of action often differ markedly between pesticides, which makes predicting the effects of mixtures difficult or impossible. Nevertheless, mixture exposure represents the environmental reality. The latter deficit in studies of neuroprotection may be due to difficulty. Neuroprotective responses

can be inferred from other studies, for example Beyers and Farmer (2001) found olfactory-mediated alarm response in Colorado pikeminnow (*Ptychocheilus lucius)* was more inhibited at 24-hr than 96-hr into copper exposure.

A variety of neuroprotective mechanisms exist to cope with contaminant exposure. The removal and inactivation of toxicants can be facilitated by biotransformation enzymes such as glutathione-S-transferases (GSTs). GST activity has mainly been measured in liver tissue, although recent studies indicate that at two (π, μ), if not three (θ), of the four GST classes may be found in fish olfactory tissue (Starcevic and Zielinski, 1995; Perez-Lopez et al., 2000; Yanagi et al., 2004). In olfactory tissue, GST performs a dual role: these enzymes facilitate the removal of both odorants and toxicants. To date, few studies have explored the changes in the olfactory GST of fishes following exposure to toxicants.

Toxicant exposure can activate the hypothalamo-pituitary-interrenal (HPI) axis in fish, resulting in an increased production and secretion of the glucocorticoid cortisol. For this reason, cortisol has often been used as a measure of stress and as a negative correlate of health (Mommsen et al. 1999). However, like GST, cortisol also represents a physiologic response that serves both neuroprotective and olfactory roles. For example, cortisol is a potent anti-inflammatory and anti-microglia agent (Drew and Chavis 2000). The anti-inflammatory action of cortisol has been exploited for years to minimize damage in a variety of tissues (Buttgereit et al, 2005). Recently, intravenous cortisol injection was shown to improve the survival of retinal ganglion cells in rats by

almost three-fold (Heiduschka and Thanos 2006). Keeping olfactory neurons alive is obviously important for olfaction, but its elevation also can serve to enhance recall of olfactory memories. Over time, the upregulation of defense mechanisms such as GST and cortisol in response to environmental pesticide exposure may help insulate fish from further pesticide harm.

In the present study, the concentrations of 70 pesticides in the Nicomeckl River, British Columbia, were measured. This river serves as both a temporary and permanent home to several species of salmonids. One species, the famous steelhead or ocean-faring rainbow trout is of conservation concern. To simulate a realistic exposure scenario, rainbow trout were exposed to a mixture of the top-ten pesticides. To gauge olfactory function, the responses of OSNs to a 100-fold change in odorant concentration were measured. To determine neuroprotective responses, the activity of olfactory GST, the concentration of plasma cortisol and the ability of trout to withstand a second, higher mixture exposure, were measured. This study contains many research firsts- these are related to our endpoints, i.e. a more physiologically-relevant olfactory sensory neuron response and the measurement of neuroprotection through cellular and endocrine responses, and our exposure design, i.e. the sub-chronic exposure to three concentrations of an environmentally-realistic pesticide mixture.

Methods

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Sun Valley Trout Farm (Mission, BC) (N= 128, mass 11.3 ± 0.2 g, length 10.5 ± 0.1 cm,

condition factor 0.966 ± 0.007). These fish had been grown in artesian spring water known to have low-to-no contaminants. At Simon Fraser University (SFU), fish were held in indoor 170 L tanks supplied with filtered, dechlorinated municipal tap water (dissolved O_2 at >90% saturation, pH 6.8, hardness 6.12 mg/l $CaCO_3$, temperature ~8°C). For holding and experiments a 12:12 light:dark photoperiod was used. Trout were fed salmon pellets (EWOS, Surrey, BC) *ad libitum* and tested under SFU animal care permit 761B.

Chemicals

All chemicals were purchased from Sigma Aldrich (Oakville, ON, Canada) and include: atrazine (2-chloro-4-(propylamino)-6-ethylamino-S-triazine; 97.4%), chlorpyriphos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl), 99.2%), diazinon (diethyl 2-isopropyl-4-methyl-6-pyrimidyl thionophosphate, 99.0%), dimethoate (O,O-dimethyl S-((methylcarbamoyl)methyl) phosphorodithioate, 99.4%), endosulfan (alpha,beta-1,2,3,4,7,7-hexachlorobicyclo(2.2.1)-2-heptene-5,6-bisoxymethylene sulfite; 99%), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea; 99%), L-serine (S-2-amino-3-hydroxypropionic acid; 99%), malathion (dicarbethoxyethyl-O,O-dimethyldithiophosphate, 97.3%), methamidophos (O,S-dimethyl phosphoramidothioate, 98.4%), parathion (O,O-diethyl O-p-nitrophenyl phosphorothioate, 98.8%), simazine (2-chloro-4,6-bis(ethylamino)-S-triazine, 99.9%), and 2-phenoxyethanol.

Field water sampling

Surface water was taken in September 2004 from the left bank of the Nicomekl River, B.C., upstream of the 40th St. Bridge at 49.0747 by 122.7928 (latitude, longitude). Water samples were collected in VWR® TraceClean™ 1 L amber glass bottles using a Supreme® MAG-DRIVE™ submersible pump and Teflon® tubing. All samples were collected from shore at a depth of 0.1 to 1 m below the water surface. To preserve the sample in the field, 100 mLs of pesticide grade dichloromethane was added to ~900 mLs of each sample directly after collection. Water samples were stored at 4°C prior to extraction and analysis.

Chemical analysis

The methods used to analyze the pesticides can be found in Ou et. al. (2007). In brief, to extract field and lab samples, a 1 L aqueous sample containing negligible visible particulates (< 1% solids) was spiked with deuterium and ^{13}C labeled analytical standards in acetone and extracted three times with 100 mL of dichloromethane (DCM). Samples were cleaned using a microsilica chromatography column consisting of 0.75 g of 10% deactivated silica in a glass wool plugged pipette. The column was first rinsed with 10 mL of hexane, then 1 mL of extract was loaded to the column drop-wise, after which the column was eluted with 5 mL of 10% methanol in DCM. All eluates were collected, reduced in volume through evaporation and spiked with labeled recovery standards before analysis. Sample analyses were conducted using high resolution gas chromatography (HRGC) and high resolution mass spectrometry (HRMS).

HRGC/HRMS was carried out using an Autospec Ultima HRMS equipped with an HP 6890 GC, a CTC auto-sampler and an Alpha data system running on Micromass software. The chromatography column was a DB-5 capillary chromatogra-phy column of 30 m × 0.25 mm i.d. and 0.1 μm film thickness. The mass spectrometer was set to operate in electron impact (EI) ionization mode using multiple ion detection and was tuned to have a static mass resolution of ≥ 8,000.

Pesticide mixture preparation

To duplicate the pesticide mixture observed in-field for laboratory exposures, the top ten pesticides by concentration (Table 4-1) were dissolved into 1 L of acetone such that 100 mLs of this stock solution diluted into 85 L glass aquaria used for exposures would yield 10× the environmentally-observed mixture concentration. For example, to duplicate 10× the 10 ng/L observed for malathion, 85 μg of malathion was added to the stock solution, and 100 mLs of this when added to an 85 L tank generated the desired concentration (100 ng/L). Acetone was used as a solvent as it was found earlier to not affect odorant response at dilutions up to ~1% (Tierney et al. 2006). The mixture stock solution was prepared immediately before experiments and kept refrigerated. For laboratory exposures, two pesticides were used in place of the breakdown products detected in field samples. Specifically, parathion was used in place of parathion-ethyl, and endosulfan (I and II) was used in place of endosulfan-sulfate.

Laboratory exposures

Groups were exposed to the environmentally observed concentration (i.e. 1×) as well as greater (10×) and lower (0.1×) mixture concentrations. To carry out exposures, one exposure group of sixteen trout (6 for electrophysiological measurements; 10 for GST and cortisol assessment) was placed into glass aquaria containing water/acetone/pesticide mixtures that were changed (90%) every 12-hr for 96-hr. One tank was sampled per day (i.e. exposures were conducted consecutively), group order was assigned randomly, and the experiment was replicated. Controls were exposed to the same concentration of acetone as the highest mixture exposure group. Tank water temperature was kept constant using an external water bath and oxygen content was monitored twice daily; tank water remained >80% saturated. To quantify the actual exposure concentrations, tank water was sampled from the second replicate after 96-hr of exposure and extracted and analyzed as for field samples.

Electrophysiology

Electro-olfactograms (EOGs) were recorded after the procedure of Evans and Hara (1985) using the apparatus described in Jarrard et al. (2004). In brief, after exposures, trout were anesthetized using 2-phenoxyethanol (0.5 ml/L induction, 0.25 ml/L maintenance), placed in a Plexiglas holder, and the outer left naris covering was removed. The exposed olfactory rosette was continuously perfused with dechlorinated water (flow rate ~1.5 ml/min). A computer controlled solenoid valve system was used to add 2-sec pulses of L-serine to this flow. EOGs were taken as the change in potential across two Ag/Ag-Cl electrodes (in

2% agar and 1M NaCl), one placed just above the caudal area of the rosette raphe (tip diameter 580 µm) and the other on the top of the head (tip diameter ~1 mm). EOGs were amplified 1000×, digitized at 200 Hz, and acquired on a computer (LabView 7, National Instruments Inc., Austin, TX). EOGs were taken as the maximum peak size in mV. All solutions were maintained at fish acclimation temperature by a Lauda chiller (Brinkmann Instruments, Westbury, NY). To monitor anesthesia, heart rate was detected using paired ventromedially placed interperitoneal silver electrodes amplified 100× and displayed on an oscilloscope.

As soon as each trout was stabilized on the apparatus, they were run through a procedure consisting of three parts: (A) the recording of baseline responses to two odorant concentrations, (B) the response to a 100-fold change in odorant intensity (for determination of a *k* value), and (C), the effect of a 5-min exposure to a high concentration of the pesticide mixture. Not every fish was successfully stabilized on the apparatus and so N numbers vary for some groups.

(A) Baseline olfactory responses to L-serine – EOGs were first recorded in response to L-serine, an odorant commonly used in olfactory studies because of its behavioral relevance (some salmonids associate this odorant with mammalian, predator skin and avoid its presence; Brett and MacKinnon, 1954; Rehnberg and Schreck, 1987). An L-serine concentration of 10^{-5} M was selected since it has been used in several similar salmon electrophysiology studies (reviewed in Baldwin and Scholz 2005), and since this appeared to be near the

lower limit of recording a clear response for the trout tested here. After recording at least two stable responses to 10^{-5} M, two to three responses were taken using 10^{-3} M L-serine. All responses were measured with at least 3-min spacing as this is sufficient time to eliminate any olfactory sensory neuron adaptation (Tierney et al. 2007a).

(B) Olfactory response to a change in odorant intensity – 3-min after attaining the last 10^{-3} M response, the odorant-free background water supply was replaced by a 10^{-5} M L-serine solution. Two EOGs, taken 2 and 5-min after the change, were again recorded in response to 10^{-3} M. Responses were taken at 5-min since earlier test trials demonstrated that the 10^{-3} M L-serine-evoked responses at 30-min did not change, and since earlier studies indicated complete adaptation in fishes can take >1-hr (Evans and Hara 1985). The relative change in the 10^{-3} M EOG (k) was calculated as the difference between the last 10^{-3} M EOG before and the average of EOGs after the change to 10^{-5} M, divided by the last before value (i.e. change in response intensity divided by the initial response intensity; $k = \Delta I / I$).

(C) Olfactory response after a second exposure to the pesticide mixture – To assess whether prior mixture exposure improved the ability of olfactory sensory neurons to withstand a subsequent exposure (such as through induction of neuroprotective mechanisms), after the above determination of k, EOGs were again recorded to 10^{-3} M L-serine in odorant-free background water, and then the clear water was switched to a high (20×) concentration of the mixture. At 2 and 5-min into this exposure, 10^{-3} M EOGs were again recorded. 5-min was chosen

as the exposure length since any pesticide impairment noted in other studies typically appeared within this timeframe. 10^{-3} M has been used previously to resolve pesticide effects (Tierney et al. 2006) as have higher (10^{-2} M; Wang and Huang 1999) and lower (10^{-5} M (Tierney et al. 2007a) and 10^{-7} M (Tierney et al. 2007b)) concentrations. The effect of the mixture is reported as the average of the 2 and 5-min values as a proportion of the last pre-exposure value.

Biochemical assessment

Trout not used in EOG testing (i.e. ten per tank; two replicates) were anesthetized using 0.2 mg/L of MS222 (Tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC) buffered 1:1 (by mass) with $NaHCO_3$ and exsanguinated. Blood was collected into 40 µL heparinized capillary tubes (VWR, Edmonton, AL, Canada) and centrifuged at 10,000× *g* for 5 min using an IEC Microcentrifuge (Fisher Scientific, Ottawa, ON, Canada). The plasma portion of these tubes was retained and frozen at -80°C for subsequent cortisol analysis. Cortisol was analyzed using an ELISA kit (Neogen, MI, USA).

Both olfactory rosettes were collected immediately from each trout after exsanguination using a scalpel with special tiny blades (#15; Almedic, MO, Canada) and a dissecting scope. This tissue was placed into ice-cold sodium phosphate buffered solution (pH 8.0) and then frozen at -80°C. After completion of the experiments, the tissue was homogenized (Glas-Col, IN, USA) and centrifuged at 10,000× g. The supernatant was collected and used for two assays: the determination of glutathione-S-transferase (GST) activity and cytosolic protein content.

GST was determined after the method of Sharma et al. (1997). Briefly, 20 µl of supernatant for each sample was added to a 96 well plate followed by 180 µl of reaction mixture, which was comprised of 0.1 M KH_2PO_4 with 1 mM EDTA (ethylenediaminetetraacetic acid), 6.25 % of 20 mM GSH (glutathione), and 20 mM CDNB (1-chloro-2,4-dinitrobenzene). The wells were then read at 340 nm every 5-min and the absorbance was used to calculate GST activity. Enzyme activity was standardized using the sample's total protein, which was assessed using the method of Bradford (1976) (kit from Bio-Rad, QE, Canada).

Statistics

Across exposure groups, differences in EOGs for 10^{-5} and 10^{-3} M L-serine, in *k*, GST activity, and for the effect of the 20× bench-top exposure, were tested against control using a one-way analysis of variance (ANOVA) followed by a Holm-Sidak *post-hoc* test. Additionally, to model the relationship between *k* and mixture exposure concentration, the individual data were fitted to a sigmoid curve. Similarly, to model the relationship between the bench-top pesticide exposure and the tank exposures, a polynomial was fitted to the raw data. A similar procedure was used for GST. The cortisol data was not normally distributed and so to detect mixture exposure effects a non-parametric Kruskal-Wallis ANOVA was used, followed by Dunn's *post-hoc* test. 5% was used as the fiducial limit of significance for all tests. Values are presented as mean ± SEM, except cortisol which is presented as median and inter quartile range (IQR). SigmaStat 3.01 (Systat Software, San Jose, CA, USA) was used for statistical analyses; SigmaPlot 9.0 (Systat Software) was used for graphing.

Results

Pesticide concentrations

For the forty pesticides in the Nicomekl R. that were within detectable range, concentrations varied from low pg/L (e.g. trifluralin 4 pg/L) to high ng/L (e.g. dimethoate at 604 ng/L) concentrations (Table 4-1). Within the top ten alone the concentration range was greater than 100-fold. Four pesticide classes were represented in the top ten, and these were organophosphorus (747 ng/L, 86.9 % of mass), triazine (91.3 ng/L, 10.6%), organochlorine (15.0 ng/L, 1.7%), and phenylurea (6.3 ng/L, 0.7%).

In the lab exposures, most of the concentrations of the measured pesticide concentrations were similar to those of the Nicomekl R. top ten, especially the triazine and phenylurea pesticides (Table 4-2). The organochlorine endosulfan concentration was comparatively low, but it played a minor role (by mass) in the mixture. Organophosphorus pesticides were typically higher than intended (e.g. 140% above normal for the 0.1×), but were very close to accurate for the realistic (1×) exposure group (Table 4-2). Overall, by class, the values were similar to those observed in field.

Olfactory responses to mixture exposure

(A) Baseline olfactory responses to L-serine – EOGs did not vary significantly across the exposure groups. The EOGs for 0, 0.1, 1 and 10× exposure groups were 1.20 ± 0.23, 0.76 ± 0.21, 1.34 ± 0.28 and 1.45 ± 0.23 mV for 10^{-5} M L-serine, and for 10^{-3} M, they were typically more than 2-fold greater at 2.77 ± 0.46, 2.49 ± 0.42, 2.28 ± 0.41 and 3.69 ± 0.34 mV.

(B) Olfactory response to a change in odorant intensity – Exposure to the mixture affected the magnitude of the olfactory sensory neuron responses when a background odorant was present ($F = 4.750$, $p = 0.007$). In particular, the *k* values were significantly greater than control for both the 1 and 10× groups (values for 0, 0.1, 1 and 10× were 0.336 ± 0.03, 0.383 ± 0.049, 0.478 ± 0.051 ($p = 0.017$), and 0.515 ± 0.034 ($p = 0.002$)) (Fig. 4-1A). This indicates that the 10^{-3} M EOGs for 1 and 10× groups were of smaller relative size when a (10^{-5} M) background odorant was present. The raw *k* values were described by a sigmoid curve ($k = 0.327 + 0.1938 / (1 + e^{-((x - 1.4153) / 0.4616)})$, where x for 0 = 0, 0.1 = 1, 1 = 2, and 10 = 3; $F = 4.7503$, $p = 0.0068$) (Fig. 4-1A).

(C) Olfactory response after exposure to a pesticide mixture pulse – The short, 5-min exposure to the 20× mixture caused similar decreases in 10^{-3} M L-serine EOGs for 0, 0.1 and 1× mixture-exposed trout (EOGs were 0.722 ± 0.035, 0.656 ± 0.090 and 0.752 ± 0.074, respectively, of their pre-exposure values) (Fig. 4-1B). However, for the 10× exposed group, the decrease (0.974 ± 0.044) was significantly ($p = 0.003$) smaller than control. A polynomial was used to describe the data ($\Delta EOG = 0.7383 - 0.1106(x) + 0.0616(x)^2$, where x for 0 = 0, 0.1 = 1, 1 = 2, and 10 = 3; $F = 6.7008$, $p = 0.0038$) (Fig. 4-1B).

Biochemical responses to mixture exposure

The GST activity in the olfactory rosettes appeared to follow an inverted U-shaped concentration-response relationship, with all mixture-exposed groups differing significantly from control ($F = 5.084$, $p = 0.003$) (Fig. 4-2A). GST activity peaked with 0.1× exposed trout, with greater exposures not eliciting greater

activity (activities: 154 ± 7, 203 ± 13, 197 ± 9 and 183 ± 10 nmol/min/mg protein for 0, 0.1, 1 and 10× respectively). As with the 20× pulse, a polynomial was used to describe the data (GST activity = 155.9 + 56.43(x) – 16.05$(x)^2$, where x for 0 = 0, 0.1 = 1, 1 = 2, and 10 = 3; F = 6.7753, p = 0.0019) (Fig. 4-2A). Plasma cortisol followed a similar relationship as it was significantly increased from control at 1× but not 10× the mixture (H = 8.890, p = 0.031) (Fig. 4-2B).

Discussion

After a 96-hr exposure to a pesticide mixture designed to resemble that observed in a BC river (i.e. the 1× treatment), a representative salmonid, the rainbow trout, experienced changes in neuroprotective and olfactory sensory neuron (OSN) responses. These changes included an increase in the activity of glutathione-S-transferase (an enzyme that facilitates xenobiotic detoxification) and a decrease in the ability of OSNs to respond to the behaviorally-relevant odorant L-serine. An exposure to one-tenth (0.1×) of the mixture resulted in a similar increase in GST activity; however OSN responses remained the same as control trout. Exposure to a ten-fold (10×) greater concentration again resulted in a similar increase in GST activity, yet OSN responses were impaired to a similar extent as the trout given the realistic (1×) mixture exposure. These data suggest upregulation in neuroprotective responses (in this case GST) may serve to limit OSN harm up to an exposure concentration equivalent to one-tenth that found in the Nicomekl River, but not to greater (i.e. close to environmental) concentrations. This was also demonstrated by the subsequent 5-min exposure

to a high (20×) concentration: trout exposed previously to 0.1 and 1× experienced the same impairment as control fish.

Pesticide concentrations

The overwhelming majority of pesticides found in the Nicomekl River were organophosphorus. For example, in the top ten pesticides, OPs made up 86.9% by mass. The next most prevalent class were the triazines, which accounted for 10.6%. In fact, insecticides and herbicides were found in the top ten in a ratio of ~8:1. Such prevalence of insecticides and herbicides is common to rivers and streams bounded predominantly by agricultural activities, although the ratio between the two can vary markedly. Harris et al. (2007) reported nearly the opposite relationship for Nathan Creek, BC, which like the Nicomekl R., is similarly home to salmon and bordered by agricultural production. For Nathan Cr., the ratio of insecticides to herbicides was ~1:10. Unlike the Nicomekl, the majority of the herbicides in Nathan were chlorophenoxy acids or esters (e.g. mecoprop and 2,4-D), and not triazines. These two waterways can also differ considerably in overall pesticide concentration (Nathan Cr. vs. the Nicomekl R. was 162 vs. 879 ng/L, respectively). The ratio variation between the two rivers likely represents differences in the adjacent crop profiles. In the region that encompasses both of these rivers, pesticide treated crops include alfalfa, corn, blueberries, potatoes, raspberries, cranberries and green beans (ENKON Environmental, 2005). Of these, insecticides are often used to a comparatively greater extent on cranberries and potatoes than blueberries.

The mixture concentration used in lab resembled the proportions of the top ten from Nicomekl R., especially for the 1× exposure group (i.e. the total concentration was 118.6% of nominal, vs. 218% for 0.1× and 162% for 10×) (Table 4-2). In toxicological studies, this amount of variation is typical. For example, in Moore and Waring's (1996a) study on diazinon, actual values varied from 13 to 220 % of nominal. A similar study (Moore and Waring 1998) likewise vetted their nominal atrazine exposure concentrations and found 8 to 72% of nominal values, with the lowest concentration (0.04 µg/L) also achieving the lowest accuracy. In the present study, the techniques used to achieve the low, ng/L, concentrations were effective with the triazines (achieving >99% accuracy), but less so with the OPs (e.g. 240% of nominal in the 0.1× group). In fact, the OPs were also high in the 10× group (i.e. 165% of nominal). The difference with OPs could have arisen from an error in preparation of their portion of the mixture stock solution. The observed and not nominal concentrations have been used in subsequent analysis.

Electrophysiology

The methods used for collecting EOG responses were efficacious, as the EOG responses to 10^{-5} M L-serine compare favorably with other studies. For example, the EOGs of control trout (1.20 ± 0.23 mV), are about the same as those of juvenile coho (*O. kisutch*) (1.86; Baldwin et al., 2003) and Atlantic salmon (*Salmo salar*) (0.90; Moore and Waring, 1996b). The 10^{-3} M responses of control trout (2.77 ± 0.46 mV) are slightly greater than double those of 10^{-5} M, which is as is typically observed (Tierney et al., 2007a). The lack of difference in

baseline EOG responses across fish exposed to control and pesticide mixtures was anticipated, since variation across fish is typically larger than pesticide-mediated impacts. For example, in the present study, EOG responses to 10^{-5} M L-serine varied from 0.188 to 3.01 mV (i.e. 16-fold). This variation in measurement greatly exceeded the average effects of pesticide exposure (27.8% decline; discussed below). This is why most studies have used bench-top pesticide exposures, where a measurement is taken for each fish, after which a short pesticide exposure is given. This permits any decline to be considered against the pre-exposure value (i.e. inter-fish EOG variation is unimportant). However, this technique does not permit use of more realistic, longer-term exposures, which is why the present study used a novel method to resolve olfactory impairment.

In the environment, odorants are rarely absent – yet they are not typically noticeable or evoke a behavioral response unless their concentration changes. This is because over time sensory systems adapt to the background, which permits resolution of signal amidst noise. In this study, trout were given a change in the concentration of a background odorant, and their ability to adapt to this change was measured and used as a correlate of nerve condition. For every trout, the EOG responses to 10^{-3} M L-serine were decreased in the presence of 10^{-5} M. This was expected since with the addition of a background odorant, the odorant intensity change was now incremental and not absolute. However, the relative responses within the background were not the same across exposure groups, indicating the ability to adapt to the background was affected by the

pesticide mixture. In fact, even at the concentration of pesticides similar to that observed in field, the adaptation ability was diminished (i.e. the 10^{-3} M peak size was relatively smaller) (Fig. 4-1A). Here the odorant used was L-serine, one that is known to be associated with predator avoidance. For this reason, the inability to adequately detect changes in L-serine concentration could equate to diminished survival. Furthermore, since a variety of salmonid behaviors depend upon properly sensing odorants (additional discussion below), the ramifications of these findings to salmonid survival are grave.

Acute exposure to the pesticide mixture

Exposure to a secondary (20×) pulse of the pesticide mixture resulted in similar reductions for 0, 0.1 and 1× exposed fish (Fig. 4-1B). For the 0.1 and 1× exposed fish, this suggests prior exposure did not prophylactically prevent further exposure damage. For the 10× exposed fish, the 20× exposure caused little if any greater EOG impairment. Two possible explanations for this exist, and they represent opposite theories with respect to neuroprotection. The observed small negative effect would be noted if either the 10× tank exposure had prepared the tissue such that further pulses were not injurious, or if the tissue was so damaged a second exposure could cause no greater EOG reduction. Given the 10× exposed trout were unable to cope with background odors as well as controls (i.e. had a significantly greater *k*) (Fig. 4-1A), it seems that the latter explanation is more likely. This is further substantiated given fish of the 1× exposure group still experienced EOG impairment (i.e. functionality, and hence capacity for responsiveness decrease, remained). Overall it appears low-

concentration exposure may not prevent damage from subsequently higher exposures.

No previous studies have tested the effects of four day exposure to a pesticide mixture on the EOG responses of fishes; however there is data for short-term (30-min) exposures to each of the four classes tested. Specifically, the OP chlorpyrifos was tested by Sandahl et al. (2004), the triazine atrazine was tested by Moore and Lower (2001) and Tierney et al. (2007b) (who tested different salmonids but found the similar relative EOG declines), endosulfan (the only organochlorine) was tested by Tierney et al. (2006), and linuron (the only phenylurea) was tested by Tierney et al. (2007a). These data permit a prediction of the effects of the 20× mixture on the L-serine EOG response, providing the relative toxicities of each pesticide are adjusted. In the present study, data from lethality studies were used to prorate the relative EOG toxicities of the pesticides to that of the known value. For example, the OP chlorpyrifos caused an EOG decline of 40% with 1 µg/L of exposure (Sandahl et al. 2004). The OP methamidophos is only 0.311% as toxic as chlorpyrifos (based on averaged lethality data from www.pesticideinfo.org; Table 4-2), therefore the adjusted EOG decline for 1 µg/L methamidophos exposure is a multiple of the two: 0.311% × 40% = 0.125 % with 1 µg/L. To account for the tank exposures, the modeled amount of mixture decline is the difference between tank and bench-top exposure.

Adjusting relative EOG toxicities using lethality data has the obvious limitation that a lethality response is organismal while the EOG response is tissue

level. Nevertheless, the assumption holds true if the mechanism causing lethality is common to that which impairs the olfactory response. If both lethality and olfactory impairments are mediated through neurological alterations, this assumption may be valid. As an example, for OPs, the majority of mixture constituents, the impairment of acetylcholinesterase (AChE) may alter ACh breakdown systemically, causing rigor, and peripherally, by increasing mucous flow (ACh acts to release mucous in the olfactory epithelium). Such altered mucous flow could either diminish or plug reception of olfactory cues (Jarrard et al. 2004).

For the model, should an unweighted summation of the contributions of each pesticide to overall EOG impairment reflect the observed impairment, the effects of the four classes would appear additive. The only case in which this reasonably occurred was for the 10× concentration group (Fig. 4-1B). And as mentioned above, fish given the 10× exposure appeared to suffer no greater negative effects following the 20× pulse likely because significant damage was already present (i.e. the model would not apply). For the other groups, the relatively greater magnitude of the impact with respect to the model suggests that synergistic interactions may have been present. Consequently, vetting a predictive EOG model for mixtures would require further experiments testing the interactive effects of the four classes. The mixture as present in field likely represents a threat that cannot be estimated on studies of single pesticide exposures.

Neuroprotection

Olfactory neurons are the only neurons regularly replaced (Brunjes and Shurling 2003), which reflects their role as exposed receptors for unknown and sometimes injurious substances. Nevertheless, neuroprotection in these cells serves to limit their harm or death. Glutathione-S-transferases are enzymes within cells such as OSNs that serve this function through conjugating solubilizing molecules such as glutathione (GSH) to toxic substances such as lipophilic pesticides. GST activity in rainbow trout olfactory epithelium has been reported at least twice, and there is variation between the two. Starcevic and Zielinski (1995) reported GST activity in rainbow trout olfactory epithelium of 478 ± 218 nmol/min per mg protein, while Pérez-López et al. (2000) noted a value of 250 ± 50 nmol/min per mg protein. Both values are greater than the control activity of this study at 153.7 ± 7.4 nmol/min per mg protein. The values of the present study are closer to those of mammalian values, for example the mouse vomeronasal organ had a value of 181.7 ± 13.4 (Green et al. 2005) and human nasal mucosa had a value of 77 ± 21 (Aceto et al. 1989). The low values here may reflect the rearing and holding conditions of the trout (artesian well water and filter dechlorinated water), which were relatively free of any contaminants or odorant buildup.

There are few studies on the effects of contaminants on olfactory GST induction in fishes. In mammals, studies have explored the effect of injury or disease, for example mice treated intraperitonealy with a compound that resembles viral RNA (polyinosinic-polycytidylic acid), experienced a 22% increase in nasal GST activity (Weech et al. 2003). There are some contaminant

studies as well, but most report data on CYP (phase I) enzymes and not GST (phase II) enzymes (e.g. Piras et al., 2003). With fish, even fewer studies exist on the effects of a stressor on olfactory GST activity. Starcevic and Zielinski (1997) found that severing the olfactory nerve of rainbow trout, which causes nerve death and regeneration over a period of three months, resulted in a rapid decrease in GST activity that returned after two months. The amount of a GST substrate, GSH, did not mirror these changes. GSH concentration remained high initially, but declined after two weeks and remained lower for the balance of the experiment.

Exposure to all concentrations of the mixture resulted in increased GST activity in olfactory tissue (Fig. 4-2A). For example, the GST activity of tissue in the 0.1× exposure group (i.e. 203.5 ± 12.6) represented a 32% increase from control. Exposure to greater concentrations of the mixture resulted in smaller increases (28 and 19% for 1 and 10×, respectively). These data alone do not necessarily demonstrate 1 and 10× exposures were associated with lowered neuroprotection, since it is the interaction between GST and its substrates (e.g. GSH) that ultimately determine detoxification ability (i.e. the enzyme is useless unless it has substrate to transfer to toxicants). The altered *k* values of 1 and 10× may suggest that the enzymes substrate was becoming depleted, since GST activity was similar for all exposed fish. Without neuroprotection, the olfactory tissue may have become damaged by the pesticide mixture. Future studies may wish to explore the interactions between contaminant exposure and GST activity and substrate concentration.

The stress hormone cortisol serves numerous functions, one of which appears to be neuroprotective. Specifically, cortisol was recently found to enhance survivorship of neurons in rats (Heiduschka and Thanos 2006). This was likely through reduced cellular destruction from inflammation and anti-microglia, which function as neurological, phagocytic cells (Drew, and Chavis, 2000). In the present study, exposure to the pesticide mixture numerically increased cortisol in all cases, and significantly in the 1× exposed trout (Fig. 4-2B). In relation to the olfactory responses to L-serine, the increase did not appear to confer a performance advantage since the ability to withstand a second pesticide pulse was no different from control. Furthermore, the *k* value was altered from 0 and 0.1×, suggesting neurological impairment was present. Exposure to a greater (10×) amount of the mixture did not cause further cortisol increase, but rather a reduced response similar to controls. Conceivably the high mixture concentration damaged the interrenal tissue responsible for producing and secreting cortisol. In future this could be tested by exposing isolated interrenal tissue to the hormone responsible for motivating cortisol production/secretion, as suggested by Hontela (1998). Overall, in this study cortisol did not appear to a good measure of neuroprotection.

Conclusion

Olfactory perception must operate within a varied and changeable environment. At a pesticide mixture concentration one-tenth that observed in field, the data suggest fish may respond with neuroprotective measures that enable retention of olfactory neuron functionality. At a concentration similar to

that observed in field, olfactory abilities were impaired, and the neuroprotective responses may not have been adequate to prevent olfactory damage. At a mixture concentration ten-times that observed in field, trout olfactory responses were likewise impaired, as was their cortisol response. In this paper we have focused on British Columbia; however, given the ubiquity of the fish and pesticides tested herein, this study could serve as a model for other regions throughout the world.

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Tables

Table 4-1 Pesticides, pesticide breakdown products and other contaminants, in order of decreasing concentration, detected in the surface water of the Nicomekl River, BC, in September 2004.

Pesticide	ng/L
Dimethoate	604
Simazine	84.5
Methamidophos	61.4
Diazinon	48.7
Chlorpyriphos	18.3
Endosulphan-Sulphate	15.0
Malathion	10.0
Atrazine	6.80
Linuron	6.27
Parathion-Ethyl	4.83
beta-Endosulphan	4.66
Cypermethrins	3.09
alpha-Endosulphan	2.18
Permethrins	1.96
Dichlorvos	1.81
Diazinon-Oxon	1.63
Fonofos	1.29
Metolachlor	0.536
Pendimethalin	0.375
HCH, gamma	0.282

Pesticide (cont.)	ng/L
HCH, beta	0.259
Metribuzin	0.237
Chlordane, gamma (trans)	0.202
Dieldrin	0.199
HCH, alpha	0.128
Chlordane, alpha (cis)	0.121
Heptachlor-Epoxide	0.104
Desethylatrazine	0.0899
Nonachlor, trans-	0.0723
Endrin	0.0585
HCH, delta	0.0539
Dacthal	0.0496
Aldrin	0.0492
Quintozene	0.0417
Endrin-Ketone	0.0161
Heptachlor	0.0120
Chlorothalonil	0.0088
Octachlorostyrene	0.006
Chlorpyriphos-Methyl	0.004
Trifluralin	0.004

Table 4-2 The experimental pesticides and their concentrations compared to those observed in the Nicomekl R. (see *Methods* for analysis information).

	Nicomekl R.			Experimental		
Pesticide (ng/L)	**0.1**	**1**	**10**	**0**	**0.1**	**1**	**10**
Dimethoate	60.4	604	6040	3.2	137	486	6620
Simazine	8.45	84.5	845	-	8.4	73.1	669
Methamidophos	6.14	61.4	614	-	-	-	67.2
Diazinon	4.87	48.7	487	-	15.7	157	1820
Chlorpyriphos	1.83	18.3	183	0.2	1.7	13.4	114
Endosulphan	1.50	15.0	150	-	1.1	4.5	30.1
Malathion	1	10	100	-	-	76.3	926
Atrazine	0.680	6.80	68.0	-	0.7	6.5	59.0
Linuron	0.627	6.27	62.7	-	-	7.2	70.8
Parathion-Ethyl	0.483	4.83	48.3	-	23.1	196	3540
concentration agreement							
total	85.977	859.77	8597.74		186	1010	13900
total agreement (% of desired)					**218%**	**119%**	**162%**
agreement by class (ng/L)							
OP					73.7	737	7370
triazine					9.1	91.3	913
OC					1.5	15.0	149.9
phenylurea					0.6	6.3	62.7
agreement by class (%)							
OP					**240%**	**116%**	**165%**
triazine					**100%**	**87%**	**80%**
OC					**0%**	**30%**	**20%**
phenylurea					**0%**	**115%**	**113%**

Table 4-3 The agreement between a model of the effects of the pesticide mixture on olfactory sensory nerve response (as measured using electro-olfactogram; EOG) and the observed responses (for a graphical presentation see Fig. 4-1B). Based on pesticide class, lethality data was used to prorate the relative EOG toxicities of the pesticides to values from other pesticide-EOG studies. The predicted EOG decline is the difference between the expected decline from the acute and chronic exposures.

Pesticide	ng/L	% by mass	Class
Dimethoate	767	89.7%	organophosphorus
Simazine	65.4	7.6%	triazine
Methamidophos	9.9	1.2%	organophosphorus
Diazinon	189	22.1%	organophosphorus
Chlorpyriphos	25.3	3.0%	organophosphorus
Endosulphan-Sulphate	6.1	0.7%	organochlorine
Malathion	97.2	11.4%	organophosphorus
Atrazine	5.3	0.6%	triazine
Linuron	7.3	0.9%	phenylurea
Parathion-Ethyl	417.2	48.8%	organophosphorus

Cont.		Reference	Adjusted	Predicted EOG impairment (%)			
LC_{50} (ug/L)	# sources used	EOG drop (% decline / ug/L)	EOG drop	0	0.1	1	10
9,100	7	Est.[a]	0.353	0.54	0.49	-1.79	0.37
72,700	22	Est.[b]	-	0.00	0.00	-0.0069	0.00
25,800	3	Est.[a]	0.125	0.00	0.00	-0.0059	0.00
964	18	Est.[a]	3.33	1.26	1.20	-4.81	0.73
80.2	25	40[a]	40	2.02	1.96	-2.52	1.49
-	-	0.25[c]	0.25	0.00	0.00	-0.0045	0.00
119	47	Est.[a]	27.0	5.25	5.25	-19.75	3.19
14,400	18	Est.[b]	-	0.00	0.00	-0.0348	0.00
-	-	3.5[d]	3.5	0.05	0.05	-0.197	0.03
1,20	21	Est.[a]	2.68	2.23	2.17	-7.26	1.71
			Predicted	11.4	11.1	-36.4	7.5
			Actual	27.8	34.4	24.8	2.6
			Difference	16.44	23.27	61.18	-4.92

LC_{50} data is the average of several sources (#'s given) as reported in an online database (www.pesticideinfo.org). References for EOG declines are: [a] Sandahl et al. 2004, [b] Tierney et al. 2007b, [c] Tierney et al. 2006, [d] Tierney et al. 2007a. All extrapolated EOG declines used a linear model, expect for the triazines, where data from Tierney et al. (2007a) permitted the use of the sigmoid curve equation: EOG decline = 104 / (1 + e^-((µg/L – 1.70) / 0.372)).

Figures

Figure 4-1 Olfactory sensory neuron (OSN) responses in juvenile rainbow trout following a 96-hr exposure to various concentrations of an environmentally-realistic pesticide mixture. Shown are the responses of OSNs to a change in background odorant strength (A), where a smaller response equates to a diminished signal amidst the noise (see inset examples of before and after OSN response traces). Also shown are the effects of a subsequent 5-min exposure to a high (20×) concentration on OSN responses (B). Responses below unity (gray line) indicate OSN impairment. A model (dashed line) is also shown. For a description of the pesticide mixture, the derivation of *k*, statistics, and measurement of OSN responses, see *Methods*; for model derivation see Table 4-3; for curve information see *Results*. (N) and p-values are given on figure. Data are given in both raw and mean±SEM; significance was tested against control.

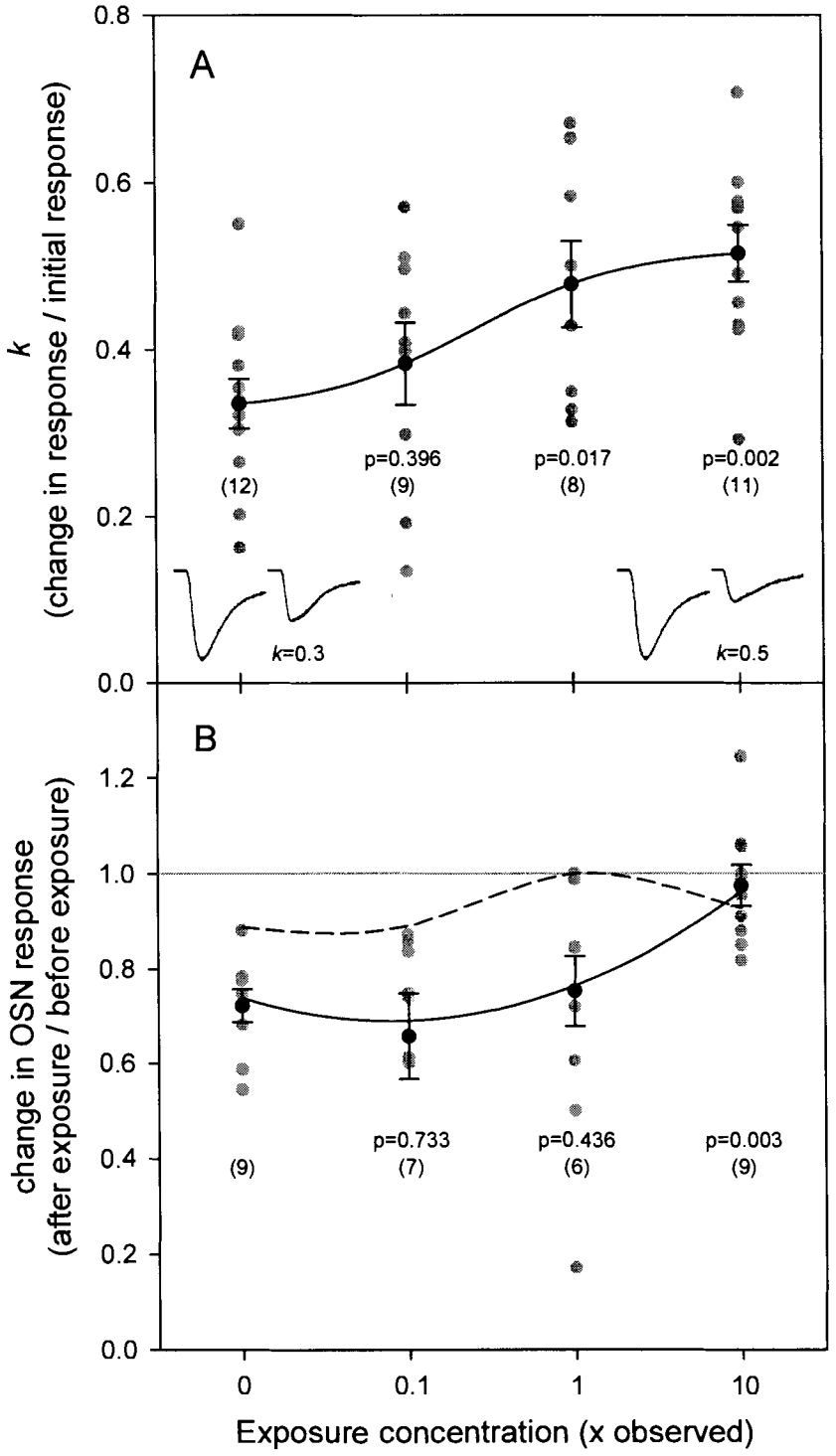
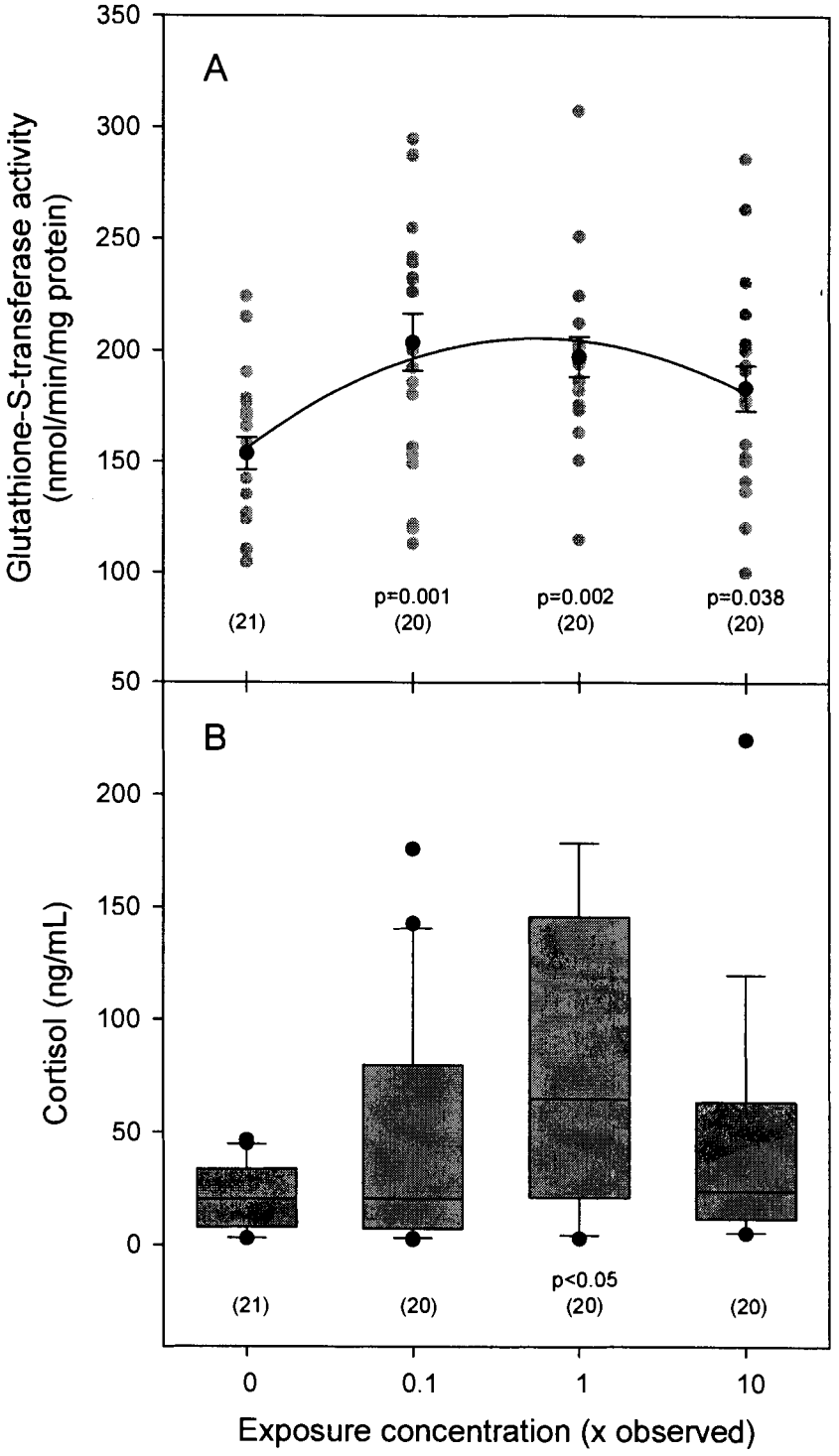

A
B
k
(change in response / initial response)
change in OSN response
(after exposure / before exposure)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
p=0.396
p=0.017
p=0.002
(12)
(9)
(8)
(11)
k=0.3
k=0.5
p=0.733
p=0.436
p=0.003
(9)
(7)
(6)
(9)
0
0.1
1
10
Exposure concentration (x observed)

Figure 4-2 The activity of the detoxification enzyme glutathione-S-transferase (GST) in the olfactory rosette tissue (A), and the concentration of plasma cortisol (B), in juvenile rainbow trout after 96-hr exposure to various concentrations of an environmentally realistic pesticide mixture. Elevated GST can indicate enhanced detoxification ability; elevated cortisol can indicate a stress response known to correlate with neuroprotection. For statistics see *Methods*; for curve information see *Results*. (N) and p-values are given on figure. Data are given in both raw and mean±SEM; significance was tested against control.


A
B
Glutathione-S-transferase activity (nmol/min/mg protein)
Cortisol (ng/mL)
350
300
250
200
150
100
50
200
150
100
50
0
p=0.001
p=0.002
p=0.038
(21)
(20)
(20)
(20)
p<0.05
(21)
(20)
(20)
(20)
0
0.1
1
10
Exposure concentration (x observed)

Chapter 5:
Relating olfactory neurotoxicity to altered olfactory-mediated behaviors in rainbow trout exposed to three currently-used pesticides

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Abstract

Odor-evoked neurophysiological responses can form the basis for behavioral responses. Here we first characterized olfactory-mediated behavioral and neurophysiological responses of juvenile rainbow trout to the amino acid L-histidine, then looked at whether there were similar responses to the carbamate antisapstain IPBC and the herbicides atrazine and Roundup®, and lastly explored how exposures to these pesticides modified the L-histidine responses. Trout were behaviorally attracted to 10^{-7} M L-histidine (as assayed in a counter-current olfactometer), but this preference behavior switched to indifference with higher histidine concentrations. Neurophysiologically, the summed electrical responses of peripheral olfactory neurons, as measured using electro-olfactogram (EOG), was 0.843 ± 0.252 mV to 10^{-7} M L-histidine. Of the pesticides, only Roundup® evoked EOGs, indicating the amino acid-based pesticide may have acted as an odorant, and generated a behavioral response: it was avoided at active ingredient [AI; glyphosate isopropyl amine] concentrations ≥ 10 mg/l. With 30-min pesticide exposures, 10^{-7} M L-histidine preference behavior was eliminated following exposure to 1 µg/l IPBC and atrazine, and 100 µg/l AI Roundup®. Similarly, 10^{-7} M L-histidine-evoked EOGs were significantly reduced by exposure to 1 µg/l IPBC, 10 µg/l atrazine, and 100 µg/l AI Roundup®. When combined together, the results demonstrate that typical preference behavior can be abolished when neurophysiological responses are reduced by >60 % of control. This asymmetry in response thresholds suggests that behavioral

responses may be more sensitive toxicological endpoints than neurophysiological responses.

Keywords

Rainbow trout; behavior; electro-olfactogram; amino acid; atrazine; Roundup

Introduction

The loss of olfactory sensory neuron (OSN) response has been used as an ecologically relevant sublethal toxicological endpoint for salmonids (Winberg et al., 1992; Baldwin et al., 2003; Jarrard et al., 2004; Sandahl et al., 2004; Baldwin and Scholz, 2005). The rationale being that since olfaction enables many indispensable behaviors throughout the salmonid lifecycle, including kin and conspecific recognition (Griffiths & Armstrong, 2000; Olsén et al., 2002), alarm response (Mirza & Chivers, 2003), feeding (Hara, 2005, 2006), imprinting and homing (Hasler & Scholz, 1983; Dittman & Quinn, 1996), gamete release synchronization (Moore & Waring, 1996a), and contaminant avoidance (Folmar, 1976; Hansen et al., 1999a,b), its impairment represents a survival threat. Given the ecological, cultural and commercial significance of salmon, and the endangered status of some stocks (COSEWIC, 2002; NMFS, 1996), understanding how contaminants alter this critical sense is of importance. Unfortunately, few studies have related changes in olfactory neurophysiological response to altered olfactory-mediated behaviors.

Several studies on a variety of teleost species have demonstrated the significance of olfaction to behaviors by outright removal of OSN input. For example, severing the OSN-brain connection eliminated the contaminant avoidance of medaka (*Oryzias latipes*) (Hidaka and Tatsukawa, 1989) and reduced the alarm response in matrinxã (*Brycon cephalus*) (Ide et al., 2003). Copper exposure caused loss of OSN tissue (verified through scanning electron microscopy) and eliminated the alarm reaction of Colorado pikeminnow

(*Ptychocheilus lucius*) (Beyers and Farmer, 2001). In tilapia (*Oreochromis mossambicus*), cauterization of male OSNs eliminated the behavioral response to pre-ovulatory females (Miranda et al., 2005). Olfactory occlusion has been shown to reduce the migratory fidelity of both chinook salmon (*Oncorhynchus tshawytscha*) (Groves et al., 1968) and black rockfish (*Sebastes inermis*) (Mitamura et al., 2005). In contrast to the above, common surface-water contaminants are known to diminish but not eliminate OSN responses, and these too can have behavioral consequences. For example, the organophosphorus insecticide diazinon has been shown to reduce Atlantic salmon (*Salmo salar*) OSN responses (Moore and Waring, 1996b) and impair two olfactory-based behaviors (anti-predator response and migratory fidelity) in chinook salmon (Scholz et al., 2000).

In the present study we took a top-down (i.e. higher to lower order) approach to explore the interaction between olfactory-based behavioral and neurophysiological responses in pesticide-exposed rainbow trout (*O. mykiss*), a ubiquitous salmonid. We assayed behavioral followed by neurophysiological responses to an amino acid, a natural odorant routinely used in fish olfaction work (see the review by Baldwin and Scholz, 2005). We then tested whether trout avoided three current-use pesticides, and whether the pesticides evoked a neurophysiological response. Finally, we used the three pesticides to help characterize the relationship between neurophysiological and behavioral impairment. Two of the pesticides that we evaluated are among the most widely used herbicides in the world: atrazine and Roundup®. Atrazine is a selective

triazine used to control broadleaf and grassy weeds. Although it is considered a next generation 'soft' pesticide (i.e. few non-target species effects and short half-life), it is a known contaminant of aquatic ecosystems (Frank et al., 1990; Pennington et al., 2001). Roundup® is a broad-spectrum, nonselective, post-emergent herbicide also used in the control of broadleaf weeds. The active ingredient of Roundup®, glyphosate isopropylamine salt, is not considered to persist or contaminate aquatic ecosystems (Giesy et al., 2000). However, we tested Roundup® because of its broad use in areas adjacent to salmon habitat in British Columbia (Verrin et al., 2004). Furthermore, the active ingredient of Roundup® is sometimes used directly in aquatic environments to control invasive weeds (Kilbride and Paveglio, 2001). The third pesticide evaluated (IPBC) is a carbamate fungicide used to control mildew (sapstain) on dimensional (i.e. milled) lumber. It is considered moderately persistent (Juergensen et al., 2000) and is highly toxic to aquatic life (Farrell et al., 1998). All three pesticides have been identified as affecting olfactory neurons in salmonids (atrazine: Moore and Lower, 2001; glyphosate: Tierney et al., 2006; IPBC: Jarrard et al., 2004), yet the manner in which the neurotoxic effects relate to behavioral change remains unknown.

Methods

Animals

Rainbow trout (mass 32.7 ± 1.2 g, fork length 14.7 ± 0.18 cm, condition factor 1.01 ± 0.01; n = 216) were purchased from Sun Valley Trout Farm (Mission, BC), and maintained in indoor 170 l tanks supplied with filtered, dechlorinated

municipal water (background water; dissolved O_2 at >90% saturation, pH 6.8, hardness 6.12 mg/l $CaCO_3$). Fish were kept under a 12 h: 12 h, light: dark photoperiod and fed commercial salmon pellets (EWOS, Surrey, BC) *ad libitum*. Room and water temperature were synchronized at ~12°C. All protocols were approved by Simon Fraser University's animal care committee.

Chemicals

All chemicals were purchased from Sigma Aldrich (Oakville, ON), except for Roundup®, which was purchased from a local retail outlet. The chemicals and their given purities where available are atrazine (2-chloro-4-(propylamino)-6-ethylamino-s-triazine; 97.4%), IPBC (3-iodo-2-propynyl N-butylcarbamate; 97%), L-histidine ($C_6H_9N_3O_2$; ≥98.5%), Roundup® concentrate (143 g/l active ingredient [AI] N-(phosphonomethyl) glycine isopropylamine salt; Monsanto Company, Canada, Lot # 130313/5), and 2-phenoxyethanol.

Behavioral and electrophysiology apparatus

Preference/avoidance trough

This apparatus is similar to that used by Saglio et al. (2001). In brief, the trough measured 30 (width) × 29.5 cm (height) × 154 (length) (136 l volume), and was constructed of 1.27 cm thick clear Plexiglas. To provide a discontinuous symmetrical polar gradient, 10 l header tanks were situated at either end, providing a flow of 22.3 l/min, which exited through a perforated center stand pipe. Trough sides were covered by 6 ml black poly. To monitor fish position, five CNL-100 CCD surveillance cameras were spaced above and evenly along

the length of the trough. To prevent outside interference, the trough and cameras were enclosed in 6 ml black polyethylene plastic. The cameras were connected to PV-140V surveillance cards in a 2 gigahertz Pentium PC running Surveillance Solutions software (Matco, St. Laurent, QC).

Neurophysiology measurements

Electro-olfactograms (EOGs), which measure changes in nasal epithelial voltage that are due to a summed generator potential response of OSNs, were recorded after Evans and Hara (1985) using the apparatus described in Jarrard et al. (2004). In brief, fish were anesthetized using 2-phenoxyethanol (0.5 ml/l induction, 0.25 ml/l maintenance) placed in a holder, and the left olfactory rosette was surgically exposed. After surgery, the rosette was kept continuously irrigated with background water or a pesticide/vehicle mixture at a flow rate of ~1.5 ml/min. EOG was measured as the relative change in voltage between two Ag/Ag-Cl electrodes (silver wire suspended in 2% agar and 1M NaCl inside a borosilicate capillary tube), one situated in the rosette and one on top of the head. The rosette electrode position was established by advancing the electrode until it depressed the mediocaudal area of the rosette raphe and then backed off until no depression was observed. In an effort to limit interpreparation variation, EOG electrode tips were not pulled and so all had a tip diameter 580 µm. EOGs were elicited using 2-sec odorant pulses delivered in the nasal water flow using a computer-controlled solenoid valve system. EOGs were DC amplified 1000×, digitized at 200 Hz, and acquired on a P4 computer running LabView 7 (National Instruments Inc., Austin, TX). Fish condition was monitored via heart rate,

measured by paired subcutaneous stainless steel electrodes amplified 100× and displayed on an oscilloscope.

Experiment 1: Behavioral and neurophysiological responses to L-histidine

To determine an olfactory-mediated behavior, the avoidance/preference responses of trout were tested to an amino acid over a 10,000× concentration range. The EOG responses were then tested over the same range. Robust EOG recordings could be taken at all L-histidine concentrations.

Behavioral L-histidine concentration-response curve

Preliminary trough experiments were conducted using L-serine since it was previously shown to elicit avoidance response in Pacific salmonids (Idler et al., 1956). However, the juvenile rainbow trout used in the present study were unresponsive to both low (i.e. 10^{-7} M; $p = 0.680$) and high (i.e. 10^{-3} M; $p = 0.592$) concentrations of this amino acid, therefore we conducted a second round of preliminary experiments with L-histidine since Shparkovskiy et al. (1983) found that 67% of Atlantic salmon (*Salmo salar*) avoided 10^{-4} M histidine (isomer not given). Some trout appeared to avoid a 10^{-3} M L-histidine pulse, and so preference/avoidance behavior was recorded for 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M L-histidine ($n = 6$ per concentration). To determine L-histidine preference/avoidance, fish were placed in the trough for 20-min and their position along the length of the trough was recorded over the next 10-min manually from the video feed. After 30-min in the trough, L-histidine was added to one of the 10 l header tanks and trout position was again recorded for 10-min. To establish the

desired concentration of L-histidine in the outflow plume, solutions in the header tanks were mixed rapidly. Nevertheless, some dilution can be expected as the plume moved down the arm of the trough. All trout roved continuously between the ends of the tanks, even so, L-histidine was always added to the side of the trough in which a trout was situated at 30-min. This side was designated the 'test' side. A similar procedure was used by Sprague (1968).

Preference/avoidance has been quantified in y-mazes using the 'reaction value', $R_V = \{(T_1 - T_2) / (T_1 + T_2)\} \times 100\%$, where T_1 was time in the 'test' side and T_2 the time in the 'blank' side (Olsén and Höglund, 1985; Ishida and Kobayashi, 1995). With this equation, preference shows as positive and avoidance, negative. Since we were interested in the response to an introduced odorant (i.e. both sides began as 'blank'), we altered this equation to simply describe the change in time spent on the test side after odorant introduction, as the 'response ratio', $R_R = (T_{after} / T_{before}) \times 100\%$. R_R values greater than unity indicate preference, while R_R values less than unity indicate avoidance.

Neurophysiological L-histidine concentration-response curve

EOGs were recorded at 10^{-7}, then 10^{-5} and lastly 10^{-3} M L-histidine under background water (n = 6). Three EOGs spaced 3-min apart were taken for each fish at each L-histidine concentration, and the average response at each concentration was considered for analysis. Three-min was used as it provided ample time for baseline return (i.e. greater times did not result in larger responses).

Experiment 2: Behavioral and neurophysiological responses to pesticides

Before determining whether the pesticides affected the base responses resolved in *Experiment 1*, we first determined whether the pesticides themselves evoked behavioral and/or neurophysiological responses.

Behavioral pesticide preference/avoidance response

As in *Experiment 1*, trout were placed in the trough, left for 20-min, and their positions were monitored for the next 10-min. After 30-min, a pesticide or vehicle control solution was added to the test side of the trough and fish position was again monitored for 10-min. R_R was calculated as above. Initial pesticide concentrations were chosen to be at or close to lethal concentrations. Specifically, we set our upper IPBC concentration at 100 µg/l, which is similar to the 96-h LC_{50} reported for coho salmon (95 µg/l; Farrell et al., 1998). The same upper concentration was selected for atrazine, a concentration which is approximately 30% of the 10-d LC_{50} for rainbow trout (340 µg/l; Davies et al., 1994). With Roundup®, an upper concentration of 100 mg/l was chosen as this was similar to a reported 96-h LC_{50} for rainbow trout (130 mg/l; Johnson and Finley, 1980). If an avoidance response was noted at these initial concentrations (i.e. $R_R<1$), then incrementally lower concentrations were tested. For exposures, IPBC was solubilized in polyethylene glycol (10 mg/ml), atrazine was solubilized in acetone (5 mg/ml) and Roundup® was added directly to background water. All pesticide solutions were prepared daily.

Neurophysiological pesticide response

As with L-histidine-evoked EOGs in *Experiment 1*, 2-s pulses of each pesticide or vehicle were delivered at the highest exposure concentration in the background water feed. If EOG responses were noted, then successively lower pesticide concentrations were tested (n = 6 per pesticide tested).

Experiment 3: Behavioral and neurophysiological responses following pesticide exposure

To determine the effect of pesticide exposure on behavioral and neurophysiological responses, separate trout were given exposures to each pesticide in both the trough and EOG apparatus and changes in L-histidine responses were measured.

Behavioral response following pesticide exposure

Fish were statically exposed in the trough for 30-min to 1, 10 or 100 µg/l IPBC or atrazine, or 10, 100 and 1000 µg/l AI Roundup® (nominal) (n = 6 per concentration). Fish were also exposed to vehicle controls for each group, i.e. acetone, polyethylene glycol and background water (n = 6 per vehicle control). To measure preference/avoidance, fish position was recorded for 10-min following 20-min of pesticide or vehicle exposure, after which water flow was restarted and 10^{-7} M L-histidine was added to one side of the trough. Fish position was recorded for a further 10-min. L-histidine at 10^{-7} M was used as it evoked an attraction response in *Experiment 1*. R_R was calculated as above.

Locomotory activity was also assessed by recording the number of times fish crossed the trough centerline from 30 to 40-min.

Neurophysiological response following pesticide exposure

To measure the effect of pesticide exposures on EOG, fish were prepared as in *Experiment 1* and exposed to pulses of 10^{-7} M L-histidine until there was ≤5% variation between two consecutive EOGs spaced 5-min apart. This spacing enabled adequate detection of any EOG response drift. After attaining a repeatable EOG, the background water feed was switched from background water to either pesticide or vehicle control solutions (same nominal concentrations as above; n = 6 per concentration). Single EOGs were then recorded at 2, 5, 10, 15, 20, 25, and 30-min during exposure, and 2, 5, 10, 15 and 20-min after exposure. This regime was designed to both resemble the trough exposure and also to reveal changes in olfaction that occurred over the course of the exposure and post-exposure period. The line washout following exposure took 30-s. Solutions were delivered in Teflon tubing; a Lauda chiller was used to maintain all solutions at the holding tank temperature (~12 °C).

Data analysis

Behavioral data

Differences in preference/avoidance were assessed in two-parts. Firstly, to test whether fish exhibited significant L-histidine preference or avoidance (i.e. R_R >1 or <1), the R_R values of each group were tested against unity using a one-sample t-test. Secondly, to test if the R_R values differed among different

pesticide exposure concentrations, a Kruskal-Wallis one-way analysis of variance (ANOVA) was carried out. To test for changes in locomotory activity, the number of trough center-line crossings was compared between control and exposed groups by the same ANOVA method.

Neurophysiological data

To test for EOG differences across L-histidine and pesticide concentration-response curves in *Experiments 1 & 2*, EOG values in mV were compared using one-way repeated measures (RM) ANOVA and a Tukey HSD *post-hoc* test. To detect differences in EOG peak amplitude size during and after exposures in *Experiment 3*, EOG (in mV) was expressed as a percent of the pre-exposure value and differences between exposure and vehicle control groups were tested using a two-way (time and treatment) RM ANOVA followed by a Tukey HSD *post-hoc* test. Vehicle control groups were not significantly different ($p = 0.626$) and so were pooled for presentation purposes.

Behavioral and neurophysiological relationships

To explore relationships between neurophysiological and behavioral changes, relative decreases in L-histidine preference responses were plotted against relative EOG decreases. Relative EOG reductions at 30-min of exposure were calculated as: $[1 - (\%\ EOG_{exposed} / \%\ EOG_{control})] \times 100\%$. Changes in relative behavioral response had to be calculated in a slightly different manner, since R_R preference response was greater than unity (e.g. preference $R_R = 1.15$, indifference $R_R = 1.00$, thus 100% of the response = 0.15). For this reason,

relative behavior change was calculated as: $[(R_{R\ control} - R_{R\ exposed}) / (R_{R\ control} - 1)] \times 100\%$ (e.g. $[(1.15 - 1.00) / (1.15 - 1)] = 100\%$ change in behavior). Linear and non-linear regressions were performed as necessary on behavior / EOG relationships and equation selection was based on best-fit models. JMP 5.0 (SAS Institute Inc., Cary, NC), SigmaStat 3.0 and SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA) were used for statistics and graphing.

Results

Experiment 1: Behavioral and neurophysiological responses to L-histidine

Behavioral L-histidine concentration-response curve

A preference response was noted to 10^{-7} M of the amino acid L-histidine as the R_R was significantly greater than unity ($p = 0.029$) (Fig. 5-1a). Because of this response, and because this concentration is within the range of some environmental amino acids (Shoji et al., 2003), 10^{-7} M L-histidine was used as the stimulus for further experiments.

Neurophysiological L-histidine concentration-response curve

EOGs approximately doubled with each 100× increase in L-histidine concentration, with 10^{-3} M L-histidine evoking significantly greater EOGs than both 10^{-5} and 10^{-7} M L-histidine (p-values of 0.031 and 0.003) (Fig. 5-1b). The EOG response to 10^{-7} M L-histidine was robust enough that this stimulus could be used to assess the impact of pesticides on EOG in *Experiment 3.*

Experiment 2: Behavioral and neurophysiological responses to pesticides

Behavioral pesticide preference/avoidance response

None of the pesticides or vehicle controls evoked preference or avoidance responses at the highest concentrations tested (Table 5-1). For example, IPBC did not differ from unity, which indicated no olfactory-mediated behavioral response (R_R = 0.953 ± 0.253, p = 0.830). However, in early range-finding experiments with Roundup®, trout avoided active ingredient concentrations incrementally greater than 1000 µg/l (10 and 100 mg/l; Table 5-1, Fig. 5-1). These concentrations were not tested for olfactory effects as they were lethal within 30-min (*n* = 5 per concentration).

Neurophysiological pesticide response

None of the pesticides evoked EOGs over the tested concentration ranges except for Roundup®, which evoked exponentially increasing EOGs in a manner similar to L-histidine (Fig. 5-1b). Specifically, the EOGS of both 10 and 100 µg/l differed with those of 1000 (p-values of 0.023 and 0.041, respectively). This indicates that over a concentration range that does not result in acute lethality, trout detect Roundup® but do not avoid it.

Experiment 3: Behavioral and neurophysiological responses following pesticide exposure

Behavioral response following pesticide exposure

Thirty-min exposures to ≥1 µg/l IPBC eliminated L-histidine preference responses (i.e. R_R values were not significantly different from unity; p-values of 0.706, 0.438 and 0.511, for 1, 10 and 100 µg/l exposures, respectively) (Fig. 5-

2a). When comparing responses across exposure groups, 10 µg/l exposed-fish also differed significantly from polyethylene glycol vehicle control ($p = 0.041$) (Fig. 5-2a). With atrazine, 1 and 100 µg/l exposures also eliminated L-histidine preference (p-values of 0.061 and 0.869, respectively), however, a significant behavioral response was noted at 10 µg/l ($p = 0.002$) (Fig. 5-2b). Intriguingly, at this exposure concentration, fish showed L-histidine avoidance instead of preference (Fig. 5-2b). Exposure to 1 and 10 µg/l atrazine also significantly increased activity (Fig. 5-2e; $p = 0.005$ and 0.023, respectively). L-histidine preference was not altered with exposure to 10 µg/l AI Roundup® ($R_R > 1$; $p = 0.015$) but was absent at greater exposure concentrations (100 and 1000 µg/l) (Fig. 5-2c). However, trout activity level was lowered by 1000 µg/l AI Roundup® (Fig. 5-2f; $p = 0.012$) and so a behavioral olfactory response may have been missed. In fact, after 30-min exposure to this concentration only 1 of 6 fish was active during the next 10-min, moving over the centerline twice compared to the pooled control average of 14.7 ± 2.4.

Neurophysiological response following pesticide exposure

IPBC reduced EOG in a concentration-dependent manner (Fig. 5-3a). Specifically, 1, 10 and 100 µg/l exposure significantly reduced EOG after 25, 5 and 2-min of exposure, respectively (p-values 0.047, 0.019 and 0.004, respectively). Atrazine exposure had similar effects on EOG, with 10 and 100 µg/l significantly reducing EOG after 10 and 2-min of exposure, respectively (p-values of 0.027 and 0.044, respectively) (Fig. 5-3b). Roundup® significantly reduced EOG after 2-min exposure to both 100 and 1000 µg/l AI (p-values 0.037

and 0.040, respectively), but had no significant effect at 10 µg/l AI (Fig. 5-3c). For all pesticides, EOG decrease after 30-min of exposure was related to exposure concentration (Fig. 5-4).

Summary: neurophysiological and behavioral toxicity relationships

Neurophysiological and behavioral response co-varied and the relative loss in preference behavior generally exceeded the relative loss in EOG response (Fig. 5-5). This was always true for IPBC and atrazine, but not for Roundup® exposure, where EOG decrease was not always associated with altered behavior. However, Roundup® was the only pesticide that evoked EOGs (Fig. 5-1b). Given the active ingredient [AI] of Roundup® (N-(phosphonomethyl) glycine) consists of a modified amino acid (glycine), it is possible that the AI bound to amino acid olfactory receptors. Because of this, the decreased L-histidine-evoked EOGs may be partially attributed to cross-adaptation of L-histidine receptors to Roundup®, rather than toxicological action. Overall, L-histidine preference behavior was absent or atypical when EOGs were reduced by 60.2, 68.2 and 70.0%, for atrazine, Roundup® and IPBC, respectively.

Discussion

The relationship between an olfactory-mediated behavior and its underlying neurophysiological response was first characterized with an amino acid stimulus, and then with pesticides that altered the response to this stimulus. An amino acid was chosen since they are natural aquatic constituents, with concentrations ranging from 10^{-12} to 10^{-7} M (Shoji et al., 2003). This range

coincides with the salmonid olfactory detection limit for amino acids of 10^{-9} to 10^{-7} (Hara, 1973). Fifty years ago it was found that an amino acid that washes off mammalian skin (L-serine) caused an avoidance response in Pacific salmonids (Brett and MacKinnon, 1954; Idler et al., 1956). This response was theorized to represent an antipredator behavior (Idler, 1956; Rehnberg and Schreck, 1987). Perhaps because of this ecologically-relevant relationship, L-serine has been used in several salmon olfactory studies (e.g. Sutterlin and Sutterlin [1971], Hara et al. [1976], Evans and Hara [1985], Rehnberg and Schreck [1987], Hansen et al. [1999b], and Sandahl et al. [2004]). However, serine has been shown to evoke the opposite response (attraction) for Atlantic salmon, with other amino acids evoking avoidance responses (Shparkovskiy et al., 1983). Specifically, Shparkovskiy et al. (1983) found that 38% of Atlantic salmon yearlings introduced into a y-maze selected an arm with 10^{-4} M (D,L) serine, vs. 1% that selected the blank arm, with the majority 61% not deciding. In contrast, 0% selected an arm containing 10^{-4} M histidine vs. 67% that selected a blank arm, with 33% not deciding. Our work confirms that L-histidine may be a stronger behavioral stimulus than L-serine for some salmon, since trout were unresponsive to L-serine. Furthermore, even though we noted an attraction response to 10^{-7} M L-histidine, our findings do not necessarily contradict Shparkovskiy et al. (1983) since it appeared the attraction response was concentration-specific.

The lack of an L-serine-based behavioral response for the rainbow trout of this study may suggest that any response to this amino acid is experientially acquired, if indeed rainbow trout ever associate L-serine with predators in a

natural setting. In general, the hatchery-reared trout of this study may have grown insensitive to predation risk, as has been noted with hatchery-reared brown trout (Álvarez and Nicieza, 2003). Additionally, should any L-serine have been introduced inadvertently to the tank water by animal maintenance activities (e.g. contamination of tank water by hand or feed), a learned attraction response may have been expected, but this was not found either.

The EOG response to a moderate L-histidine concentration (10^{-5} M) compares favorably with other studies using 10^{-5} M L-serine (e.g. 1.97 ± 0.54 mV herein, 0.95 ± 0.07 mV for Atlantic salmon parr [Moore and Waring, 1996b], and ~2 mV for coho salmon parr [Baldwin et al., 2003]). This comparability in spite of our use of a different amino acid is expected, given that L-histidine and L-serine were noted earlier to be of nearly identical stimulatory efficacy in rainbow trout (10^{-4} M L-histidine evoked a bulbar response 97.8 ± 18.7% that of L-serine; Hara, 1973), and validates our electrophysiological techniques.

The pesticide concentrations in the present study were chosen to establish concentration-response relationships within our exposure period (30-min), as well as to have ecological relevance. For example, IPBC has been detected in runoff in concentrations of up to 370 µg/l (Envirochem, 1992). Glyphosate (the active ingredient of Roundup®) has been detected in spills at concentrations of 112 µg/l (Frank et al., 1990), and in spite of its tendency to bind sediments (Giesy et al., 2000), it can also be present in runoff. For example, Edwards et al. (1980) found 5.2 mg/l in runoff within 1-d of an application of 8.96 kg/ha and that 2 µg/l was still present in runoff up to 4-mo post-application.

Atrazine has been detected in rural Ontario ponds from runoff at 6 and spills at 200 µg/l (Frank et al., 1990), and in surface waters of the US at 69.4 µg/l (Waldron, 1974). Furthermore, the pesticides tested are all routinely used around salmonid habitat, thus the experiments conceivably have ecological relevance. Nevertheless, future experiments would benefit from longer exposure periods to lower concentrations and mixtures.

Both pulsatile pesticide release (such as that simulated here) and continuous pesticide release have the potential to create environmental contaminant gradients. Fish may reduce their pesticide exposure if they have the sensory input, be it olfactory or gustatory, across any such gradients. For example, 2,4-D, copper sulfate, dalapon, xylene and acrolein can cause avoidance responses in teleosts (Folmar, 1976). Hansen et al. (1999c) also showed that rainbow trout may avoid certain mixtures of metals, further demonstrating that salmonids have a sensitive capacity to detect environmental contaminants. Low, sublethal contaminant concentrations (e.g. 5 µg/l) may be all that is needed to elicit an avoidance response (see the review by Scott and Sloman, 2004). However, not all pesticides generate a sensory response, and even if they were to cause a response, there is no guarantee that the response would be interpreted as unpleasant. For example, prochloraz, bentazone and nicosulfuron caused preference behavior in goldfish (*Carassius auratus*) (Saglio et al., 2001). In the present study, just Roundup® was capable of evoking EOGs, but it was only avoided at concentrations greater than 1 mg/l – concentrations

which coincided with acute lethality. Sublethal, olfactory and locomotory effects were noted at lower concentrations.

Environments with low and constant background pesticide concentrations may afford salmon little ability to modulate exposures, and these exposures could conceivably affect preference/avoidance behaviors to natural odorants. In the present study, L-histidine preference behavior was altered by each of the pesticides tested: IPBC and atrazine reduced preference behavior with exposure of ≥1 µg/l, while Roundup® did so at a 100-fold greater active ingredient concentration. The decreased attractiveness to certain amino acids may translate to, among other things, decreased foraging success and therefore decreased animal fitness.

Our results were not unanticipated since these pesticides had previously been identified as negatively affecting olfactory sensory neurons. We not only confirmed that these current-use pesticides affect neurophysiological responses, but also demonstrated that they negatively affect behavioral responses based on olfaction. With respect to regulatory limits, IPBC toxicity was noted below the limit set by the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CWQG) (i.e. 1 µg/l vs. 1.9 µg/l limit [CCME, 1999]). Similarly, sublethal effects were noted for atrazine at concentrations below Canadian guidelines (i.e. 1 µg/l vs. 1.8 µg/l [CCME 1999]). In contrast, effects for Roundup® were noted at 100 but not 10 µg/l active ingredient concentration, which suggests the CWQG for this herbicide (65 µg/l) may be acceptable, if applied in acute exposures to trout.

Hyperactivity was observed in trout exposed to 1 and 10 but not 100 µg/l atrazine. In agreement with this, the burst swimming behavior of goldfish was increased 24-h following exposure to 0.5 µg/l atrazine (Saglio and Trijasse, 1998). The lack of hyperactivity at highest exposure concentration may indicate that any stimulatory effects of atrazine were lost with increasing exposure concentration, as can be observed in hormetic dose-response relationships (Calabrese and Blain, 2005). In contrast to atrazine, trout exposed to Roundup® were hypoactive. Given that mortality was observed below a lethality study (i.e. 100% mortality was observed within 30-min of exposure to 10 mg/l, compared to a 96-h LC_{50} of 130 mg/l for juvenile rainbow trout; Johnson and Finley, 1980), the Roundup® we tested may have had added toxicity due to formulation ingredients. One such ingredient may have been the surfactant polyoxyethylene amine (POEA), a chemical that greatly enhances Roundup®'s toxicity to aquatic life (Tsui and Chu, 2003). Both hyper- and hypoactivity are sublethal effects that may result in mortality in the field, such as through increasing susceptibility to predation or decreased predator avoidance.

In this study, our primary goal was to report on the behavioral and neurophysiological responses of rainbow trout to odorants, and how those responses were altered by pesticide exposure. By looking at alterations in responses from two levels of organization, we can simultaneously address two questions, one from a basic and the other from an applied research standpoint: how do the two levels relate and which of the two is a more sensitive toxicological endpoint? The expectation was that one of three relationships

would be found between EOG reduction and loss of preference behavior: (1) that EOG and behavior response would co-vary perfectly (i.e. EOG reduction = behavior reduction); (2) that EOG reduction would exceed behavioral reduction; or (3) that behavioral reduction would exceed EOG reduction. If any toxicological effects were extrapolated from neurophysiological to behavioral endpoints, relationship (1) would suggest a neurophysiological endpoint may adequately reflect behavioral endpoints, whereas (2) and (3) would suggest an over and underestimation, respectively, of the significance of any impairments.

Other studies have explored the effects of pesticides on olfaction and olfactory-mediated higher-order responses; however, these are generally complicated by different exposure periods. For example, the EOG responses of Atlantic salmon to $PGF_{2\alpha}$ (a priming pheromone) were reduced by 70% following 30-min exposure to 10 µg/l carbofuran, while a 5-d exposure of the whole animal to a similar concentration (13.9 µg/l) and $PGF_{2\alpha}$ caused a ~71% drop in expressible milt and a ~58% reduction in plasma testosterone titres (Waring and Moore, 1997). Ignoring the differences in exposure method and length, there appears reasonable agreement between the decrease in olfactory and olfactory-physiologic responses mediated by carbofuran. Similar results were noted with atrazine, with the same caveats applying: 30-min exposure to 10 µg/l reduced $PGF_{2\alpha}$-evoked EOG by 34% and 5-d exposure to 14 µg/l reduced expressible milt by 58% and plasma testosterone titre by 75% (Moore and Waring, 1998).

In the present study, amino acid preference response was absent well before EOG response was completely eliminated. In quantitative terms, an EOG

loss exceeding ~60% removed 100% of olfactory-mediated behaviors (Fig. 5-5). Furthermore, small EOG losses can abolish a disproportionately larger amount of olfactory-mediated behavior (e.g. the 10.9% EOG loss observed following 1 µg/l atrazine exposure equated to 45.4% loss of L-histidine preference response). Sensory input does not guarantee a response unless it is above some threshold. When the signal is no longer perceived as ecologically relevant (e.g. is too weak to be worth searching for) it may be ignored. Therefore, near the behavioral response threshold a fish may increasingly rely on other sensory inputs to make decisions. Since amino acids are known to trigger feeding behavior (e.g. surfacing; Hara, 2006), falling below a threshold could incorrectly lead to discontinuation of food searching behavior, which would equate to underutilization of local resources. Furthermore, if the sensory input is not associated with positive behavioral reinforcement such as a reward, fish may make incorrect decisions or not make them at all. This is evident with our Roundup® testing: fish perceived the pesticide, but did not avoid concentrations that impacted their activity.

Conclusions

Using pesticide exposures we demonstrated that a behavioral response can be lost well before the underlying neurophysiological response is eliminated. This relationship suggests that neurophysiological and behavioral response thresholds can be asymmetrical. For toxicological studies using olfactory neural endpoints alone, toxicity data may need to be considered a conservative estimate of the potential impact if extrapolated to higher-order – and ultimately

more biologically/ecologically relevant – responses such as behavior. Future studies could further enhance relevancy with chronic exposures to lower contaminant concentrations.

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Table

Table 5-1 The preference or avoidance responses (Response Ratio, R_R) of rainbow trout to various concentrations of IPBC (iodocarb), atrazine, Roundup® and vehicle control solutions. R_R values significantly less than unity (one-sample t-test) indicate avoidance.

	exposure concentration		R_R mean ± SEM	p-value
IPBC	100	µg/l	0.953 ± 0.253	0.830
[1]vehicle control	100	µl	1.00 ± 0.05	0.951
atrazine	10	µg/l	1.14 ± 0.16	0.500
	100		0.707 ± 0.186	0.112
[2]vehicle control	200	µl	0.974 ± 0.070	0.719
Roundup®	1		1.14 ± 0.20	0.423
	10	mg/l*	0.793 ± 0.136	0.048
	100		0.571 ± 0.098	0.001
[3]vehicle control	7	ml	0.998 ± 0.095	0.989

*Roundup® exposure concentration was for the active ingredient, glyphosate isopropyl amine salt. Vehicle controls: 1 = polyethylene glycol, 2 = acetone, 3 = background water.

Figures

Figure 5-1 Olfactory mediated (a) behavioral and (b) neurophysiological responses of rainbow trout to concentrations of the amino acid L-histidine (≥98.5%) and the herbicide Roundup® (143 g/l of active ingredient glyphosate). The behavioral response ratio (R_R) was determined as the time spent on the test side after divided by before odorant introduction over 10-min, as $R_R = (T_{after} / T_{before}) \times 100\%$. Electro-olfactograms (EOGs) were recorded as maximum change in the voltage of olfactory tissue in response to a 2-sec pulse of either L-histidine or Roundup. For (a), significant difference from unity (i.e. a behavioral response) is denoted by an asterisk (one-sample t-test; $n = 6$ per concentration); in (b), EOGs with dissimilar alpha (Roundup) or numeric (L-histidine) symbols are significantly different (one-way RM ANOVA, Tukey HSD; $n = 6$). Behavior curve equations: for L-histidine $R_R = 0.409 - 0.119 \times (\log [\text{L-histidine}])$, $p = 0.0104$, $R^2 = 91.7\ \%$; for Roundup $R_R = 0.740 + 0.260 \times (\log [\text{Roundup}]) + 0.0625 \times (\log [\text{Roundup}])^2$, $p < 0.001$, $R^2 > 99.9$. Neurophysiological curve equations: for L-histidine EOG (mV) $= 21.8 \times 1.61^{(\log[\text{L-histidine}])}$, $p = 0.0159$, $R^2 > 99.9$; for Roundup EOG (mV) $= 51.9 \times 1.66^{(\log[\text{Roundup}])}$, $p = 0.0159$, $R^2 > 99.7$.

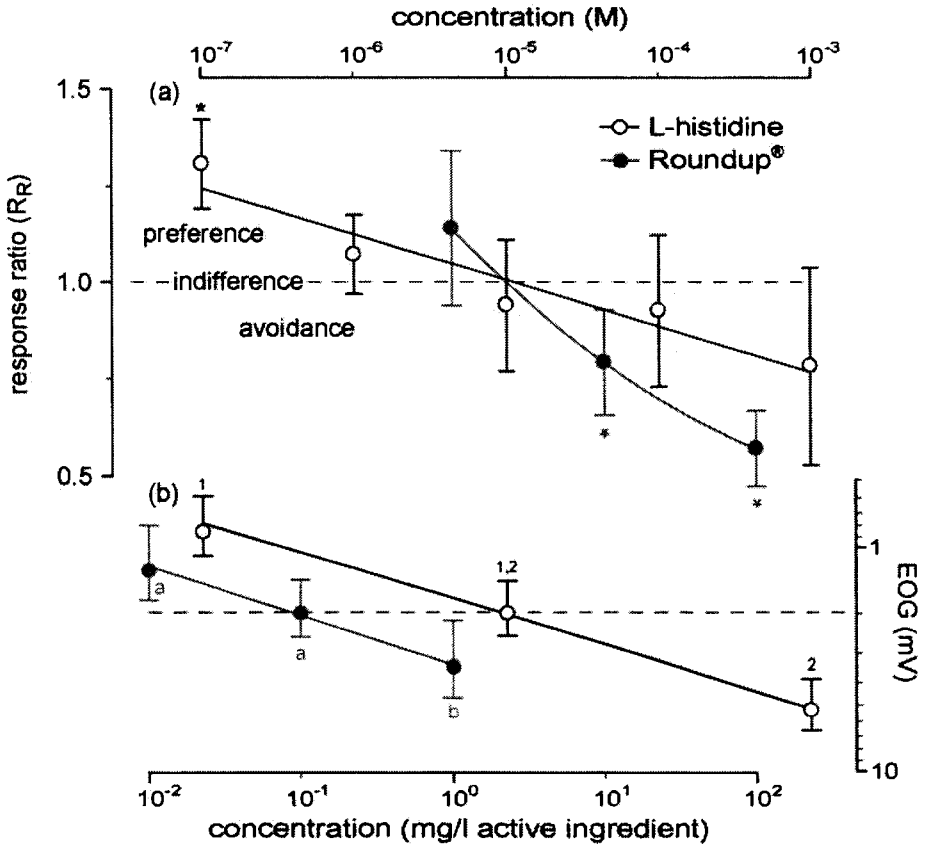


Figure 5-2 The preference/avoidance response (response ratio; see Fig. 5-1) of rainbow trout (a,b,c) and their activity level (d,e,f) following a 30-min exposure to concentrations of IPBC, atrazine, and Roundup® in an avoidance trough (n = 6 per concentration). Exposure groups differing significantly from unity (i.e. a behavioral response; one-sample t-test; green and red shading indicate attraction and repulsion, respectively) are denoted by asterisks; exposure groups differing from each other are denoted by crosses (ANOVA). Dashed line indicates first value differing from unity. Activity was determined as the number of trough centerline crossings during the 10-min following exposure. Significant differences from control are shown with asterisks (one-way ANOVA, Dunn's Test). Pooled control fish mean activity ±95CIs are shown as green shading (d,e,f).

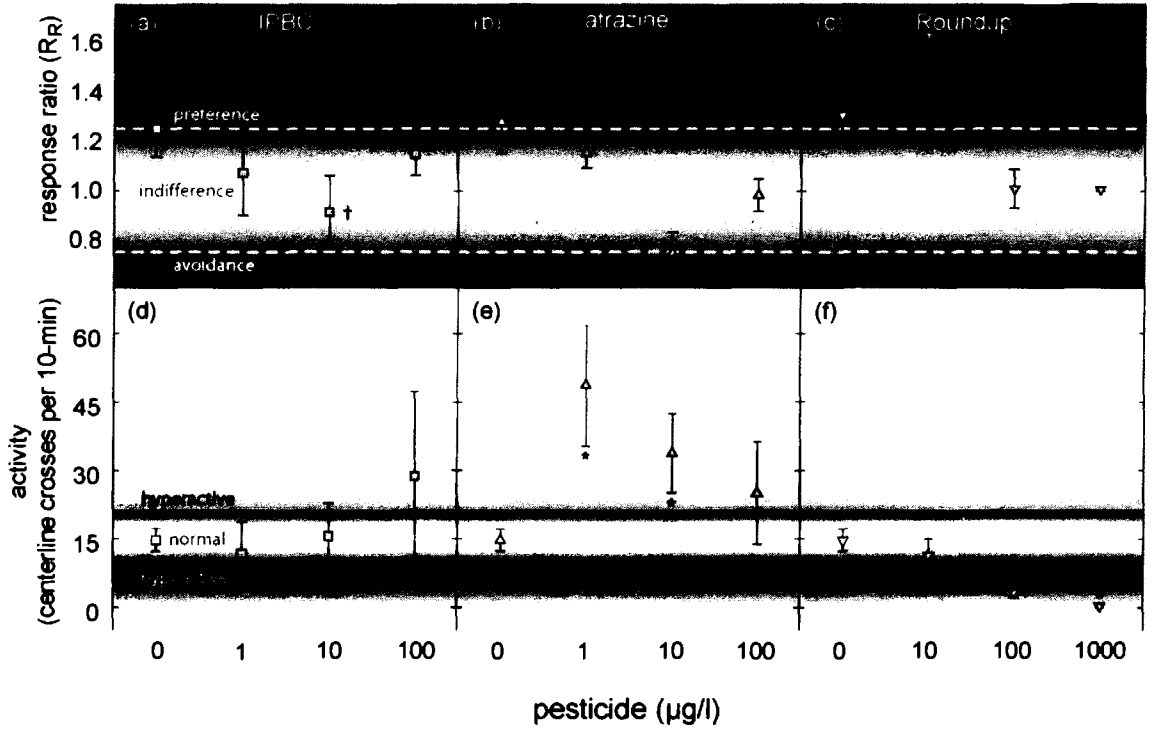


Figure 5-3 Changes in L-histidine-evoked electro-olfactogram during and following a 30-min exposure to various concentrations of IPBC, atrazine, and (active ingredient) Roundup®. Dashed line indicates pooled vehicle (PEG, acetone and DDW) controls (n = 18). Significant difference from pooled control is indicated with dots (two-way RM ANOVA, Tukey HSD, n = 6 per concentration).

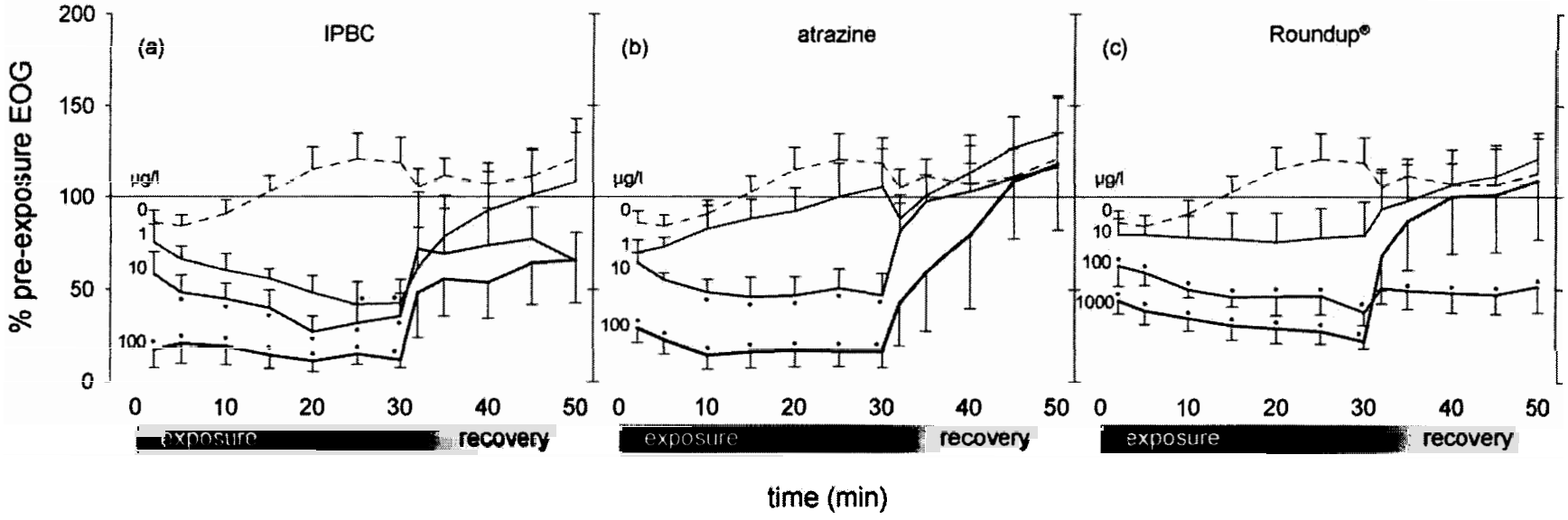


Figure 5-4 The reduction in L-histidine-evoked EOG (compared to pre-exposure value) after 30-min exposure to various concentrations of IPBC atrazine, and (active ingredient) Roundup®. Curve equations on figure are perfect-fit (IPBC: % EOG = 43.5 – 0.729 × ([log IPBC] + 1)$^{3.43}$; for atrazine: % EOG = -17.2 + (123 × 0.524)$^{[\log \text{ atrazine}]}$; for Roundup®: % EOG = 12.2 + (175 × 0.382)$^{[\log \text{ Roundup}]}$).

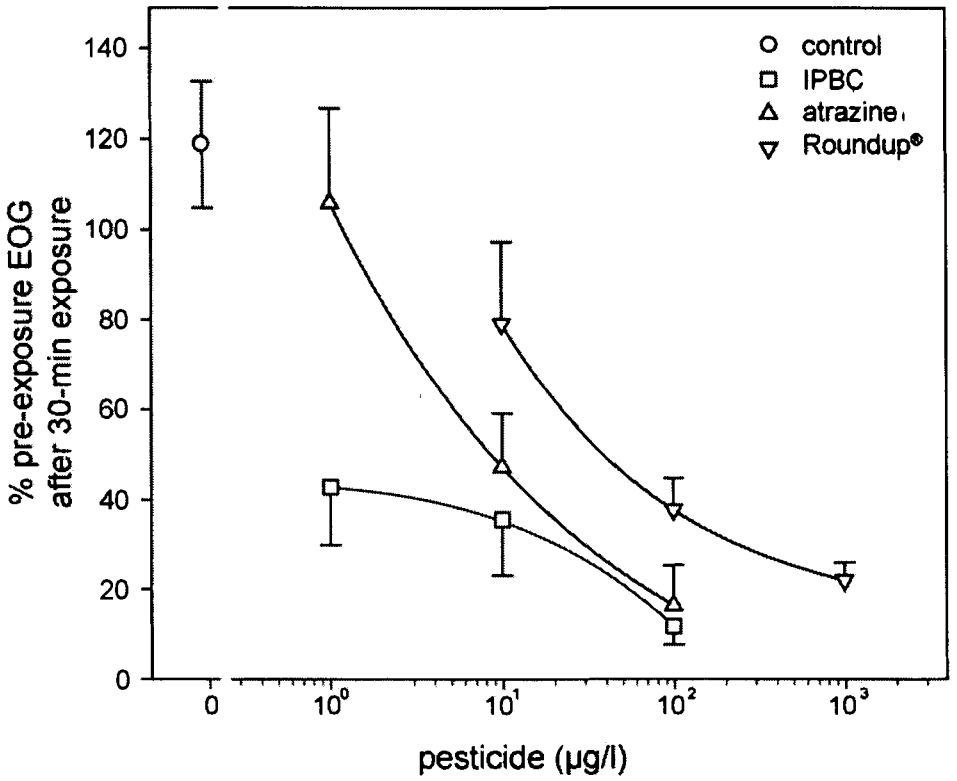
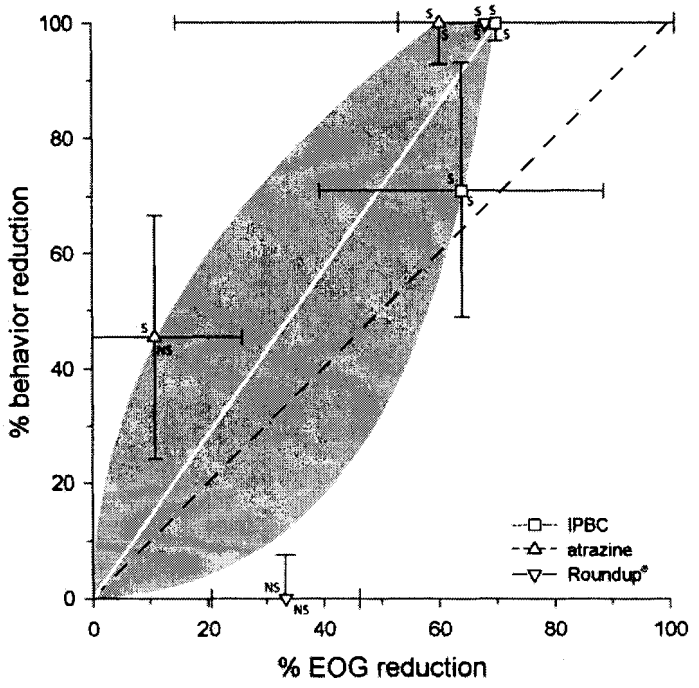


Figure 5-5 The degree to which changes in neurophysiological and behavioral response due to 30-min pesticide exposure vary with each other. Perfect co-variation is shown as the 45° solid line. The shaded region is bounded by a more and a less conservative estimate of the potential for changes in neurophysiological response to alter olfactory-mediated behavior (perfect fit model for atrazine: % behavior reduction = 15.1 × (% EOG reduction) $^{0.462}$; and IPBC: % behavior reduction = 2.06 × 1.06 $^{(\%\ EOG\ reduction)}$ – 2.06; and a linear curve-fit to IPBC and atrazine data through the origin (white line): % behavior reduction = 1.42 × (% EOG reduction); R^2 = 78.8%). Values are mean ± SEM; significant difference from control is shown for each point (S = significant, NS = not significant).



Chapter 6:
The alarm reaction of coho salmon parr is impaired by the carbamate fungicide IPBC

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Abstract

To determine whether the carbamate fungicide IPBC alters the olfactory-mediated behavioral and physiologic alarm responses of coho salmon parr (*Oncorhynchus kisutch*), groups of coho salmon were exposed to skin extract (an alarm pheromone source) under a variety of conditions. In the 3 min following skin extract exposure, freezing behavior was significantly increased (Δ calculated as: [(time (s) after / time (s) before) -1] × 100%) under darkness (IR lighting) but not ambient lighting (25.3 ± 2.6% and 7.5 ± 5.7%, respectively), and so IR was used for further experiments. Physiologically, following skin extract exposure, plasma cortisol concentration was increased at 0.5 h (58.1 ± 14.6 vs. 4.32 ± 1.31 ng/ml, exposed vs. control), hematocrit (Hct) was increased at 2 h (50.4 ± 1.0 vs. 41.7 ± 1.6%), and leucocrit (Lct) was decreased at 0.5 and 2 h (0.534 ± 0.114 and 0.13 ± 0.01 vs. 1.23 ± 0.20%). After 0.5 h exposures to 0, 1, 10 and 100 µg/l IPBC and skin extract, the time spent dashing (>5 cm/s) increased significantly (323 ± 118%) in the first minute after skin extract exposure, but was absent in IPBC-exposed coho. Freezing behavior increased after skin extract exposure with control and 1 µg/l IPBC exposures (11.0 ± 3.0% and 17.7 ± 11.0%, respectively), but was absent after 10 µg/l and decreased after 100 µg/l IPBC. Physiologically, Hct and plasma lactate concentration were significantly increased above controls after 1 µg/l IPBC exposure (Hct: 45.7 ± 1.6% vs. 34.0 ± 1.6%, lactate: 12.8 ± 1.2 vs. 3.30 ± 1.2 mM). After 10 µg/l exposure, IPBC alone elicited a stress response similar to skin extract. However in the 100 µg/l treatment group the stress parameters were not different from controls. These

findings suggest that the behavioral and physiologic alarm responses of juvenile salmonids may be impaired by acute exposure to ≥1 µg/l IPBC.

Keywords: coho salmon; *Oncorhynchus kisutch*; olfaction; alarm response; behavior; cortisol; carbamate; pesticide; IPBC

Introduction

For salmonids, olfaction facilitates indispensable behaviors such as migration and predator avoidance. By necessity, olfactory sensory neurons (OSNs) have a relatively unprotected environmental interface, which may leave them at risk during contaminant exposure. Environmentally realistic concentrations of metals and pesticides have been shown to impair salmonid OSNs (e.g. Hara et al., 1976; Borie et al., 1981; Waring and Moore, 1997; Baldwin et al., 2003; Jarrard et al., 2004; Sandahl et al., 2004). By impairing OSNs, contaminants may also affect olfactory-mediated behavioral and physiologic responses. For example, pesticides toxic to OSNs can lead to reduced sex steroid production (Moore and Waring, 1996a; Moore and Waring, 2001) and altered migration (Scholz et al., 2000). Compared with tests of OSN function, olfactory-mediated behavioral and physiologic response tests are more ecologically meaningful (Scott and Sloman, 2004), but they are also more technically difficult to assess.

Carbamate pesticides can rapidly impair salmonid OSNs at exposure concentrations ≤1 µg/l (Jarrard et al., 2004; Tierney et al., 2006). For example, 1 µg/l exposure to the carbamate antisapstain wood preservative IPBC (3-iodo-2-propynyl-N-butyl carbamate) significantly impaired coho salmon OSNs in 25 min (Tierney et al., 2006). The manner in which carbamates impair olfaction is unknown; most carbamate pesticides (i.e. N-methyl carbamates such as carbaryl or carbofuran) impair their targets is through addition of a carbamoyl moiety (NH_2CO) to the ser-OH esteratic site of the enzyme acetylcholinesterase. IPBC's

mode of fungicidal action is unknown, although it has been hypothesized to be due to the terminal iodine (Juergensen et al., 2000). Structurally, IPBC resembles other carbamates in that it contains a core of carbamic acid (see Fig. 6-1), but it is not an N-methyl carbamate. IPBC has received attention in part because it can be present in runoff from lumber storage and milling sites located on salmon-producing rivers (Envirochem, 1992; Juergensen et al., 2000). Currently, the Canadian regulatory IPBC guideline for freshwater is 1.9 µg/l (CCME, 1999); US guidelines would be present on product labeling (FIFRA, 1996). The Canadian limit is well below acute lethality values for juvenile salmonids (96 h LC_{50} of 67 and 95 µg/l for rainbow trout [*Oncorhynchus mykiss*] and coho salmon [*O. kisutch*], respectively [Bailey et al., 1999; Farrell et al., 1998]), but it is not below concentrations that cause olfactory toxicity (i.e. 0.1 µg/l, Jarrard et al., 2004). Although IPBC has been identified as toxic to salmonid OSNs, it is not known how this may translate to altered olfactory-mediated physiologic or behavioral responses.

This study examined the olfactory-mediated alarm responses of coho salmon parr following a sublethal IPBC exposure. The alarm response consists of physiologic and behavioral responses, characteristic of 'fight-or-flight' reactions (Cannon, 1914). Specifically, following sensory input, the physiologic stress responses ensues by central command neurons of the autonomic nervous system (Jansen et al., 1995). These responses may include hypothalamo-sympathetic-chromaffin axis-mediated release of catecholamines (e.g. epinephrine) and hypothalamo-pituitary-interrenal axis-mediated release of

glucocorticoids (e.g. cortisol) (Mommsen et al., 1999). The former providing rapid (i.e. ≥seconds) enhancement of glucose and oxygen delivery to tissues such as muscle and brain, and the latter providing a following up (i.e. ≥minutes) that also enhances glucose availability, but in addition, enhances its storage (Mommsen et al., 1999). In the teleost alarm response, both primary (e.g. cortisol increases) and secondary (e.g. glucose increase) responses typically occur (Scott et al., 2003; Rehnberg and Schreck, 1987). In non-olfactory-based stress studies, other blood sample based measures including packed red and white blood cell volume (hematocrit [Hct] and leucocrit [Lct], respectively) have been used to assess secondary physiologic stress responses. For example, Hct increased from 37 to 42% (Tierney et al., 2004a) and Lct increased from 0.57 to 0.82% (Tierney et al., 2004b) in coho salmon parr 96 h after a cold-shock (18 to 8 °C transfer).

Physiologic changes may underlie behavioral alarm responses, which usually involve energy expenditure. For example, when the ostariophysan matrinxã (*Brycon cephalus*) perceives alarm pheromone, a period of hyperactivity (i.e. 'dashing') precedes hypoactivity (i.e. 'hiding') (Ide et al. 2003). In salmonids, a variety of alarm response studies have reported a period of hypoactivity or increased time spent frozen in alarmed fish (in rainbow trout: Brown and Smith, 1997; Brown and Smith, 1998; Mirza and Chivers, 2001; Mirza and Chivers, 2003; Leduc et al., 2004; in chinook salmon (*O. tshawytscha*): Berejikian et al., 1999; Scholz et al., 2000; Berejikian et al., 2003). As the magnitude of the alarm

signal increases, the amount of inactivity increases (Mirza and Chivers, 2003). Thus far, dashing behavior has not been observed in coho salmon.

In the present study, swimming behaviors were observed and primary and secondary measures of stress were taken in coho salmon parr following skin extract exposure. Typically, salmonid alarm response behavior is examined in individuals; however, we chose to measure it in groups, which may improve ecological relevance. This also facilitates physiologic measures, which tend to be measured in five or more tank-exposed fish. To help limit any non-olfactory-based alarm reaction (e.g. visual alarm reaction propagation; Smith, 1992), a novel infrared camera and lighting system was designed to allow testing under darkness. To help resolve a suitable sampling time and skin extract concentration, the physiologic responses were measured at 0, 0.5, 1 and 2 h after exposure to three concentrations of skin extract. Finally, both the physiologic and behavioral responses of coho exposed to skin extract were characterized following exposure to three concentrations of IPBC.

Materials and methods

Animals

Coho salmon parr were obtained from the Fisheries and Oceans Canada (DFO) Capilano Fish Hatchery (North Vancouver, BC) in January 2005 (n = 370, mass 10.0 ± 0.13 g, length 10.0 ± 0.04 cm, fork length condition factor 0.995 ± 0.006; Adams et al., 1993). Coho were held for at least three weeks prior to experimentation in a 500 l flow-through tank supplied with dechlorinated municipal water (dissolved oxygen >90% saturation, pH 6.8, hardness 6.12 mg/l

$CaCO_3$), using a 12:12 h light:dark photoperiod, and were fed commercial salmon feed *ad libitum* (EWOS Pacific, Surrey, BC).

Skin extract and pesticide preparation

Skin extract was prepared as in Brown and Smith (1998). In brief, fish were sacrificed by cervical dislocation, the skin was then removed, rinsed with distilled deionized water (DDW), massed and placed on ice. In the first experiment with lighting, 50 mg/l of skin extract was used as this concentration was comparable to that used in a similar study with rainbow trout (i.e. 43 mg/l; Scott et al., 2003). To achieve the desired skin extract exposure concentration, 1.7 g of skin was homogenized, filtered through glass wool and brought to a final volume of 50 ml in DDW and then frozen at -20 °C. Thus, when added to the 34 l tanks (dimensions of 50 [length] × 25 [width] × 27 [height] cm), the final skin extract concentration was ~50 mg/l. In the second experiment, to determine the effect of skin extract concentration on alarm response, concentrations bracketing this concentration were also tested (i.e. 5 and 500 mg/l).

IPBC (3-iodo-2-propynyl-N-butyl carbamate; 97%; Sigma Aldrich, Oakville, ON) was solubilized in polyethylene glycol (10 mg/ml) before being added to tanks.

Video surveillance equipment and light sources

For behavior analysis, video was acquired and captured using CNL100 CCD cameras (1 per tank) streaming to PV-140V surveillance cards (Matco, St.

Laurent, QC) in a 2 gigahertz Pentium PC. Cameras were placed 15 cm above the water, which permitted capture of the complete tank area.

For tests under ambient lighting (first experiment only), tanks were illuminated using standard, fluorescent lighting. For all other tests, individual tanks were illuminated using two custom-built infrared (IR) light sources, each consisting of a parallel array of four strings of six TSAL5100 940 nm wavelength diodes (Vishay, Shelton, CT) and two 22 Ω resistors (total 48 LEDS per tank). IR (i.e. wavelengths between ~≥750 nm and ≤1 mm) cannot be detected by other salmonids (e.g. rainbow trout [Allen and Munz, 1983] and Atlantic salmon [Ali, 1961]), and so it is likely that coho cannot detect IR either. Here, the IR light illuminated the tanks and was relayed by the cameras, computer and software as white-light.

Experiment 1: The alarm reaction under different lighting conditions

To determine whether the behavioral alarm response could be measured in the absence of visual light, coho were tested under ambient and infrared lighting. Coho were held for 24 h in 34 l tanks, with control and treatment tanks for each lighting condition (5 fish per tank; 4 tank replicates per lighting condition). Each tank, camera, and lighting arrangement was completely shielded in black plastic to eliminate outside visual disturbances. Vehicle control (DDW) or skin extract was injected into tanks through a glass inflow tube and chased by 15 ml of DDW to ensure complete delivery. For IR experiments, the fluorescent lights were turned off and IR lights were turned on 0.5 h before injections. Video was recorded for 5 min before and after vehicle or skin extract

exposure for subsequent behavioral analysis. All experiments were conducted at the same time of day.

Experiment 2: Skin extract concentration and sampling time

As in Experiment 1 (Exp. 1), fish were placed into 34 l flow-through tanks and acclimated for 24 h before testing (n = 5 per tank). To help determine an appropriate skin extract concentration and post-skin extract exposure time that would yield a significant physiologic stress response, fish were exposed to 0, 5, 50, and 500 mg/l skin extract and sampled at 0, 0.5, 1 and 2 h post skin extract or DDW, with the entire experiment performed in duplicate. Water flow was discontinued 0.5 h prior to the delivery of the skin extract.

At each sampling time, fish were sacrificed by over anesthetization with 0.3 g/l MS-222 (Syndel Laboratories, Vancouver, BC) buffered 1:1 with $NaHCO_3$. Fish were then massed, lengthed and blood samples were collected via severed caudal peduncle into 40 µl heparinized Hct tubes. Hct tubes were centrifuged at 10,000× g for 5 min, and Hct and Lct were measured within 10 min using digital calipers and a dissecting microscope. Hct and Lct measurements were performed in triplicate for most fish. Plasma was isolated and stored at -80 °C until analyzed for cortisol concentration. Each sampling was conducted between 10:00 am and 12:00 pm to minimize the possibility of diurnal cortisol fluctuations (Peter et al., 1978). Cortisol concentrations were measured in triplicate using ELISA kits (Neogen Corporation, Medicorp, Montréal, QC).

Experiment 3: The alarm reaction after IPBC exposure

As in Exps. 1 and 2, fish were placed into 34 l flow-through tanks and allowed to acclimate for 24 h (n = 5 per tank). To determine whether IPBC altered the alarm response, fish were statically exposed to 0, 1, 10 and 100 (nominal) µg/l IPBC for 0.5 h, with the experiment performed in hexuplicate. Controls received the same amount of vehicle as the highest concentration (i.e. 0.1 ml/l or 0.34 ml PEG per tank). Tanks were then given 50 mg/l skin extract, a concentration shown in Exp. 2 to cause significant changes in primary and secondary stress response parameters, or vehicle. After a 0.5 h skin extract or vehicle exposure (total IPBC exposure of 1 h), fish were sacrificed and blood samples were taken and analyzed as above, except that plasma glucose and lactate concentrations were also measured (kits from Stanbio, Boerne, TX; Trinity Biotech, St. Louis, MO).

Behavioral and statistical analysis

The swimming speed of each fish in the horizontal plane was determined manually with the help of 1 cm^2 black gridlines on white plexiglas tank bottoms for every second of 3 min before and after skin extract or vehicle exposure. This behavioral scoring was conducted blind. For Exp. 1, since early observations indicated that fish were either still or in motion, the proportion of time spent either active (>0 cm/s) or inactive (frozen; ~0 cm/s) for the 3 min before and after skin extract exposure was determined within each tank. A similar quantification of activity was used by Lindsay and Vogt (2004) and Scholz et al. (2000). Differences in the average time spent frozen before and following skin extract

exposure were compared using two-way repeated measures analysis of variance (RM ANOVA) followed by Holm-Sidak post-hoc test. Additionally, to determine if the change in the time spent frozen was significant for each lighting condition, the change in freezing, calculated as [after – before; time in seconds], was tested against zero using a two-tail t-test.

For Exp. 3, to better capture fish behavior, the 'active' category of Exp. 1 was split into 'active' (>0 and <5 cm/s) and 'dashing' (>5 cm/s). Since dashing has typically been observed prior to the freezing portion of the alarm response (Ide et al., 2003), the amount of time spent dashing during the first minute and the amount of time spent frozen during the third minute was compared to the average for the 3 min pre-exposure period. To ease reporting, the % change ([after/before -1] × 100%) is also provided. Within all tanks, there was at least 1 s of each activity over the pre- and post-skin extract 3-min periods, and so the equation was never undefined. As in Exp 1, a two-tail t-test of the change in activity against zero was used to detect significant changes in behavior.

For physiologic data, comparisons were made between replicate tanks, and if no differences were found, data was pooled and individual fish were considered (and thus n = 10 and 30 per treatment, Exps. 2 and 3, respectively). For Exp. 2, two-way ANOVA followed by a Holm-Sidak test was used to test for differences and interactions between skin extract concentration (0, 5, 50, 500 mg/l) and exposure time (0, 0.5, 1, 2 h) for stress parameters. For Exp. 3, the same statistical method was used to detect differences in alarm response at each IPBC concentration. All percent data were arcsine transformed prior to

statistical analysis (Zar, 1999). Statistical significance was set at $p < 0.05$, and unless stated otherwise, reported values are mean ± S.E.M. JMP 5.0 (SAS, Toronto, ON), SigmaStat 3.0 and SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA) were used for statistical analysis and graphing.

Results

Experiment 1: The alarm reaction under different lighting conditions

Fish were significantly more active in darkness, spending 29.9 ± 4.5% of the time active under infrared (IR) light (i.e. an average 269 ± 40 s of the total 900 possible seconds [5 fish × 3 min × 60 s/min] was spent at >0 cm/s) compared to 10.0 ± 6.6% (or 90.2 ± 59.0 s of total) under ambient lighting ($p = 0.038$) (Fig. 6-2). However, following skin extract exposure, activity levels were similar (4.08 ± 1.62% IR vs. 7.33 ± 1.06% ambient) (Fig. 6-2). Freezing behavior increased in both lighting conditions (25.3 ± 2.6% under IR vs. 7.54 ± 5.7% under ambient), but the increase was only significant under IR ($p = 0.008$). Subsequent experiments were conducted under IR.

Experiment 2: Skin extract concentration and sampling time

Skin extract exposure elicited significant changes in all of the measured primary and secondary stress response parameters compared to controls. Values for time 0 and vehicle control groups (no skin extract) were not significantly different and so were pooled and are reported as mean ± 95% CI (Fig. 6-3). Plasma cortisol concentration was significantly increased over controls at 0.5 h following skin extract exposure ($p = 0.001$) (Fig. 6-3a), whereas

Lct was significantly decreased at 0.5 and 2 h, but not at 1 h (p values of 0.04, 0.001 and 0.193, respectively) (Fig. 6-3c). For both plasma cortisol concentration and Lct, the amount of skin extract used (5, 50, 500 mg/l) did not influence the response and so values are reported pooled (p values of 0.426 and 0.207, respectively) (Fig. 6-3a, c). With Hct, there were some differences between skin extract exposure concentrations, although overall, Hct values were significantly greater than control at 2 h post exposure ($p < 0.001$) (Fig. 6-3b). Since a sampling time of 0.5 h resulted in significant changes in two of three measured stress parameters (i.e. cortisol concentration increased and Lct decreased), and as 50 mg/l is a concentration similar to that used previously (i.e. 43 mg skin/l, in Scott et al., 2003), this time and concentration were used for experiments with IPBC.

Experiment 3: The alarm reaction after IPBC exposure

In vehicle-exposed tanks, dashing was significantly ($p = 0.002$) increased in the first minute follow skin-extract exposure. Specifically, during the 3 min pre-skin extract exposure, there was an average of 2.69 ± 1.44 s of dashing compared 9.83 ± 6.92 s of dashing in the 1 min following, resulting in a 323 ± 118% increase (Fig. 6-4a). Freezing was significantly ($p = 0.024$) increased in the third minute following skin extract exposure (11.0 ± 3.0% increase, or 264 ± 10 s before vs. 292 ± 3 s after skin extract exposure) (Fig. 6-4b). In all IPBC-exposed fish, the time spent dashing was completely eliminated (Fig. 6-4a). The time spent freezing was not altered with 1 µg/l IPBC (17.7 ± 11.0%; $p = 0.030$), but was absent with 10 (4.06 ± 2.22%; $p = 0.065$) and decreased with 100 µg/l

IPBC (-23.6 ± 11.4%; p = 0.030; i.e. skin extract caused an increase in activity at 100 µg/l IPBC) (Fig. 6-4b).

In skin extract-exposed fish, cortisol concentration increased (p = 0.020) and Hct decreased (p = 0.008) compared to vehicle controls (Fig. 6-5a, b, respectively). The stress responses were altered differently by IPBC at each exposure concentration. Specifically, in the 1 µg/l IPBC treatment group, skin extract exposure caused significant increases in both Hct (p = 0.010) and plasma lactate concentration (p = 0.009) (Fig. 6-5b, c, respectively). With a 10 µg/l IPBC exposure, IPBC exposure alone caused an increase in stress parameters; skin extract caused no further change or was not different from control. For example, Hct was significantly increased from control (p = 0.029), but was not influenced by skin extract exposure (p = 0.513) (Fig. 6-5b). No stress parameters were significantly different from control following 100 µg/l IPBC exposure. No changes were detected in Lct and or glucose for any groups.

Discussion

The goal of this research was to determine if exposure to the carbamate fungicide IPBC impaired the behavioral and physiologic olfactory-based alarm reaction in coho salmon parr. Certain coho stocks are endangered in Canada and the US, and these and other salmonids are important to aboriginal culture, recreational and commercial fisheries, marine ecosystems (e.g. food for resident killer whales, *Orcinus orca*) and terrestrial ecosystems (i.e. nutrients for temperate rainforests and terrestrial predators). Salmonids are particularly vulnerable to the possible effects of currently-used pesticides, with their large-

scale migrations and their reliance on multiple habitat types at different life stages.

The olfactory-mediated alarm response was selected for study since it can be crucial to successful negotiation of a predator attack (Kats and Dill, 1998). For the juvenile life stage tested here, the alarm response is of obvious ecological relevance. However, the alarm response is not the only function in which olfaction is essential to survival; olfaction is indispensable throughout the salmonid life cycle, enabling imprinting and return migration (Dittman and Quinn, 1996), and the synchronization of gamete release (Moore and Waring, 1996b). Since many of these behaviors are based on olfaction, a reduction in alarm response performance may indicate a reduction in others. The carbamate IPBC was chosen for study both because of its proximal use to major salmon producing rivers in British Columbia and elsewhere, and because of its established toxicity to salmon OSNs (Jarrard et al., 2004; Tierney et al., 2006). Furthermore, IPBC may serve as a model for other current-use pesticides that are acutely toxic to salmonid OSNs. The IPBC concentrations used in this study (1, 10 and 100 µg/l) are potentially environmentally realistic, since IPBC has been reported to vary from non-detectable to 370 µg/l in stormwater (Envirochem, 1992). Furthermore, the method of pulse exposures used here may also be typical of IPBC exposures in the field since it is moderately soluble, does not appreciably adsorb to sediments, and can remain in solution for extended periods (Table 6-1).

To determine if testing could be conducted under darkness (in order to remove visual alarm propagation that can occur in groups of fish), coho were tested under infrared and fluorescent lighting. Coho salmon were more active under darkness, a finding that agrees with other studies. For example, chinook salmon alarm-induced activity increased with water turbidity, which limits vision (Gregory, 1993; Gregory and Northcote, 1993). On a larger scale, the migration activity of brown trout (*Salmo trutta*) was found to be greater at night (Bendall et al., 2005). Consequently, it is possible that for salmonids, activity is greater under the cover of darkness because the perceived predation risk is lower. This agrees with the present study: because of the greater activity under darkness, the relative increase in freezing behavior was significant only in the absence of light. For coho tested under fluorescent lighting, the increase in freezing was not significant, which suggests that these coho may have been already somewhat in predator avoidance mode. After skin extract exposure, activity was the same regardless of lighting, which may indicate skin extract induced a risk level unrelated to lighting. Similarly, Vainikka et al. (2005) noted that the antipredator responses (which included decreased activity and shoaling) of perch (*Perca fluviatilis*) did not differ with risk level. In contrast, Hartman and Abrahams (2000) noted that fathead minnows (*Pimephales promelas*) exhibited an increased dashing response following alarm substance exposure under turbid conditions. They propose that since the minnows were unable to visually assess their surroundings, the predation risk was assumed to be greater and thus there was a commensurate increase in alarm response. The difference between salmonids

and minnows in this regard is unclear. Darkness was used in the present study to help isolate a chemosignal from photosignals, however, it does not remove all non-olfactory based sensory input, e.g. the mechanoreception of the lateral line system (Dijkgraaf, 1962; Ćurčić-Blake and van Netten, 2006).

The experimental procedure used was not stressful since control cortisol concentrations (4.32 ± 1.31 ng/ml) compare favorably with other studies using similar fish (e.g. 2-5 ng/ml in Atlantic salmon [*Salmo salar*] parr [Shrimpton and McCormick, 1998] and 5 and 10-15 ng/ml in juvenile rainbow trout [Scott et al., 2003 and Toa et al., 2004, respectively]). However, for the final set of experiments with IPBC (Exp. 3), which were conducted in April and approximately one month following the initial measurements (Exp. 2), the control values were higher (61.8 ± 6.0 ng/ml). This may have been from natural increases in this stress hormone coincident with the approaching smoltification period (Shrimpton and McCormick, 1998; McCormick et al., 2000). Regardless of this baseline concentration, skin extract exposure elicited a plasma cortisol increase. In the coho tested earlier, skin extract exposure caused an approximately ten-fold increase in plasma cortisol concentration in 0.5 h; in the coho tested later, the difference was closer to two-fold. As a general rule, stressful events such as handling or contaminant exposure can cause increases in plasma cortisol concentration in 1 h (reviewed by Mommsen, 1999).

The concentration of skin extract used (5, 50 or 500 mg/l) did not appear to influence the cortisol response, which may indicate that these concentrations were all above the HPI axis response threshold. Therefore, to facilitate a

comparison with a similar study on rainbow trout that used 43 mg/l (Scott et al., 2003), 50 mg/l was used in the final set of experiments. In Scott et al. (2003) and the present study, skin extract caused significant increases in plasma cortisol concentrations within 0.5 h. In contrast, Ide et al. (2003) found that skin extract did not increase cortisol in matrinxã. Other antipredator cues, such as predator scent, can also increase plasma cortisol concentration. For example, plasma cortisol concentration was increased in coho salmon following exposure to tank water of both northern squawfish (*Ptychocheilus oregonensis*) and largescale sucker (*Catostomus macrocheilus*) (Rehnberg and Schreck, 1987). Secondary measures of stress may also indicate an olfactory-mediated alarm response. Here, we report for the first time that both Hct and Lct can change following skin extract exposure, with decreases in both noted 0.5 h following exposure. These changes may have their origins in altered cortisol concentrations since this hormone is the teleost mineralocorticoid and is leucocytolytic (Weyts et al., 1998). The olfactory-mediated physiologic response likely provides energy for antipredator behaviors, since an Hct increase can enhance blood oxygen carrying capacity (Gallaugher and Farrell, 1998), and leucocyte destruction may make protein available for gluconeogenesis (McLeay, 1973).

Coho exhibited a biphasic behavioral alarm response to skin extract (i.e. dashing occurred prior to freezing). As stated in the introduction, dashing has not been observed previously in salmonids. The difference in the present study may have been due to the testing procedure, which consisted of five coho together under IR in small tanks, or may have been influenced by the life stage

used. Alternately, the concentration of skin extract was sufficient to evoke an as yet unseen behavioral response. As Mirza and Chivers (2003) noted, the magnitude of the behavioral alarm response can be related to skin extract concentration. If this is the case, it suggests that some components of the alarm response may differ in thresholds, seeing as the cortisol response was unrelated to skin extract concentration. This idea is further supported with our IPBC exposures: dashing was impaired by a lower concentration of IPBC than freezing (i.e. 1 vs. 10 µg/l, respectively), which may indicate that dashing behavior has a higher response threshold.

Other pesticides can impair alarm responses at similar concentrations, for example, in chinook salmon, Scholz et al. (2000) found that the freezing response was impaired by 2 h exposure to 1 µg/l of the insecticide diazinon. Furthermore, environmental contaminants other than pesticides can impair the alarm response; Scott et al. (2003) found that the normal decreases in line crossings and feeding caused by skin extract exposure did not occur in rainbow trout exposed to 2 µg/l Cd for one week. Furthermore, contaminant exposure can reduce the alarm response in other types of fishes. For example, Saglio and Trijasse (1998) found that the grouping response exhibited by goldfish following skin extract exposure was decreased 24 h after exposure to 5 µg/l of the herbicides atrazine or diuron. The results of the present study together with those of others clearly establish that olfactory-mediated behavior can represent a sensitive sublethal toxicity endpoint in fish. Moreover, because of the importance of olfactory-mediated behaviors such as the alarm response, a pesticide-

associated impairment of performance may have profound consequences for survival.

The physiologic alarm response of coho salmon parr was also altered by IPBC exposure. The expectation was that a decrease in this stress response would occur since a 1 µg/l IPBC exposure has been shown to impair olfactory neurons (Jarrard et al., 2004; Tierney et al., 2006) and olfactory-mediated behaviors. This was true for Hct, where skin extract alone caused an Hct decrease that was eliminated by 1 µg/l IPBC exposure, but not necessarily true for lactate, where skin extract appeared to cause a significant increase only in the presence of IPBC. It is possible that the lactate increase was motivated by multiple stressors (i.e. IPBC and skin extract together). Both Hct and plasma lactate concentration may be increased in juvenile salmonids following stressful events such as contaminant exposure (Tierney et al., [2004a] and Wood et al., [1996], respectively). These findings are significant because they indicate that olfactory toxicity may need to be considered along with other measures of toxicity.

Skin extract exposure did not appear to contribute to the physiologic stress response at an IPBC exposure concentration of 10 µg/l or greater. However, three stress parameters (plasma cortisol and lactate concentration, and Hct) were increased. These IPBC-mediated increases may represent meaningful digressions from homeostasis, as Hct was increased beyond the normal healthy salmonid Hct range (i.e. from 41.3 ± 2.2% to 48.2 ± 2.2%; normal range 35-41%, Gallaugher and Farrell, 1998). Curiously, after 100 µg/l IPBC

exposure, parameters were not different from 0 µg/l IPBC control. These findings are in keeping with Farrell et al. (1998), who found no change in a similar suite of primary and secondary stress parameters in juvenile rainbow trout after 24 h to 140 µg/l Polyphase P-100 (97% IPBC). Since stress parameters were increase by a concentration between 1 and 100 µg/l, IPBC may have had a hormetic effect (reviewed by Calabrese, 2005). Although the mechanism of IPBC toxicity is unknown (Juergensen et al., 2000), perhaps at the highest concentration, IPBC had a sedatious effect or it negatively impacted the hypothalamo-pituitary-interrenal (HPI) axis. In any event, the non-linear response complicates an extrapolation of IPBC effects from higher to lower concentrations.

Even though a physiologic stress response was absent after a 100 µg/l IPBC exposure, coho still exhibited a behavioral response to skin extract, indicating that there was sensory input from olfactory neurons. However, skin extract exposure at this IPBC concentration caused a decrease in freezing behavior, instead of the typical increase. It is possible that by impairing olfactory neurons, a 100 µg/l IPBC exposure effectively increased the alarm response threshold, to such a degree that normal alarm behaviors did not ensue (Mirza and Chivers, 2003). Alternately, the behavioral response may have deviated because a physiologic stress response was absent. This would indicate that a physiologic response is necessary for normal alarm behaviors to occur. Future studies could test this interplay by blocking the stress response with β-blockers (e.g. propranolol) or cortisol blockers (e.g. metyrapone).

Conclusions

Both behavioral and physiologic aspects of the olfactory-mediated alarm reaction were altered by environmentally realistic concentrations of the carbamate fungicide IPBC. 1 µg/l IPBC exposure eliminated dashing and aggravated the physiologic stress response. After 10 µg/l IPBC exposure, the freezing response did not occur, and increases in various physiological measures of stress were observed. In fact, on its own, stress parameters were increased after 10, but not 1 or 100 µg/l IPBC exposure, indicating a non-linear or complex, multifaceted mechanism of action which precludes a traditional concentration-response evaluation. After 100 µg/l exposure, alarm behavior was deviant (i.e. in place of a freezing response, skin extract caused an increase in activity), and a physiologic stress response was absent. Since alarm response is an important antipredator behavior, exposures to IPBC at concentrations at or below the existing Canadian regulations of 1.9 µg/l may render exposed salmon more susceptible to predation.

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Table

Table 6-1 General information and some physico-chemical properties of IPBC[1].

Property		Value
Name		3-iodo-2-propynyl butyl carbamate
Chemical formaula		$C_8H_{12}INO_2$
CAS number		55406-53-6
Uses		antisapstain antimicrobial mildewcide
Products		Kop-Coat NP-1 Troysan KK 108-A Troysan Polyphase P-100
Molecular mass		281 $g \cdot mol^{-1}$
Specific gravity		1.58 $g \cdot ml^{-1}$
Water solubility		156 $mg \cdot L^{-1}$
Hydrolysis half-life	pH 5	stable
	pH 7	139 d
	pH 9	0.947 d
Vapor pressure	20 °C	0.002 Pa
	30 °C	0.007 Pa
Melting point		54 °C
Log octanol-water partition coefficient (K_{ow})		2.81
Log organic-carbon adsorption coefficient (K_{oc})		2.05±0.12[2]
Bioconcentration factor		< 4.5

[1]Taken from the review by Juergensen et al. (2000)
[2]Average for various sediments

Figures

Figure 6-1 The chemical structure of (A) IPBC (3-iodo-2-propynyl-N-butyl carbamate) and that of the N-methyl carbamate (B) carbaryl (1-naphthyl-N-methyl carbamate).

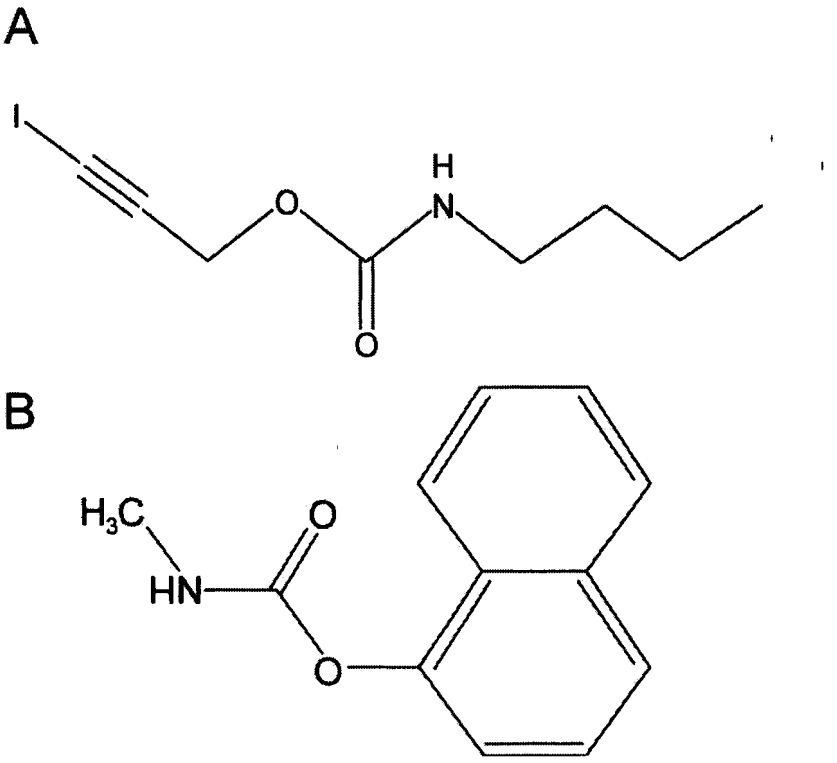


Figure 6-2 The percent of time juvenile coho salmon spent either active (>0 cm/s) or inactive (~0 cm/s) 3 min before and following skin extract exposure under either ambient or infrared lighting (darkness). Asterisk indicates significant difference (2-way ANOVA, Holm-Sidak, n = 4 tanks per lighting condition, 5 fish per tank).

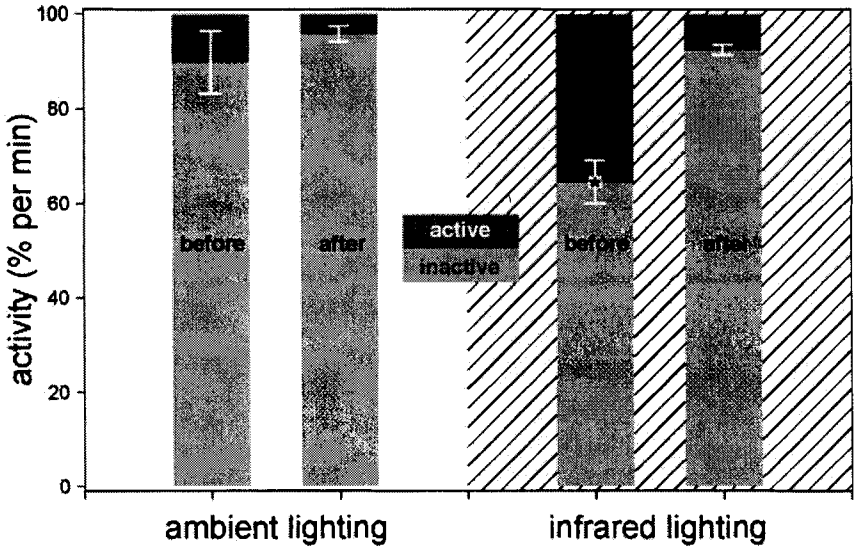


Figure 6-3 (a) plasma cortisol concentration, (b) hematocrit and (c) leucocrit of coho salmon parr at 0, 0.5, 1 and 2 h following exposure to 0, 5, 50, 500 mg/l of skin extract, an alarm pheromone source. Plasma cortisol concentration and leucocrit were pooled as there no differences due to skin extract concentration. Similarly, time 0 and distilled water controls were pooled and are shown as mean ± 95% CI (dashed and dotted lines, respectively). Like alphanumeric superscripts indicate values that are not significantly different; asterisk indicates difference from control (2-way ANOVA, Holm-Sidak, n = 10 per group).

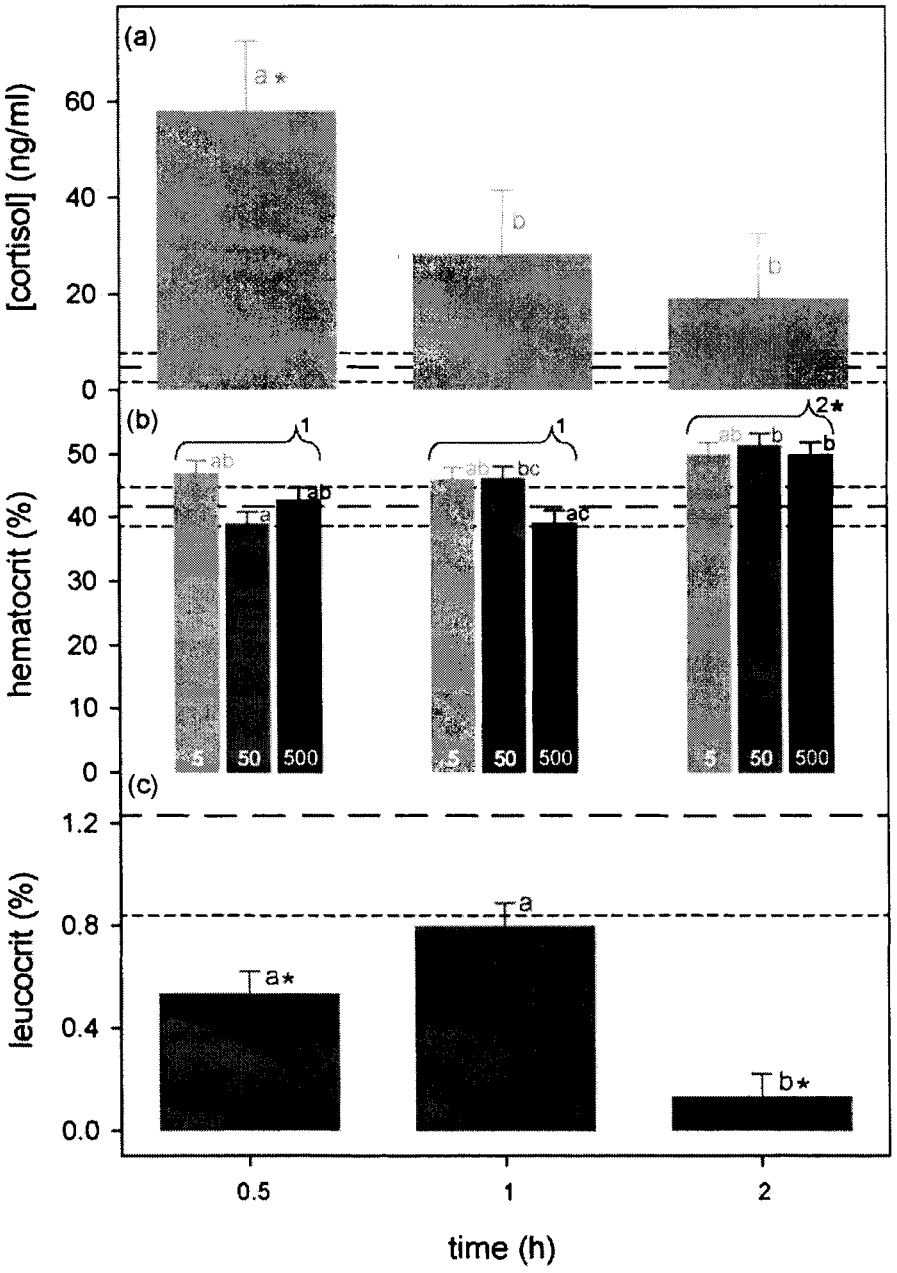


Figure 6-4 (a) changes in the proportion of the first minute spent dashing (>5 cm/s) and (b) of the third minute spent frozen (~0 cm/s) for coho salmon after 0.5 h exposure to concentrations of the carbamate pesticide IPBC and either a distilled water control (○) or 50 mg/l skin extract (●). Percent activity change was calculated as (after/before - 1) × 100%. Values differing from zero (i.e. no change) are denoted with asterisk (2-sample t-test, n = 6 tanks of 5 fish per point).

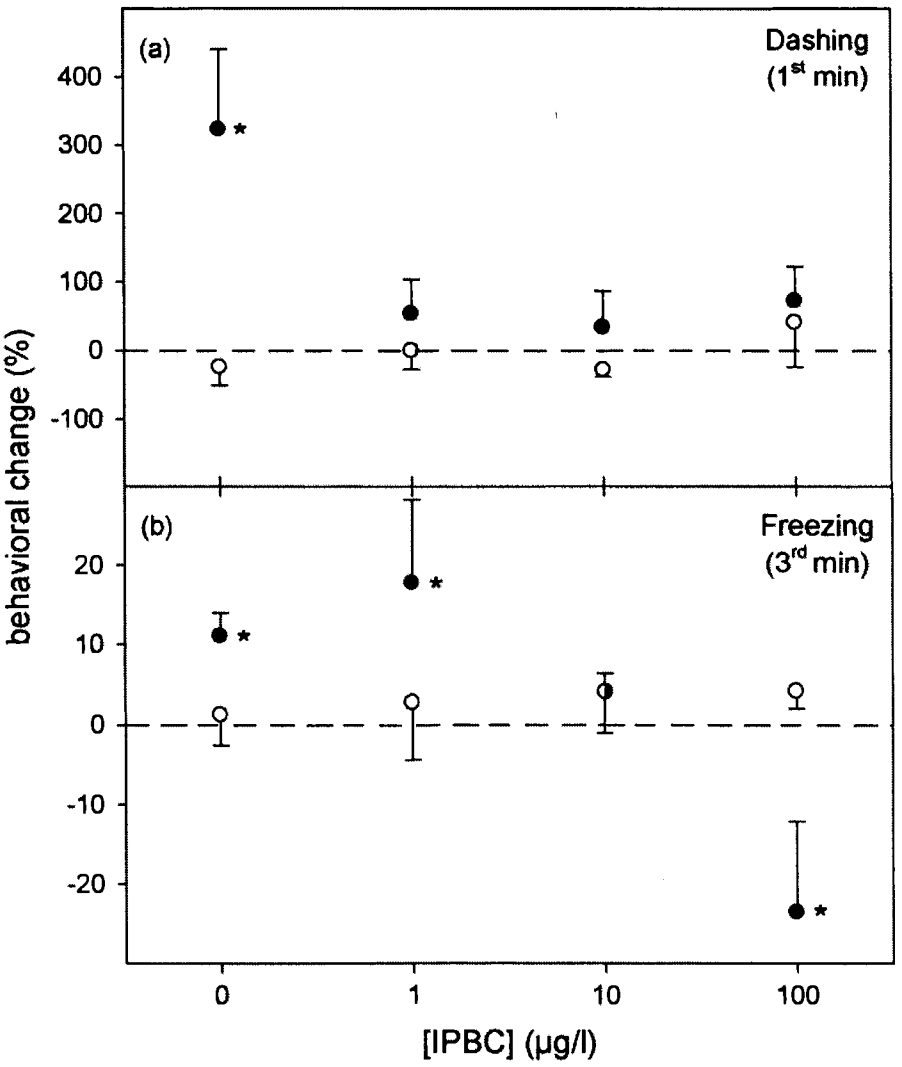
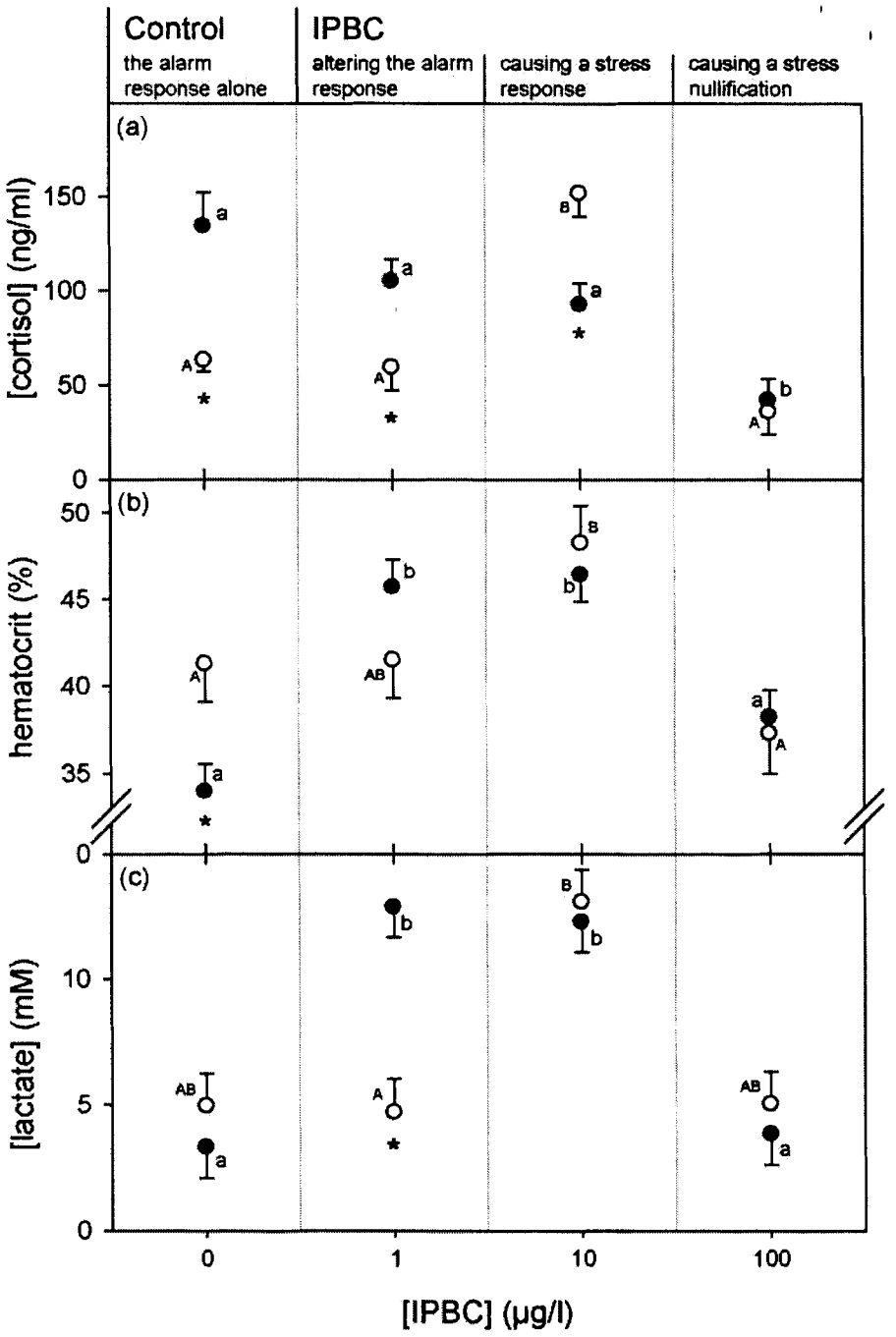


Figure 6-5 (a) plasma cortisol concentration, (b) hematocrit and (c) lactate concentration in coho salmon parr 1 h after IPBC exposure and 0.5 h after either distilled water control (○) or 50 mg/l skin extract (●). Like letters indicate values that are not significantly different from each other; differences between control and skin extract-exposed groups at each IPBC concentration are denoted with asterisks (2-way ANOVA, Holm-Sidak, n = 30 per point).



Chapter 7:
The relationship between cholinesterase inhibition and two types of swimming performance in chlorpyrifos-exposed coho salmon (*Oncorhynchus kisutch*)

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Abstract—Brain acetylcholinesterase (AChE) activity was evaluated after two different swimming tests in coho salmon (*Oncorhynchus kisutch*) (238 ± 5 g) given 96-h exposures to 0, 5, 10, 20, or 40 µg/L chlorpyrifos. Brain AChE activity decreased in a concentration-dependent manner (AChE activities were 81.8, 52.2, 37.3, and 21.3% of the control value for 5, 10, 20, and 40 µg/L exposures, respectively) whereas swimming performance was impaired after a threshold of AChE impairment was reached. Specifically, for swimming performance (U_{crit}) measured using the established ramp-U_{crit} test (152 ± 8 min duration), this threshold occurred with AChE activity of 68.5 ± 18.1% of control. For a rapid acceleration test ($U_{\Delta V}$) (where V represents velocity) (27.6 ± 0.8 min) this value was 52.6 ± 15.4% of control. Both swim protocols resulted in similar maximum swim speeds (control ramp-U_{crit} and $U_{\Delta V}$ values: 3.44 ± 0.09 and 3.71 ± 0.13 body lengths per second), and performance was significantly reduced after 20 and 40 µg/L exposures in both groups (ramp-U_{crit} values of 86.4 and 83.6 % of control; $U_{\Delta V}$ values of 85.2 and 77.8% of control). Although both tests yielded similar swim speeds, post-exercise plasma lactate concentrations were greater for the $U_{\Delta V}$ test (i.e., 11.3 ± 0.6 vs 8.6 ± 0.5 mmol/L), indicating a greater anaerobic effort. This increase was exaggerated after 10 µg/L chlorpyrifos (i.e., 14.6 ± 1.3 mmol/L); indicating anaerobic muscle was used to attain the same speed. Given the threshold relationship between AChE inhibition and swimming performance, coho appear able to maintain integrated swimming activity despite significant impairment of an underlying neurological control mechanism.

Keywords— Chlorpyrifos Acetylcholinesterase Salmon Swimming Performance

Introduction

Acetylcholinesterase (AChE) inhibition disables the main mechanism that terminates cholinergic synaptic transmission in the brain and neuromuscular junctions of both vertebrates and invertebrates [1,2]. This inhibition can have biological consequences such as hyperactivity [3], tetanus [4], and death [5]. The first nerve agent discovered (Tabun gas) was an anti-AChE agent, so it is no surprise that it gave rise to a series of agents designed for medical, pest control and warfare applications. As a result, bioassays of AChE inhibition are a routine indicator of nontarget species anticholinesterase poisoning [6].

As with many other subcellular toxic effects, it can be difficult to ascribe physiological or ecological relevance to sublethal AChE inhibition. The role of AChE in coordinating muscle performance through both central and neuromuscular activity is an obvious choice for understanding how its inhibition affects higher-order processes. With fish, their ability to swim provides an ecologically relevant endpoint. Swimming has been assessed in a variety of ways: spontaneous swimming [7], sprint swimming or burst swimming [8], volitional swimming against flow [9], and the most widely used, critical swimming (U_{crit}) ability [10-13]. Factors known to impact U_{crit} include water temperature [14,15], salinity [16], and pH [17], fish health [18-20], life history stage [21], and contaminant exposure [22-24]. To our knowledge, only three studies have examined U_{crit} following anticholinesterase exposure [25-27], and only one has endeavored to make comparisons between AChE inhibition and U_{crit}. Specifically, Cripe et al. [26] found that exposure to the organophosphorus (OP)

insecticide ethyl (p-nitrophenyl) benzenethiophosphonate (EPN) resulted in significant decreases in both AChE and U_{crit} in sheepshead minnow (*Cyprinodon variegatus*). However, AChE activity and U_{crit} were measured in different fish after differing exposure times. Furthermore, Cripe et al. [26] found azinphosmethyl (another OP) also inhibited AChE but did not alter U_{crit}. More commonly, anticholinesterase studies have looked at spontaneous swimming activity [7,28-30]. Some of these have noted hyperactivity [28,29], while another noted concentration-dependent activity decreases [30], and another still found no change at all [7].

In the present study we examined the relationship between chlorpyrifos-induced AChE inhibition and the impairment of critical swimming performance in coho salmon. Chlorpyrifos was chosen since it is a contaminant of significant concern, both for its potent AChE-inhibiting properties, and owing to its presence in salmon producing waterways. For example, chlorpyrifos has been detected in the Hood River, Oregon, USA (e.g., at 0.482 µg/L in 1999) [31] and in the Nicomekl River, British Columbia, Canada (e.g., at 18.3 ng/L, 2004) (M. Sekela, Environment Canada, Vancouver, British Columbia, unpublished data). Coho salmon were used as a test animal because it is an endangered species in some areas of Canada [32] and the United States [33]. Furthermore, its juvenile life history places it in ditches directly adjacent to agricultural activities. We tested juvenile coho using a ramp-U_{crit} test which yields similar performance as the standard U_{crit} test but with a shorter test time [24,34]. In addition, we used a rapid acceleration test we named $U_{\Delta V}$, which is more likely to exhaust fish (i.e.,

necessitate a lengthy recovery of energy reserves) since the U_{crit} test may measure fatigue (i.e., a short term transient energy depletion) or a behavioral endpoint [9,35]. The goals of the present study were to first determine the effects of chlorpyrifos on two different measures of coho locomotion, a sublethal endpoint that relies on the integration of the nervous and muscular systems, and to next relate any locomotory changes to AChE inhibition.

Materials and methods

Animals

Coho salmon (*Oncorhynchus kisutch*) were obtained as parr from the Fisheries and Oceans Canada (DFO) Chilliwack Hatchery (Chilliwack, BC, Canada) and raised to young adults (n = 67, mass 238 ± 5 g, length 28.1 ± 0.2 cm, condition factor 1.08 ± 0.01) in outdoor 1,200 L tanks supplied with filtered, dechlorinated municipal freshwater (dissolved O_2 at >90% saturation, pH 6.8, hardness 6.12 mg/L $CaCO_3$) under natural photoperiod and water temperature. Fish were tested from the beginning of March through the end of May (ambient water temperature 6-8°C). Although coho of this age are often at sea, they are amenable to being landlocked [36] and have been used in other recent studies of swimming performance (e.g., [37]). Coho were fed commercial salmon pellets (Ewos, Surrey, BC, Canada) ad libitum.

Chemicals

All chemicals were purchased through Sigma (Sigma Aldrich, Oakville, ON, Canada) and included acetylthiocholine iodide (AChI; ≥98%), Bradford

reagent, chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate; 99.2%), clove oil, and 5,5′ – dithiobis (2-nitro-benzoic acid; ≥98%).

Exposures

To sublethally expose coho to chlorpyrifos, we selected an initial exposure concentration less than the lowest reported salmonid 96-h LC50 value (i.e. the concentration causing 50% mortality). Specifically, Macek et al. [38] reported a 96-h LC50 of 51.0 µg/L for 0.6 to 1.5 g rainbow trout, while Johnson and Finley [39] reported a 96-h LC50 value of 7.10 µg/L for the same species, with a similar mass of 1.4 g. Therefore, we set our lowest exposure concentration at 5 µg/L (nominal) and doubled this until an impairment of swimming performance was detected.

To carry-out exposures, coho were placed into 100 L glass aquaria and chlorpyrifos dissolved in acetone (2 mg/ml) or acetone alone was added with the water changes twice daily for 4 d. Fresh chlorpyrifos stock solutions were prepared weekly and kept refrigerated. Controls received the same amount of acetone as the highest chlorpyrifos exposure group. Tank water temperature was held constant at acclimation temperature by an external water bath. Two fish were held in each tank and tank oxygen content was monitored twice daily; hypoxia was not noted. It was our goal to have approximately six fish in each exposure group, with twice that number in controls. In all, 70 fish were tested (for U_{crit} tested fish, n = 12, 6, 5, 3, and 6; for $U_{\Delta V}$ tested fish, n = 15, 6, 6, 5, and 6; exposure to 0, 5, 10, 20, and 40 µg/L chlorpyrifos, respectively).

Swim tunnel

Details of the swim tunnel are found elsewhere [40]. In brief, the tunnel was a 272 L, impellor-driven respirometer based on the design of Brett et al. [10]. In the present study, the frequency drive control was updated with a Siemens digital controller (Siemens, Richmond, BC, Canada) that permitted rapid and precise velocity changes from a remote PC. The frequency drive was regularly calibrated to water velocity using a Valeport flow-probe (Valeport Marine Scientific, Dartmouth, Devon, UK).

Exercise protocols

For the swim tests, coho were lightly anesthetized using clove oil (50 mg/L), measured for mass and length, placed in the chamber (20.3 cm diameter × 1.86 m length) and left to recover for 30-min at 0.3 body lengths per second (BL/s). Each fish was then given one of two swimming protocols. The ramp-U_{crit} test is described elsewhere [24,34], but in brief coho were brought to 75% of their max (using an initial estimate from test fish not included in analysis) in 5-min steps after which 20-min steps were given until fish became impinged on the rear electrified grid for at least 20 s. All steps were of the same height of 0.2 BL/s. For the acceleration test ($U_{\Delta V}$), all 0.2 BL/s steps were of 2-min duration. The U_{crit} and $U_{\Delta V}$ values were calculated as the speed of the last step completed plus the temporal fraction of the incomplete step [10]. We anticipated that both measurements might result in the same final velocity since Peake and Farrell [9,35] noted that the transparent length-restricted tube generally used to permit fish observation (e.g., [35]) can prevent the use of any supervening unsteady

swimming gait. Specifically, fish typically require a much longer open distance in order to use burst-and-coast swimming. A burst within the tube we used could take the fish from the back of the chamber to the front within a second.

Tissue analyses

Immediately following swim tests, coho were sacrificed via cervical dislocation, blood was sampled via cardiac puncture and whole brains were removed, weighed, and frozen at -80°C. Four 40 µl heparinized capillary tubes were filled from each fish, spun at 10,000× *g* for 5 min and the height of the packed red and white cells, as well as the total height (red, white, and plasma), were taken with the aid of digital calipers and a dissecting microscope within 5-min of test completion. Hematocrit (Hct) and leucocrit (Lct) were then calculated as: (red / total) × 100% and (white / total) × 100%, respectively. The average value of the four tubes was used for most fish. Following Hct and Lct measurement, the plasma portion of the tubes was transferred into 0.5 ml Eppendorf tubes and frozen at -80°C for subsequent lactate analysis. Lactate was analyzed in duplicate using kits (Trinity Biotech, St. Louis, MO, USA).

Brain AChE was measured as in Sandahl and Jenkins [31]. In brief, each brain was homogenized in a sodium phosphate buffered solution (pH 8.0). The homogenate was then diluted to concentrations of 5 and 10 mg tissue/ml buffer and centrifuged at 45× *g* for 10 min. Aliquots of 250 µl of supernatant were placed in triplicate in 96-well plates. Acetylthiocholine iodide was introduced as the AChE substrate and the absorbances of the aliquots were read at 420 nm every 20 s for 10 min. The AChE activity was calculated from the resulting

slopes; triplicate averages were used for most coho. Activities were standardized against total protein, which was measured on the supernatant of each sample according to Bradford [41]. Analyses were conducted without experimenter's knowledge of the test animal.

Statistics

Differences in U_{crit} in cm/s across exposure groups and test types were tested using analysis of covariance (ANCOVA) with fish length as a covariate, followed by a Tukey's post-hoc test. Although fish length was not a significant covariate, U_{crit} is reported in body lengths per second (BL/s) to facilitate comparisons with other salmon swimming data in the literature. Differences in plasma lactate, Hct and Lct were detected using two-way analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test. The Hct and Lct values were arc-sine transformed prior to analysis. Differences in AChE activity (in nmol/min/mg protein) were detected using a Kruskal-Wallis (i.e., nonparametric) one-way ANOVA followed by Dunn's post-hoc test. To model the relationship between AChE activity and chlorpyrifos concentration, the individual data were then fitted with an exponential curve. A break-point segmented regression was used to estimate the point at which changes in AChE activity were associated with altered swimming performance for each swim test type. Regressions were fitted to the raw data using the segmented package [42] in R, version 3.2.1 [43]. The statistical theory for determining the breakpoints or segments is outlined in Muggeo [44]. Five percent was used throughout as the limit of significance; SigmaStat 3.01 (Systat Software, San Jose, CA, USA) and JMP 5.0 (SAS,

Toronto, ON, Canada) were used for all tests except the breakpoint analysis. SigmaPlot 9.0 (Systat Software) and Adobe Illustrator 10.0 (Adobe Systems, San Jose, CA, USA) were used for graphing.

Results

AChE activity

Chlorpyrifos exposure caused a concentration-dependent exponential decrease in AChE activity (Fig. 7-1) (AChE activities for 5, 10, 20, and 40 µg/L exposures were 81.8, 52.2, 37.3, and 21.3% of the control value). Significant differences in AChE activity from the control value (283 ± 18 nmol/min/mg protein) occurred with exposures of ≥ 10 µg/L ($p < 0.05$).

Swimming performance and blood chemistry

Ramp-U_{crit} tests lasted 152 ± 8 min; $U_{\Delta V}$ tests were five times shorter (27.6 ± 0.8 min). The ramp-U_{crit} and $U_{\Delta V}$ fatigue values were not significantly different for either control fish (3.44 ± 0.09 and 3.71 ± 0.13 BL/s; $p = 0.160$), or any chlorpyrifos exposure group ($p = 0.186$) (Fig. 7-2A). However, compared to controls, both measures of swimming performance were significantly reduced after exposure to 20 and 40 µg/L chlorpyrifos (p values of 0.018 and <0.001) (Fig. 7-2A).

For control fish, neither Lct nor Hct were different following the two swim tests (U_{crit} and $U_{\Delta V}$ Lct values: 0.8 ± 0.1 and 0.7 ± 0.1%; $p = 0.417$; Hct values: 40.7 ± 0.9 and 42.1 ± 1.0 %; $p = 0.632$). Post-exercise plasma lactate concentration for control fish was significantly higher for the $U_{\Delta V}$ test compared

with the ramp-U_{crit} swim test (10.5 ± 0.8 vs 8.2 ± 0.7 mmol/L; p = 0.031; Fig. 7-2B), indicating a greater anaerobic swimming effort for the acceleration swim test.

Leucocrit was unaffected by chlorpyrifos exposure. Hematocrit was significantly (p <0.001) reduced in the 40 µg/L chlorpyrifos exposure group (32.9 ± 1.1%). The elevated post-exercise plasma lactate concentration for coho given the $U_{\Delta V}$ swim test compared to the ramp-U_{crit} swim test was evident in most chlorpyrifos exposure groups (Fig. 7-2B). In addition plasma lactate values for the 10 µg/L exposure group were significantly higher (p = 0.003) than control fish, suggesting a greater anaerobic swimming effort at this intermediate chlorpyrifos exposure concentration. However, this response subsided at higher exposures.

Relationship between AChE activity and swimming performance

According to the mean U_{crit} and $U_{\Delta V}$ values, swimming ability appeared to decline at 20 µg/L. However, with break-point analysis, it is evident that swimming ability began to decline at exposure concentrations closer to 10 µg/L (Fig. 7-3). The estimated breakpoints for the two tests occurred at different levels of AChE impairment (Fig. 7-3). Specifically, for ramp-U_{crit}, the breakpoint occurred when an AChE activity was 209 ± 55 nmol/min/mg protein or 68.5 ± 18.1% of control, which relates to an interpolated chlorpyrifos exposure concentration of 6.91 µg/L. For $U_{\Delta V}$, the breakpoint occurred with greater AChE inhibition, at an AChE activity of 160 ± 47 nmol/min/mg protein or 52.6 ± 15.4% of control, which relates to chlorpyrifos exposure concentration of 12.0 µg/L.

Discussion

The present study is the first to determine the relationship between acetylcholinesterase (AChE) inhibition and critical swimming performance. Neurotransmission within the musculature that enables swimming is mediated by acetylcholine (ACh), the breakdown of which is accomplished by AChE. This enzyme class is routinely measured, especially in toxicological studies, albeit in brain tissue (e.g., [6,31,45]). However, AChE inhibition within brain and muscle tissues is strongly correlated (i.e., slope ≈ 1, r^2 =85%; [30]). Furthermore, other enzyme classes that catalyze ACh cleavage (e.g., butyrylcholinesterases) are likewise inhibited by anticholinesterase agents and thus could not serve in place of impaired AChE; a finding demonstrated using AChE knockout mice [46]. To date, three other studies have measured U_{crit} following anticholinesterase exposure, but none of which looked at AChE activity following swimming performance.

AChE activity and chlorpyrifos exposure

In the present study, the brain AChE activities of control coho were similar to those of the coho from Jarrard et al. [47] (i.e., 283 ± 18 vs 150 to 250 nmol/min/mg protein). These AChE activities are higher than Sandahl et al. [30] reported for 4.5 g coho (i.e., ~25 nmol/min/mg protein) and Sandahl and Jenkins [31] reported for steelhead (i.e., 34.1 nmol/min/mg protein). The difference between our values and some of the others may be due to size [48], lab [49], or sampling [48] techniques, or simply natural variation [50].

Swimming performance

The control U_{crit} values of the young adult coho in the present study compare favorably with those reported elsewhere. For example, Lee et al. [14] tested Chehalis River (BC, Canada) coho adults (≥2.3 kg) in a similar swim tunnel and determined U_{crit} to range from 1.64 to 1.68 BL/s. Cech et al. [51] found adult seawater acclimated coho (~760 g) had U_{crit} values of approximately 1.18 BL/s. With juvenile coho, Besner and Smith [52] noted that 17 g coho could maintain approximately 4 BL/s and higher at approximately 5.5 BL/s with training. Brauner [16] found similar results with 21 g coho, at 5.5 BL/s. The 238 g of the fish in the present study places their size between the adult and juvenile studies above, and so does the average control U_{crit} value of 3.44 BL/s.

The speed of the steady swimming gait that U_{crit} captures may reflect swimming speeds in the wild and so have eco-relevance. For example, the U_{crit} values Farrell et al. [40] reported for adult sockeye are similar to migration speeds assessed using telemetry (i.e., 2.5 BL/s) [53]. With respect to physiologic relevance, U_{crit} was originally thought to indicate fatigue – a point where energy reserves no longer support the level of swimming activity [9]. However, recent work has suggested that U_{crit} may not reflect a deficit in available energy, but rather the point where the size of the swim tube may preclude the use of any supervening aerobic, unsteady swimming gait [35]. If this were true, U_{crit} would indicate gait transition and so shortening the test would not affect the value. We found this to be the case: coho given U_{crit} tests and $U_{\Delta V}$ acceleration tests performed to the same maximum velocity.

Although there were no differences between the maximum velocities attained using the ramp-U_{crit} and $U_{\Delta V}$ tests, an important post-exercise metabolic difference was seen. For any given maximum velocity, plasma lactate should be constant if the recruitment of red (aerobic) and white (anaerobic) muscle utilization remains constant. Here we noted that at $U_{\Delta V}$, control coho had a 30% higher plasma lactate concentration. An increase in plasma lactate can be interpreted as greater white muscle activity, which suggests that even though coho reached similar velocities in both tests, white muscle was used to a larger extent at $U_{\Delta V}$. Consistent with this conclusion is the fact that control $U_{\Delta V}$ plasma lactate values (10.5 ± 0.8 mmol/L) were lower than reported in a 5-min chase test (e.g., ~35 mmol/L in 30 g Atlantic salmon) [54], but higher than after ramp-U_{crit} tests given to adult (2.24 kg) sockeye (4 mmol/L) [24], and the coho of the present study (8.2 ± 0.7 mmol/L).

Just as important is the finding that the highest post-exercise plasma lactate value was found in 10 µg/L chlorpyrifos-exposed fish (i.e., 14.6 ± 1.3 mmol/L). Although contaminant exposure alone could account for increases in plasma lactate concentrations [55,56], this increase in plasma lactate was not maintained in fish receiving greater chlorpyrifos exposures. Furthermore, the swim speeds of the 10 µg/L-exposed fish were no different from control. Therefore, we propose an alternative explanation for these exposure-related lactate dynamics. We suggest that the 10 µg/L-exposed fish compensated for partial AChE blockade (hence muscle recruitment) by increasing white muscle recruitment from the reserve capacity to maintain maximum swimming

performance. However, at higher levels of AChE inhibition (i.e., chlorpyrifos exposure concentrations >10 µg/L) it was no longer possible to recruit white muscle to compensate for neuromuscular blockade and so swimming performance fell as did plasma lactate concentrations.

The relationship between AChE activity and swimming

The inhibition of AChE has often been associated with increased swimming activity or hyperactivity. For example, Kumar and Chapman [57] found increased swimming activity in eastern rainbow fish (*Melanotaenia duboulayi*) exposed to the OP pesticide profenofos for 10-d. Bretaud et al. [3] found similar results with goldfish (*Carassius auratus*) following 4-h exposures to carbofuran; in fact, increased locomotor activity was detectable at an exposure concentration that was lower than the concentration which inhibited AChE activity. Work with salmon and swimming activity is in contrast to the above studies. Coho exposed to chlorpyrifos for 96-h did not display hyperactivity, but rather a concentration-dependant decrease in swimming activity [30] (Fig. 7-4). Indeed, significant decreases in swimming activity were seen at an exposure concentration as low as 0.6 µg/L, which corresponded to a relative AChE activity of 77.2% of control. Little et al. [25] likewise found that the swimming activity decreased incrementally for rainbow trout given 96-h exposures to 0.01 and 0.1 µg/L of methyl parathion (an OP insecticide), but found that U_{crit} was not affected.

Based on the activity-based outcomes discussed above (either an increase or concentration-dependent decrease), it would be reasonable to consider that any chlorpyrifos-caused hyperactivity would alter coordination and

cause a concentration-dependent U_{crit} decrease, or alternately that U_{crit} would not be affected until a threshold of AChE inhibition was reached. Our results found the latter. This threshold relationship is supported even by the equivocal results from earlier studies. Specifically, Cripe et al. [26] found that 2.2 µg/L of EPN (an OP insecticide) reduced the AChE activity of sheepshead minnow (*Cyprinodon variegatus*) to 17% of control (measured on exposure days 161 and 235) and U_{crit} to 56.7% of control (measured on exposure days 257 to 263) (Fig. 7-4). However, in the same study, 0.5 µg/L of azinphosmethyl (also an OP insecticide) caused a similar amount of AChE inhibition (i.e., 22% of control; measured at days 14 and 107) but U_{crit} (measured at days 164-169) was unaffected. Similarly, Van Dolah et al. [27] found that azinphos-methyl caused decreases in the U_{crit} of red drum (*Sciaenops ocellatus*) at roughly 2.4× the 24-h AChE EC50 (i.e. the effective concentration causing 50% inhibition) (an exposure concentration of 12 µg/L) but not in mummichog (*Fundulus heteroclitus heteroclitus*) even exposures approximately 24× the EC50. In spite of the apparently contradictory findings of the two earlier studies, given swimming performance must decline at some point of cholinergic inhibition, there appears to be a threshold relationship between AChE inhibition and swimming performance.

In our work, we found that the AChE inhibition threshold was determined by the type of muscle used for any given method of swimming. For critical swimming speed, which is powered primarily by aerobic muscle [9], this threshold occurred with an AChE activity 68.5 ± 18.1% of control. For swimming powered

by more anaerobic means (i.e., the $U_{\Delta V}$ acceleration test), this threshold was much higher: AChE activity had to be less than 52.6 ± 15.4% of control before swimming performance decreased. The higher inhibition threshold for anaerobic swimming can be explained by the difference in the amount of red and white tissue. Specifically, salmonid white muscle mass is nearly 20× that of red muscle [58], and so even with the same proportion of AChE inhibition, a greater amount of white tissue would be available to support swimming. The cost would be increased lactate production, which was the case. Although this oxygen debt did not appear to reduce swim speed, successive swimming efforts might have been impaired, and this could be tested in future using a repeat swimming protocol [20,24].

Overall, critical swimming performance remains available even when AChE inhibition reduces spontaneous swimming activity (Fig. 7-4). This suggests that any anticholinesterase exposure impairing spontaneous swimming, a swimming mode that might enable foraging, may still leave fish with maximum swimming performance, a swimming mode powered by more white muscle that could enable behaviors such as predator evasion. Furthermore, although our results indicate that the sustained swimming adult salmon may use for migration might be unaffected by low-level anticholinesterase exposure, anticholinesterase pesticides have been shown to alter the olfactory sensory neurons that guide migration [7]. Additionally, should lower concentrations cause hyperactivity (Fig. 7-4) [28,29], fish may experience increased predation through inability to remain inconspicuous. Given predators tend to preferentially select areas that permit

easier prey capture [59], fish populations in areas of organophosphorus pollution may experience increased predation.

Conclusion

Sloman and Scott [60] pointed out that understanding how contaminants alter mechanisms underlying higher order processes will greatly enhance and add depth to other studies that use more ecologically relevant behavioral endpoints. Here we demonstrated that 96-h exposures to ≥ 10 μg/L of the insecticide chlorpyrifos can impair the cholinergic nervous system and lead to a proportional increase in the use of white muscle that may differentially impair swimming behaviors such as predator avoidance.

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Figures

Figure 7-1 Brain acetylcholinesterase (AChE) activity of coho given 96-h exposures to chlorpyrifos. Curve-fit information is given on the figure. Significant differences across groups are shown by dissimilar letters (one-way Kruskal-Wallis analysis of variance, Dunn's test, p <0.05).

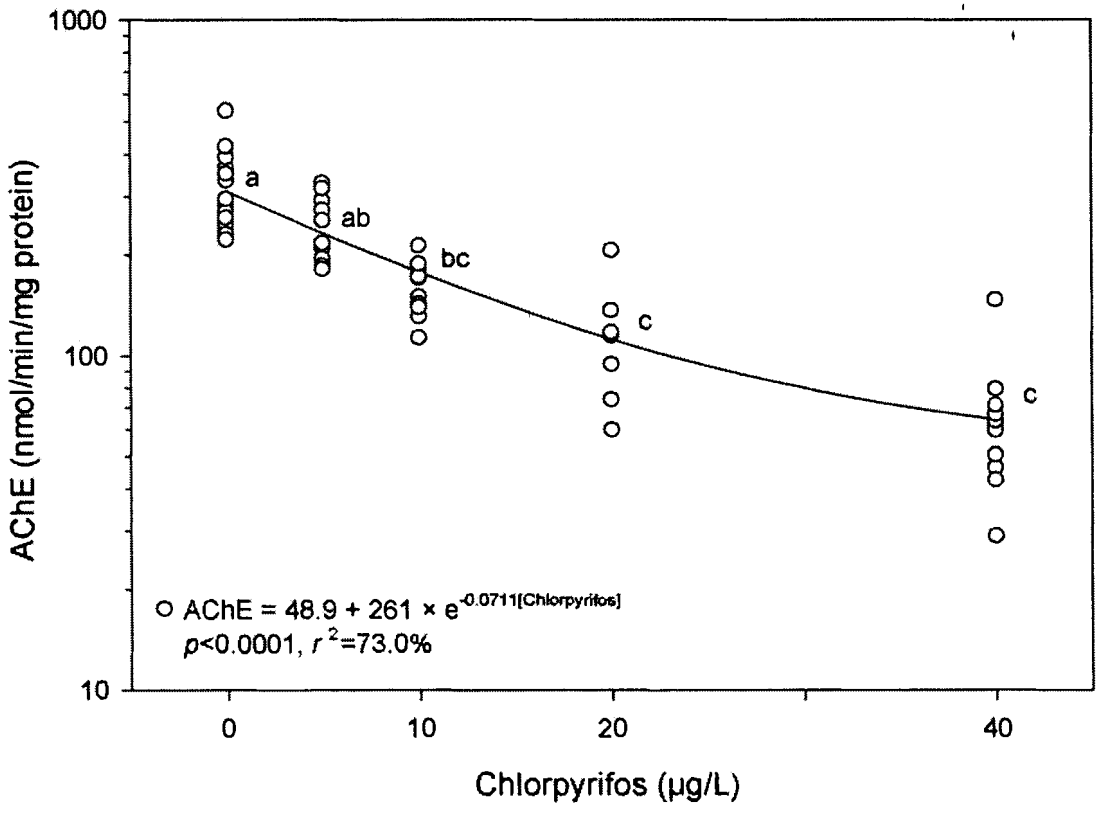


Figure 7-2 (A) The swimming performance and (B) post-exercise plasma lactate concentration (+ one standard error of the mean; SEM) of coho given a standard ramp-U_{crit} test (duration 152 ± 8 min) and an acceleration test ($U_{\Delta V}$) (27.6 ± 0.8 min) after 96-h exposures to various concentrations of chlorpyrifos. Swim speeds are given in body lengths per second (BL/s). Overall plasma lactate mean ± SEM for each test type are shown as solid and dotted lines. Significant differences across groups (*n* values given in the bars of (A)) are shown by dissimilar alphanumeric characters and within groups by asterisks ((A) two-way analysis of covariance, Tukey's test p <0.05; (B) analysis of variance, Holm-Sidak p <0.05).

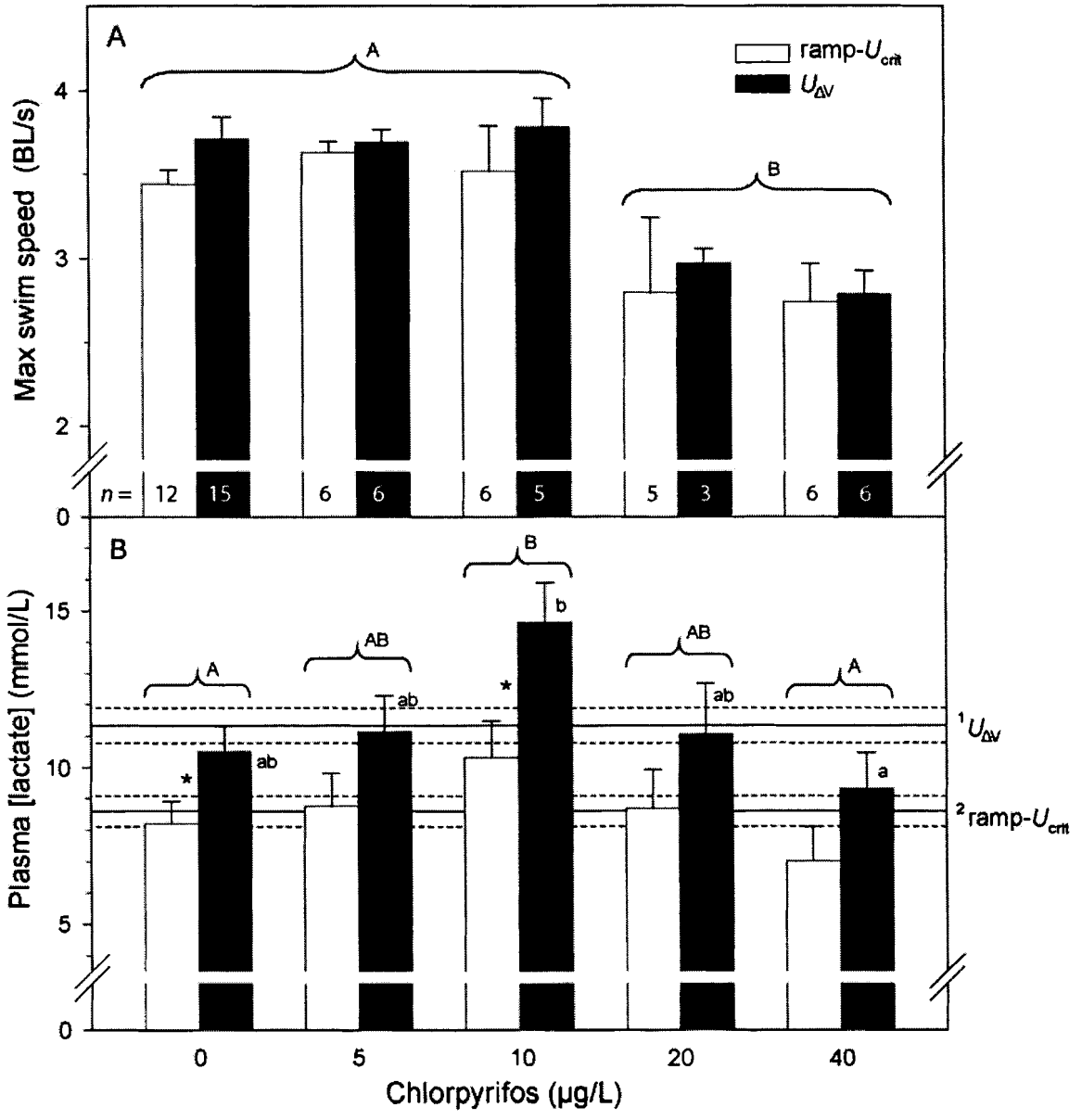


Figure 7-3 Swim speed versus brain acetylcholinesterase (AChE) activity for coho given two different swim tests after exposure to various chlorpyrifos concentrations. Breakpoint analysis was used for each test to determine where further AChE inhibition would impair swimming for an acceleration test ($U_{\Delta V}$) and a ramp-U_{crit} test. Breakpoints are shown as solid vertical lines ± standard error ($U_{\Delta V}$ test, big dash; ramp-U_{crit} test, small dash).

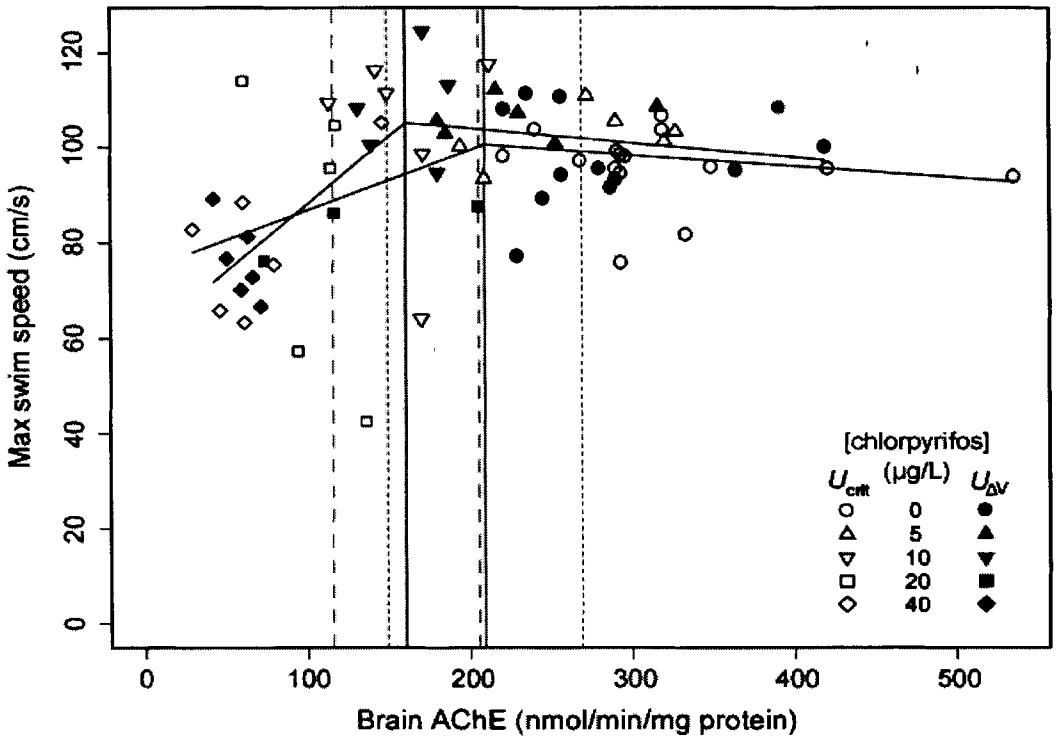
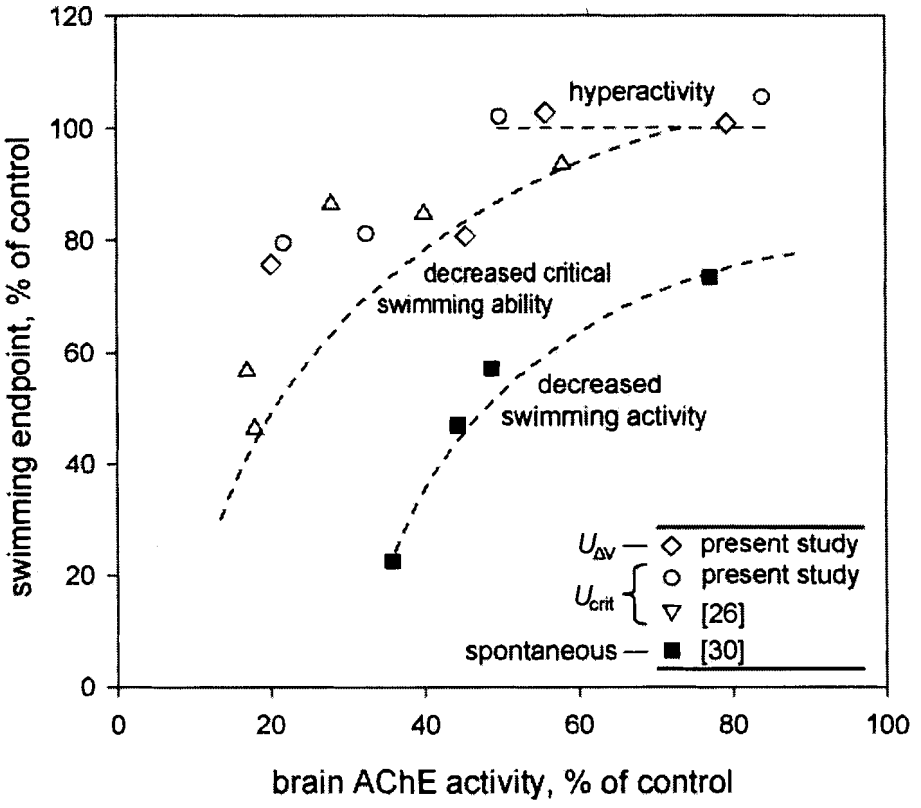


Figure 7-4 The relationship between acetylcholinesterase (AChE) inhibition and reduction in a swimming-based endpoint. Shown are data from a rapid acceleration test ($U_{\Delta V}$) and critical swimming performance test (ramp-U_{crit}) from the present study (i.e., 96-h chlorpyrifos-exposed coho), ethyl (p-nitrophenyl) benzenethiophosphonate (EPN)-exposed sheepshead minnow [26], and chlorpyrifos-exposed coho [30]. Thresholds where AChE inhibition impairs critical swimming ability (U_{crit}), swimming activity, and causes hyperactivity were made by drawing an arc through [30] data; the upper U_{crit} threshold was made by drawing an arc through the lower U_{crit} data points.



Chapter 8:
REVIEW
Olfactory toxicity in fishes

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Introduction

Animals employ a variety of sensory modalities to gather environmental information. In air, vision can provide animals with an unrestricted view over many kilometers. In water, dissolved material, depth and the density of water itself conspire to restrict vision considerably. For example, in river water, organic material and sediment may limit vision to less than one meter, and in seawater, visible light typically does not penetrate past ten meters. In such a restricted environment, the olfactory system can provide biotic and abiotic information from the near to the far.

Dissolved odorants can provide directional, conditional, tactical and relational information. Directional information may come from static or dynamic sources, a well-known static example is the homing salmon exhibit to the odorant bouquet of their natal stream (Scholz et al. 1976); a dynamic example is the searching behavior a shark exhibits up a concentration gradient of blood (Gilbert 1977). Conditional information can indicate the status of either biotic or abiotic sources; a biotic example is the ability of male sticklebacks to discriminate between males and ovulated females (McLennan 2004), an abiotic example is the ability of goldfish (*Carassius auratus*) to sense changes in environmental calcium (a correlate of salinity) (Hubbard et al. 2000). Tactical information may concern the presence of prey (Hara 2006a), or predators, through release of alarm pheromone from a nearby damaged fish (Brown 2003) or through predator scent (Rehnberg and Schreck 1987). Relational information can enable sibling

(Quinn and Hara 1986) or conspecific identification (Rajakaruna et al. 2006). With such a variety of behaviors relying upon, or at least integrating olfactory cues, it is clear why toxicological studies have explored how waterborne contaminants alter the sense of olfaction or the various behaviors it governs.

This review collates and summarizes known studies of fish olfactory toxicity in order to bring researchers up to date and identify research gaps. This review also compares and relates olfactory toxicity endpoints measured at different levels of biological organization to reveal differences in the sensitivity of the levels and determine if lower level responses can be used to predict responses from higher, more ecologically relevant-responses such as behavior.

Fish olfaction

Basic fish olfaction has been extensively reviewed (e.g. Zippel 1993; Laberge and Hara 2001; Zielinski and Hara 2001; Belanger et al. 2006; and Hamdani and Døving 2007). Here, to facilitate understanding of the impact of contaminants on olfaction, a brief description of fish olfaction is provided.

Of the sensory inputs, the well-developed olfactory tissues that most teleosts possess constitute an invaluable chemical information receiver. The sensitivity of fish olfaction is odorant dependent, but in general, fish can detect odorants in the parts per billion to trillion Belanger et al. (2006). This level coincides with concentrations of both natural odorants, such as amino acids (Shoji et al. 2003), and contaminants (Harris et al. 2007).

Olfactory perception begins with a molecular 'handshake' between an olfactory sensory neuron (OSN) and a ligand. The sensory neuron receives ligands in G-protein coupled receptors (GPCRs). In fish, this metabotropic receptor superfamily includes a diverse array of approximately 100 distinct receptors, with each neuron expressing just one type (Mombaerts 1999). Fish have a complement of olfactory receptor proteins that respond largely to dissolved odorants. Not all fish are have the same set of receptors; within catfishes (genera *Ictalurus* and *Ameiurus*) for example, differences exist in the ability to perceive the amino acid L-isoleucine from L-valine (Valentincic 2005).

GPCRs have been classified into subfamilies, which include OR, V2R and GFB (reviewed in Hamdani and Døving 2007). These subfamilies belong to morphologically distinct OSNs, in these cases, to ciliated, microvillus and crypt OSNs, respectively. Each OSN type can be distinguished microscopically, and are generally named after appearance: ciliated cells have cilia protruding from a knob, microvillus cells have larger, unciliated protruberances, while crypt cells have an apically focused ciliary grouping (Zielinski and Hara 2001; Schmachtenberg 2006). These classes have differential responses to five odorant classes: amino acid, bile salt, steroid, prostaglandin and nucleotide (Laberge and Hara 2001). OSN classes are dispersed across the olfactory epithelium (OE) into distinct regions, although OSNs expressing the same GPCR send their axons to converge on the olfactory bulb (OB) at discrete locations referred to as glomeruli or neuropils.

The various OSN classes will have one of two types of heterotrimeric GPCRs; those that stimulate phospholipase C (PLC), which produces IP_3, and those that stimulate adenylyl cyclase, which produces cAMP (Sorensen and Sato 2005). Both signaling molecules bind and affect the opening of cation (sodium or calcium) channels, and the influx of calcium activates calcium-gated chloride channels (Zhainazarov and Ache 1995). In fishes, these OSN responses have predominantly been characterized using a technique known as electro-olfactogram (EOG). Here the generator potentials of OSNs in response to ligand binding and GPCR activation are recorded. The EOG technique is time-tested, as it has been in use for greater than fifty years (Ottoson 1956) (reviewed in Scott and Scott-Johnson 2002). The terminal, glomerular responses to odorants at the OB have been recorded using a similar field potential measurement referred to as electro-encephalogram (EEG).

Following OSN response and bulbar reception, signals can be conducted from the glomeruli back to the higher brain, the telencephalon, thus permitting integration with a variety of other internal and external input. Olfaction can serve as the foundation for many complex behaviors, including alarm and avoidance response, feeding, migration, kin and conspecific recognition and mating synchronization, to name a few. Some of these responses involve physiologic components, for example the reception of priming pheromone by male salmon can lead to an increase in plasma testosterone that upregulates milt production (Waring et al. 1996). In this example, the olfactory-mediated response corresponded to a distinct stage of maturity, which is often the case. For salmon,

there are at least seven distinct behaviors (Fig. 8-1). In general, olfaction ties fish to their biotic and abiotic environment, permits survival and helps to facilitate reproduction.

Olfactory toxicity

The reception of odorants, their processing and integration with other neural and sensory input, through to the physiological and behavioral responses that can ensue constitutes a complex, invaluable system that may be interfered with by foreign agents in several ways (Fig. 8-2). The olfactory system directly transduces environmental information regarding dissolved substances via exposed OSNs. This leaves the olfactory epithelium relatively exposed to a highly variable environment, one which can bring injurious substances. There are three categories used to describe olfactory alteration: 1) anosmia, the inability to smell, 2) hyposmia, a lowered ability to smell, and 3) dysosmia, a condition where odorants are not perceived normally. Most toxic effects result in hyposmia, but since the degree of olfactory impairment is typically related to exposure concentration, increased exposure may result in functional anosmia.

Biochemical and molecular olfactory toxicity

Few studies have used biochemical or molecular techniques to determine olfactory toxicity of contaminants in fish. This is regrettable, since these techniques potentially enable a mechanistic understanding of toxicity. Some of the available endpoints include measurements of cellular enzymatic reactions,

DNA or RNA adducts, DNA mutations, or effects on cellular receptors or signal amplification proteins. One drawback of measurements at biochemical and molecular levels is that extrapolating effects to organismal and population health or survival may be difficult to impossible.

Several studies have noted that anti-acetylcholinesterase (AChE) agents such as organophosphorus and carbamate pesticides can cause decreased OSN responses (Table 8-1); some have proposed that a decrease in AChE activity is at fault (e.g. Jarrard et al. 2004; Tierney et al. 2007b). The rationale here is that since mucous production is upregulated by peripheral secretion of acetylcholine (ACh) (Inglis et al. 1997), anti-AChE agents may alter mucous dynamics. However, some environmental conditions irritate the tissue and likewise promote increased mucous secretion (e.g. low pH in rainbow trout (*Oncorhynchus mykiss*); Miller and Mackay 1982, Klaprat et al. 1988). This suggests that if mucous secretion can affect OSN responses, there may be multiple pathways in which this toxicity could occur. Pesticide exposure can affect other enzymes within olfactory tissue. For example, 1 mg/L diazinon exposure reduced the amount of the enzyme tyrosine hydroxylase (TH) (which facilitates catecholamine production) in the olfactory bulb of Japanese medaka (*Oryzias latipes*) (Shin et al. 2001). The relevance of such enzyme impairment to decreased olfactory responses, either at the epithelium or bulbar tissue remains unclear.

Genotoxic compounds have the capacity to form DNA adducts. Although we are unaware of work on fish olfactory DNA adducts, examples exist for mammals exposed to toxicants. For instance, in Fischer rats (*Rattus norvegicus*)

given aerial exposure to 500 ppm propylene oxide for 20-d, 58.5 out of 106 guanine nucleotides were converted to the adduct 7-(2-hydroxypropyl) guanine (7-HPG) (Segerback et al. 1998). Similarly, 20-min exposure to dimethyl sulfate formed N7-methylguanine and N3-methyladenine within 30-min of exposure (Mathison et al. 1995). Since the toxicity of carcinogenic compounds typically evolves over long-term or repeated exposures, olfactory dysfunction through genotoxic mechanisms may take time to develop.

Receptor level effects hold promise for determining toxicity mechanisms, since many neurotoxic agents mediate their toxicity through receptor modification (Tierney and Kennedy 2007). At least one study has already noted that OSNs expressing different GPCR proteins can be differentially sensitive to certain pesticides (Tierney et al. 2007b). Further studies could use biochemical techniques (e.g. inhibit or stimulate known portions of the GPCR signaling pathway) or molecular techniques (e.g. alter receptor protein expression) to further determine pesticide targets. Another potential avenue for research is into possible contaminant-caused modification of receptor protein expression, since certain fish exhibit expression changes during the lifecycle. For example, receptor protein DNA transcripts increased 50-fold during the parr-smolt transformation in Atlantic salmon (*Salmo salar*) (Dukes et al. 2004). Numerous potential molecular toxicity avenues remain open for study.

Electrophysiological olfactory toxicity

In situ preparations of tissues such as the olfactory rosette or bulb can be used to relatively rapidly identify compounds or agents that may cause olfactory toxicity. In the below sections, the effects of injurious agents on cells of the olfactory rosette and bulb are considered in order pH, metals, pesticides and other organics. Data from both rosette (EOG) and bulbar (EEG) recording locations typically vary proportionately with each other (Baldwin et al. 2003, Sandahl et al. 2004; Hara 2006b), and are considered together below.

pH

Both acidity and alkalinity appear to alter fish OSN responses, and the effects do not appear specific to OSN class. The EOGs responses of male Atlantic salmon to testosterone and dilute female urine were reduced in a concentration-dependent manner within 5-min of exposure to pH changes > or < 7.5 (Moore 1994) (Table 8-1). Chronic acid exposure also results in EOG impairment, as after a two-week exposure to pH 4.7, L-serine responses of rainbow trout to were reduced by ~50% (Klaprat et al. 1988). Together, these data suggest pH can cause acute and persisting impairment of olfactory function.

Metals

Metals are well-known for their effectiveness as blockers of ion channels, such as sodium or calcium channels (reviewed in Florea and Busselberg (2006)). In studies of fish olfactory toxicity, copper has received the most attention (Table 8-1). This metal can cause a concentration-dependent decrease in EOG/EEG, with negative effects occurring at ≤10 µg/L. For example, the perfusion of copper

caused a steady decline in coho salmon (*Oncorhynchus kisutch*) OSN responses over 30-min exposures (Baldwin et al. 2003). In particular, 5-min into a 10 µg/L copper exposure, EOG responses were ~80% of the pre-exposure value. At 30-min, the value was ~30% of pre-exposure. Recovery was not rapid, as even 90-min following exposure cessation, the responses were only ~60%. Recovery over several days was explored in chum salmon (*Oncorhynchus keta*), here EOG responses appeared to return to control within 1-d following exposure to concentrations less than 24 µg/L (Sandahl et al. 2006). The toxicity of copper does not appear specific to OSN receptor class, as exposure caused similar decreases in L-serine as it did in TChA responses (Baldwin et al. 2003). These findings imply that copper exposure can have a general, potentially necrotizing effect on olfactory tissue, as suggested by Thompson and Hara (1977).

Copper OSN toxicity can be offset by increasing calcium (Bjerselius et al. 1993), which indicates copper's toxicity may at least partially be exerted through calcium channel alteration and not the olfactory receptors themselves (Rehnberg and Schreck 1986). Furthermore, lowering the availability of the copper ions through addition of sodium bicarbonate mitigates copper toxicity (Table 8-1). For example, in 4 mM bicarbonate, 10 µM copper decreased Atlantic salmon EOG to 50% within 5-min, while without bicarbonate, this exposure eliminated EOG response (Winberg et al. 1992). Species-specific differences in metal sensitivities exist, as rainbow trout were more sensitive than chinook to copper exposure (Hansen et al. 1999b).

Although copper has received much attention, it was not the first metal investigated for olfactory toxicity. The very first salmon EOG study used mercuric chloride ($HgCl_2$) as a blocking agent for Atlantic salmon OSN responses to a various amino acids (Sutterlin and Sutterlin 1971). Here 10^{-4} M (~27 mg/L) mercuric chloride effectively eliminated all amino acid responses up to 1-hr post-exposure. Other studies have continued to use $HgCl_2$ for this purpose, including, Hara et al. (1976), Thompson and Hara (1977) and Baatrup et al. (1990). The last of these studies found that within 2-min of exposure to 10^{-5} M $HgCl_2$, the EOG responses of Atlantic salmon to 340 µM ($10^{-3.47}$ M) L-alanine was reduced to 35% of the pre-exposure value. With cessation, these responses recovered to 50%. Methylation appears to enhance mercury's toxicity, as EOG responses did not recover within 10-min of exposure to the same concentration of methyl mercury (CH_3HgCl) (Baatrup et al. 1990).

Aluminum is also likely toxic to OSNs, as Klaprat et al. (1988) found that when added to low pH, it caused reductions in rainbow trout EOG that were greater than pH caused alone (i.e. 35% greater). To confirm its toxicity, aluminum will need to be tested on its own.

An investigation into the toxicity of lakes contaminated with mining effluent consisting of cadmium, copper, nickel and zinc, to Arctic char (*Salvelinus aplinus*) determined that cadmium but not nickel or zinc also can impair OSNs (Thompson and Hara 1977). In comparison to copper ($CuSO_4$), which reduced EEG to ~10% of pre-exposure within 2-min, cadmium ($CdCl_2$) reduced EEG by ~50% over a 10-min period. The effects of cadmium also recovered more rapidly, as near-

normal responses returned within 2-min, whereas those of copper took 10-min. There is indication that other heavy metals pose a threat to olfaction. Gold reduced the responses of palatal (mouth) chemoreceptors of carp (*Cyprinus carpio*), in some cases by as much as 50% following exposure to $10^{-4.3}$ M (~18 mg/L) $NaAuCl_4$ (Hidaka 1970). In comparison to copper, most other metals negatively affect fish EOG/EEG in the mg not µg/L concentration range.

Pesticides and other organics

Several classes of pesticides affect fish olfactory responses (Table 8-1). Those that do typically have a rapid effect on OSN responses (<2-min), which in some cases can be specific to a particular OSN class. Pesticide-mediated OSN impairments often remain constant throughout the exposure period, with responses generally recovering from 30-min exposures within 5 to 20-min of exposure. These data suggest that the mechanisms affecting pesticide toxicity differ from the general effects of copper. Below, available data have been summarized according to pesticide class, which often indicates mechanism of action.

Carbamate – EOG responses can be rapidly decreased by exposure to µg/L concentrations of carbamates (Table 8-1). The fungicide IPBC is the most toxic examined thus far, as a 30-min exposure to just 0.1 µg/L reduced coho EOG by as much as 40% (Jarrard et al. 2004). Other carbamates appear less toxic; carbofuran, for example, required almost 10 µg/L to reach the same impairment (Jarrard et al. 2004). Concordantly, the mechanism of toxicity for IPBC is suspected to differ from other carbamates (i.e. may not be mediated

through anti-AChE effects) (Juergensen et al. 2000). Carbaryl impaired both L-serine and TChA-evoked EOGs of three salmonids (*O. kisutch*, *mykiss* and *nerka*) within 30-min, although the TChA responses appeared to be more resistant (Tierney et al. 2007b). Here the differences across OSN class were not large enough to suggest that the mechanism of toxic action of carbamates may specific to certain OSNs. Yet, with OSN classes often differing in morphology, receptor and transduction machinery, it would be surprising if some pesticides did not have OSN class-specific effects.

Organophosphorus (OP) – As with carbamates, these anti-AChE agents can rapidly reduce EOG responses (Table 8-1). Chlorpyrifos appears the most toxic; 30-min exposure to 625 ng/L chlorpyrifos exposure reduced coho L-serine and TChA-evoked EOGs and EEGs to 75% of control (Sandahl et al. 2004). A third receptor class, those that respond to pheromones, was also tested with an OP. Here 30-min exposure to diazinon caused a concentration-dependent decrease in EOG responses to the priming pheromone prostaglandin F2α ($PGF_{2\alpha}$) in Atlantic salmon (Moore and Waring 1996b) (Table 8-1). Per concentration, the decreases OP exposure can cause in EOG responses are comparable to those of carbamates, which may be related to their similar mechanism of toxic action.

Phenylurea – One phenylurea herbicide has been tested, and it is the first example of marked OSN class-specific toxicity (Table 8-1). Exposure to the phenylurea herbicide linuron caused toxicity to one class of OSNs but not another (Tierney et al. 2007b). Specifically, the L-serine-evoked EOG responses

of three salmonids (*O. kisutch*, *mykiss* and *nerka*) were reduced from 50 to 80% of their pre-exposure values following 30-min exposure to 10 µg/L, yet the bile salt (TChA) -evoked responses were not affected for any of the species, even by 100 µg/L exposure. Since ciliated OSNs respond to three odorant classes (pheromone, amino acid and bile salt) and microvillar respond to just amino acids (Sato and Suzuki 2001), it is possible that microvillar OSNs are more susceptible to this herbicide. This differential toxicity highlights the potential dissimilarity between the general effects of metal exposure and the potentially specific effects of pesticides.

Pyrethroids – There is reason to expect that these pesticides will affect salmon olfaction *a priori*, since they act by delaying closure of sodium channels (Narahashi 1996). In fact, effects on salmon EOG have been noted in separate studies of cypermethrin and esfenvalerate, although very different results were found for each. A strict comparison cannot be made though, as the concentration and exposure periods were considerably different. Acute (30-min) effects were not noted in OSN responses of coho following exposure to 0.2 µg/L of esfenvalerate, however, at the olfactory bulb, an increase was noted in low basal activity (≤10 Hz frequency bursts increased) (Sandahl et al. 2004). With cypermethrin, EOG effects were noted, but over a sub-chronic (4-d) exposure (Moore and Waring 2001). For exposure to <4 ng/L, PGF2α evoked EOGs were only 12% of control, and L-serine evoked EOGs were 17%. These decreases are the highest thus far noted in an EOG study, and they were caused by the lowest exposure concentration. This finding highlights the danger of

extrapolating a short, lab-based study to the environment, where low concentration, continuous exposure may occur.

Triazines – As with carbamates, OPs and phenylurea pesticides, triazine herbicides are efficacious at impairing EOG in the µg/L range (Table 8-1). Moore and Waring (1998) noted decreases in Atlantic salmon EOGs evoked by $PGF_{2\alpha}$ with atrazine exposures greater than 2 µg/L. Amino acid responses can be similarly affected, as decreased EOGs evoked by both L-serine and L-histidine have been noted (Moore and Lower 2001 and Tierney et al. 2007c, respectively). For example, in Atlantic salmon, 30-min exposure to 2 µg/L reduced L-serine-evoked EOGs to ~70% of pre-exposure, and for rainbow trout, 30-min exposure to 10 µg/L reduced L-histidine 45% of pre-exposure (Moore and Lower 2001). Simazine and atrazine likely share a mechanism of EOG toxicity since in combination their effects were additive (Moore and Lower 2001) (Table 8-1).

Other pesticides – There are numerous other current-use pesticides and pesticide formulations that potentially have relevance to fish olfaction (i.e. are found in salmon-bearing waters). Of those that have been tested, few appear to affect olfactory responses in the low µg/L range that coincides with the upper end of some environmentally-observed concentrations. The 2,6-dinitroaniline herbicide trifluralin and organochlorine insecticide endosulfan affected coho EOG, but marginally so and only at concentrations similar to their solubility (i.e. high µg/L concentrations) (Tierney et al. 2006a) (Table 8-1). Exposure to a high (100 mg/L) concentration of the phenoxyacetic herbicide 2,4-D appeared to eliminate coho EOG responses altogether, and cause a persisting damage

(Tierney et al. 2006a). A similar concentration of glyphosate also eliminated EOG, but recovery followed (Tierney et al. 2006a). Given glyphosate resembles an amino acid (glycine), the reductions and recovery in L-serine-evoked EOGs may have been caused by some receptor specificity overlap. The pesticide formulation in which glyphosate serves as the active ingredient, Roundup, is considerably (>10×) more toxic than the active ingredient alone (Tierney et al. 2007c). In fact, an exposure of 100 µg/L (of active ingredient in formulation) resulted in a persisting (>20-min) 50% EOG impairment in rainbow trout (Tierney et al. 2007c). The greater effects observed with Roundup may have been due to 'inert ingredients' such as surfactants since these are known to be olfactory toxicants (Sutterlin et al. 1971).

Surfactants – The second electrophysiology study into the effects of contaminants on fish olfaction tested the effect of 150 surfactants on the EEG responses of Atlantic salmon (Sutterlin et al. 1971). Because of the volume of tests it is not possible to present the results for each surfactant. Rather, the effects of several classes can be presented (Table 8-1). Examples of EEG effects include anionic surfactants such as alkyl benzene sulfonates (ABS), diamines and quaternary ammonia compounds. A subsequent EOGs study found sodium laurel sulfonate (SLS) depressed L-serine evoked responses in whitefish (*Coregonus clupeaformis*) by 50% following exposure to 0.5 mg/L (Hara and Thompson 1978). Given surfactants are sometimes used as pesticide formulations, and since a pesticide formulation (Roundup) was found to be of at least 10× greater toxicity than its active ingredient alone (Tierney et al. 2007c), it

would be beneficial to report inert ingredients on pesticide formulations, as well as perhaps monitor environmental surfactant levels.

Anatomical olfactory toxicity

The effects of toxic agents may be evident in changes to internal or external cellular appearance, or in cellular death or growth. Unlike electrophysiological techniques, quantification of the extent of alteration through histochemical means represents a challenge (Bernet et al. 1999). Nevertheless, as with *in situ* cellular responses such as EOG/EEG, histology temporally captures a condition, which may be affected by toxic agents.

pH

Moderate acid (pH 4.7) exposure alone does not appear to cause ciliary loss (Klaprat et al. 1988); data are unavailable for the effects of alkalinity on the olfactory epithelium. Given pH alterations have an effect on EOG responses, conceivably not all pH-mediated effects are apparent through structural observation.

Metals

Metals are the focus of several histological studies, with mercury and copper dominating as with EOG/EEG studies. Copper toxicity has been explored histochemically in a variety of ways, and the effects are concentration-dependant. The number of OSNs in chum salmon (*O. keta*) taking up fluorescent dye increased following 3 and 8 µg/L exposures but decreased with 24 and 58 µg/L 4-hr exposures (Sandahl et al. 2006), which suggests membrane function

may be affected. Recovery was not complete even after 10-d. For brown trout (*Salmo trutta*), an intermediate concentration (18 µg/L) caused ciliary loss within a day, and recovery took up to 8-d (assessed using TEM and SEM) (Moran et al. 1992). For rainbow trout, a similar concentration (20 µg/L) caused changes in OSNs consistent with apoptotic responses with 15-d exposure (Julliard et al. 1996). Copper exposure concentrations of ≥50 µg/L reduced the number of ciliated and microvillar cells in chinook and rainbow trout within 1 to 4-hr of exposure (Hansen et al. 1999b). This exposure was associated with loss of cilia and rupture of microvillar cells. Lengthy copper exposure can alter cell growth and death. For example, increases were noted in the number of globlet cells and degenerating cells in sections of rainbow trout olfactory epithelium chronically (40-wk) exposed to 20 and 40 µg/L copper (Saucier and Astic 1995). These changes gradually reversed, with 6 and 14-wks required for recovery from the respective concentrations. Combining these various results for copper exposure, it appears that copper causes anatomical changes in the olfactory epithelium that range from slight to severe following low to high µg/L range exposure, respectively.

Aluminum may cause anatomical alteration in the olfactory epithelium at similar concentration range as copper. Klaprat et al. (1988) found that aluminum (9.5 µg/L) in combination with moderate acid (pH 4.7), caused significant ciliary destruction in rainbow trout. Further studies could test aluminum on its own to determine its effects.

Metal accumulation may occur in the olfactory epithelium, which may give rise to longer term metal toxicity. Mercuric chloride ($HgCl_2$) accumulated around the cellular borders of OSNs in Atlantic salmon, while methyl mercury (CH_3HgCl) given in food accumulated in OSN lysosomes and inclusion bodies (Baatrup and Døving 1990). Furthermore, through anterograde (forward moving) transport up the axons, such metals can make their way into the olfactory bulb (Tallkvist et al. 1998), potentially causing impairment in bulbar olfactory responses.

Pesticides and other organics

There do not appear to be any anatomical studies of pesticide effects on the olfactory epithelium of fish, but there are studies of other organics. In fact, the first study into the effects of contaminants on fish olfactory epithelium did not use metals, but rather surfactants. Here yellow bullheads (*Ictalurus natalis*) exposed to hard (degradation resistant) and soft (degradable) alkyl benzene sulfonate (ABS) surfactants experienced a thickening of the OSNs that was not repaired within 6-wks of exposure to 4 and 5 µg/L (Bardach et al. 1965). This resulted in impaired olfaction, as exposed fish were unable to locate distant food pellets as well as control fish. Exposure to 0.03 to 0.1% of the non-ionic detergent Triton X-100 caused loss of olfactory epithelium cells in channel catfish (*Ictalurus punctatus*) and regeneration was apparent within 4 days (Cancalon 1983). These authors suggested that membrane receptor proteins were solubilized by the detergent.

As with metal exposure, the anesthetic tricaine methanesulfonate (MS222) was determined to destroy cilia in channel catfish, with repair taking up to 28

days (Lewis et al. 1985). This is may be cause for concern given MS222's wide use in electrophysiological studies and the importance of cilia to OSN responses.

Hydrocarbon exposure appears to alter cellular turnover in olfactory tissue. For tidewater silverside (*Menidia beryllina*) exposed for seven-days to whole crude oil (WHO) and water-soluble fractions (WSF) of crude oil, aberrant growth (hyperplasic; i.e. increase in cell number) and death of supporting (sustentacular) olfactory cells, as well as death of sensory cells, occurred at concentrations of 5 mg WHO and 5% WSF (21-30-d exposure) (Solangi and Overstreet 1982). The hogchoker (*Trinectes maculates*) exhibited similar cell death, however at higher, 100 mg/L WHO and 50% WSF exposure concentrations (Solangi and Overstreet 1982). Increased cell death may be attributable to the oxidative stress that hydrocarbons impart (Xue and Warshawsky 2005). Differences across cell types may be partially due to intrinsic differences in the expression of enzymes that protect against such stress (e.g. GSTs).

While anatomical studies can effectively illustrate the effects contaminants can have at the cellular and tissue level, they cannot demonstrate how these effects may translate to alterations in animal behavior.

Behavioral olfactory toxicity

Behavioral responses are intended to improve an organism's position. Like other measures of toxicity, behavioral responses can be affected by various endogenous factors, such as developmental stage and sex, and exogenous

factors, such as temperature and salinity. Unlike the preceding levels of olfactory-based responses, these responses potentially integrate many inputs – those of the present and those of the past. Olfactory-mediated behaviors may be innate or based on memorized, acquired responses. Both sources rely on olfactory input, and therefore either type is amenable to olfactory toxicity testing. However, with potentially wide signal integration, non-olfactory-based input may figure into behavioral responses, which represents a consideration for attributing olfactory causality to certain behavioral responses. Consider an organism exposed to a neurotoxic contaminant may experience altered olfactory perception, and through the uptake, distribution and metabolism of the contaminant, also experience other toxic effects. There are various examples of this; recently Tierney et al. (2007c) found that juvenile rainbow trout exposed to (1µg/L) atrazine experienced a decrease in L-histidine preference response and an increase in swimming activity. Such alteration of swimming activity has also been observed in goldfish following (>5 µg/L) carbofuran exposure (Bretaud et al. 2002). These alterations are neither behavioral, although sometimes referred to as such, nor olfactory based. In the below discussion of ethotoxicity, care has been given to focus discussion on those contaminants that affect behavior chiefly through olfactory modification.

Behavioral responses to contaminants

Nervous input from olfactory, gustatory and solitary chemosensory cells can supply environmental chemical information and modulate contaminant exposure. Unpleasant or painful stimuli will stereotypically and reflexively evoke

avoidance behavior. However, there is no guarantee that nociception (pain) is associated with contaminant exposure. For example, fish sometimes exhibit attraction response to pesticides (e.g. Saglio et al. 2001; Table 8-2). Overall, any attraction or repulsion is proportional to a contaminant's perceived toxicity, which is dependant on its concentration and detection threshold. Below, preference/avoidance responses to various contaminants, which have been measured since at least 1915 (Shelford and Powers 1915), are summarized and categorized according to contaminant class.

pH

Preference/avoidance response to pH change remains largely untested. Avoidance of acidity has been noted for at least one species, as Jones et al. (1985b) found arctic char avoided water flows of pH ≤5.5. Remaining data on pH is for its impact on behavioral responses to other odorants (which is given later in the text).

Metals

Fish avoid many metals. Specifically, arsenic, cadmium, chromium, cobalt, copper, iron, mercury, nickel, selenium, and zinc are avoided to varying degrees (Table 8-2). Avoidance thresholds for some of these exist in the µg/L range (e.g. copper and nickel), while others are in the mg/L range (e.g. iron and mercury).

Copper was avoided by chinook and rainbow, with chinook (*O. tshawytscha*) exhibiting higher sensitivity (0.7 vs. 1.6 µg Cu/L) (Hansen et al. 1999a). The avoidance of copper can also be concentration specific: in two

cases fishes avoided low but not high concentrations (Giattina et al. 1982, Hansen et al. 1999a). Since copper can impair the olfactory epithelium within minutes (Baldwin et al. 2003), conceivably a copper plume could impair neurological detection before the behavioral response threshold is reached.

Nickel, as with copper, evokes avoidance/attraction responses that can depend on concentration. However, in this case, rainbow trout were attracted to low (6 µg/L) but avoided higher (24 µg/L) concentrations (Giattina et al. 1982). With zinc, avoidance responses were noted for rainbow trout at concentrations greater than 5.6 µg/L (Sprague 1968). Zinc, along with copper, cadmium and lead, were constituents of mixture designed to resemble a river (Clark Fork River, MO, USA) (Hansen et al. 1999c). Here rainbow avoided a concentration similar to only 10% (6.6 µg/L) strength river water (Table 8-2). Rainbow trout exhibited a lower avoidance threshold than brown trout (Hansen et al. 1999c). The major constituents of the mixture were copper and zinc, both of which can evoke avoidance at concentrations similar to or lower than those observed with 10% of the mixture. Fathead minnow also avoided a mixture designed to resemble a river (New River, Virginia, USA), and here the avoidance was found to depend on the season (Hartwell et al. 1987b) (Table 8-2).

Cobalt is avoided at higher concentrations than either copper or zinc (e.g. 24 µg/L for chinook) (Hansen et al. 1999a) (Table 8-2). Like copper, species-specific sensitivities exist: for rainbow, the threshold was 7.5× greater. Higher (mg/L) amounts of iron were aversive to coho (Updegraff and Sykora 1976)

(Table 8-2). Similarly, avoidance of mercuric chloride occurred at a high (272 mg/L) concentration in ninespine stickleback (*Pungitius pungitius*) (Jones 1947).

One study demonstrated just how aversive responses can affect wild fish populations. A mixture of copper and zinc ≥ 35-43% of LC_{50} (proportions not given) caused an increase in the number of Atlantic salmon that returned downstream rather than continue upstream during return migration (Saunders and Sprague 1967). At 80% of LC_{50}, upstream movement was eliminated. The authors noted these 'avoidance' thresholds were higher than other lab-based studies, but pointed out that the lifestage likely provided a motivational force that may have effectively increased the avoidance response threshold.

Pesticides and other organics

With metals, certain environmental concentrations are likely benign or even beneficial, as they serve a variety of roles such as helping to maintain ionic balances across exposed membranes, which may help explain why metal attraction responses exist. With pesticides, it is more challenging to conceive of any health benefits from their exposure, and so attraction responses should not be considered useful. Yet, some pesticides do evoke attraction responses.

Various organophosphates and carbamates evoke avoidance responses (Table 8-2). For example, fenitrothion was avoided by goldfish (Scherer 1975) and medaka (Hidaka and Tatsukawa 1989) at 10 and 90 µg/L, respectively. Not all OPs and carbamates evoke avoidance, at least for all species, as sheepshead minnow (*Cyprinodon variegatus*) did not avoid malathion or carbaryl formulation (Hansen 1969). Mosquito fish (*Gambusia affinis*) avoided a similar suite except

for a low (0.01 mg/L) concentration of endrin (Hansen et al. 1972). In another study, a higher concentration endrin was avoided, as were the pesticides DDT, toxaphene and parathion (Kynard 1974). In a population of mosquitofish that had been captured near agricultural activities, the avoidance of parathion was reduced from 0.2 to 1 mg/L (Kynard 1974), suggesting either neuroprotection (adaptation) or persisting damage.

Glyphosate, the active ingredient of Roundup, was not avoided by rainbow trout even at a concentration of 10 mg/L (Folmar 1976). However, another paper found Roundup was avoided by rainbow trout, at the same active ingredient concentration (10 mg/L) (Tierney et al. 2007c). 'Inert' ingredients in Roundup are known to have sometimes included surfactants such as POEA (polyethoxylated tallow amine). Given surfactants are among the most avoided and toxic chemicals to OSNs (see below), the avoidance response to the formulation is unsurprising.

Alarmingly, some pesticides evoke attraction responses. Saglio et al. (2001) noted goldfish were attracted to prochloraz and nicosulfuron concentrations of 1 and 10 mg/L, and bentazone concentrations of 0.01 and 10 mg/L (Table 8-2). The implication of this finding to an environmental setting is that not only may fish fail to leave an impacted site when given the choice, but they may chose to occupy areas of pesticide pollution. The ramification of this counterintuitive response to survival is axiomatic.

Hydrocarbons and some of their constituents can evoke avoidance responses (Blaxter and Hallers-Tjabbes 1992) (Table 8-2). For example, coho

salmon avoided 3.2 mg/L of PAHs (Weber et al. 1981). The avoidance threshold of hydrocarbons for coho appeared dependent on lifestage. Specifically, coho parr avoided concentrations of 3-4 mg/L of monocyclic hydrocarbons while smolts avoided ≤2 mg/L (Maynard and Weber 1981). Curiously, three mixture constituents (benzene, toluene and O-xylene) had lower thresholds (Table 8-2), especially O-xylene (0.2 mg/L). A similar concentration (1.7 mg/L) of coal distillates (total phenols) evoked avoidance in fathead minnow (*Pimephales promelas*) (Dauble et al. 1985). Carbon dioxide also exerts concentration-specific avoidance/attraction in Arctic char, with the avoidance threshold at >50 µM (Jones et al. 1985b). Ninespine stickleback were found to avoid ethanol, chloroform and formalin, but all at fairly high concentrations (Table 8-2) (Jones 1947).

PCBs appear to evoke avoidance, although a considerable species-specific difference exists in the available data. In the avoidance of Aroclor (a PCB mixture), pinfish (*Lagodon rhomboids*) avoided 10 mg/L, mosquitofish avoided 0.01 mg/L, and sheepshead minnows didn't respond at all (up to 10 mg/L) (Hansen et al. 1974) (Table 8-2). This interspecies response variation (>1000×) is great, but not as large as observed for a surfactant SLS. With SLS, Ishida and Kobayashi (1995) noted an avoidance response at 0.01 µg/L for carp whereas Hara and Thompson (1978) noted an *attraction* response at 0.1 mg/L for whitefish. This variation is unprecedented in the data set, and highlights the difficulty in predicting avoidance responses across fishes.

Other contaminants

The earliest avoidance/preference study to contaminants that the authors are aware of explored the avoidance of hydrogen sulfide by herring (*Clupea pallasii*) (Shelford and Powers 1915). Unfortunately, methods limited the resolution of concentration. Nevertheless, hydrogen sulfide did appear to evoke an avoidance response. In a more recent study (Hall et al. 1984), striped bass (*Morone saxatilis*) and Atlantic menhaden (*Brevoortia tyrannus*) avoided low mg/L hydrogren sulfite concentrations, and the avoidance threshold appeared to decrease with increasing temperature.

Chlorine on its own or in other compounds or mixtures can evoke avoidance responses (Table 8-2). Both chlorine and chloramine (at concentrations ≥70 µg/L) were avoided by dace (*Rhinichthys atratulus*) (Fava and Chu-Fa 1978). Similarly, coho and shiner perch (*Cymatogaster aggregate*) avoided chlorine, albeit at a higher concentration (Stober et al. 1980). Intriguingly, with low concentration and elevated temperature, shiner perch exhibited an attraction response (Stober et al. 1980). Both bleached (i.e. with chlorine) an unbleached kraft pulpmill effluent (KME and BKME, respectively) can be aversive at low concentrations (Table 8-2). Atlantic salmon avoided 0.001% (Sprague and Drury 1969), while pinfish and gulf killifish (*Fundulus grandis*) avoided 0.1% BKME (Lewis and Livingston 1977). Two components within pulp mill effluent were avoided by herring, albeit in the mg/L range (Wildish et al. 1977) (Table 8-2). Given chlorine is also BKME constituent, the avoidance responses may be partially due to its presence.

Behavioral responses following contaminant exposure

Exposure to contaminants can cause the reduced, altered or eliminated perception of odorants, which can lead to changes in behaviors. Fewer studies have tested behavioral modification following exposure than for outright attraction or avoidance of exposure. Many of the contaminants identified as toxic using EOG/EEG response have also been associated with impaired behavioral responses. The types of exposure-driven behavioral changes discussed below include modification of migration, preference/avoidance responses, both to contaminants or natural odorants, and the alteration of alarm response.

As with physiological measurements, alterations in behavior lend themselves to quantification. However, in some cases, it can be difficult to separate olfactory toxicity from other forms of toxicity. For instance, should a fish no longer respond to a food cue, it may appear that olfactory impairment is at cause. However, in many cases, food cues are visual. The lack of response to a visual cue is likely due to the inability or unwillingness to respond to the cue, perhaps through systemic neurotoxicity, and is not from olfactory toxicity. For this reason, predator-prey and food cue experiments using vision are not considered here.

pH

Decreased pH appears capable of altering both preference and avoidance responses. With a 30-min exposure to pH 5.1 (a pH decrease of 2.5 units), Atlantic salmon lost a preference response to L-glycine and an avoidance response to L-alanine switched to a preference response (Royce-Malmgren and

Watson 1987). With a 14-d exposure to pH 4.5 to 4.75, Actic char exhibited decreased attraction to a food odorant (Jones et al. 1985a). Given lowered pH can alter OSN responses in a concentration-dependent manner (Moore 1994), these data affirm that the perceived concentration of a behaviorally-relevant odorant may determine its preference or avoidance.

Metals

Available studies show metal exposure can alter preference/avoidance (Table 8-3). As would be expected, copper exposure has the capacity to subsequently inhibit the avoidance of other substances. For instance, ninespine stickleback exposed to 635 mg/L copper for at least 5-min ceased to avoid chloroform and formalin (Jones 1947). At lower metal concentrations, adaptation may be possible and this may permit retention of sensory discriminatory abilities. After a lengthy (45-d) exposure to a metal mixture, rainbow trout chose clean water over the metal mixture (Hansen et al. 1999c). Similarly, chronic exposure of coho to iron (1.20 mg Fe/L, to ~4 cm fry) did not alter the subsequent avoidance response to greater (4.25-6.45 mg/L) amounts of iron (Updegraff and Sykora 1976). In contrast to this, a study of fathead minnow exposed to a simulated metal-impacted stream found that the preference/avoidance to greater metal mixture concentrations was dependent on the length of exposure, with fish preferring three-times the exposure concentration after 3-mos, avoiding five-times the amount at 6-mos, and losing all response to ten-times the amount at 9-mos (Hartwell et al. 1987a).

Alarm response behavior has been the focus of several basic research studies (e.g. Berejikian et al. 1999, Brown and Smith 1997, Pollock et al. 2003, reviewed in Døving et al. 2005) and has received some toxicological application. Alarm behaviors represent a means to limit predation, and can include dashing and hiding. This behavior can arise from innate sources, which may or may not include an olfactory component, and acquired sources, such as the scent of a damaged conspecific in conjunction with predator scent. The scent of a damaged conspecific, referred to as 'alarm pheromone' is known for some fishes (e.g. goldfish), but unknown for the vast majority (Brown 2003). Alarm response can represent a molecular reflexive altruism since it can be proportional to relatedness (Mirza and Chivers 2002). Of the metals, thus far cadmium has been determined to alter alarm response (Scott et al. 2003). Pesticides also alter components of this responses, and they are given below.

Pesticides and other organics

An ecologically relevant example of an impaired behavioral response was for the fidelity of return migrating chinook following exposure to diazinon (Scholz et al. 2000). This response, which can be considered a preference response occurring over distance, was reduced by 20% even with 24-hr exposure to 0.1 µg/L diazinon. Greater exposure reduced fidelity further. This work demonstrates that exposure may alter the subsequent perception and behavioral response to an attractant, in this case even at a 2 km range.

Unlike the Scholz et al (2000) study, behavioral toxicity responses are typically measured in more confined spaces. The attraction response and

foraging behavior following contaminant exposure has been studied enough to warrant its own review (Kasumyan 2001). However, this review did not specifically isolate olfactory effects from other physiological impairments, and so here discussion is given on those changes attributable to olfactory-based impairments.

Pesticide exposure can reduce or eliminate attraction (preference) responses (Table 8-3). For goldfish, 4-hr exposure to 1 µg/L carbofuran reduced food odor attraction by 38% (Saglio et al. 1996). Curiously, longer (12-hr) exposure caused less impairment. Perhaps with longer exposure the olfactory tissue has time to adjust and compensate for exposure. With brief (30-min) exposures, three other currently-used pesticides altered rainbow trout preference behavior towards an amino acid, L-histidine (Tierney et al. 2007c). Specifically, preference behavior was eliminated by 1 µg/l IPBC and atrazine, and 100 µg/l AI Roundup. In future, longer-term exposures may be used to determine whether adaptation is possible to these and other pesticides.

Exposure to an avoided chemical can alter the avoidance of another. For example, the avoidance threshold carp exhibit to the three pesticides was modified by the addition of the SLS (Ishida and Kobayashi 1995). On their own, avoidance thresholds for fenitrothion and SLS were 490 and 0.01 µg/L, respectively. With 1% SLS in the fenitrothion solution, the fenitrothion avoidance threshold was decreased to 1 µg/L.

Thus far diuron, atrazine (Saglio and Trijasse 1998), diazinon (Scholz et al. 2000) and IPBC (Tierney et al. 2006b) have been found to alter alarm

behavior (Table 8-3). Typically any altered behavior is reported as changes in the freezing portion of the response. With chinook salmon, the freezing was incrementally reduced with exposures in excess of 1 µg/L of diazinon (Scholz et al. 2000). Similar findings were noted for the potent olfactory toxicant IPBC and another salmonid (rainbow trout) (Tierney et al. 2006b). An exception is for goldfish; in this case, following 24-hr exposure to 5 µg/L of either diuron or atrazine, the grouping behavior goldfish perform in response to skin homogenate was decreased (Saglio and Trijasse 1998). The difference in behavioral response likely reflects variation in alarm response between species. A diminished alarm response suggests fish may not negotiate a predator attack and so suffer higher mortality.

Integrating electrophysiological, physiological, and behavioral data

Few olfactory toxicological studies have endeavored to relate effects across organizational levels. Nevertheless, those that have can be divided into those that relate EOG/EEG to other physiological responses or to behavioral responses, and those that relate olfactory-mediated physiologic responses to behavioral responses. Determining relationships across these levels may highlight differential sensitivities (e.g. is OSN or behavioral response a better indicator of toxicity?) or thresholds (e.g. at what point of OSN impairment does contaminant avoidance response fail?); both of which may be used to gauge the usefulness of toxicity data to predicting organismal performance.

Relationships between olfactory neurological and other physiological responses

Toxicant exposure can be used to help determine mechanistic relationships between biochemical or molecular responses and olfactory neuron function. Studies continue to suggest cholinesterase impairment as a potential mechanism of olfactory toxicity (e.g. Jarrard et al. 2004, Tierney et al. 2007b). The relationship was explored by Jarrard et al. (2004) by recording EOGs and measuring AChE activity in coho rosette tissue following carbamate exposure (Table 8-4). The data from carbofuran suggests such a relationship exists since EOG decreases agreed closely with AChE impairment (i.e. 30-min exposure to 10 µg/L caused 50% decreases in both; 200 µg/L caused ~20%). However, debate remains since the presence of AChE in the olfactory epithelium has not been conclusively demonstrated.

Several endocrine responses associated with mating are downstream of and initiated by olfactory neuron responses. Measurements of both OSN and endocrine responses facilitate understanding the ramifications of olfactory impairment to critical behaviors. Furthermore, armed with knowledge of how pesticides alter both responses, the effect pesticide exposure may have on reproductive parameters can be estimated in future through use of measurements of OSN function.

With Atlantic salmon, the effects of OSN impairment on priming responses (i.e. milt and hormonal production) of males by female urine have been tested in a series of five papers (Moore and Waring 1996b, Waring and Moore 1997,

Moore and Waring 1998, Moore and Lower 2001, Moore and Waring 2001; Table 8-4). In interpreting OSN-physiological relationships of these studies, a consideration is that exposure periods usually differed for each endpoint. Even so, in most cases, as expected, pesticide exposure was associated with reduced EOG responses and lower levels of plasma testosterone, 11-ketotestosterone (another androgenic hormone), 17, 20βP (which increases secretion of gonadotropin II (GtH II) (Zheng et al. 1997)), and expressible milt. These hormonal responses typically varied similarly.

Overall, olfactory mediated hormonal responses appear to be more sensitive to pesticide exposure than OSN response. In most cases, there is a greater than five-fold difference in sensitivity between EOG impairment and altered testosterone response (Fig. 8-3). This indicates that there may be a threshold between OSN responses and downstream hormonal responses. As a particular example, consider that with diazinon exposure, the maximum milt reduction occurred at an exposure concentration of 0.8 µg/L (Moore and Waring 1996b) (Table 8-4). In contrast, EOG responses declined in a concentration dependent manner from 1 to 20 µg/L. This suggests that small impairments in OSN response may translate to larger declines in milt production.

Like carbofuran and diazinon, the triazines atrazine and simazine often caused hormonal decreases (Table 8-4). For example, in Atlantic salmon exposed to ≥6 µg/L atrazine for 5-d, the milt and three reproductively active hormones (testosterone, 11-KT and 17, 20βP) were not increased (i.e. primed) to the same extent as unexposed fish (Moore and Waring 1998). In fact, milt was

not increased to the same extent after just 0.04 µg/L atrazine exposure. Here, OSN responses were significantly decreased after 2 µg/L. Again the data suggests that endocrine processes downstream of OSN responses are highly sensitive to OSN impairment.

An important difference occurred with some cases of triazine exposure. For instance, in male Atlantic salmon, plasma testosterone was increased (244% of control) following exposure to 0.5 µg/L of simazine (Moore and Lower 2001) (Table 8-4). In contrast, four times this concentration (2 µg/L) decreased EOG. This suggests simazine may be causing a non-olfactory-based effect. Supporting this, triazines are known to induce aromatase activity, which converts testosterone to estradiol and so it has anti-androgen properties (Hayes et al. 2006). The observed androgen increase indicates testosterone secretion may have been elevated to offset increased plasma estradiol. This could be investigated in future by measurements of both hormones.

Relationships between olfactory neurological and behavioral responses

There are at least two ways in which OSN responses and behavioral response relationships have been established: through correlations between pesticide-evoked EOGs and avoidance/preference responses, and between pesticide-impaired EOG responses and behavioral responses. With the latter category, the potential for non-olfactory mediated effects is larger, since fish receive exposure through surrounding water.

Of the few studies that enable comparison of contaminant avoidance and EOG/EEG response, the data indicates that OSN detection typically occurs in

advance of avoidance (Fig. 8-4). For example, the OSN response thresholds for carp to benthiocarb and isoprothiolane were 10 and 1000 fold-fold greater, respectively, than the avoidance thresholds (Ishida and Kobayashi 1995, Ishida et al. 1996) (Fig .4). A similar finding was noted with the pesticide formulation Roundup: its presence (and the detection thereof) was not sufficient for avoidance (Tierney et al. 2007c). This suggests the obvious: that responses likely need to be perceived as unpleasant to evoke a behavioral avoidance response. Not all pesticides or their formulations are going to be perceived as such; recall Saglio et al. (2001) found pesticide preference responses. These findings together indicate that fish may not avoid pesticide exposure, and so potentially injury, even when given the choice.

For fish exposed to contaminants (i.e. no opportunity for avoidance), behavioral responses appear to be lost well before neurological responses are. This was evident with three pesticides (atrazine, IPBC and Roundup), as concentration-specific decreases were observed in both preference and OSN responses yet the behavioral decreases were absent with OSN impairments of 60% or greater (Tierney et al. 2007c). The implication of this finding to gauging pesticide-mediated toxicity from OSN data alone is that the toxicity may be too conservative: small OSN impairments may cause disproportionately large effects on behaviors.

Relationships between olfactory-mediated physiological and behavioral responses

There have been many examples thus far of how stereotypical physiological and behavioral responses ensue following odorant detection. The relationships discussed to this point have included mainly EOG/EEG responses, yet other examples of olfactory-based physiological impairments exist. As in the above section, determination of how physiological impairments relate to behavioral alterations has more than basic research value: the relative sensitivity of toxicity endpoints can be determined across biological levels of organization.

Physical damage to OSNs can impair alarm response behavior. Beyers and Farmer (2001) related the OSN ciliary loss copper causes to changes in the behavioral component of the alarm response in Colorado pikeminnow (*Ptychocheilus lucius*). The findings were unexpected: behavioral alarm responses appeared more affected after 24-hr than 96-hr for copper concentrations above 66 µg/L. The lack of a concentration-dependent behavioral decrease was theorized by the authors to be from neuroprotective responses, such as mucous generation.

The alarm response is sometimes associated with a stress response, and so certain stress hormones may provide good correlates of alarm behavior. Scott et al. (2003) monitored changes in rainbow trout alarm behavior and plasma cortisol, a stress hormone that often increases in concentration with alarm response (Rehnberg and Schreck 1987). In unexposed fish, alarm substance caused plasma cortisol to increase 4-fold, whereas in fish exposed to

2 µg/L cadmium, the elevation was only 2-fold. Behaviorally, this alteration corresponded to impaired alarm response: fish no longer froze. Since the behavioral response was lost in advance of the physiological response, the data here suggests a threshold exists between cortisol secretion and freezing response, if the physiological response figures into motivating the behavioral responses. A similar study was conducted with the carbamate fungicide IPBC and the alarm response of coho salmon (Tierney et al. 2006b). Here, IPBC exposure on its own was associated with increased cortisol. For this reason, using some physiological endpoints as correlates of olfactory toxicity is not necessarily prudent.

Challenges in separating physiologic and olfactory responses

As was outlined at the outset of the behavioral section, separating olfactory-mediated behavioral toxicity from other types of behavioral toxicity (e.g. swimming activity) can be difficult. For instance, when feeding behavior decreases (e.g. with 0.1 ppm fenitrothion exposure (Bull and McInerney 1974)), it may be due to decreases in the sense of smell and taste, since food can be rejected if the taste is off (e.g. food contaminated with the same pesticide was rejected by *S. salar* (Symons 1973)), or from a decrease in locomotory ability. A parallel could be drawn with mating responses: often these responses involve complex displays and other courtship behaviors, many of which are impaired by contaminant exposure (reviewed in Jones and Reynolds 1997). Similarly again, 3-d following a 7-d exposure to 1µg/L DDT, goldfish did not regroup into schools

as rapidly as control (Weis and Weis 1974). Such behaviors may or may not include an olfactory component. Nevertheless, from an ecological perspective, such measures of integrated sensory and neurological response may bring increased ability to extrapolate to field conditions and thus improve estimation of environmental impact assessment.

Endpoints related to olfactory toxicity

Various measures exist to determine how olfactory tissue can resist or cope with toxicant exposure. These neuroprotective responses include the measurement of inactivation/detoxification (i.e. Phase I and II biotransformation) enzymes within the mucous, globlet (mucous-producing cells), OSN and other cells of the olfactory epithelium. There are several studies of Phase I changes in mammalian tissues, for instance the substituted benzene herbicide dichlobenil induces expression of the cytochrome P450 isozyme 2A5 (CYP2A5) in mouse olfactory tissue (Piras et al. 2003). In fish, exploration of CYP induction in fish olfactory cells is rare (Saucier et al. 1999). In rainbow trout given a 4-d exposure to the CYP inducer β-naphthoflavone, CYP1A1 was expressed in OSNs, cells in which it had not previously been detected (Saucier et al. 1999). Similarly, the polycyclic aromatic hydrocarbon and model carcinogen, benzo[a]pyrene, induced CYP1A1 expression after 48-hr in the headwater livebearer (*Poeciliopsis monacha*) (Smolowitz et al. 1992).

Even less data exists on the activity of Phase II enzymes in fish olfactory tissue. A study into glutathione-S-transferase (GST) found that killing OSNs

through severing bulbar innervation caused a rapid decrease in the GST of the olfactory epithelium in rainbow trout (Starcevic and Zielinski 1997). The enzyme activity returned after two months, while full tissue regeneration required three. However, GST activity tells only a portion of the neuroprotective equation: substrate must be present. The substrate glutathione (GSH) had an alternate recovery profile: GSH remained initially high, but dropped to a lower level after 2-wks where it remained to the experiment's conclusion (96-d). Neuroprotective responses may also be measured upstream of enzyme activity through determination of Phase I mRNA (Chung-Davidson et al. 2004; Gillner et al. 1987), or of expression promoter proteins such as c-fos that are involved with neuroprotection (Salierno et al. 2006, Tierney and Kennedy 2007).

Neuronal loss can indicate exposure to a damaging agent, and the regrowth of the olfactory epithelium can be monitored. In mammals suffering complete loss of olfactory tissue, this would necessitate monitoring regeneration using histology or other techniques over at least 4 to 6 weeks (Schwob 2005). In fish, regrowth can be more lengthy, as complete regeneration can take up to 7 months in rainbow trout at 11°C (Evans and Hara 1985) and slightly less time (~6.4 months) in goldfish at 18°C (Zippel et al. 1997). During regrowth, the tissue progresses from redevelopment of the apical surfaces of ciliated and microvillar cells through to axonal reconnection in the olfactory bulb (Zippel et al. 1997).

Olfactory toxicity endpoints vs. other toxicity endpoints

Toxicity is related to the level of biological organization measured: with increasing levels come greater toxicity thresholds (Fig. 8-5). This review has focused on toxicity to OSNs and the downstream relevance of that toxicity to behavior, since OSNs are exposed and sensitive to contaminants, and since the behaviors they support are often quite critical to survival. However, the significance of measures of olfactory toxicity to organismal survival and beyond remains poorly understood. Furthermore, placing olfactory toxicity endpoints in context of other toxicity endpoints to determine what constitutes a meaningful negative effect is not trivial. In this section, OSN toxicity is considered against other measures of contaminant effects, which helps to address if OSN toxicity is more or less sensitive and meaningful than other measures.

Consider that acetylcholinesterase (AChE) impairing insecticides inhibit both olfaction (Sandahl et al. 2004) and muscle performance (Tierney et al. 2007a). For a fish exposed to an anti-AChE agent, it could be difficult to determine whether the ability to escape a predator is more impaired than the ability to smell a predator. Yet if each physiologic system is differentially sensitive, and the representative system may be ranked according to its survival importance, then perhaps risk can be calculated.

With the anti-AChE insecticide chlorpyrifos, three recent studies on coho salmon have been conducted on both olfactory and swimming impairment. OSN responses were reduced by 20% following 30-min exposure to 0.72 µg/L (Sandahl et al. 2004) and spontaneous swimming (a correlate of foraging ability)

was decreased 27% with 0.6 µg/L (Sandahl et al. 2005). Critical swimming performance (a correlate of predator escape ability) was impaired at a higher concentration of ≥10 µg/L (Tierney et al. 2007a). Lethality values (96-hr LC_{50}) are typically higher than OSN or swimming impairment thresholds (e.g. 15 µg/L) (Macek et al. 1969). With this example, olfactory ability and spontaneous swimming ability seem approximately equal in sensitivity to chlorpyrifos, and critical swimming ability appears comparatively less so. If an exposure of ~1 µg/L were to occur in an ecological setting, perhaps fish would have reduced ability to detect food and forage for it, yet remain capable of escaping predators. In the short-run, such a scenario may not increase the likelihood of death, but in the long-run, lead to decreased energy reserves and so potentially cause 'ecological death', i.e. not outright mortality, but a much-increased likelihood of death from ecological processes such as predation (Kruzynski and Birtwell 1994). Ideally, the relevancy of any sublethal measure needs to be tied to its potential for enhancing the likelihood of ecological death.

In summary, the impairment of neurological systems is potentially a double-edged sword: not only may pesticide exposure cause incorrect perception of information critical to survival (such as predator scent), but it may also impair the proper response (such as through impairment of muscle innervation). Given the upstream importance of sensory perception, it arguably trumps other physiological responses that are based on external stimuli. Nevertheless, the importance of either to survival can ultimately only be decided over time *in situ*: olfaction is not always critical to survival.

Research directions

In olfactory toxicity work, two aspects, each representing opposite ends of biological order, remain relatively unexplored: the mechanisms of toxicity of the various contaminants, and the effects of olfactory toxicity on survivorship. The former can be remedied by molecular studies and more in-depth electrophysiology (e.g. patch clamping), while the latter can be remedied by more behavioral testing, with field studies, mesocosm studies, lab studies with field links (such as through the use of pesticide mixtures), and modeling. Ultimately linking mechanistic effects to altered survivorship will help to permit predicting the effects of contaminant exposure on fish populations in the environment (Fig. 8-6).

These research directions are met with several challenges. The determination of mechanism of toxicity for each of the myriad pesticides, inert ingredients and other contaminants is near to impossible. Not only is the number of contaminants huge, but so is the number of fish species (~30,000). The variation contaminants evoke in fish responses can be marked, as discussed. Furthermore, many fishes have complex life histories and behaviors that may bring them through various environments, each with their own contaminant concerns. Measuring the impact of single contaminants on behaviors critical to survival is challenge enough – extrapolating to the environment and its contaminant mixtures will be difficult.

Nevertheless, such studies, including those on mixtures, need to be conducted. Two studies have demonstrated that olfactory impairment limited the success of return migrating salmon (Saunders and Sprague (1967); Scholz et al.

(2000)). In each case, the contaminant was tied to the environment and to olfactory impairment. With accurate environmental survey data, information on fish movement and olfactory sensitivity, it may be possible to increasingly make mechanistically-based predictions on the impact of contaminants on fish species, even those with complex life histories.

Conclusion

Across all levels of olfactory organization and response discussed above, the recurring theme is that a variety of pesticides, surfactants, metals and hydrocarbons alter the function and structure of olfactory sensory neurons and the higher-order behavioral responses they support. For any toxicity study to be useful, the findings should relate to growth, reproduction or recruitment, which together represent levels of survivorship relevant to population. Relating the importance of a decrease in olfactory neuron response from a short-term pesticide exposure to a population-level impact is a challenge rife with uncertainty.

Several studies indicate that thresholds exist between neurological, physiological and behavioral responses. The ramifications here for extrapolating neurological and physiological data to behavioral, and likely ecological, impact are straightforward: lower order measures may underestimate the impact of toxicity to higher order biological responses. Sublethal measures such as the EOG or stress response may translate to disproportionately larger alterations in behaviors that fish rely upon for survival. For this reason, olfactory toxicity

studies must continue to link mechanistic understandings to behavioral responses that can be related to changes in survivorship, especially at the ecosystem level. Each of these links remain information-poor, however the last step remains the most difficult and unstudied.

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Table 8-1. The effects of pH and various contaminants on the olfactory responses of several fishes.

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
pH										
pH	*S. salar*	EOG	Testosterone	10^{-7} M	9.5	5-min	5%			(Moore 1994)
					8.5		62%			
					7.5		100%			
					6.5		76%			
					5.5		38%			
					4.5		5%			
					3.5		0%			
			Ov. female urine	1 in 10^{-4} dilution	9.5		48%			
					8.5		64%			
					7.5		176%			
					6.5		100%			
					5.5		80%			
					4.5		20%			
					3.5		0%			
	O. mykiss	EEG	L-serine	10^{-5} M	4.7	2-wk	50%			(Klaprat et al. 1988)
Metals										
Aluminum	*O. mykiss*	EEG	L-serine	10^{-5} M	pH 4.7+ 20 µm/L	2-wk	15%			(Klaprat et al. 1988)
$CdCl_2$	*S. alpinus*	EEG	L-serine	10^{-5} M	0.1 mg/L	10-min	50%	10-min	80%	(Thompson and Hara 1977)
$CuCl_2$	*O. kisutch*	EOG	L-serine	10^{-5} M	1 µg/L	30-min	75%			(Baldwin et al. 2003)
					2 µg/L		70%			
					5 µg/L		50%			
					10 µg/L		30%	90-min	65%	
		EOG	TChA	10^{-6} M	10 µg/L	30-min	33%			

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
$CuCl_2$	*O. keta*	EOG	L-serine	10^{-3} M	3 µg/L	4h	86%	1-d	100%	(Sandahl et al. 2006)
					8 µg/L		71%		100%	
					24 µg/L		36%		71%	
					58 µg/L		11%		100%	
	O. tshawytscha		L-serine	10^{-3} M	25 µg/L	1-hr	50%			(Hansen et al. 1999b)
					50 µg/L		50%			
					100 µg/L		10%			
					200 µg/L		10%			
	O. mykiss		L-serine	10^{-3} M	25 µg/L	1-hr	50%			(Hansen et al. 1999b)
					50 µg/L		20%			
					100 µg/L		10%			
					200 µg/L		10%			
	S. salar	EOG	L-alanine	10^{-3} M	4 mM	5-min	50%	30-min	90%	(Winberg et al. 1992)
	$CuCl_2$	10 mM	in concentrations of HCO_3^-		0.4 mM		10%	30-min	70%	
					0.04 mM		0%	30-min	50%	
					0.00 mM		0%	30-min	30%	
$CuSO_4$	*O. mykiss*	EEG	L-serine	10^{-5} M	0.01 mg/L	4h	90%			(Hara et al. 1976)
					0.05 mg/L	4h	50%			
					0.1 mg/L	4h	15%			
					0.1 mg/L	10-min	20%	10-min	80%	(Thompson and Hara 1977)
$HgCl_2$	*S. salar*	EEG	DL-serine	10^{-3} M	$\geq 10^{-4}$ M	10-s	0%	>1-hr		(Sutterlin and Sutterlin 1971)
	O. mykiss	EEG	L-serine	10^{-5} M	0.25 mg/L	1h	78%	20-min	100%	(Hara et al. 1976)
						2h	67%	50-min	100%	
						3h	47%	60-min	60%	
						4h	29%	60-min	55%	
	S. salar	EOG	L-alanine	340 µM	10^{-5} M	2-min	35%	NA	50%	(Baatrup et al. 1990)

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
Pesticides										
2,4-D	*O. kisutch*	EOG	L-serine	10^{-3} M	1 mg/L	30-min	100%			(Tierney et al. 2006a)
					10 mg/L		100%			
					100 mg/L		0%	>60-min		
	O. mykiss	EOG	L-histidine	10^{-5} M	1 µg/L	30-min	100%			(Tierney et al. 2007c)
					10 µg/L		45%	2-min		
					100 µg/L		20%	2-min		
Atrazine	*S. salar*	EOG	$PGF_{2\alpha}$	10^{-9} M	1 µg/L	30-min	100%			(Moore and Waring 1998)
					2 µg/L		91%			
					5 µg/L		79%			
					10 µg/L		66%			
					20 µg/L		58%			
	S. salar	EOG	L-serine	10^{-5} M	2 µg/L	30-min	53%			(Moore and Lower 2001)
			$PGF_{2\alpha}$	10^{-9} M	1 µg/L		86%			
Carbaryl	*O. kisutch*	EOG	L-serine	10^{-5} M	100 µg/L	30-min	70%	>20-min		(Tierney et al. 2007b)
			TChA		100 µg/L		75%	5-min		
	O. mykiss		L-serine		100 µg/L		80%	>20-min		
			TChA		100 µg/L		90%	10-min		
	O. nerka		L-serine		100 µg/L		50%	>20-min		
			TChA		100 µg/L		80%			
Carbofuran	*O. kisutch*	EOG	L-serine	10^{-5} M	2 µg/L	30-min	67%			(Jarrard et al. 2004)
					10 µg/L		52%			
					20 µg/L		48%			
					200 µg/L		20%			
Carbofuran	*S. salar*	EOG	$PGF_{2\alpha}$	10^{-9} M	0.1 µg/L	30-min	61%			(Waring and Moore 1997)
					1 µg/L		88%			
					2 µg/L		73%			
					5 µg/L		82%			
					10 µg/L		67%			

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
Chlorothalonil	*O. kisutch*	EOG	L-serine	10^{-3} M	1 mg/L	30-min	100%			(Tierney et al. 2006a)
Chlorpyrifos	*O. kisutch*	EOG	TChA	10^{-5} M	0.625 µg/L	30-min	75%			(Sandahl et al. 2005)
					0.625 µg/L		75%			
			L-serine	10^{-4} M	0.625 µg/L		75%			
					0.625 µg/L		75%			
			TChA	10^{-5} M	1.25 µg/L		30%			
					1.25 µg/L		30%			
			L-serine	10^{-4} M	1.25 µg/L		50%			
					1.25 µg/L		50%			
			TChA	10^{-5} M	2.5 µg/L		40%			
					2.5 µg/L		50%			
			L-serine	10^{-4} M	2.5 µg/L		65%			
					2.5 µg/L		45%			
Cypermethrin	*S. salar*	EOG	$PGF_{2\alpha}$	10^{-8} M	< 4 ng/L	5-d	12%			(Moore and Waring 2001)
			L-serine	10^{-5} M	< 4 ng/L	5-d	17%			
Diazinon	*S. salar*	EOG	$PGF_{2\alpha}$	10^{-9} M	0.1 µg/L	30-min	100%			(Moore and Waring 1996b)
					1 µg/L		78%	4.5-hr	80%	
					2 µg/L		65%			
					5 µg/L		51%			
					10 µg/L		26%			
					20 µg/L		21%			
Endosulfan	*O. kisutch*	EOG	L-serine	10^{-3} M	10 µg/L	30-min	100%			(Tierney et al. 2006a)
					100 µg/L		60%	2-min		
Glyphosate	*O. kisutch*	EOG	L-serine	10^{-3} M	0.1 mg/L	30-min	100%			
					1 mg/L		66%	>60-min		
					10 mg/L		44%	5-min		
					100 mg/L		0%	>60-min		

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
IPBC	*O. kisutch*	EOG	L-serine	10^{-5} M	0.047 µg/L	30-min	70%			(Jarrard et al. 2004)
					0.47 µg/L		51%			
					4.7 µg/L		35%			
					47 µg/L		24%			
					1 µg/L		72%	30-min		(Tierney et al. 2006a)
					10 µg/L		54%	>60-min		
					100 µg/L		58%	>60-min		
	O. mykiss	EOG	L-histidine	10^{-5} M	1 µg/L	30-min	45%	2-min		(Tierney et al. 2007c)
					10 µg/L		40%	2-min		
					100 µg/L		20%	2-min		
Linuron	*O. kisutch*	EOG	L-serine	10^{-5} M	100 µg/L	30-min	40%			(Tierney et al. 2007b)
			TChA		100 µg/L		100%			
	O. mykiss	EOG	L-serine	10^{-5} M	100 µg/L		85%			
			TChA		100 µg/L		100%			
	O. nerka	EOG	L-serine	10^{-5} M	100 µg/L		50%			
			TChA		100 µg/L		100%			
Mancozeb	*O. kisutch*	EOG	L-serine	10^{-5} M	0.22 mg/L	30-min	57%			(Jarrard et al. 2004)
					2.2 mg/L		50%			
Roundup	*O. mykiss*	EOG	L-histidine	10^{-5} M	10 µg/L	30-min	70%	2-min		(Tierney et al. 2007c)
					100 µg/L		40%	2-min	50%	
					1000 µg/L		25%	2-min		
Simazine	*S. salar*	EOG	L-serine	10^{-5} M	2 µg/L	30-min	50%			(Moore and Lower 2001)
			$PGF_{2\alpha}$	10^{-9} M	0.1 µg/L		101%			
					2 µg/L		72%			
Simazine + Atrazine	*S. salar*	EOG	L-serine	10^{-5} M	1+1 µg/L		51%			
			$PGF_{2\alpha}$	10^{-9} M	0.5+0.5		82%			
				10^{-9} M	1+1		70%			
trifluralin	*O. kisutch*	EOG	L-serine	10^{-3} M	30 µg/L	30-min	100%			(Tierney et al. 2006a)
					300 µg/L		70%	2-min		

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
Surfactants										
SLS	*C. clupeaformis*	EEG	food extract	NA	0.1 mg/L	15-min	20%			(Hara and Thompson 1978)
					0.5 mg/L		50%			
					1 mg/L		70%			
					5 mg/L		60%			
					10 mg/L		80%			
			L-serine	10^{-5} M	0.1 mg/L		10%			
					0.5 mg/L		50%			
					1 mg/L		55%			
					5 mg/L		60%			
					10 mg/L		90%			
S. salar/ EEG / 10^{-5} M DL-alanine										(Sutterlin et al. 1971)
Alkyldimethyl-3,4-dichloro-benzyl ammonium chloride					1 mg/L	15-s	90%			
Alkyldimethyl-3,4-dichloro-benzyl ammonium chloride					10 mg/L		0%			
B-hydroxyethylbenzyl coco imidazolinium chloride					1 mg/L		50%			
B-hydroxyethylbenzyl coco imidazolinium chloride					10 mg/L		0%			
B-hydroxyethylbenzyl stearyl imidazolinium chloride					1 mg/L		90%			
B-hydroxyethylbenzyl stearyl imidazolinium chloride					10 mg/L		27%			
Branched sodium dodecylbenzene sulfonate					10 mg/L		60%			
Calcium dodecylbenzene sulfonate					10 mg/L		60%			
Di hydrogenated tallow dimethyl ammonium chloride					10 mg/L		90%			
DI-coco dimethyl ammonium chloride					1 mg/L		39%			
DI-coco dimethyl ammonium chloride					10 mg/L		0%			
Lauryldimethylbenzyl ammonium chloride					10 mg/L		85%			
Linear sodium dodecylbenzene sulfonate					10 mg/L		50%			
Methyldodecylbenzyl-trimethyl ammonium chloride					1 mg/L		70%			
Methyldodecylbenzyl-trimethyl ammonium chloride					10 mg/L		0%			
N-coco-propylenediamine					10 mg/L		30%			
N-soya-propylenediamine					10 mg/L		18%			

Contaminant	Species[a]	OSN Test[b]	Odorant[c]	[odorant]	[contam.]	Exposure duration	Response (of pre-exp.)	Recovery (time)	(%)	Reference
N-tallow-propylenediamine					10 mg/L		26%			
Octylcresoxyethoxyethyldimethylbenzyl ammonium chloride					1 mg/L		33%			
Octylcresoxyethoxyethyldimethylbenzyl ammonium chloride					10 mg/L		0%			
Octylphenoxyethoxyehtyldimethyl benzyl ammonium chloride					1 mg/L		85%			
Octylphenoxyethoxyehtyldimethyl benzyl ammonium chloride					10 mg/L		0%			
Sodium kerylbenzene sulfonate					10 mg/L		80%			
Sodium toluene sulfonate					10 mg/L		80%			
Sodium tridecylbenzene sulfonate					10 mg/L		60%			
Stearyldimethylbenzyl ammonium chloride					10 mg/L		90%			
Triethanolammonium dodecylbenzene sulfonate					10 mg/L		48%			
Other contaminants										
Hydrocarbons (monocyclic aromatic)	*O. kisutch*	EEG	L-serine	10^{-3} M	4 mg/L	20-min	NS			(Maynard and Weber 1981)
Morpholine	*O. mykiss*	EEG	L-serine	10^{-5} M	10 g/L	2-min	70%			(Hara 1974)

[a]Fish key: *C. clupeaformis* = Lake whitefish, *O. keta* = Chum salmon, *O. kisutch* = Coho salmon, *O. mykiss* = Rainbow trout, *O. nerka* = Sockeye salmon, *O. tshawytscha* = Chinook salmon, *S. alpinus* = Arctic char, *S. salar* = Atlantic salmon.
[b]Two types of olfactory neuron tests are included: EOG (electro-olfactogram), which are field potentials taken from the nasal tissue, and EEG (electro-encephalogram), which are field potentials taken from the olfactory bulb (i.e. brain).
[c]Various odorants were used to evoke EOG and EEG responses, these include amino acids (L-serine, L-histidine, DL-alanine), bile salt (taurocholic acid; TChA), food extract, hormones (testosterone), ovulated (Ov.) female urine and pheromones (prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$).

Table 8-2 The preference or avoidance responses of a variety of fishes to pH and various contaminants.

Contaminant		Concentration	Response	Species[a]	reference
pH					
pH	pH	5.5	avoidance	*S. alpinus*	(Jones et al. 1985a)
Metals					
Arsenic	Na_2AsO_2 (Ar III)	28 µg/L	avoidance	*N. crysoleucas*	(Hartwell et al. 1989)
Cadmium	$CdCl_2$	68 µg/L	NR	*N. crysoleucas*	
Chromium	$K_2Cr_2O_7$ (Cr IV)	73 µg/L	avoidance	*N. crysoleucas*	
Cobalt	$CoCl_2$	180 µg/L	avoidance	*O. mykiss*	(Hansen et al. 1999a)
		24 µg/L	avoidance	*O. tshawytscha*	
Copper	$CuCl_2$	0.7 µg/L	avoidance	*O. tshawytscha*	(Hansen et al. 1999a)
		1.6 µg/L	avoidance	*O. mykiss*	
		26 µg/L	avoidance	*N. crysoleucas*	(Hartwell et al. 1989)
		330 µg/L	attraction	*O. mykiss*	(Giattina et al. 1982)
		6.4 µg/L	avoidance	*O. mykiss*	
	$CuSO_4$	0.1 µg/L	avoidance	*O. mykiss*	(Folmar 1976)
		16 mg/L	avoidance	*P. pungitius*	(Jones 1947)
Iron	Fe (total dissolved sp.)	4.25-6.45 mg/L	avoidance	*O. kisutch*	(Updegraff and Sykora 1976)
Mercury	$HgCl_2$	272 mg/L	avoidance	*P. pungitius*	(Jones 1947)
Nickel	$NiCl_2$	23.9 µg/L	avoidance	*O. mykiss*	(Giattina et al. 1982)
		6 µg/L	attraction	*O. mykiss*	
Selenium	Na_2SeO_3	3489 µg/L	NR	*N. crysoleucas*	(Hartwell et al. 1989)
Zinc	$ZnSO_4$	48 mg/L	avoidance	*P. pungitius*	(Jones 1947)
		5.6 µg/L	avoidance	*O. mykiss*	(Sprague 1968)
Mixture	Cu:Co mixture	1.0:0.9 µg/L	avoidance	*O. tshawytscha*	(Hansen et al. 1999a)
		2.6:2.4 µg/L	avoidance	*O. mykiss*	
Mixture	12 Cu: 1.1 Cd: 3.2 Pb: 50 Zn	6.6 µg/L (total)	avoidance	*O. mykiss*	(Hansen et al. 1999c)
Mixture	1 Cu: 0.54 Cr: 1.85 Ar: 0.38 Se	29 µg/L (in lab)	avoidance	*P. promelas*	(Hartwell et al. 1987a)

Contaminant		Concentration	Response	Species[a]	reference
Mixture	1 Cu: 0.54 Cr: 1.85 Ar: 0.38 Se				
	Spring (simulated stream)	71.1 µg/L (in field)	avoidance	*P. promelas*	(Hartwell et al. 1987b)
	Summer (simulated stream)	34.3 µg/L (in field)	avoidance	*P. promelas*	
	Summer (natural)	73.5 µg/L (in field)	avoidance	*P. promelas*	
Pesticides					
2,4-D	Herbicide	0.1 mg/L	avoidance	*C. variegatus*	(Hansen 1969)
		1 mg/L	avoidance	*G. affinis*	(Hansen et al. 1972)
		1 mg/L	avoidance	*O. mykiss*	(Folmar 1976)
Acrolein	Algaecide	0.01 mg/L	avoidance	*O. mykiss*	(Folmar 1976)
Bentazone	Herbicide	0.01 and 10 mg/L	attraction	*C. auratus*	(Saglio et al. 2001)
Benthiocarb	Herbicide	1.7 µg/L	avoidance	*C. carpio*	(Ishida and Kobayashi 1995)
Dalapon	Herbicide	<50 µM	attraction	*O. mykiss*	(Folmar 1976)
DDT	Insecticide	0.005 mg/L	avoidance	*C. variegatus*	(Hansen 1969)
		0.1 mg/L	avoidance	*G. affinis*	(Hansen et al. 1972)
		10 mg/L	avoidance	*G. affinis (near agriculture)*	(Kynard 1974)
		10 mg/L	NR	*G. affinis (~pristine)*	
Dursban (chlorpyrifos)	Insecticide	0.01 mg/L	NR	*C. variegatus*	(Hansen 1969)
		0.1 mg/L	avoidance	*C. variegatus*	
		0.1 mg/L	avoidance	*G. affinis*	(Hansen et al. 1972)
		0.1-0.25 mg/L	avoidance	*C. variegatus*	(Hansen 1969)
		0.5-1 mg/L	NR	*C. variegatus*	
		10 mg/L	avoidance	*C. variegatus*	
Endrin	Insecticide	0.01 mg/L	NR	*G. affinis*	(Hansen et al. 1972)
		0.25 mg/L	avoidance	*G. affinis (~pristine)*	(Kynard 1974)

Contaminant		Concentration	Response	Species[a]	reference
		1 mg/L	avoidance	*G. affinis (near agriculture)*	
		0.001 mg/L	avoidance	*C. variegatus*	(Hansen 1969)
Fenitrothion	Insecticide	10 µg/L	avoidance	*C. auratus*	(Scherer 1975)
		490 µg/L	avoidance	*C. carpio*	(Ishida and Kobayashi 1995)
		90 µg/L	avoidance	*O. latipes*	(Hidaka and Tatsukawa 1989)
Isoprothiolane	Fungicide	6.7 µg/L	avoidance	*C. carpio*	(Ishida and Kobayashi 1995)
Malathion	Insecticide	0.05 mg/L	avoidance	*G. affinis*	(Hansen et al. 1972)
		1 mg/L	NR	*C. variegatus*	(Hansen 1969)
Nicosulfuron	Herbicide	1 - 10 mg/L	attraction	*C. auratus*	(Saglio et al. 2001)
Parathion	Insecticide	0.2 mg/L	avoidance	*G. affinis (~pristine)*	(Kynard 1974)
		1 mg/L	avoidance	*G. affinis (near agriculture)*	
Prochloraz	Fungicide	1 - 10 mg/L	attraction	*C. auratus*	Saglio et al., 2001
Roundup	Herbicide	10 mg/L (A.I.)	avoidance	*O. mykiss*	Tierney et al., 2007c
Sevin (carbaryl)	Insecticide	10 mg/L	NR	*C. variegatus*	(Hansen 1969)
		10 mg/L	avoidance	*G. affinis*	(Hansen et al. 1972)
Toxaphene	Insecticide	0.25 mg/L	avoidance	*G. affinis (near agriculture)*	(Kynard 1974)
		0.25 mg/L	avoidance	*G. affinis (~pristine)*	
Surfactants					
Surfactant	POE-ether	500 µg/L	avoidance	*O. latipes*	(Hidaka and Tatsukawa 1989)
	SLS	0.01 µg/L	avoidance	*C. carpio*	Ishida and Kobayashi, 1995
	SLS	10 µg/L	avoidance	*O. latipes*	(Hidaka and Tatsukawa 1989)
	Sodium lauryl sulfate (SLS)	0.1 mg/L	attraction	*C. clupeaformis*	(Hara and Thompson 1978)

Contaminant		Concentration	Response	Species[a]	reference
Hydrocarbons					
Hydrocarbon (HC)	Benzene	1.9 mg/L	avoidance	*O. kisutch (parr)*	(Maynard and Weber 1981)
	Chloroform	0.01-0.02%	avoidance	*P. pungitius*	(Jones 1947)
	Coal distillate	1.7 mg/L	avoidance	*P. promelas*	Dauble et al., 1985
	Ethanol	1%	avoidance	*P. pungitius*	(Jones 1947)
	Formalin	0.1-0.4%	avoidance	*P. pungitius*	
	Monocyclic aromatic HCs	1.4 mg/L	avoidance	*O. kisutch (smolt)*	(Maynard and Weber 1981)
		3.7 mg/L	avoidance	*O. kisutch (parr)*	
	O-xylene	0.2 mg/L	avoidance	*O. kisutch (parr)*	
	Toluene	0.9 mg/L	avoidance	*O. kisutch (smolt)*	
		1.4 mg/L	avoidance	*O. kisutch (parr)*	
	Xylene	1 mg/L	avoidance	*O. mykiss*	(Folmar 1976)
Other					
Chloramine		≥70 µg/L	avoidance	*R. atratulus*	(Fava and Chu-Fa 1978)
Chlorine	Freshwater	≥70 µg/L	avoidance	*R. atratulus*	
	Seawater	10-100 µg/L (16, 20°C)	preference	*C. aggregata*	(Stober et al. 1980)
		175 µg/L (13°C)	avoidance	*C. aggregata*	
		2 µg/L	avoidance	*O. kisutch*	
Hydrogen sulfide	H_2S	2.2 mg/L (15°C)	avoidance	*M. saxatilis*	(Hall et al. 1984)
		2.3 mg/L (20°C)	avoidance	*M. saxatilis*	
		2.9 mg/L (25°C)	avoidance	*B. tyrannus*	
		3.0 mg/L (25°C)	avoidance	*M. saxatilis*	

Contaminant		Concentration	Response	Species[a]	reference
		3.0 mg/L (30°C)	avoidance	*B. tyrannus*	
		3.2 mg/L (15°C)	avoidance	*B. tyrannus*	
		3.5 mg/L (30°C)	avoidance	*M. saxatilis*	
		3.6 mg/L (20°C)	avoidance	*B. tyrannus*	
		not given	avoidance	*C. pallasii*	(Shelford and Powers 1915)
PCB	Aroclor (a PCB mix)	0.01 mg/L	avoidance	*G. affinis*	(Hansen et al. 1974)
		10 mg/L	NR	*C. variegatus*	
		10 mg/L	avoidance	*L. rhomboides*	
Pulp mill effluent	BKME	0.001%	avoidance	*S. salar*	(Sprague and Drury 1969), (Lewis and Livingston 1977)
		0.1%	avoidance	*L. rhomboides*	
		0.1%	avoidance	*F. grandis*	(Myllyvirta and Vuorinen 1989)
		0.13-0.25%	avoidance	*C. albula*	(Sprague and McLeese 1968)
		12-15%	avoidance	*S. salar*	
	Humic acid	0.1-0.2 mg/L	avoidance	*C. harengus*	(Wildish et al. 1977)
	KME	2.50%	avoidance	*O. tshawytscha*	(Jones et al. 1956)
		10%	NR	*O. kisutch*	(Campbell and Bettoli 1992)
		10%	NR	*S. canadense*	
		10%	NR	*I. punctatus*	
		10%	NR	*M. chrysops*	
	Sodium lignosulfonate	0.1-0.3 mg/L	avoidance	*C. harengus*	(Wildish et al. 1977)

[a]Fish key: *B. tyrannus* = Atlantic menhaden, *C. aggregate* = Shiner perch, *C. albula* = vendace, *C. auratus* = Goldfish, *C. carpio* = Carp, *C. clupeaformis* = Lake whitefish, *C. harengus* = Atlantic herring, *C. pallasii* = Pacific herring, *C. variegates*

= Sheepshead, minnow, *F. grandis* = Gulf killifish, *G. affinis* = Mosquitofish, *I. punctatus* = channel catfish, *L. rhomboids* = Pinfish, *M. chrysops* = striped bass, *M. saxatilis* = striped bass, *N. crysoleucas* = golden shiner, *O. kisutch* = Coho salmon, *O. latipes* = Medaka, *O. mykiss* = Rainbow trout, *O. tshawytscha* = Chinook salmon, *P. promelas* = fathead minnow, *S. alpinus* = Arctic charr, *S. canadense* = sauger, *S. salar* = Atlantic salmon.

Table 8-3 The alteration of fish behaviors following exposures to various contaminants for varying lengths.

Contaminant	Species[a]	Exposure			Odorant	Behavior	Reference
		Concentration	Duration	Effect (%)			
pH							
pH	*S. alpinus*	4.5, 4.75	14-d	–	food extract	deceased attraction	(Jones et al. 1985a)
Metals							
Copper	*P. pungitius*	0.01 M (635 mg/L)	5-15-min	100%	chloroform	decreased avoidance	(Jones 1947)
					formalin	decreased avoidance	
Iron	*O. kisutch*	1.2 mg Fe/L	birth to 4cm	–	4.25-6.45 mg Fe/L	avoidance	(Updegraff and Sykora 1976)
Metal mix	*O. mykiss*	12 Cu: 1.1 Cd: 3.2 Pb: 50 Zn 66.3 µg/L	45-d		≥4x	avoidance	(Hansen et al. 1999c)
	P. promelas	1 Cu: 0.54 Cr: 1.85 Ar: 0.38 Se					(Hartwell et al. 1987a)
		98 µg/L	3-mo	–	294 µg/L	preference response	
			6-mo	–	490 µg/L	avoidance response	
			9-mo	–	980 µg/L	NR[b]	
		1 Cu: 0.54 Cr: 1.85 Ar: 0.38 Se					(Hartwell et al. 1987b)
		98 µg/L in an artificial stream	3-mo	–	1470 µg/L	loss of avoidance	
		98 µg/L in a natural stream		–	2940 µg/L	loss of avoidance	
Pesticides							
Atrazine	*C. auratus*	5 µg/L	24-hr	-80%	skin extract	decreased sheltering	(Saglio and Trijasse 1998)

Contaminant	Species[a]	Exposure			Odorant	Behavior	Reference
		Concentration	Duration	Effect (%)			
				-60%		decreased grouping	
Cabofuran	*C. auratus*	1 µg/L	4-hr	-38%	food extract	attraction	(Saglio et al. 1996)
		10 µg/L		-64%			
		100 µg/L		-84%			
		1 µg/L	8-hr	-27%	food extract	attraction	
		10 µg/L		-30%			
		100 µg/L		-64%			
		1 µg/L	12-hr	-5%	food extract	attraction	
		10 µg/L		-16%			
		100 µg/L		-46%			
Diazinon	*O. tshawytscha*	0.1 µg/L	24-hr	-20%	NA	return migration	(Scholz et al. 2000)
		1 µg/L		-20%			
		10 µg/L		-40%			
		0.1 µg/L	2-hr	+50%	skin extract	alarm response (activity)	
		1 µg/L		+125%			
		10 µg/L		+75%			
Diuron	*C. auratus*	5 µg/L	24-hr	-40%	skin extract	decreased grouping	(Saglio and Trijasse 1998)
IPBC	*O. kisutch*	10 µg/L	30-min	-100%	skin extract	alarm response	(Tierney et al. 2006b)
		100 µg/L		-135%			

Contaminant	Species[a]	Exposure			Odorant	Behavior	Reference
		Concentration	Duration	Effect (%)			
Parathion	*C. auratus*	330 µg/L	24-hr	–	food extract	attraction	(Rand et al. 1975)
Other							
BKME	*C. albula*	0.13%	1-wk	–	0.13% BKME	preference	(Myllyvirta and Vuorinen 1989)
		2.25%		–	0.75-4.5%	avoidance	
		4.5%		–	0.75-4.5%	avoidance	
Chlorine	*S. alpinus*	>19 µg/L	6-d	–	food extract	deceased attraction	(Jones and Hara 1988)

[a]Fish key: *C. albula* = vendace, *C. auratus* = Goldfish, *O. kisutch* = Coho salmon, *O. mykiss* = Rainbow trout, *O. tshawytscha* = Chinook salmon, *P. promelas* = Fathead minnow, *P. pungitius* = Ninespine stickleback, *S. alpinus* = Arctic charr.

[b]NR = no response

Table 8-4 The relationships between olfactory sensory nerve (OSN) impairment and impairment of olfactory-mediated physiological and behavioral responses. Shown are (A) relationships between OSN responses as measured using electro-olfactogram (EOG), and the impairment of acetylcholinesterase (AChE), (B) priming of male plasma hormones and milt, and (C) amino acid preference response. Also shown is (D) the impairment relationship between physiological and behavioral components of the olfactory-mediated alarm response.

(A) OSN to acetylcholinesterase (AChE) impairment

		OSN		Fish[a]	
		Exposure (µg/L)	EOG (% control)	Exposure (µg/L)	AChE (%) impairment
(Jarrard et al. 2004)					
Pesticide	Carbofuran	2	67%	2	
Species	*O. kisutch*	10	52%	10	50%
EOG	10^{-5} M	20	48%	20	
[stim]	L-serine	200	20%	200	25%
OSN exp.[b]	30-min				
Fish exp.[c]	30-min				

(B) OSN to priming response impairment

						Plasma value (% of control)			Milt
					17, 20βP	GtH-II	testosterone	11-KT	(mg/g body)
(Waring and Moore 1997)									
Pesticide	Carbofuran								
Species	*S. salar*	0.1	61%	1.1	52%		62%	50%	80%
EOG	$PGF_{2\alpha}$	1	88%	2.7	28%		46%	44%	32%
[stim]	10^{-9} M	2	73%	6.5	12%		23%	22%	36%
OSN exp.	30-min	5	82%	13.9	4%		15%	11%	4%
Fish exp.	5-d	10	67%	22.7	8%		0%	17%	12%
				22.7	0%		4%	14%	-16%

(B) OSN to priming response impairment						Plasma value (% of control)			Milt
					17, 20βP	GtH-II	testosterone	11-KT	(mg/g body)
(Moore and Waring 1996a)									
Pesticide	Diazinon								
Species	*S. salar*	0.1	100%	0.3	57%	31%	68%	89%	36%
EOG	$PGF_{2\alpha}$	1	78%	0.8	-29%	12%	42%	37%	45%
[stim]	10^{-9} M	2	65%	1.7	-21%	0%	21%	26%	9%
OSN exp.	30-min	5	51%	2.7	7%	14%	47%	47%	55%
Fish exp.	5-hr	10	26%	5.6	-21%	17%	53%	53%	45%
		20	21%	13	-29%	2%	50%	55%	18%
				28	-29%	21%	37%	37%	36%
				45	-21%	17%	32%	39%	36%
(Moore and Waring 2001)									
Pesticide	Cypermethrin								
Species	*S. salar*	0.004	12%	0.004	59%		116%	75%	63%
EOG	$PGF_{2\alpha}$			0.004	77%		41%	63%	6%
[stim]	10^{-8} M			0.015	59%		33%	38%	0%
OSN exp.	5-d			0.028	-2%		17%	25%	0%
Fish exp.	5-d			0.038	5%		0%	0%	6%
				0.33	-14%		-4%	-25%	13%
(Moore and Lower 2001)									
Pesticide	Simazine	0.1	101%	0.1	200%		167%	-400%	21%
Species	*S. salar*	2	72%	0.5	82%		244%	-1350%	-58%
EOG	$PGF_{2\alpha}$			1	82%		200%	-350%	-42%
[stim]	10^{-9} M			2	282%		211%	-150%	-37%
OSN exp.	30-min								
Fish exp.	5-d								

(B) OSN to priming response impairment						Plasma value (% of control)			Milt
					17, 20βP	GtH-II	testosterone	11-KT	(mg/g body)
Pesticide	Atrazine								
Species	*S. salar*	1	86%	0.5	245%		167%	0%	153%
EOG	$PGF_{2\alpha}$			2	109%		144%	100%	147%
[stim]	10^{-9} M								
OSN exp.	30-min								
Fish exp.	5-d								
(Moore and Waring 1998)									
Pesticide	Atrazine								
Species	*S. salar*	1	100%	0.04	125%		134%	108%	80%
EOG	$PGF_{2\alpha}$	2	91%	3.6	100%		86%	108%	55%
[stim]	10^{-9} M	5	79%	6	49%		57%	25%	45%
OSN exp.	30-min	10	66%	14	15%		0%	25%	25%
Fish exp.	5-d	20	58%						
(Moore and Lower 2001)									
Pesticide	Atra.+ Sim.								
Species	*S. salar*	0.5+0.5	82%	0.5+0.5	291%		133%	-350%	-158%
EOG	$PGF_{2\alpha}$	1+1	70%	1+1	200%		144%	300%	-226%
[stim]	10^{-9} M								
OSN exp.	30-min								
Fish exp.	5-d								

(C) OSN to behavioral impairment relationships

			EOG (% pre)	Behavior (% pre)
(Tierney et al. 2007c)				
Pesticide	Atrazine			
Species	*O. mykiss*	1	89%	89%
EOG	L-histidine	10	40%	40%
[stim]	10^{-7} M	100	14%	14%
OSN exp.	30-min			
Fish exp.	30-min			
Pesticide	IPBC			
Species	*O. mykiss*	1	36%	29%
EOG	L-histidine	10	30%	0%
[stim]	10^{-7} M	100	10%	0%
OSN exp.	30-min			
Fish exp.	30-min			
Pesticide	Roundup			
Species	*O. mykiss*	10	67%	100%
EOG	L-histidine	100	32%	3%
[stim]	10^{-7} M	1000	19%	2%
OSN exp.	30-min			
Fish exp.	30-min			

(D) Physiological to behavioral impairment relationships

Alarm response tests				
(Scott et al. 2003)			plasma cortisol	Δ line crossings
Metal	Cadmium		(% of control)	
Species	*O. mykiss*	0		
OSN exp.	7-d	2	61%	-50%
Fish exp.	15-min	3	30%	100%

Notes:
The response after 2 µg/l is negative since fish became active and did not freeze
After 3 µg/l, the response was the same as control

(Tierney et al. 2006b)				Δ freezing
Pesticide	IPBC	0		
Species	*O. kisutch*	1	85%	0%
OSN exp.	30-min	10	77%	reduced 100%
Fish exp.	30-min	100	38%	reduced 135%

[a]Fish key: *O. kisutch* = Coho salmon, *O. mykiss* = Rainbow trout, *S. salar* = Atlantic salmon.

[b]'OSN exp.' is the exposure period for the olfactory rosette tissue

[c]'Fish exp.' is the exposure period for the whole animal

Figures

Figure 8-1 The lifecycle of migratory salmonids as related to olfactory-mediated behaviors. The outer ring shows the physiological/behavioral phase, the middle ring shows corresponding developmental stage, and the inner ring indicates the emergence of olfactory mediated behaviors of: (1) kin and conspecific recognition; (2) alarm response, first innate (to damaged siblings) then acquired (from a predator scent); (3) food location; (4) imprinting to home stream olfactory bouquet (HSOB); (5) return migration to HSOB; (6) mate attraction/repulsion; and (7) priming and synchronization of gamete release.

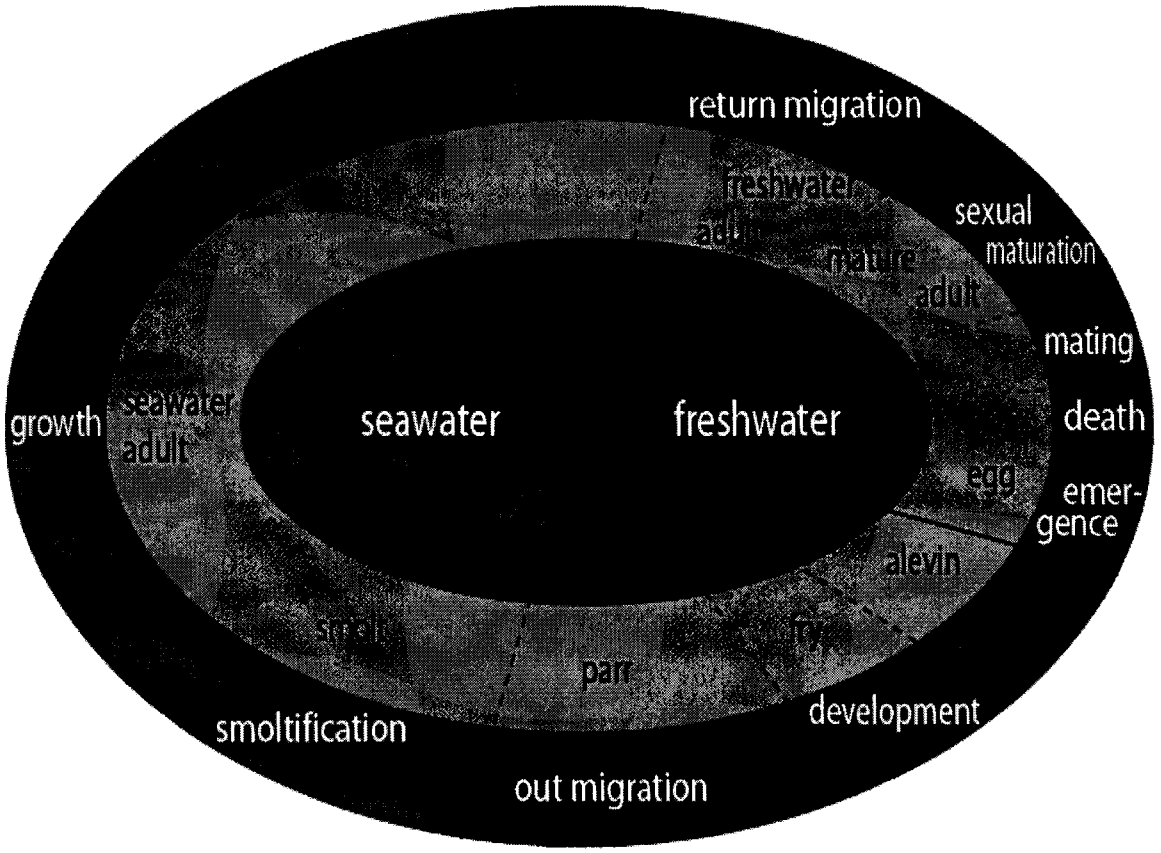


Figure 8-2 Odorants are perceived by sensory neurons, the input is then processed and integrated with other sources, which can lead to physiological and/or behavioral responses. Physiological and behavioral responses can feed back on each other, and be integrated into further processing (adapted from Scott and Sloman 2004). Contaminants can (a), act as odorants or modify odorant perception, and/or (b), act on the nervous system through other pathways, and/or (c), alter other physiologic responses, all of which potentially translate into altered behavior.

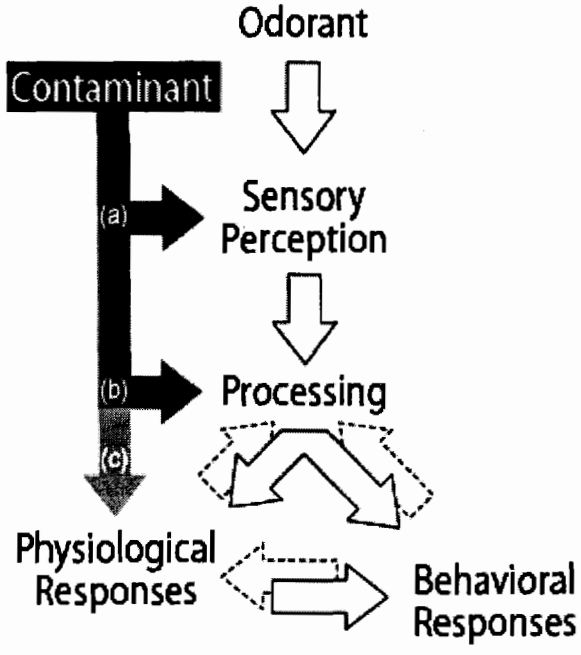


Figure 8-3 The relative difference in sensitivity between an olfactory sensory neuron (OSN) response and an olfactory-mediated hormonal response following exposure to various pesticides. The relative difference was calculated between the pesticide concentration required to cause a 50% change in OSN response (as measured using electro-olfactogram) and testosterone concentration. The 50% values shown on the y-axis were interpolated from regression models fitted to data in Table 8-4.

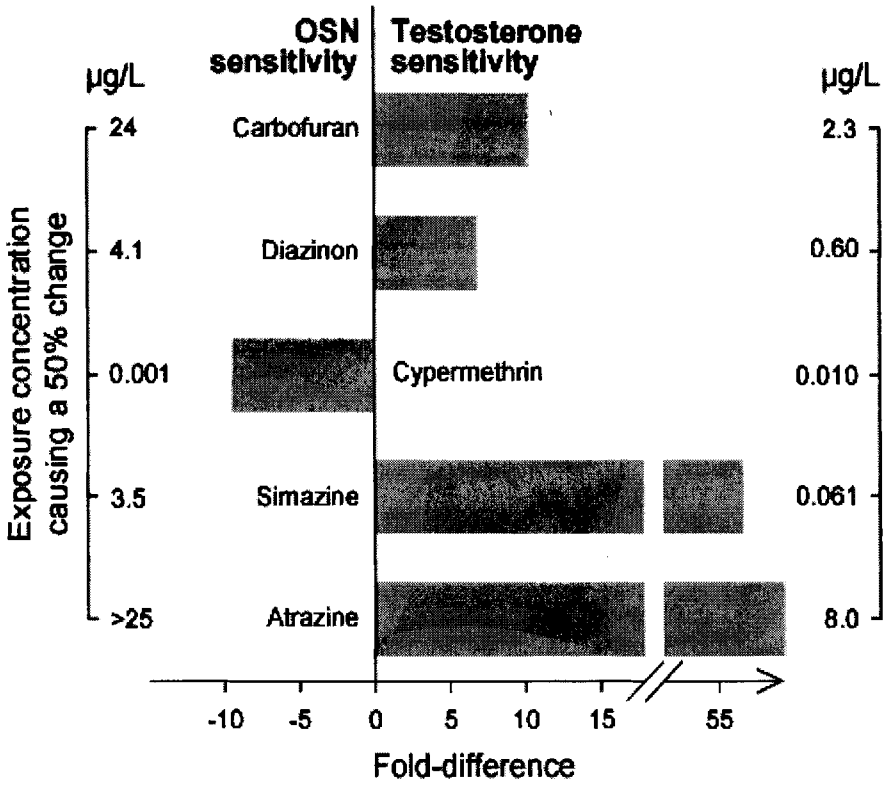


Figure 8-4 The relative difference in sensitivity between olfactory sensory neuron (OSN) detection of pesticides and avoidance of pesticides. The y-axis values indicate the threshold concentrations at which OSN and avoidance responses were no longer detected. Benthiocarb, fenitrothion and isoprothiolane values are from Ishida and Kobayashi (1995), and Ishida et al. (1996); Roundup values are from Tierney et al. (2007c); hydrocarbon values are from Maynard and Weber (1981).

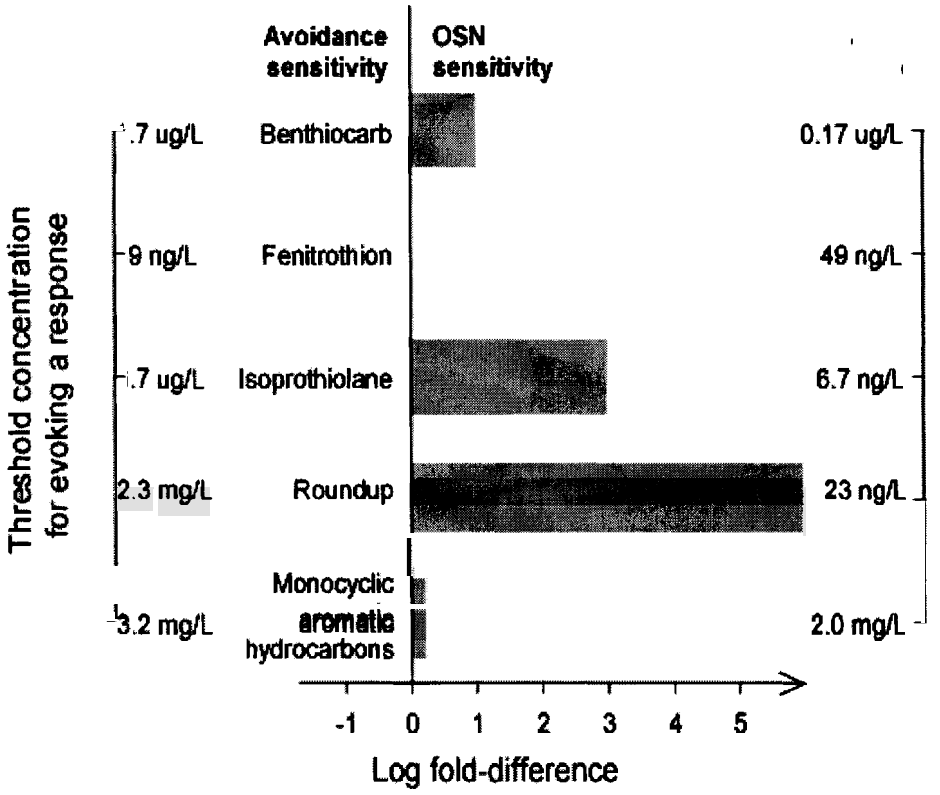


Figure 8-5 Various methods exist for measuring toxicity, including median time to lethality (LC_{50}) and median time to inhibition (IC_{50}), a sublethal measure. Increasing the exposure concentration brings more rapid effect for both endpoints. With olfactory toxicity, IC_{50} can be determined from the concentration-dependent decreases pesticides can cause in olfactory sensory neuron response to an odorant. For complete organismal protection, a maximum contaminant level (MCL) would need to be below IC_{50}.

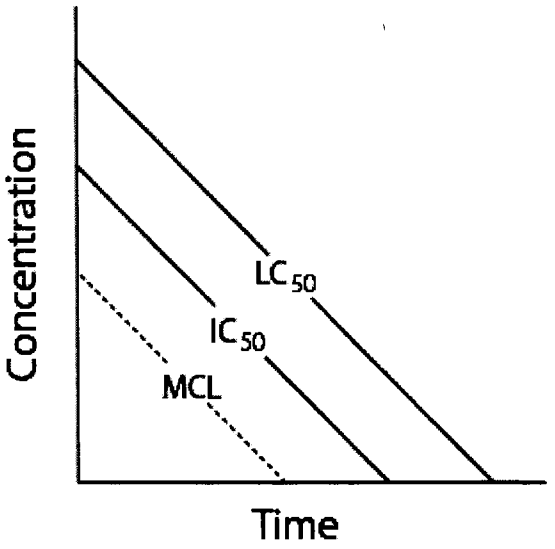
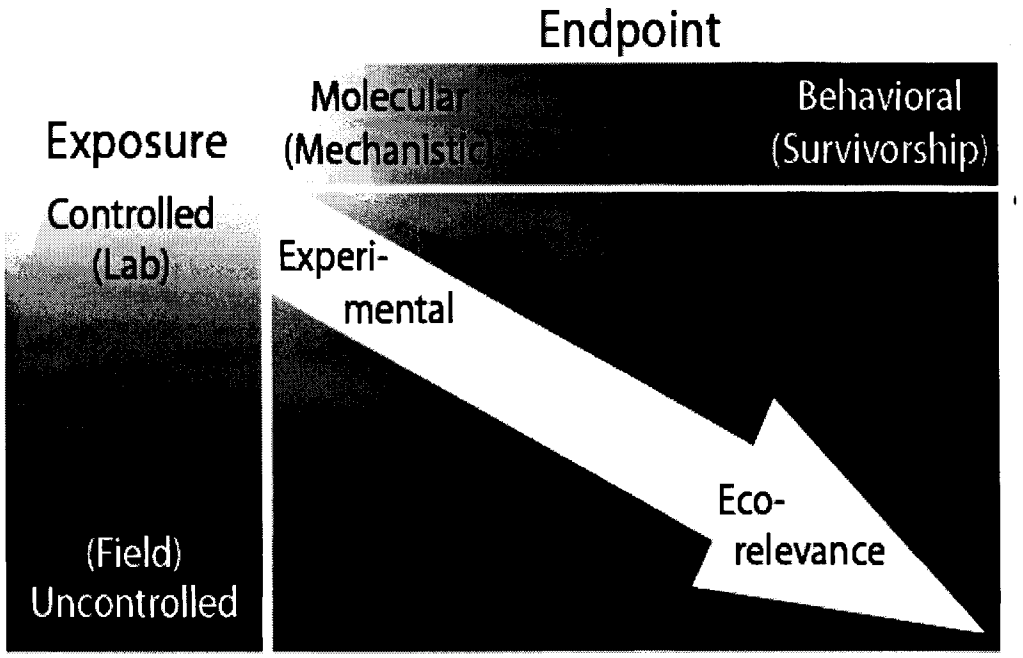


Figure 8-6 Toxicity endpoints relate to the ecological relevance. While lab based studies may elucidate toxicity mechanisms, they may lack in simulating the complex behavioral alterations that can occur following exposure to pesticide mixtures.



Appendix

Aquatic Non-Scents (cover story)

Science News

Week of Jan. 27, 2007; Vol. 171, No. 4

Repercussions of water pollutants that mute smell

Janet Raloff

People complain about the way that fish smell. But it's the fish that should be doing the grumbling. In pristine waters, the animals smell quite well, thank you. Those tiny holes near fishes' mouths are, in fact, nostrils through which the animals draw in water to pump over olfactory nerves. By distinguishing scents, fish find food and mates and avoid predators.

These rainbow trout and some other aquatic creatures can temporarily lose their sense of smell—and ability to detect mates, food, and predators—when exposed to any of several common water pollutants.

Studies decades ago, for instance, showed that mechanically plugging the nostrils of adult salmon prevented them from locating their natal streams when they attempted to return home to spawn. The fish as juveniles had recorded memories of smells as they went to sea. Without detecting the olfactory signposts, the fish couldn't retrace their routes, says Nathaniel L. Scholz, a zoologist at the National Oceanic and Atmospheric Administration's (NOAA's) Northwest Fisheries Science Center in Seattle.

In a series of studies over the past 6 years, his group has demonstrated that metals and pesticides—at concentrations commonly found in streams—can impair a salmon's sense of smell just as effectively as plugging the nostrils did. Meanwhile, other scientists have shown that such pollutants block the sense of smell in other organisms.

"What we're finding," says Scholz, is that "even short-term exposure to many of these pollutants—on the order of hours—can interfere with olfaction."
Researchers have reported that the impairment can disrupt the animals' normal behaviors in several ways. Fish use their keen sense of smell not only to navigate dark and cloudy waters but also to nose out scents indicating danger, such as chemicals from a predators' skin.

The studies are establishing that aquatic animals exposed to pollutants miss chemical cues that have life-and-death consequences, says ecotoxicologist Gregory G. Pyle of Nipissing University in North Bay, Ontario.

Pesticidal nose plugs

Atrazine, North America's most widely used herbicide for corn and cotton blunts a fish's sense of smell, according to work by Keith Tierney and his colleagues at Simon Frasier University in Burnaby, British Columbia.

A 30-minute exposure to a 1 parts per billion (ppb) concentration of atrazine reduced the activity of olfactory neurons in coho salmon (Oncorhynchus kisutch) by 11 percent, the researchers reported last November at the annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC) in Montreal. The animals' neural responses to alarm odors dropped by 45 percent. Higher doses of the herbicide triggered greater losses in smell; 100 parts per million atrazine eliminated any response to a predator's scent. River concentrations up to 20 ppb can occur briefly near farms that apply it, says Tierney.

Pure glyphosate, the active ingredient in another widely used herbicide that's sold under a series of trade names, including Roundup, caused similar changes in salmon olfaction, although only at far higher doses than were required of the commercial herbicide formulation. At the November SETAC meeting, these researchers presented data showing that Roundup was 100 times as powerful at blocking fishes' sense of smell as was an equal quantity of pure glyphosate.

Roundup contains a variety of ingredients added to glyphosate to increase the herbicide's adhesion to leaves and to retard its breakdown. Although these ingredients are listed as inert components on herbicide labels, Tierney's team concludes that they aren't inert as far as fish olfaction is concerned.

"I'd like to find out what those inerts are," Tierney says, but he notes that pesticide manufacturers regard them as part of their proprietary recipes.

Tierney isn't alone in his concern over supposedly inert ingredients. Some "4.1 billion pounds of inert [pesticide] ingredients are applied annually" to the U.S. environment, Christian E. Grue of the University of Washington in Seattle and his colleagues reported at the SETAC meeting.

Because these compounds aren't lethal to untargeted organisms, they don't require identification on labels, the Seattle researchers note—even though the inerts may exert a subtle but substantial toxic effect on aquatic life. Grue argues that "a new regulatory strategy is needed," which would require toxicity analyses of any supposedly inert ingredients.

Atrazine isn't the only chemical pesticide that can suppress a fish's ability to smell. Tierney's group showed that at exposures of about 10 ppb, the fungicidal wood-preservative known as IPB turned off olfaction in coho salmon. The researchers described that finding in the August 2006 Aquatic Toxicology.
They also reported in the October 2006 Environmental Toxicology and Chemistry that the insecticide endosulfan and the herbicides trifluralin and 2,4-D can impair a fish's sense of smell.

Scholz' group, too, has made contributions to the list of pesticides that affect fish olfaction. Six years ago, that team showed that diazinon significantly impaired responses by Chinook salmon (Oncorhynchus tshawytscha) to alarm scents and reduced their success in finding their natal pools.

More recently, Scholz' team has shown that low concentrations of the insecticide chlorpyrifos can impair olfaction in coho salmon and that the insecticide esfenvalerate can trigger abnormal neural signals in response to food scents.

Copper masks

Copper runs off agricultural lands, lawns, and urban streets. It's an active ingredient in many pesticides, and brake pads often shed copper as they wear down. Dissolved copper diminishes a fish's sense of smell, several researchers reported at the SETAC meeting.

Scholz' team, for instance, reported that just 3 hours of exposure to waterborne copper reduced responses of juvenile coho salmon to a typical alarm odor, a scent that came from the skin of a wounded coho. Such chemical alarms typically make young coho dive for cover.

Juvenile salmon that had been kept in clean tanks reacted properly to the cue. They stopped swimming, ignored food, and sought refuge. Other juveniles, which had spent 3 hours in copper-tainted water, swam placidly and continued feeding after researchers spiked their water with an alarm scent. Scholz characterizes the copper-treated fish as "totally oblivious" to this whiff of danger.

The scientists went further in establishing that copper impairs the sense of smell. From several fishes' nostrils, they recorded electrical signals carrying information

to the brain from olfactory neurons. Compared with the untreated fish, copper-treated coho produced much weaker—or no—responses to the alarm scents. Scholz says that his group found "a very tight relationship" between the fishes' behavior and olfactory neuron signals.

Reehan Mirza, Pyle, and their colleagues at Nipissing University have witnessed the same phenomenon in fish exposed to copper in the wild. These Canadian researchers worked with yellow perch (Perca flavescens) taken from Ontario lakes, some of which were contaminated by runoff from a copper mine. "Fish from the clean lakes responded to alarm cues," Mirza told Science News, "while perch from the contaminated lakes did not."

Mirza's team exposed the fish to chemicals extracted from the skin of a wounded perch or the skin of a rainbow trout, a perch predator. Odor sensitivity in perch from lakes with less than 2 parts per billion (ppb) copper was 2.5 times that in fish from lakes containing at least 18 ppb copper.

Dissolved copper also seems to prevent female fish from sizing up prospective mates. In seeking males that are fat and healthy, female fathead minnows (Pimephales promelas) assess a Romeo's qualities "exclusively from chemical information," says Pyle.

To test copper's effects on this aspect of smell, Pyle and his colleagues raised males for a month on either a low- or a high-calorie diet. The results were emaciated, sickly looking males and specimens of minnow machismo.

A male from each category was then placed at the end of each branch of a Y-shaped maze. Egg-swollen females placed at the start of the maze couldn't see their potential mates but could pick up the males' scents. In nearly every case where the female had come from a clean lake, she headed toward the larger male. Females from copper-tainted lakes, by contrast, showed no preference between the candidates.

The researchers then built a glass cage in which the females could see but not smell potential mates. In this case, females from both the clean and copper-tainted lakes showed no preference for robust males. Pyle concludes that females can't compensate visually for their lack of smell.

"This can have implications on the subsequent gene pool," he told Science News. "If you're not picking the optimal mate, there's an increasing probability that that next generation won't be as successful in their environment."

Beyond fish

Pyle and his colleagues have also shown effects of copper-mine runoff on leeches and water fleas. The team began investigating the metal's impact on

leeches after hearing reports from anglers who observed that leeches, aquatic relatives of the earthworm, appeared to inhabit only the lakes around Sudbury, Ontario, that weren't polluted by copper-mine wastes.

Pyle explains that the local leech, Nephelopsis obscura, is "effectively blind—and therefore relies almost exclusively on chemical information [scents] to locate food."

The researchers collected leeches from clean lakes and then put some in water with copper at concentrations of either 10 or 20 ppb. After 2 to 16 days of such exposure, each leech was placed in a clean-water tank containing a Y-shaped maze. One arm of the maze was baited with beef liver.

Copper-exposed leeches had trouble identifying the path to the food, and the longer they had been exposed to the metal the less likely they were to succeed. Leeches unexposed to copper invariably found the meal unless the researchers laced the maze water with copper. Then, Pyle notes, these leeches, too, swam "randomly, no longer able to discriminate food scents."

Even more telling, Pyle says, were tests in which his group placed a leech in a fishbowl of clean water along with a dead minnow. If the leech had been living in clean water, it immediately swam to the fish and began feeding. However,

leeches that had spent a week in water with at least 5 ppb copper had trouble locating the minnow.

Pyle says that some Sudbury-area lakes have up to 30 ppb copper concentrations—and no leeches. Unable to smell their next meal, the animals there may have all starved, he concludes.

His team has also studied copper's effect on a tiny water flea (Daphnia pulex)—a crustacean about 2.5 millimeters long—that serves as the bottom rung on many lake-animal food ladders. Larvae of an insect known as the phantom midge (Chaoborus americanus) are among Daphnia's predators. The larvae release an odor when they feed on the water fleas. Young Daphnia that pick up the scent respond, over several days, by developing neck spines that make the tiny animals too big for a larval midge's mouth.

In lab tests, however, Daphnia housed in water containing as little as 5 ppb copper developed few if any protective neck spines in response to the midge scent.

Growing concerns

Pesticides typically reach streams via runoff when rains hit a recently treated farm, forest, or lawn. Roadways, storm drains, and paved and plowed land

facilitate quick pollutant runoff into surface waters, Scholz says. His NOAA team now simulates such intermittent pollutant exposures.

In these circumstances, a pollutant-induced shutdown of fishes' sense of smell may last only a few hours. However, in some regions, pulses of polluted storm water "may come through so frequently that fish never effectively recover," Scholz says. Increasing runoff in areas of growing urban and rural development may explain why salmon are disappearing from streams throughout the West, he proposes.

Pyle notes that pesticides and copper at concentrations similar to those in the environment knock out olfactory communications in every species tested to date—whether water fleas, leeches, or fish. He told Science News, "The apparent ubiquity of this phenomenon is, well, quite disturbing."

Corrections:

This article misidentified glyphosate. This herbicide is the active ingredient not in atrazine but in a range of products such as Roundup. Studies in Canada have shown that both atrazine and glyphosate can impair fish olfaction.

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