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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 the present study, we investigated 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-olfactogram 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-dichlorophenoxyacetic acid. With the relatively insoluble pesticides endosulfan and trifluralin, decreases in EOG amplitude were only apparent at relatively high concentrations (100 and 300 μg/L, respectively) following 20 min of exposure and were 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 postexposure. Dichlorophenoxyacetic acid 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 greater than the 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 exposures in the low-μg/L range.

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 the sublethal olfactory toxicity of carbamates [3], chlorpyrifos [4], copper (a constituent of pesticides; e.g., 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, because some stocks are endangered in Canada [8] and the United States [9]. Ideally, water-quality guidelines are in place to protect species from injurious chemical concentrations. However, guidelines often are 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 median lethal concentration (LC50) for another salmonid, rainbow trout (*O. mykiss*) [10], yet simazine is permitted at 10 μg/L in Canada [11] (http://www.ccme.ca). Similarly, the carbamate fungicide iodocarb (IPBC), which has a maximum allowed freshwater 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 the LC50 [12]. These studies demonstrate that salmonid olfaction can be impaired at toxicant concentrations substantially less than the LC50—and less than existing water-quality guidelines. Because olfaction is tantamount to survival for anadromous salmonids, this sublethal toxicity endpoint would need to be considered in determining the 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.

In the present study, we examine five agricultural pesticides identified as being of concern to the aquatic environment: Chlorothalonil, endosulfan, glyphosate, trifluralin, and 2,4-dichlorophenoxyacetic acid (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 (especially by the forestry industry) herbicide, with more than 120,000 kg (acid and isopropylamine forms) used in British Columbia (Canada) during 2003 [14] (http://www.env.gov.bc.ca/epd/epdpa/ipmp/technical_reports/pesticide_survey2003/survey_2003.html).

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Trifluralin is a pre-emergent herbicide used to control broadleaf weeds and annual grasses. Like glyphosate, 2,4-D is used to control broadleaf and aquatic weeds. Because the selected pesticides are currently used in agriculture, the potential exists for their presence in coho-bearing streams bordering land with agricultural activity. The fungicide IPBC was included in our testing despite being previously identified as an olfactory toxicant [3], because we used a new testing protocol. In British Columbia, IPBC is used routinely (>10,000 kg sold 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 the EOG 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 ± 0.86 g; length, 11.4 ± 0.25 cm; 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, >90% saturation; pH 6.8; hardness, 6.12 mg/L as $CaCO_3$) under a 12:12-h light:dark photoperiod with a 30-min dawn: dusk transition and a temperature of approximately 10°C. Coho salmon parr were fed commercial salmon pellets (EWOS, Surrey, BC, Canada) ad libitum.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). The specific pesticides and their given purities were chlorothalonil (2,4,5,6-tetrachloro-1,3-dicyanobenzene; purity, 99.1%), endosulfan (α,β-1,2,3,4,7,7-hexachlorobicyclo(2.2.1)-2-heptene-5,6-bisoxymethylene sulfite; purity, 99%), glyphosate acid (*N*-(phosphonomethyl) glycine; purity, 99%), IPBC (purity, 97%), trifluralin (α,α,α-trifluro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine; purity, 99.5%), and 2,4-D (purity, 98%). 2-Phenoxyethanol was used for anesthesia, and L-serine (serine; purity, 99%) was used as an odorant.

Electrophysiology

Electro-olfactograms were recorded according to the procedure described by Evans and Hara [2] using the apparatus described by Jarrard et al. [3]. Briefly, fish were anesthetized using 2-phenoxyethanol (induction, 0.4 ml/L; maintenance, 0.2 ml/L) and placed in a Plexiglas holder, and the gills were 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 the holding-tank temperature (~10°C) by a Lauda chiller (Brinkmann Instruments, Westbury, NY, USA). Fish heart rate was monitored by paired internal, ventromedially placed, stainless-steel electrodes, amplified 100×, and displayed on an oscilloscope. Tests in which arrhythmia was observed were not included for analysis. An Ag/Ag-Cl electrode in 2% agar (1 M NaCl) inside a pulled-glass pipette (tip diameter, ~150 μm) was advanced until it was observed to be 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 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 1,000-fold, 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. 1A; for example traces, see Fig. 1B). Two-second pulses of concentrations greater than 10^{-2} M required more than 2 min to restore to 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 previous electrophysiological work with salmonids (*O. kisutch* and *O. mykiss* [15]), is greater than the 10^{-5} M used in some EOG studies (see, e.g., [3,7]) but lower than the 10^{-2} M used in another [16]. In the present study, 10^{-3} M was used because 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 (e.g., with carcass decay).

To limit EOG variation during exposure and postexposure periods, fish were acclimated to the apparatus, and pre-exposure EOGs were taken until the variation between two EOGs determined 5 min apart was 5% or less. 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% of the LC50s for salmonids (Table 1). To establish a concentration–response relationship, incrementally higher and lower concentrations also were tested. With glyphosate, because formulations generally have higher toxicity as a result of surfactants (e.g., 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, for which 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 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 first had to 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 (10 mg/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 evok-

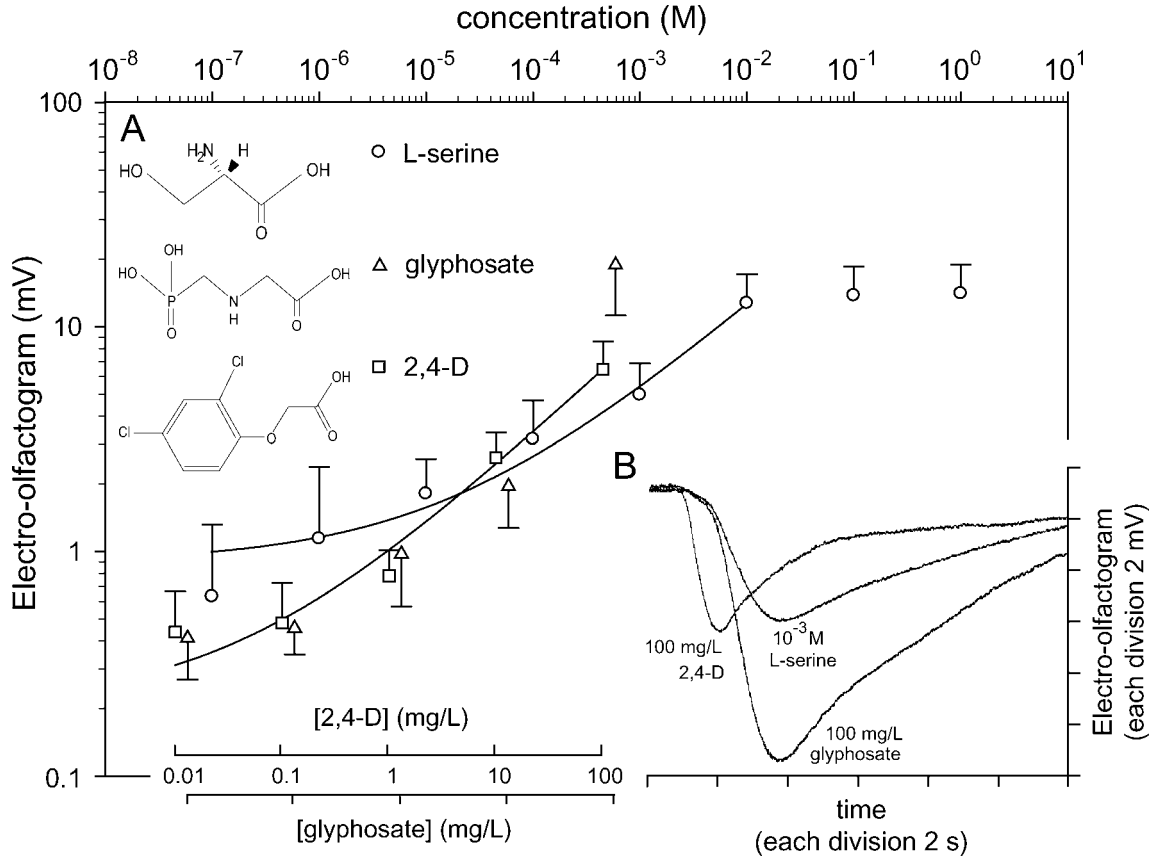

Fig. 1. (**A**) Electro-olfactogram (EOG) responses and (**B**) example EOG traces taken from the nasal epithelium of coho salmon in response to 2-s pulses of various concentrations of L-serine, glyphosate acid, and 2,4-dichlorophenoxyacetic acid (2,4-D) (in **A**, $n = 6$ per group; in **B**, serial EOG traces were taken 10 min apart from one fish). Curve equations for **A** are as follows: L-Serine, EOG = 0.896 + 79.0·2.59$^{\log[\text{serine}]}$, $r^2 = 99.3\%$, $p = 0.0005$; 2,4-D, 0.207 + 193·2.78$^{\log[2,4\text{-D}]}$, $r^2 = 99.6\%$, $p = 0.0037$; glyphosate, EOG = 0.559 + 67,300·12.7$^{\log[\text{glyphosate}]}$, $r^2 = 99.9\%$, $p = 0.0005$.

ing EOGs, each pesticide was first tested at its maximum exposure concentration using the same protocol as that for 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 at 2, 5, 10, 15, 20, 30, 40, 50, and 60 min postexposure. 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 postexposure 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 followed by a Holm-Sidak post hoc test. Because no differences were found, control groups were pooled, and pesticide-exposure groups were tested against the control using the same method as described above.

For glyphosate, the median inhibitory concentration (IC50) 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 (average values for control, 0.1, 1, 10, and 100 mg/L at each time point), a three-parameter exponential decay model (inhibition = $y_o + a \cdot e^{b[\text{glyphosate}]}$) was used for simplicity, because a polynomial model did not provide a better fit. To establish 95% confidence intervals (CIs), curves also were fitted to twofold the standard error above and below each point. To view interactions in IC50 and exposure duration, the 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 occurred 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 the mean ± standard error, 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 bpm). Across all fish, EOG acclimation took 49.3 ± 2.2 min, and pre-exposure EOGs were 4.31 ± 0.19 mV (range, 0.496–10.6 mV). The EOG acclimation time was positively correlated to fish mass (range, 7.53–42.8 g) [(min) = 1.4(g) + 30, $r^2 = 15.6\%$, $p = 0.0015$].

Table 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

Pesticide[a]	Test animal	Size (g)	Temp (°C)	LC50	95CI	Test length	Form[b]	Reference	Solubility,[c] half-life	Water standards (Canadian)[d]	EOG (coho salmon)		EOG sensitivity	
											Screen range	Effect	<LC50	<Standards
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][e]	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]						
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 139 d	1.9 μg/L	1–100 μg/L	1	Yes	Yes

[a] 2,4-D = dichlorophenoxyacetic acid; IPBC = iodocarb.
[b] I = pure material; T = technical material; F = formulation, U = unknown.
[c] From Wauchope et al. [19] except for IPBC, which was taken from Juergensen et al. [45].
[d] Canadian Water-Quality Guidelines for the Protection of Aquatic Life, freshwater guidelines. In the United States, the Federal Insecticide, Fungicide, and Rodenticide Act controls exposure through labeling restrictions on pesticide applications based on modeled environmental concentrations.
[e] U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC (http://www.cerc.cr.usgs.gov/pubs/center/pdfDocs/90301.pdf).

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. 1).

With 30-min exposures, vehicle control EOG peak amplitude did not vary significantly over the 90-min testing procedure (Fig. 2A). For IPBC, exposure to 1 μg/L caused a significant decrease in EOG 25 min into the 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. 3). At the end of exposure, EOG responses differed from control for all concentrations, with treatments at 1, 10, and 100 μg/L reaching 71.8 ± 16.5%, 54.1 ± 5.14%, and 57.9 ± 13.6%, respectively, of control. At 60 min postexposure, all IPBC-exposure groups remained significantly depressed from control except the lowest concentration, which returned to the 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. 2B), so other concentrations were not explored. Endosulfan at 100 μg/L caused significant EOG decreases with 20 to 30 min of exposure, but EOG returned to control levels by 2 min postexposure (Fig. 2C). Endosulfan at 10 μg/L did not cause any difference compared to controls. Similarly, with exposure to high-concentration (300 μg/L) trifluralin, EOG was decreased at 30 min but recovered within 2 min of exposure cessation (Fig. 2D). As with endosulfan, EOG effects were absent when trifluralin exposure was reduced by 10-fold (30 μg/L).

At exposures of 1 and 10 mg/L, 2,4-D did not affect serine-evoked EOGs (Fig. 2E). However, in the presence of 100 mg/L of 2,4-D, the EOG response was atypical and in the opposite direction. Because 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), because 2,4-D was not included in the stimulant pulse. For the purposes of the present paper, reversed washout EOGs were considered as EOG loss and as 0% of pre-exposure EOGs. Two minutes following exposure to 100 mg/L, EOG appeared to recover to 53.2 ± 9.4%, and it remained at a similar level up to 60 min postexposure. A clear concentration–response relationship was not established with 2,4-D, because both 1 and 10 mg/L exposures did not alter EOG significantly from the control values.

Glyphosate reduced EOG in a concentration-dependent manner (Figs. 2F and Fig. 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). Exposure to 100 mg/L of glyphosate, as with exposure to 100 mg/L of 2,4-D, caused small reversed EOGs as a result of washout. After 30 min of exposure, EOGs recorded in fish exposed to 1, 10, and 100 mg/L were 66.1 ± 8.0%, 44.4 ± 13.9%, and 0 ± 0%, respectively, of their pre-exposure level. Exposure at 0.1 mg/L did not affect EOG during or after exposure. Unlike the 100 mg/L of 2,4-D exposure, EOG recovery after 100 mg/L was time-dependent (Fig. 2F). By 60 min postexposure, 1 and 100 mg/L exposures remained significantly different from control, at 74.5 ± 11.7% and 72.8 ± 11.0%, respectively, of their pre-exposure EOG.

The point at which EOG was 50% of the pre-exposure value (IC50) was interpolated for glyphosate. At 2 min into the exposure, the IC50 was 10.9 mg/L, and at 5 min, it dropped to 8.17 mg/L and then remained at a similar level for the balance of the exposure (IC50 = $7.66 \cdot e^{[0.218/(\text{time}-1.39)]}$, r^2 = 88.4%, p = 0.01; upper 95% CI = $9.76 + 11.5 \cdot e^{(-0.289 \cdot \text{time})} + 0.322 \cdot \text{time}$, r^2 = 96.3%, p = 0.01; lower 95% CI = $4.91 + 28.5 \cdot e^{(-1.32 \cdot \text{time})} - 0.102 \cdot \text{time}$, r^2 = 95.8%, p = 0.01).

The IC50s were not calculated for other pesticides, because greater than 50% inhibition was not reached except for 2,4-D. However, the IC50 was not calculated with this pesticide, because 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 typically were noted in the high-μg/L to mg/L concentration range except for IPBC, for which 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 1). First, we asked if an acute, 30-min pesticide pulse alters olfaction, setting our pulse concentrations according to either maximum solubility (because potentially little likelihood exists that a salmon will encounter a pulse of a concentration > 100% solubility) or an estimate of sublethal exposure concentration (50% of the 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 equal to or less than the concentration at which 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, because 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 antipredator behavior [21]. Recently, it was demonstrated that amino acids also can function as ''home stream olfactory bouquet'' constituents for chum salmon [17]. Thus, pesticides that reduce amino acid–elicited EOGs also may impair imprinting. However, as mentioned earlier, the amino acid concentrations of salmon EOG studies likely are greater than environmental amino acid concentrations, which hampers real-world extrapolation.

We found decreases in serine EOG amplitude with IPBC similar to those reported by Jarrard et al. [3]. Those authors found a 50 and 70% decrease in EOG 30 min following a exposure at 1 and 10 μg/L, respectively; we noted a decrease of 30 and 45%, respectively, for the same concentrations. The more conservative impairment estimate of the present study may be attributable to a higher L-serine stimulus concentration (10^{-3} M in the present study and 10^{-5} M in Jarrard et al. [3]) or differences in the exposure system. If the difference is caused by the greater stimulus concentration, this suggests that EOG alteration, as assayed through EOG peak size, may be-

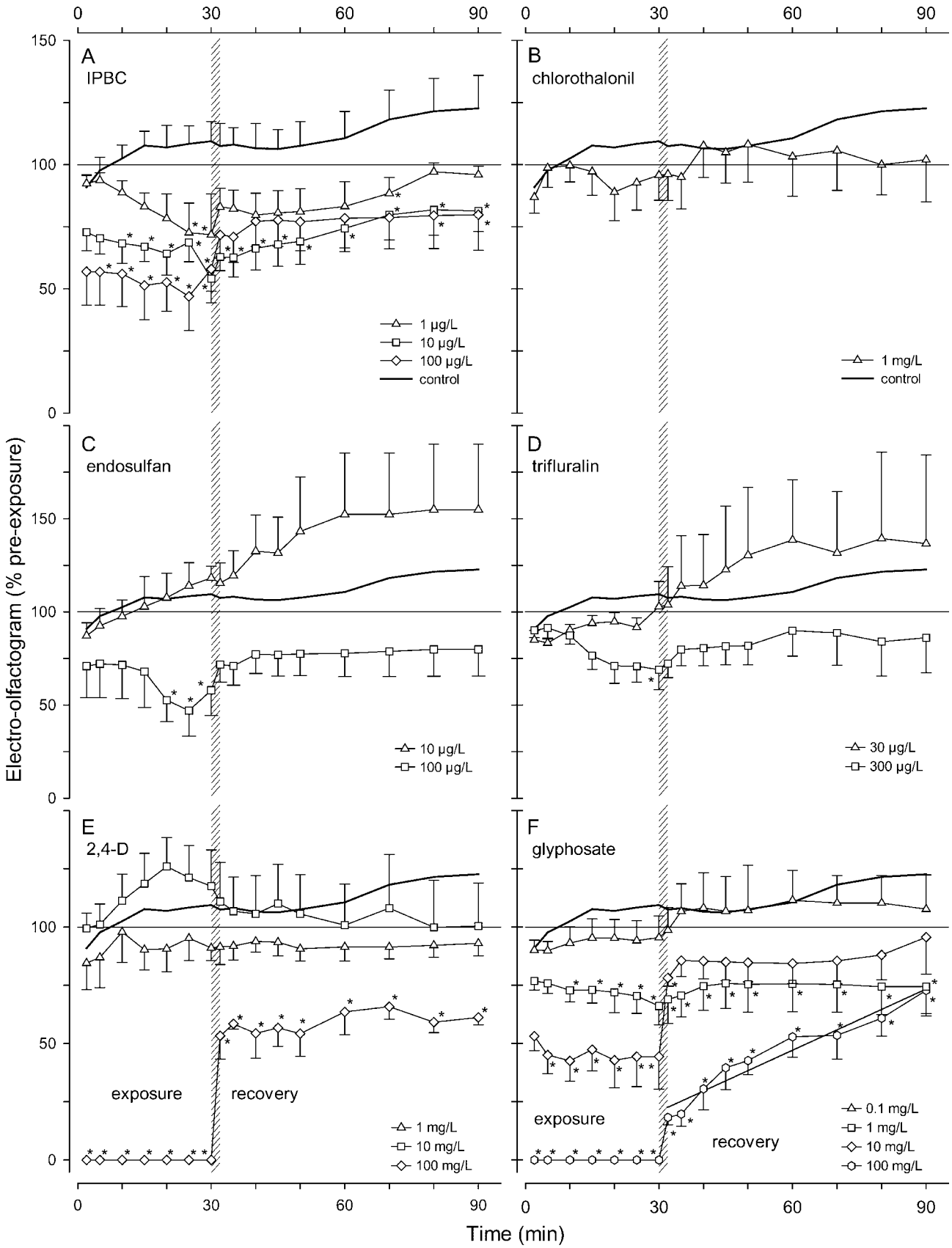

Fig. 2. Changes in coho salmon electro-olfactogram (EOG) during 30-min exposure and 60-min recovery from (**A**) control ($n = 18$), the carbamate fungicide iodocarb (IPBC), and (**B**) chlorothalonil, (**C**) endosulfan, (**D**) trifluralin, (**E**) 2,4-dichlorophenoxyacetic acid (2,4-D), and (**F**) glyphosate ($n = 6$ per exposure concentration). Hatched area represents the exposure washout and transition period. For reference, the EOG of control fish are shown as a solid line. Line equation for 100 mg/L of 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, $p < 0.05$).

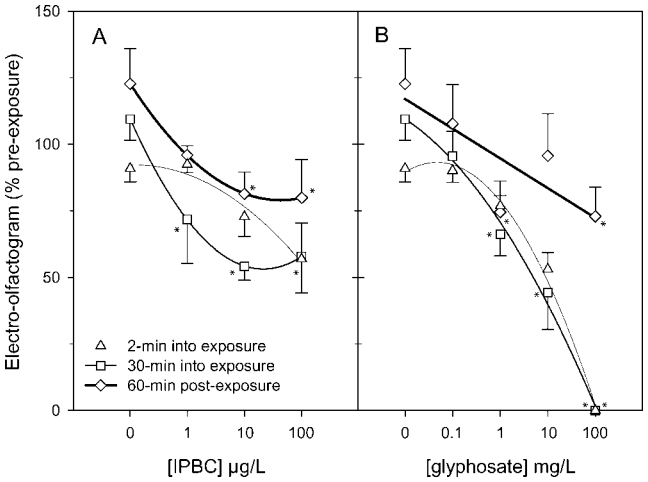

Fig. 3. Changes in electro-olfactogram (EOG) at 2 and 30 min into and 60 min after (**A**) iodocarb (IPBC) and (**B**) glyphosate exposure. Polynomial curves were fitted for visualization purposes only. Significant differences from control are denoted with asterisks (two-way repeated-measures analysis of variance, Holm-Sidak, $p < 0.05$).

come 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. In any event, iodocarb is clearly toxic to coho salmon olfaction at concentrations less than those that cause lethality (Table 1). The Canadian Water-Quality Guidelines for the Protection of Aquatic Life (CWQG) [11] set a limit at 1.9 µg/L. Based on the present results and those of Jarrard et al. [3], the guidelines may not provide an 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 causing lethality and the CWQG of 0.18 µg/L (Table 1). Chlorothalonil was tested just above its theoretical maximum solubility (0.6 mg/L [19]), which is a concentration well above the 96-h LC50 range of 14.3 to 17.1 µg/L [23,24], so chronic effects or other sublethal effects are likely to develop before 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 a 4-d exposure [25,26]. That we only noted olfactory effects after exposure to a 200-fold higher concentration suggests either that 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, because the time to EOG impairment increased with decreasing concentration for both IPBC and glyphosate exposures, acute and chronic endosulfan olfactory toxicities likely differ in their 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 the present study, we found a greater than 20-fold range in EOG peak size before 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 (0.02 µg/L) (Table 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, it only caused a small effect. Because lethality occurs at a much lower concentration (Table 1), and because the CWQG is 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 exposure to low-concentration (1 and 10 mg/L) 2,4-D. 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 the set acted on by 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] and *Solea senegalensis* [28]). At high concentration (100 mg/L), 2,4-D eliminated serine-evoked EOGs. This effect may result from 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 exposure to 100 mg/L of 2,4-D as it did after exposure to 100 mg/L of glyphosate. The approximately 50% recovery observed could be caused by persistent effects or actual physical damage resulting from prolonged depolarization.

Folmar [29] found that rainbow trout avoided 2,4-D concentrations of 1 and 10 mg/L but not 0.1 mg/L, suggesting that salmonids may behaviorally reduce 2,4-D exposure where possible. Our results suggest that 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 the 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 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 of 10.9 mg/L (95% CI, 6.72–16.8 mg/L) 2 min into the exposure. However, glyphosate also evoked EOGs in a concentration-dependent manner. This is not surprising given the similarity of glyphosate to the amino acid glycine. Thus, during exposure, the reduced serine EOG peak size may indicate that glyphosate was acting as an agonist; consequently, there may have been receptor adaptation. Several minutes after glyphosate washout, it was unlikely that significant residual receptor binding (i.e., antagonist or agonist action) occurred. However, because the serine-evoked EOG did not return to control levels for all exposure concentrations (1 and 100 mg/L), evidence suggests either persisting adaptation or toxic effects on OSNs or other cells of the olfactory rosette.

In our tests, we used pure glyphosate acid, despite product formulations generally having elevated toxicity because of the 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 (WA, USA), 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, in addition to its high solubility, make exploring its olfactory toxicity of interest, even though it tends not to persist unbound (for review, see Giesy et al. [32]).

The present study noted that glyphosate has sublethal neurophysiological effects (i.e., salmonid olfaction would be impaired before lethality would be expected) (Table 1). Fish may be able to reduce any sublethal effects, because our data suggest that fish can detect it via OSNs. However, a previous study suggested that this may not be the case, because rainbow trout did not avoid glyphosate over a concentration range of 0.1 to 10 mg/L [29]. Thus, the present results as well as those of the previous study [29] suggest that salmonid OSNs detect both 2,4-D and glyphosate but do not avoid glyphosate. Regardless, in view of the existing CWQG for glyphosate (65 μg/L), the relatively high (1 mg/L) concentration required to significantly decrease EOG, and the physicochemical properties of glyphosate, acute exposures of glyphosate are unlikely to be 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 organophosphorous insecticide diazinon [6], atrazine [7], and the carbamate fungicide IPBC [3], have acetylcholinesterase (AChE)-inhibiting properties. Acetylcholine is of central importance, because 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 AChE will cause elevated acetylcholine, 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. Because our exposure system was designed to isolate pesticide exposure to the naris, altered heart rate would not have been expected, even if a pesticide had AChE-inhibiting properties.

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. Thus, runoff pulses of this semipersistent pesticide should be considered as hazardous to olfaction in migratory salmonids. The existing CWQG would likely better protect salmonid olfaction if set at the NOAEC, which for the olfactory epithelium would be approximately 10-fold lower than the present value. 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 organophosphorous pesticides. Electro-olfactograms could be of greater use and have better real-world applicability if new methods could be evolved to use the EOG to detect any olfactory effects arising from longer-term exposures.

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REFERENCES

1. Ottoson D. 1956. Analysis of the electrical activity of the olfactory epithelium. *Acta Physiol Scand* 35:1–83.
2. Evans RE, Hara TJ. 1985. The characteristics of the electro-olfactogram (EOG): Its loss and recovery following olfactory nerve section in rainbow trout (*Salmo gairdneri*). *Brain Res* 330:65–75.
3. Jarrard HE, Delaney KR, Kennedy CJ. 2004. Impacts of carbamate pesticides on olfactory neurophysiology and cholinesterase activity in coho salmon (*Oncorhynchus kisutch*). *Aquat Toxicol* 69:133–148.
4. Sandahl JF, Baldwin DH, Jenkins JJ, Scholz NL. 2004. Odor-evoked field potentials as indicators of sublethal neurotoxicity in juvenile coho salmon (*Oncorhynchus kisutch*) exposed to copper, chlorpyrifos, or esfenvalerate. *Can J Fish Aquat Sci* 61:404–413.
5. Baldwin DH, Sandahl JF, Labenia JS, Scholz NL. 2003. Sublethal effects of copper on coho salmon: Impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. *Environ Toxicol Chem* 22:2266–2274.
6. Moore A, Waring CP. 1996. Sublethal effects of the pesticide diazinon on olfactory function in mature male Atlantic salmon parr. *J Fish Biol* 48:758–775.
7. Moore A, Lower N. 2001. The impact of two pesticides on olfactory-mediated endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. *Comp Biochem Physiol B* 129: 269–276.
8. Committee on the Status of Endangered Wildlife in Canada. 2002. COSEWIC assessment and status report on the coho salmon *Oncorhynchus kisutch* (Interior Fraser population) in Canada. CW69-14/289-2003E-IN. Ottawa, ON, Canada.
9. National Marine Fisheries Service. 1996. Endangered and threatened species: Threatened status for central California coast coho salmon evolutionarily significant unit (ESU). *Fed Reg* 61:56138–56149.
10. Bathe R, Sachsse K, Ullmann L, Hormann WD, Zak F, Hess R. 1975. The evaluation of fish toxicity in the laboratory. *Proc Eur Soc Toxicol* 16:113–124.
11. The Canadian Council of Ministers of the Environment. 1999. Canadian Environmental Quality Guidelines. Manitoba Statutory Publications, Winnipeg, MB.
12. Farrell A, Stockner E, Kennedy CJ. 1998. A study of the lethal and sublethal toxicity of Polyphase P-100, an antisapstain fungicide containing 3-iodo-2-propynyl butyl carbamate (IPBC), on fish and aquatic invertebrates. *Arch Environ Contam Toxicol* 35: 472–478.
13. Verrin SM, Begg SJ, Ross PS. 2004. Pesticide use in British Columbia and the Yukon: An assessment of types, applications, and risks to aquatic biota. *Can Tech Rep Fish Aquat Sci* 2517: 1–209.
14. ENKON Environmental Limited. 2005. Survey of pesticide use in British Columbia: 2003. Project 1004-011. Environment Canada, Ministry of Environment, Lands and Parks. Surrey, BC, Canada.
15. Hansen JA, Rose JD, Jenkins RA, Gerow KG, Bergman HL. 1999. Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to copper: Neurophysiological and histological effects on the olfactory system. *Environ Toxicol Chem* 18:1979–1991.
16. Wang D-Y, Huang B-Q. 1999. TBT (tributyltin) toxicity to the visual and olfactory functions of tigerperch (*Terapon jarbua* Forsskål). *Zool Stud* 38:189–195.

17. Shoji T, Yamamoto Y, Nishikawa D, Kurihara K, Ueda H. 2003. Amino acids in stream water are essential for salmon homing migration. *Fish Physiol Biochem* 28:249–251.
18. Tsui MTK, Chu LM. 2004. Comparative toxicity of glyphosate-based herbicides: Aqueous and sediment pore-water exposures. *Arch Environ Contam Toxicol* 46:316–323.
19. Wauchope RD, Buttler TM, Hornsby AG, Augustijn-Beckers PWM, Burt JP. 1992. The SCS/ARS/CES pesticide properties database for environmental decision-making. *Rev Environ Contam Toxicol* 123:1–157.
20. Scholz N, Truelove NK, French BL, Berejikian BA, Quinn TP, Casillas E, Collier TK. 2000. Diazinon disrupts antipredator and homing behaviors in chinook salmon (*Oncorhynchus tshawytscha*). *Can J Fish Aquat Sci* 57:1911–1918.
21. Idler DR, Fagerlund UHM, Mayoh H. 1956. Olfactory perception in migrating salmon I. l-Serine, a salmon repellent in mammalian skin. *J Gen Physiol* 39:889–892.
22. Rehnberg BG, Jonasson B, Schreck CB. 1985. Olfactory sensitivity during parr and smolt developmental stages of coho salmon. *Trans Am Fish Soc* 114:732–736.
23. Davies P, White R. 1985. The toxicology and metabolism of chlorothalonil in fish I. Lethal levels for *Salmo gairdneri*, *Galaxias maculatus*, *G. truttaceus*, and *G. auratus* and the fate of ^{14}C-TCIN in *S. gairdneri*. *Aquat Toxicol (Amsterdam)* 7:93–106.
24. Davies P. 1987. Physiological, anatomic, and behavioral changes in the respiratory system of *Salmo gairdneri* Rich on acute and chronic exposure to chlorothalonil. *Comp Biochem Physiol C* 88: 113–119.
25. Park D, Hempleman SC, Propper CR. 2001. Endosulfan exposure disrupts pheromonal systems in the red-spotted newt: A mechanism for subtle effects of environmental chemicals. *Environ Health Perspect* 109:669–673.
26. Park D, Propper CR. 2002. Endosulfan affects pheromonal detection and glands in the male red-spotted newt, *Notophthalmus viridescens*. *Bull Environ Contam Toxicol* 69:609–616.
27. Hubbard PC, Barata EN, Canário AVM. 2003. Olfactory sensitivity to catecholamines and their metabolites in the goldfish. *Chemical Senses* 28:207–218.
28. Velez Z, Hubbard PC, Barata EN, Canário AVM. 2005. Evidence for functional asymmetry in the olfactory system of the Senegalese sole (*Solea senegalensis*). *Physiol Biochem Zool* 78:756–765.
29. Folmar L. 1976. Overt avoidance reaction of rainbow trout fry to nine herbicides. *Bull Environ Contam Toxicol* 15:509–514.
30. Ishida Y, Yoshikawa H, Kobayashi H. 1996. Electrophysiological responses of three chemosensory systems in the carp to pesticides. *Physiol Behav* 60:633–638.
31. Kilbride KM, Paveglio FL. 2001. Long-term fate of glyphosate associated with repeated Rodeo applications to control smooth cordgrass (*Spartina alterniflora*) in Willapa Bay, Washington. *Arch Environ Contam Toxicol* 40:179–183.
32. Giesy JP, Dobson S, Solomon KR. 2000. Ecotoxicological risk assessment for Roundup® herbicide. *Rev Environ Contam Toxicol* 167:35–120.
33. Burleson ML, Milsom WK. 1995. Cardio-ventilatory control in rainbow trout: II. Reflex effects of exogenous neurochemicals. *Respir Physiol* 101:289–299.
34. Inglis SK, Corboz MR, Taylor AE, Ballard ST. 1997. In situ visualization of bronchial submucosal glands and their secretory response to acetylcholine. *Am J Physiol Lung Cell Mol Physiol* 272:L203–L210.
35. Davies PE, Cook LSJ, Goenarso D. 1994. Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. *Environ Toxicol Chem* 13:1341–1354.
36. Johnson W, Finley MT. 1980. Handbook of acute toxicity of chemicals to fish and aquatic invertebrates: Summaries of toxicity tests conducted at Columbia National Fisheries Research Laboratory, 1965-78. Report 137. U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC.
37. Das RC, Bani S. 1993. Effect of endosulfan and malathion on the ovarian steroidogenesis and brain acetylcholinesterase activity of the catfish, *Clarias batrachus* (Linnaeus). *Veterinarski Arhiv* 63:75–83.
38. Neškovic NK, Poleksi V, Elezovi I, Karan V, Budimir M. 1996. Biochemical and histopathological effects of glyphosate on carp, *Cyprinus carpio* L. *Bull Environ Contam Toxicol* 56:295–302.
39. Mitchell D, Chapman PM, Long TJ. 1987. Acute toxicity of Roundup® and Rodeo® herbicides to rainbow trout, chinook, and coho salmon. *Bull Environ Contam Toxicol* 39:1028–1035.
40. Macek KJ, Hutchinson C, Cope OB. 1969. The effects of temperature on the susceptibility of bluegills and rainbow trout to selected pesticides. *Bull Environ Contam Toxicol* 4:174–183.
41. Cope OB. 1966. Contamination of the freshwater ecosystem by pesticides. *J Appl Ecol* 3:33–44.
42. Bandt HJ. 1957. The toxicity of herbicides to fish. *Z Fisherei (N.F.)* 6:121–125.
43. Gómez L, Masot J, Martínez S, Durán E, Soler F, Roncero V. 1998. Acute 2,4-D poisoning in tench (*Tinca tinca* L.): Lesions in the hematopoietic portion of the kidney. *Arch Environ Contam Toxicol* 35:479–483.
44. Meehan WR, Norris LA, Sears HS. 1974. Toxicity of various formulations of 2,4-D to salmonids in southeast Alaska. *J Fish Res Board Can* 31:480–484.
45. Juergensen L, Busnarda J, Caux P-Y, Kent R. 2000. Fate, behavior, and aquatic toxicity of the fungicide IPBC in the Canadian environment. *Environ Toxicol* 15:201–213.