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Salmon Olfaction is Impaired by an Environmentally Realistic Pesticide Mixture

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Many of the salmon-producing waterways of the world contain pesticides known to harm olfactory sensory neurons (OSNs) that are critically important throughout the salmon lifecycle. The ability of OSNs to retain functionality after exposure to complex pesticide mixtures remains unknown. Here we show that a 96-h exposure to an environmentally realistic concentration of a mixture made from the ten most frequently occurring pesticides in British Columbia's Nicomekl River reduced the OSN responses of rainbow trout to a behaviorally relevant odorant. Odor-evoked responses were not altered by exposure to one-fifth of the realistic concentration, and this may have been due an upregulation in detoxification enzymes, since glutathione-S-transferase activity reached a maximum (>32% above control) at this concentration. Mixture exposure did not help to prevent OSN impairment from a second, brief (5 min) exposure to a higher (20×) concentration of the mixture, suggesting longer-term, low-concentration exposures may not prevent damage from brief, high-concentration pulse exposures. This study demonstrates that environmentally observed pesticide mixtures can injure salmon olfactory tissue, and by extension, contribute to the threatened and endangered status of many salmon stocks.

Introduction

For many salmon, such as the famous ocean-going steelhead rainbow trout (*Oncorhynchus mykiss*), olfaction enables critically important behaviors. Since the 1970s, a series of studies have shown pesticides and some other waterborne contaminants impair the ability of salmonid olfactory sensory neurons (OSNs) to respond to odorants (reviewed in ref (*1*)). These studies have typically used brief (e.g., 30-min) exposures to single contaminants. This method, while mechanistically strong, does not represent the environmental reality in at least two significant ways: environmental exposures may be lengthy in duration, and they may involve complex contaminant mixtures.

Over sustained contaminant exposures, cells such as OSNs may maintain their ability to respond to odorants by adjusting to the contaminants. One of the adjusting mechanisms may be the upregulation of proteins involved with contaminant detoxification. Biotransformation enzymes such as glutathione-S-transferases (GSTs) are likely candidates since they facilitate contaminant detoxification and since two (π, μ), if not three (θ), of the four GST classes can be found in fish olfactory tissue (*2–4*). To date no studies have explored whether fish olfactory GST can be upregulated to offset any impact of waterborne contaminants on OSN odorant responses. Over lengthy contaminant exposures, upregulated GST expression may help fish OSNs retain their odorant responses in spite of contaminant presence.

Here we measured the concentrations of 40 contaminants, consisting mostly of pesticides, in the Nicomekl River, British Columbia (BC). This river has been the focus of restoration activity, owing in large part to its status as a temporary and permanent home to several species of salmonids, including steelhead rainbow trout. To simulate a realistic exposure scenario, trout were exposed for 96 h in laboratory to a mixture of the top-ten pesticides at concentrations intended to be lower, realistic, and higher than those observed in the Nicomekl River. Following exposure, the functionality of OSNs was assessed by their odor-evoked responses to different concentrations and relative intensities of a behaviorally relevant amino acid. To gauge tissue responses intended to retain OSN function after pesticide exposure, the activity of olfactory GST and the ability of trout to withstand a second, higher mixture exposure, were measured. Our expectations were that following mixture exposure, odor-evoked responses and GST activity would experience concentration-dependent decreases and increases, respectively, and that prior exposure to a low concentration of the mixture would lessen any impairment from a subsequent exposure.

Experimental Procedures

Animals. 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). At Simon Fraser University (SFU; Burnaby, BC), 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), and included 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 (α,β-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 and Analysis. Surface water was taken in September 2004 from the left bank of the Nicomekl

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TABLE 1. Contaminants, Consisting Mainly of Pesticides or Pesticide Breakdown Products, Detected in the Surface Water of the Nicomekl River, BC, in September 2004 (Sample Detection Limits (SDL) are given in parentheses)

pesticide	ng/L	(SDL)	pesticide (cont.)	ng/L	(SDL)
Dimethoate	604	(0.436)	HCH, β	0.259	(0.0037)
Simazine	84.5	(0.329)	Metribuzin	0.237	(0.12)
Methamidophos	61.4	(1.38)	Chlordane, γ (trans)	0.202	(0.011)
Diazinon	48.7	(0.285)	Dieldrin	0.199	(0.0217)
Chlorpyriphos	18.3	(0.208)	HCH, α	0.128	(0.0027)
Endosulphan-Sulfate	15.0	(0.0097)	Chlordane, α (cis)	0.121	(0.0131)
Malathion	10.0	(0.453)	Heptachlor-Epoxide	0.104	(0.067)
Atrazine	6.80	(0.0732)	Desethylatrazine	0.0899	(0.0068)
Linuron	6.27	(0.428)	Nonachlor, trans-	0.0723	(0.017)
Parathion	4.83	(0.0163)	Endrin	0.0585	(0.0254)
β-Endosulphan	4.66	(0.102)	HCH, δ	0.0539	(0.0104)
Cypermethrins	3.09	(0.0585)	Dacthal	0.0496	(0.0016)
α-Endosulphan	2.18	(0.133)	Aldrin	0.0492	(0.0081)
Permethrins	1.96	(0.137)	Quintozene	0.0417	(0.0263)
Dichlorvos	1.81	(0.19)	Endrin-Ketone	0.0161	(0.0104)
Diazinon-Oxon	1.63	(0.0175)	Heptachlor	0.0120	(0.001)
Fonofos	1.29	(0.0021)	Chlorothalonil	0.0088	(0.0039)
Metolachlor	0.536	(0.0214)	Octachlorostyrene	0.006	(0.001)
Pendimethalin	0.375	(0.0878)	Chlorpyriphos-Methyl	0.004	(0.001)
HCH, gamma	0.282	(0.0035)	Trifluralin	0.004	(0.0025)

River, BC, upstream of the 40th St. Bridge at 49.0747 by 122.7928 (latitude, longitude). A single grab water sample was collected in a VWR TraceClean 1 L amber glass bottle using a Supreme MAG-DRIVE submersible pump and Teflon tubing. The sample was collected from shore at a depth of 0.1 to 1 m below the water surface. To preserve the sample in the field, 100 mL of pesticide grade dichloromethane (DCM) was added to ~900 mL of each sample directly after collection. The water sample was stored at 4 °C prior to extraction and analysis.

To extract field and laboratory 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 DCM. Samples were cleaned using a microsilica chromatography column consisting of 0.75 g of 10% deactivated silica in a glass wool plugged pipet. The column was first rinsed with 10 mL of hexane, then 1 mL of extract was loaded to the column dropwise, 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 autosampler, and an Alpha data system running on Micromass software. The chromatography column was a DB-5 capillary chromatography 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 ionization mode using multiple ion detection and was tuned to have a static mass resolution of ≥8,000. Sample detection limits are given in Table 1.

Pesticide Mixture Preparation. To simulate the pesticide mixture observed in-field for laboratory exposures, the top-ten pesticides by concentration (and not toxicity to rainbow trout) (Table 1) were dissolved into 1 L of acetone such that 100 mL of this stock solution diluted into the 85 L glass aquaria used for exposures would yield 1000% (i.e., 10×) of the environmentally observed mixture concentration. Acetone was used as a solvent as it was found earlier to not affect odorant response at dilutions of ~1% (*5*). The mixture stock solution was prepared immediately before experiments and kept refrigerated. For laboratory exposures, endosulfan (I and II) was used in place of endosulfan-sulfate. This substitution likely had little impact on the toxicity of the mixture since the pesticides appear to have similar toxicities (*6*) and since its relative contribution (by mass) to the mixture was low (i.e., a target of 1.7%).

Laboratory Exposures. Groups of fish were exposed to concentrations intended to be lower than, similar to,and greater than the environmentally observed concentration. To carry out exposures, one exposure group of 16 trout (6 for electrophysiological measurements; 10 for GST assessment) was placed into glass aquaria containing water/acetone/pesticide mixtures that were changed (90%) every 12 h for 96 h. 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 h of exposure and extracted and analyzed as for field samples.

Odorant Responses. The odor-evoked responses of trout were measured using the electro-olfactogram (EOG) technique as described in Evans and Hara (*7*) and the apparatus and techniques described in Jarrard et al. (*8*). The EOG is a measure of the ligand (odorant) receptor binding induced ionic flux across the olfactory epithelium of multiple neurons that serves as a generator potential (*7*). With sufficient generator potential, action potential(s) will be propagated back to the brain for processing. To measure EOG 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-s 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 1 M 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). Recorded EOG values were 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, it was taken through a procedure consisting of three parts: (A) recording of baseline responses to two odorant concentrations, (B) response to a 100× change in odorant intensity, and (C) effect of a 5-min exposure to a high concentration of the pesticide mixture. Not every fish was successfully stabilized on the apparatus, so *N* varies for some groups (for control, low, medium, and high concentration groups, 0, 25, 33, and 8.3% of fish were not stabilized).

(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; *9, 10*). An L-serine concentration of 10^{-5} M was selected since it has been used in several similar salmon electrophysiology studies (reviewed in ref (*1*)), and since this appeared to evoke clear responses from 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 3-min spacing as this is sufficient time to eliminate any olfactory sensory neuron adaptation (*11*).

(B) Olfactory Response to a Change in Odorant Intensity. Odorants are typically perceived in the presence of background odorants. For this reason, it is likely the perception of relative changes in odorant strength that guides the migratory route, not just odorant presence (*12*). In the present study, we tested the ability of fish to adapt to an increase in background odorant strength. For trout, 3 min after attaining the last 10^{-3} M response, the background water supply was replaced by a 10^{-5} M L-serine solution. At 2 and 5 min into the change, EOGs were again recorded in response to 10^{-3} M. Responses were taken up to 5 min since earlier test trials demonstrated that the 10^{-3} M L-serine-evoked responses at 30 min were no different, and since earlier studies indicated complete OSN adaptation in fishes can take >1 h (*7*). To determine the relative change in the 10^{-3} M EOG (given as *k*), the difference between the last 10^{-3} M EOG before and the average of EOGs after the increase in background odorant were divided by the EOG value before (i.e., $k = \Delta$EOG response/initial EOG response × 100%).

(C) Olfactory Response after a Second Exposure to the Pesticide Mixture. To assess whether prior mixture exposure improved the ability of OSNs to withstand a subsequent exposure, after the above determination of *k*, EOGs were again recorded to 10^{-3} M L-serine in clear background water, and then this source was switched to a high (20×) concentration of the mixture. At 2 and 5 min into this second exposure, 10^{-3} M EOGs were recorded. Five min was chosen as the exposure length since any pesticide impairment noted in other studies typically appeared within this time frame (*11*). 10^{-3} M has been used previously to resolve pesticide effects (*5*), as have higher (10^{-2} M) (*13*) and lower (10^{-5} M (*11*) and 10^{-7} M (*14*)) 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 (10 per tank; 2 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. After exsanguination, both olfactory rosettes were collected immediately from each trout and 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) and centrifuged at 10,000*g*. The supernatant was collected and used for the determination of glutathione-S-transferase (GST) activity and cytosolic protein content. GST was determined after the method of Sharma et al. (*15*) and standardized using the sample's total protein, which was assessed using the method of Bradford (*16*) (kit from Bio-Rad, QE).

Statistics. Across exposure groups, differences in EOGs evoked by 10^{-5} and 10^{-3} M L-serine, in *k*, GST activity, and the effect of the secondary (20×) benchtop exposure, were tested against control using a one-way analysis of variance (ANOVA) followed by a Holm-Sidak posthoc 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 benchtop pesticide exposure and the tank exposures, a polynomial was fitted to the raw data. A similar procedure was used for GST. Five % was used as the fiducial limit of significance for all tests. Values are presented as mean ± standard error (SEM). SigmaStat 3.5 and SigmaPlot 10 were used for statistical analyses and graphing (Systat Software, San Jose, CA).

Results

Pesticide Concentrations. For the forty contaminants in the Nicomekl River 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 1). The range of measured concentrations was greater than 100-fold for the ten most abundant pesticides. Four pesticide classes were represented in the top ten, and these were organophosphate (OP) (747 ng/L, 86.9% of mass), triazine (91.3 ng/L, 10.6%), organochlorine (OC) (15.0 ng/L, 1.7%), and phenylurea (6.3 ng/L, 0.7%).

In the laboratory exposures, most of the concentrations of the measured pesticide concentrations were similar to those of the Nicomekl River top ten, especially the triazine and phenylurea pesticides (Table 2). The OC endosulfan concentration was comparatively low, but it played a minor role (by mass) in the mixture. OPs were typically higher than intended (e.g., 140% above normal for the low concentration group), but were close to accurate for the realistic exposure group (Table 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.* Following tank exposure, EOGs did not vary significantly across the groups. The EOGs for control, low, realistic, and high 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 OSN 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 realistic and high concentration groups (values for control, low, realistic, and high groups were 33.6 ± 3.0, 38.3 ± 4.9, 47.8 ± 5.1 ($p = 0.017$), and 51.5 ± 3.4% ($p = 0.002$)) (Figure 1A). This indicates that the 10^{-3} M EOGs for the realistic and high concentration groups were of smaller relative size when a (10^{-5} M) background odorant was present. Across the tested mixture concentrations, the *k* values were described by a sigmoid curve ($F = 4.7503$, $p = 0.0068$) (Figure 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 control, low, and realistic exposure groups (EOGs were 72.2 ± 3.5, 65.6 ± 9.0, and 75.2 ± 7.4%, respectively, of their pre-exposure values) (Figure 1B). However, for the high concentration group, the decrease (97.4 ± 4.4%) was significantly ($p = 0.003$) smaller than that of control. Across the tested mixture

TABLE 2. Experimental Pesticide Concentrations (ng/L) Compared to Those Found in the Nicomekl River (Low and High Values Were Intended to Be 10-Fold Higher and Lower than Those Observed in the Field)

		experimental			
pesticide	Nicomekl River observed	control	low	realistic	high
Dimethoate	604	3.2	137	486	6620
Simazine	84.5		8.4	73.1	669
Methamidophos	61.4				67.2
Diazinon	48.7		15.7	157	1820
Chlorpyrifos	18.3	0.2	1.7	13.4	114
Endosulphan-sulfate[a]	15.0		1.1	4.5	30.1
Malathion	10			76.3	926
Atrazine	6.80		0.7	6.5	59.0
Linuron	6.27			7.2	70.8
Parathion	4.83		23.1	196	3540
concentration agreement[b]					
total	859.77	3.4	186	1010	13900
total agreement (% of desired)		0%	218%	119%	162%
agreement pesticide class					
Organophosphate			240%	116%	165%
Triazine			100%	87%	80%
Organochlorine			0%	30%	20%
Phenylurea			0%	115%	113%

[a] For experimental exposures, endosulfan (I and II) was used in place of endosulfan-sulfate. See Experimental Procedures for details. [b] Experimental concentrations, which were designed to be 0.1, 1, and 10× the observed field concentration of 859.77 ng/L, if in excess of the intended amount are shown as >100%.

concentrations, a polynomial was used to describe the data ($F = 6.7008$, $p = 0.0038$) (Figure 1B).

Biochemical Response 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 ($F = 5.084$, $p = 0.003$) (Figure 2A). GST activity peaked with the trout exposed to the lowest mixture concentration, with greater exposures not eliciting any greater activity (activities: 154 ± 7, 203 ± 13, 197 ± 9, and 183 ± 10 nmol/min/mg protein for control, low, realistic, and high concentration groups). As with the 20× pulse, a polynomial was used to describe the data ($F = 6.7753$, $p = 0.0019$) (Figure 2).

Discussion

This study demonstrates that a pesticide mixture similar to that observed in a BC river can impair the critically important olfactory responses of a representative and well-known salmonid, the rainbow trout. The inability to withstand exposure may have in part been due to the olfactory detoxification responses, which were no greater than those observed at a concentration just one-fifth of that found in the river. Without correct olfactory responses, avoiding predators and return migration may not adequately occur (*17, 18*), and this suggests water quality may need to be addressed before any restorative action with salmon will attain success. In this paper we have focused on British Columbia; however, given the widespread presence of the fish and pesticides tested herein, this study could serve as a model for other regions throughout the world.

Pesticide Concentrations. In the environment, especially in agricultural waterways, pesticides are known to exist in complex mixtures (USGS Pesticide National Synthesis Project; http://water.usgs.gov/nawqa/pnsp/). In the Nicomekl River, OP insecticides constituted the overwhelming majority of the contaminants (86.9% by mass of the top ten), with triazine herbicides accounting for the next most prevalent class (10.6%). Overall, 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. For example, the near opposite of this ratio (i.e., ~1:10) was found in Nathan Creek, another salmon-producing waterway in the same region (*19*). 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 (*20*). Of these, insecticides are often used to a comparatively greater extent on cranberries and potatoes than blueberries.

The concentrations of the pesticides in the laboratory exposures were generally similar to those of the Nicomekl River. Overall, the concentrations that were intended to be 10, 100, and 1000% of the measured values were determined to be 21.8, 119, and 1620%. The lower exposure concentration used here, although greater than intended, has environmental relevance as well. For example, Nathan Creek had a total pesticide concentration of 158 ng/L (as measured using a protocol similar to that of this study) (*19*), which is only 15.0% lower than the low concentration group (i.e., 186 ng/L; Table 2). By comparison, our control exposure (i.e., 3.4 ng/L) resembles a "pristine setting" from northern BC (Koeye River; 2.2 ng/L) (*19*). Because of our QA/QC procedures, we are highly confident in the river and laboratory water pesticide concentrations. Over time, through flow and other changes, the river could experience greater or smaller total pesticide concentrations or mixture proportions. It would be impossible to know how the mixture in this river or others vary over time and space, although many of the higher concentration pesticides may remain present over time, which suggests our findings may be applicable over extended periods.

Odorant Responses. The odor-evoked responses of control trout (i.e., 1.20 ± 0.23 mV) compare favorably with those of other similarly sized salmonids given the same concentration of the same odorant (i.e., 10^{-5} M L-serine), such as of juvenile coho (*O. kisutch*) (1.86 mV) (*21*) and Atlantic salmon (*Salmo salar*) (0.90 mV) (*22*). Furthermore, the control responses to a 100-fold greater odorant concentration (i.e.,

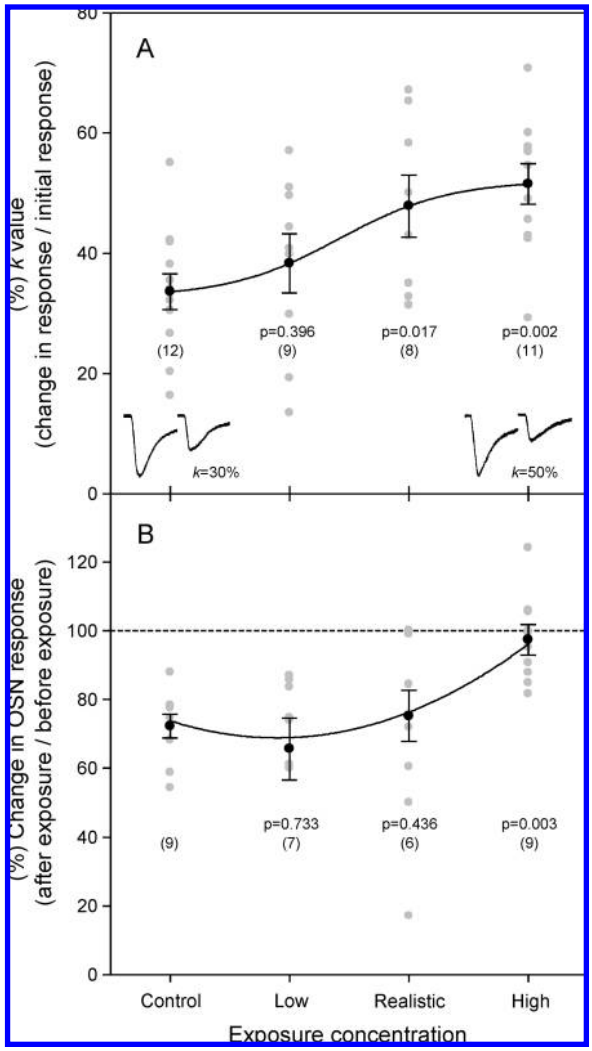

FIGURE 1. Olfactory sensory neuron (OSN) responses of juvenile rainbow trout following a 96-h exposure to control (0%), low (21.8%), realistic (119%), and high (1620%) concentrations of a pesticide mixture resembling that in the Nicomekl River, a salmon-producing waterway in British Columbia. Shown are the relative responses of OSNs to 10^{-3} M L-serine after a change in background L-serine strength (from none to 10^{-5} M) (A); a smaller response equates to a diminished signal amidst the noise (see inset OSN responses for before and after background addition). Also shown are the effects of a subsequent 5-min exposure to a high (2000%) concentration on OSN responses (B). Responses below unity (gray line) indicate OSN impairment. For a description of the pesticide mixture, the derivation of *k*, statistics, and measurement of OSN responses, see Experimental Procedures; *N* and *p*-values are given on figure.

10^{-3} M) were approximately double (2.77 ± 0.46 mV) those of the lower concentration, which is typical (*11*). The responses of trout exposed to the pesticide mixture did not vary significantly from the control values at either odorant concentration, but this lack of difference was not unanticipated. The variation in baseline odor-evoked responses can be great and so necessitate very large sample sizes to detect any subtle pesticide-mediated impacts (*5*). Furthermore, an assessment of how a toxicant alters OSN odorant responses before and after a brief (typically 30-min) exposure does not test how OSNs adapt to altered odorant intensity, which is a more environmentally and physiologically relevant end point. In the present study, the odor-evoked responses during a change in background odorant concentration were used as a correlate of the trout's ability to resolve changes in odorant concentration.

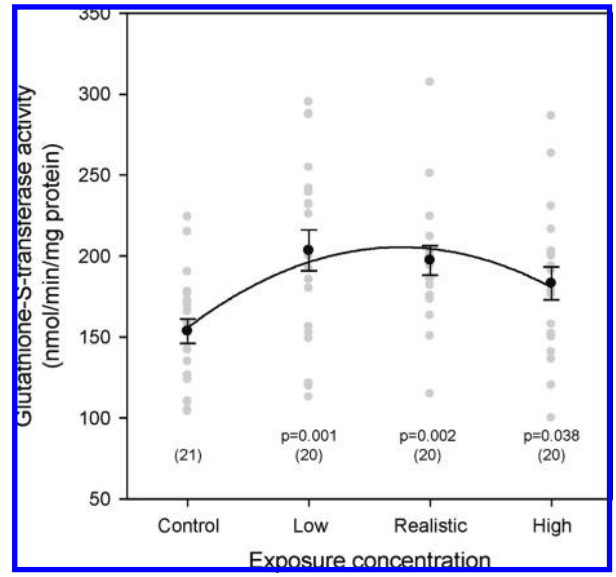

FIGURE 2. Activity of the detoxification enzyme glutathione-S-transferase (GST) in the olfactory rosette tissue in juvenile rainbow trout after 96-h exposure to control (0%), low (21.8%), realistic (119%), and high (1620%) concentrations of a pesticide mixture resembling that in the Nicomekl River, a salmon-producing waterway in British Columbia. Elevated GST can indicate enhanced detoxification ability. For statistics see Experimental Procedures; *N* and *p*-values are given on figure.

In all tested trout, increasing the background odorant concentration reduced the OSN responses, as expected. However, there were differences across groups due to mixture exposure (Figure 1A). In particular, both the realistic and high concentration groups were significantly different from control. This indicates that the (10^{-3} M L-serine) EOG peak responses for these groups were of smaller relative size when a background odorant (10^{-5} M L-serine) was present. Brief, benchtop exposures have previously been associated with reducing EOG peak response magnitude (e.g., ref (*11*)); however, here we show that longer-term exposures reduce EOG peak responses in the presence of a background odorant. This new OSN assessment technique may be useful in determining olfactory impairment in future long-term studies of contaminant exposure.

The amino acid L-serine was selected as an odorant because it is associated with predator avoidance in some salmon. For this reason, the reduced ability to detect changes in L-serine concentration could equate to diminished predator detection and hence survival. Furthermore, since a variety of other salmonid behaviors such as imprinting and food location depend upon properly sensing amino acids, the ramifications of our findings to salmon survivorship, especially in the Nicomekl River, may be serious.

Pesticide Pulse Exposure. The short, 5-min exposure to a secondary (20×) pulse of the pesticide mixture caused similar decreases in odor-evoked responses for control, low, and realistic mixture-exposed trout (Figure 1B). This suggests prior mixture exposure may not have prevented alteration from further exposures. For the high concentration mixture-exposed fish, the pulse 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 EOG decrease would be noted if either the high concentration tank exposure had prepared the tissue such that additional pulses were not injurious, or if the tissue was so impaired that a second exposure could cause no further EOG reduction. Given the high concentration mixture-exposed trout were unable to respond to an increase in background odor concentration as

well as other groups (Figure 1A), it seems that the latter explanation is more likely. This is further substantiated given fish of the realistic exposure group still experienced EOG impairment (i.e., functionality, and hence capacity for diminished responsiveness, remained). Overall it appears low-concentration exposure may not prevent impairment from subsequently higher exposures.

Detoxification Response. Without pesticide mixture exposure, the detoxification enzyme activities of trout appeared normal. In the olfactory rosette tissue of control trout, the activity of GST detoxification enzymes (154 ± 7 nmol/min per mg protein) was lower than two other studies of rainbow trout olfactory GST (478 ± 218 (*2*) and 250 ± 50 (*3*) nmol/min per mg protein), and similar to those of some mammalian values (e.g., mouse vomeronasal organ 181.7 ± 13.4 (*23*) and human nasal mucosa 77 ± 21 (*24*)).

There are no studies on the effects of pesticides on olfactory GST induction; most olfactory studies report CYP (phase I) enzymes and not GST (phase II) enzymes (*25*). Although not a study of the effects of pesticides on olfactory GST activity, one study found that severing the olfactory nerve of rainbow trout, which caused nerve death and regeneration over a period of three months, resulted in a rapid decrease in GST activity that returned after two months (*26*). In the present study, pesticide mixture exposure caused increases in GST activity of all groups (Figure 2A). Surprisingly, the highest GST activity (i.e., 32% increase) was found in the trout exposed to the lowest amount of pesticide mixture. These data suggest that GST activity and so any neuroprotection it may afford reached a maximum with one-fifth of the concentration of pesticides found in the Nicomekl River.

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