

## COMPARATIVE THRESHOLDS FOR ACETYLCHOLINESTERASE INHIBITION AND BEHAVIORAL IMPAIRMENT IN COHO SALMON EXPOSED TO CHLORPYRIFOS

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**Abstract**—Chlorpyrifos is a common organophosphate insecticide that has been widely detected in surface waters that provide habitat for Pacific salmon in the western United States. Although chlorpyrifos is known to inhibit acetylcholinesterase (AChE) in the brain and muscle of salmonids, the relationship between sublethal AChE inhibition and more integrative indicators of neuro-behavioral impairment are poorly understood. This is particularly true for exposures that reflect the typical range of pesticide concentrations in the aquatic environment. To directly compare the effects of chlorpyrifos on AChE activity and salmon behavior, we exposed juvenile coho salmon (*Oncorhynchus kisutch*) to chlorpyrifos (0–2.5 µg/L) for 96 h. A computer-assisted, three-dimensional video imaging system was used to measure spontaneous swimming and feeding behaviors in control and chlorpyrifos-exposed fish. After the behavioral trials, brain and muscle tissues were collected and analyzed for AChE activity. Chlorpyrifos inhibited tissue AChE activity and all behaviors in a dose-dependent manner. Moreover, brain AChE inhibition and reductions in spontaneous swimming and feeding activity were significantly correlated. Benchmark concentrations for sublethal neurotoxicity (statistical departure values) were <0.5 µg/L and were similar for both neurochemical and behavioral endpoints. Collectively, these results indicate a close relationship between brain AChE inhibition and behavioral impairment in juvenile coho exposed to chlorpyrifos at environmentally realistic concentrations.

**Keywords**—Salmon Pesticide Organophosphate Behavior Acetylcholinesterase

## INTRODUCTION

Acetylcholinesterase (AChE) inhibition is a classical biomarker of exposure to certain organophosphate and carbamate insecticides in fish. It has been more than 40 years since Ellman et al. [1] developed a relatively simple method for quantifying AChE activity in vertebrate tissues and Weis [2] demonstrated that organophosphates inhibit AChE activity in the fish brain. Numerous studies in the decades since have shown that laboratory exposures to organophosphates or carbamates result in a concentration-dependent inhibition of AChE activity in the nervous system or muscle of various fish species. In addition, field surveys have documented depressed AChE activity in the brains of fish collected from aquatic systems contaminated with anticholinesterase insecticides [3,4]. Accordingly, AChE inhibition is now generally accepted as a biomarker of exposure for pesticides that share the anticholinesterase mechanism of action [5].

The extent to which AChE inhibition is a reliable or meaningful biomarker of effect is considerably less clear. The quantitative or direct relationship between AChE inhibition and physiological or behavioral impairment has not been widely investigated in fish, and relatively few studies have explored the effects of pesticides at ecologically relevant concentrations (i.e., at concentrations that have actually been detected in freshwater or estuarine habitats). Acetylcholinesterase is an enzyme that regulates cholinergic signaling by hydrolyzing the transmitter acetylcholine at central and peripheral synapses in the vertebrate nervous system [6]. Organophosphate and carbamate insecticides are both potent inhibitors of fish AChE activity. At relatively high concentrations, such as those re-

sulting from an accidental spill, these anticholinesterases cause hyperactivity, muscle twitching, loss of equilibrium, and ultimately death in fish (reviewed by Zinkl et al. [7]). At sublethal concentrations, anticholinesterases impair several important physiological and behavioral processes including swimming performance [8–10], swimming stamina [11,12], prey capture [13], predator detection [14], predator avoidance [15], migration [14], learning [16], and conspecific social interactions [17–19]. However, with a few exceptions [9–12], these previous studies have not specifically related observed changes in fish behavior to AChE inhibition in brain or muscle. Also, certain species of fish can withstand as much as 80% acute AChE inhibition in the brain without lethality and recover their AChE activity after exposure ends [2,11]. Given the wide range over which AChE inhibition can be considered sublethal, and the lack of data relating degrees of inhibition to specific toxicological outcomes, caution has been urged in the use of AChE inhibition alone as a biomarker of effect [5].

To address the question of whether anticholinesterase insecticides inhibit AChE activity in fish at environmentally realistic concentrations, we recently investigated the effects of short-term (96-h) exposures to the organophosphate chlorpyrifos on brain AChE activity of juvenile steelhead (*Oncorhynchus mykiss*) [20]. Using a benchmark concentration (BMC) approach [21], we found that chlorpyrifos significantly inhibits enzyme activity at concentrations below a part per billion. This threshold is within or near the range of chlorpyrifos detections in many watersheds that provide freshwater rearing habitat for threatened or endangered populations of salmonids (i.e., chinook, coho, sockeye, steelhead, chum, and other species belonging to the genus *Oncorhynchus*) in the western United States [22–25]. Importantly, however, the higher-order con-

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sequences of a relatively small but measurable inhibition of brain AChE activity have not been explored at these relatively low exposure concentrations.

In the present study, we compare the effects of chlorpyrifos exposures on AChE activity and behavior in juvenile coho salmon (*O. kisutch*). A three-dimensional digital video data acquisition system was used to monitor swimming and feeding behavior in chlorpyrifos-exposed animals. After behavioral trials, the brains and muscle of coho were analyzed for AChE inhibition. In this way, both biochemical and behavioral measures were obtained from the same individual fish, allowing for a direct evaluation of the effects of chlorpyrifos at two different scales of biological organization. From these data, benchmark calculations were used to compare relative changes in brain and muscle AChE activity, spontaneous swimming, and feeding behavior. We find that thresholds for AChE inhibition, as estimated by using the BMC approach, are reasonably predictive of behavioral impairments that could potentially affect the foraging behavior and growth of juvenile salmonids.

## MATERIALS AND METHODS

### Animals

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA, USA) at the eyed egg stage and were raised at the Northwest Fisheries Science Center's hatchery facility (Seattle, WA, USA) under natural photoperiod conditions. Coho fry were maintained in tanks supplied with filtered, dechlorinated municipal water (hereafter referred to as hatchery water; 120 mg/L total hardness as CaCO<sub>3</sub>, pH 6.6, dissolved oxygen 8.1 mg/L, temperature 11–13°C) on a single-pass flow-through system. Fish were raised on standard commercial salmon pellets until one month before experiments, at which point the diet was changed to frozen brine shrimp (Hikari, Hayward, CA, USA). Fish were four to five months old, with an average size ( $\pm$  one standard deviation [SD]) of  $4.5 \pm 0.3$  cm and  $0.7 \pm 0.2$  g.

### Chlorpyrifos exposure and analysis

Analytical grade chlorpyrifos (99.3% purity; *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinol)-phosphorothionate) was purchased from Chem Service (West Chester, PA, USA). Chlorpyrifos stocks were prepared in ethanol, and added in 100- $\mu$ L volumes to 25 L of water in glass aquaria (30 L) to produce nominal chlorpyrifos concentrations of 0, 0.6, 1.2, 1.8, and 2.5  $\mu$ g/L. The final (estimated) ethanol concentration in the exposure tanks (for all treatments including controls) was 0.004% of the total volume. Fish were exposed in groups ( $n = 5$  or 6) for 96 h (unfed) by using a static-renewal (12-h) regimen. For each group, individual fish were partitioned within a single exposure tank. Exposures were staggered, with intervals between animals to accommodate individual behavioral trials at the end of the 96-h exposure period. Each exposure concentration was replicated in triplicate ( $n = 15$ –17 total fish per concentration). No changes in water temperature, pH, or dissolved oxygen were observed over the exposure period. Six water samples from each chlorpyrifos exposure group (three at the onset and three at the end of static-renewal intervals) were analyzed to compare nominal and measured values, and to determine if chlorpyrifos concentrations decreased over the course of the static exposure interval. Samples were collected in acid-washed, amber glass bottles fitted with Teflon<sup>®</sup>-coated lids and refrigerated at 4°C.

Table 1. Nominal and measured chlorpyrifos concentrations ( $\mu$ g/L). Exposure solutions ( $n = 3$  each) were sampled at the beginning (initial measured) and end (12-h measured) of static renewal periods. Concentrations are mean  $\pm$  one standard deviation

Nominal	Chlorpyrifos concentration ( $\mu$ g/L)	
	Initial measured	12-h Measured
0	0 $\pm$ 0	0 $\pm$ 0
0.6	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1
1.2	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1
1.8	1.2 $\pm$ 0.1	1.1 $\pm$ 0.1
2.5	1.7 $\pm$ 0.3	1.6 $\pm$ 0.1

Extractions and analyses of water samples were conducted three weeks after collection. Analyses were performed by gas chromatography–mass spectrometry by using previously established methods [20]. Hatchery water did not contain detectable amounts of chlorpyrifos (detection limit 0.02  $\mu$ g/L). Recovered initial chlorpyrifos ranged from 50 to 68% of nominal values, and no additional loss occurred at the end of the 12-h exposure period (Table 1). Because chemical recoveries from the exposure solutions were somewhat lower than expected, the chlorpyrifos stocks also were analyzed. The stocks were determined to be (mean  $\pm$  one standard error [SE])  $98 \pm 5\%$  of expected concentrations. Accordingly, the differences between the nominal and measured concentrations were likely due to loss during exposure, sample handling, storage, or the extraction–analytical process. Thus, chlorpyrifos exposures are reported here as nominal concentrations with the realization that actual concentrations were likely somewhat lower.

### Acetylcholinesterase analysis

In some species of fish, brain and muscle tissues can contain substantial amounts of both AChE and butyrylcholinesterase (BuChE) [26]. Although the physiological role of AChE is well established, the function of BuChE is less well understood [27], and it is important to distinguish between the two enzymes when reporting the inhibitory effects of anticholinesterase compounds in fish. The relative contributions of AChE and BuChE to total cholinesterase activity in brain and muscle has not been determined for coho salmon, although steelhead do not appear to express substantial amounts of BuChE in brain or muscle tissues [20].

A pharmacological approach was used to determine if brain and muscle samples from juvenile coho contained AChE activity, BuChE activity, or both. Triplicate composite homogenates ( $n = 5$  or 6 fish per composite) were treated with either BW284c51 (1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide) or iso-OMPA (tetraisopropylpyrophosphoramide) as specific inhibitors of AChE and BuChE, respectively. The inhibitory effects of BW284c51 and iso-OMPA were evaluated over a range of  $10^{-9}$  to  $10^{-4}$  M and  $10^{-8}$  to  $10^{-4}$  M, respectively. Control homogenates received a treatment of distilled water only. The treated and control homogenates ( $n = 3$  each) were incubated at room temperature (20–25°C) for 30 min. The rate of substrate hydrolysis by total cholinesterase was determined by using the colorimetric method of Ellman et al. [1], as modified by Sandahl and Jenkins [20]. Measurements were performed on a SpectroMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and all reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Acetylthiocholine iodide was used as the enzyme substrate and 5,5'-dithiobis-(2-nitro-benzoic acid) was

used as the reactive chromogen. Final concentrations of tissue and reagents in the microplate wells used for the assay were brain or muscle at 1 mg/ml, 3 mM acetylthiocholine iodide, and 0.25 mM 5,5'-dithiobis-(2-nitro-benzoic acid). Reactions were carried out in 0.1 M sodium phosphate buffer (pH 8.0) at 25°C. Activities of AChE are expressed in  $\mu\text{mol}/\text{min}/\text{g}$  tissue. For in vivo measurements of acetylcholinesterase inhibition after behavioral trials (see below), chlorpyrifos-exposed fish were sacrificed by immersion in a lethal dose (200 mg/L) of MS-222 (tricaine methanesulfonate, Sigma Chemical) after behavioral trials. Brain and muscle tissues were collected and immediately frozen at  $-80^\circ\text{C}$ . Enzymatic activity was analyzed within two weeks of tissue collection by using the procedures outlined above.

#### *Quantification of swimming and feeding behaviors*

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

After exposures to chlorpyrifos, individual fish were transferred to the observation tank and allowed to acclimate for 30 min. After acclimation, spontaneous swimming was recorded over a 3-min interval. Subsequently, 30 adult brine shrimp (thawed after being previously frozen) were injected into the circulation system (time  $[t] = 0$  s). The swimming speed of fish after the introduction of food (feeding swimming rate) was then measured for 1 min. A digital video camcorder (Canon DR45, Canon, Tokyo, Japan) with a front view of the tank was used to monitor individual food strikes, and the video was later analyzed to measure latency to first strike and the total number of strikes during the 1-min feeding interval.

Spatial movements of the fish were monitored by two orthogonally positioned Firewire digital cameras (Fire-i, Uni-brain, San Ramon, CA, USA) connected to a laptop computer (iBook, Apple Computer, Cupertino, CA, USA). One camera was positioned to view the front of the tank, whereas the second viewed the left side. A custom software program (written in REALbasic, REAL software, Austin, TX, USA) displayed simultaneously acquired frames from the cameras at 12 frames/s, recorded a pair of frames every 2 s, and continuously recorded keyboard input. Semiautomated computer video analysis of each pair of frames (custom scripts in VideoScript, Videoscript, Corrales, NM, USA) was used to locate the position of the fish in both two-dimensional views. A macro in Excel (Microsoft®, Redmond, WA, USA) was used to correct for refraction and to triangulate the three-dimensional location of the fish. The time of brine shrimp introduction to the tank and subsequent strikes at the food items were indicated by keystrokes on the laptop computer.

#### *Statistical analyses*

The effects of chlorpyrifos on AChE activity and behavioral measures were analyzed by using either one-way analysis of

variance (ANOVA) to test for differences between groups (followed by a Dunnett's test for comparisons with controls), or regression analysis to test for concentration-dependent relationships. Correlations were determined by using the Pearson correlation procedure. Benchmark concentration analyses were conducted by using U.S. Environmental Protection Agency benchmark dose software (Ver 1.3.2; available on the Internet at <http://cfpub.epa.gov/ncea/cfm>).

The BMC is the estimated exposure concentration that results in a benchmark adverse response (e.g., an exposure at the BMC10 would be expected to produce a 10% decrease in AChE activity or swimming rate). The BMC method has several advantages over more traditional toxicological measures, such as the lowest-observed-effect concentration (LOEC) or no-observed-effect concentration (NOEC) [21,28,29]. Although the LOEC and NOEC are constrained to individual experimental doses, the BMC method is based on a regression of the data, and thus considers the entire dose-response relationship. Moreover, the BMC method incorporates control values in the regression. This allows BMCs to be estimated (or interpolated) for chemical concentrations that fall between the control and the lowest experimental exposure dose. Benchmark responses can be based on statistical departures or relative departures [20]. A statistical departure is the point at which the exposure can be statistically differentiated from controls. In the present study, the statistical departure is defined as the 95% lower confidence interval of the control mean. A relative departure is the point at which the exposure produces a specified percent difference from the controls, an arbitrary number that doesn't necessarily account for biological variability or sample size. Relative departures are useful for comparing treatment effects (e.g., AChE inhibition) between studies. In the present study, a relative departure of 10% was chosen for brain AChE inhibition, spontaneous swimming rate, and total food strikes in fish exposed to chlorpyrifos.

## RESULTS

### *Concentration-dependent AChE inhibition in brain and muscle*

In vitro treatments of cholinesterase extracts from control fish tissues with BW284c51 (specific AChE inhibitor) and iso-OMPA (specific BuChE inhibitor) indicated that AChE is the primary form of cholinesterase expressed in the brain and muscle of coho salmon (Fig. 1A). Incubation with iso-OMPA up to  $10^{-4}$  M had no effect on the rate of substrate hydrolysis, indicating that BuChE does not make a measurable contribution to the overall rate of cholinesterase activity in either tissue. However, BW284c51 was a potent inhibitor of enzyme activity in both tissues, with inhibitory, concentration-dependent curves that were very similar over the range of  $10^{-9}$  to  $10^{-4}$  M. In both tissues, activity was abolished at the highest concentration. Thus, as previously reported for steelhead trout [20], the predominant source of cholinesterase activity in coho brain and muscle can be attributed to AChE alone.

Consistent with previous results for steelhead [20], 96-h in vivo exposures of juvenile coho to chlorpyrifos significantly inhibited AChE activity in brain and muscle tissues at all chlorpyrifos levels tested (ANOVA,  $df = 4$ , Dunnett's test,  $p < 0.05$ ; Table 2) in a concentration-dependent manner (regression analysis,  $p < 0.001$ ; Fig. 1B). For control fish, baseline AChE activity in the brain was lower than that of muscle tissue (mean  $\pm$  SE;  $23.2 \pm 0.5$  and  $38.9 \pm 1.4$   $\mu\text{mol}/\text{min}/\text{g}$ , respectively). At the lowest chlorpyrifos concentration tested (0.6  $\mu\text{g}/\text{L}$ ),

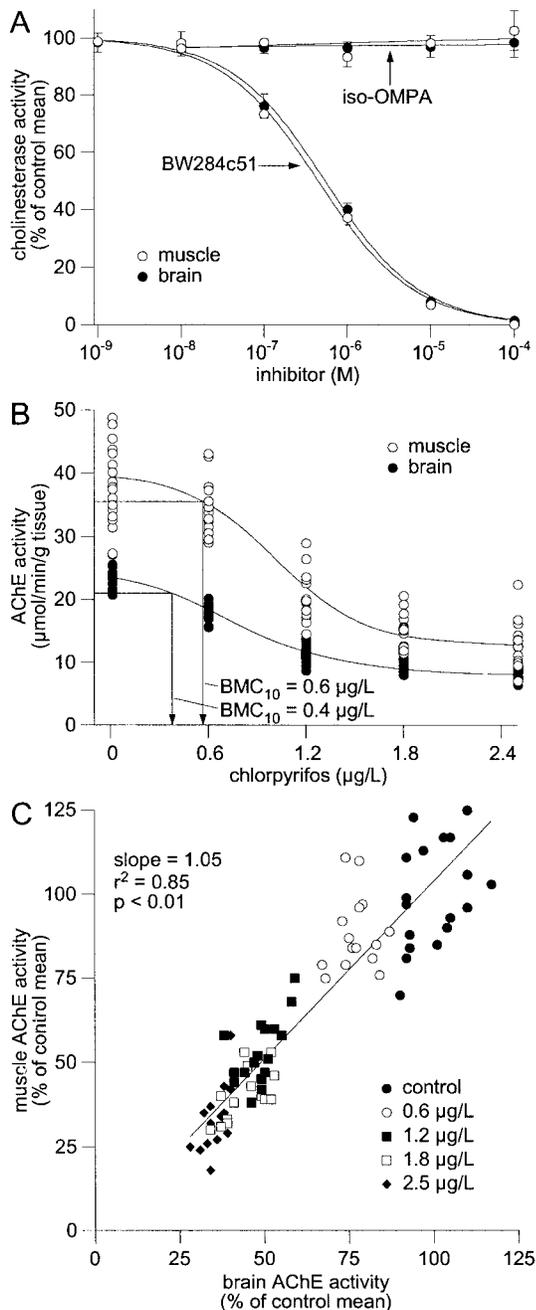


Fig. 1. Acetylcholinesterase (AChE) is the primary acetylcholine-metabolizing enzyme present in brain and muscle tissue of coho fry, and enzyme activities in both tissues are sensitive to the inhibitory effects of chlorpyrifos. (A) Incubation of brain and muscle homogenates ( $n = 3$ ; triplicate composites of  $n = 5$  or  $6$  fish each) with inhibitors of AChE (1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide [BW284c51]) and butyrylcholinesterase (tetraiso-propylpyrophosphoramidate [iso-OMPA]) indicates that AChE is the predominant cholinesterase present in these tissues. Error bars represent 95% confidence intervals of group means. (B) Chlorpyrifos inhibits the activity of brain and muscle AChE in fish ( $n = 15$ – $17$  per exposure) over a range of concentrations. As an example of benchmark concentration analysis, the horizontal line represents a 10% reduction in AChE activity benchmark concentration analyses (BMC<sub>10</sub>). This corresponds to estimated BMC<sub>10</sub> values of chlorpyrifos exposure concentrations of  $0.4 \mu\text{g/L}$  and  $0.6 \mu\text{g/L}$  for brain and muscle, respectively. (C) Brain and muscle AChE activities were compared in individual fish exposed to chlorpyrifos (each point represents AChE activities of a single fish). The AChE activities in both tissues were significantly correlated (Pearson correlation,  $r^2 = 0.85$ ,  $p < 0.01$ ), and shown to be similarly sensitive to the inhibiting effects of chlorpyrifos (slope = 1.05).

brain and muscle AChE activities were inhibited (mean  $\pm$  SE)  $23 \pm 1\%$  and  $12 \pm 3\%$ , respectively, as compared to controls. Although fish at the highest exposure concentration ( $2.5 \mu\text{g/L}$ ) had mean brain and muscle AChE activity inhibited by 64% and 67%, respectively, no mortalities occurred in this treatment group. As shown in Figure 1C, a significant correlation was found between brain and muscle AChE activities within individual fish (slope = 1.05,  $r^2 = 0.85$ ,  $p < 0.01$ ), indicating that AChE in both tissues were similarly inhibited by chlorpyrifos over the range of concentrations tested.

Both data sets (brain and muscle AChE inhibition) were fit by using a sigmoid logistic model. For BMC estimates, the 95% lower confidence interval of the respective control mean was used as the statistical departure value. For brain AChE inhibition, this departure corresponded to a 4% reduction in activity (or  $-1.0 \mu\text{mol/min/g}$ ). For muscle AChE activity, this departure corresponded to an 8% reduction in activity (or  $-3.0 \mu\text{mol/min/g}$ ). The relative departures for brain and muscle AChE inhibition were chosen to be a 10% reduction in activity. An example of the BMC approach is shown in Figure 1B, and the calculated BMC estimates are shown in Table 3. The BMC<sub>10</sub> (relative departure) estimates for brain and muscle AChE inhibitions were found to be similar (chlorpyrifos at  $0.4$  and  $0.6 \mu\text{g/L}$ , respectively).

#### *Chlorpyrifos reduces swimming and feeding behaviors in juvenile coho*

In behavioral trials, spontaneous swimming rate, swimming rate during feeding, latency to first strike, and total food strikes during a fixed time interval (1 min) were each significantly altered in chlorpyrifos-exposed fish relative to controls (Table 2), consistent with previous studies showing that swimming and feeding behaviors are sensitive indicators of sublethal anticholinesterase neurotoxicity in fish [9,30]. Typical examples of the swimming activity of individual control and chlorpyrifos-exposed ( $1.2 \mu\text{g/L}$ ) fish are shown in Figure 2, with  $t = 0$  indicating the time that brine shrimp were added to the observation tank. Control fish generally remained in motion throughout the entire trial (i.e., they swam actively both before and after the introduction of food into the tank). By contrast, chlorpyrifos-exposed fish displayed periods of relative inactivity ( $<1$  cm of movement per second) before food was introduced into the tank, with a transient increase in activity after food was introduced. The frequency and duration of inactivity increased at higher chlorpyrifos exposure concentrations.

The average swimming rates for the different treatment groups ( $n = 15$ – $17$  fish per group) before and after the addition of food are shown in Figure 3A. Exposures to chlorpyrifos significantly reduced spontaneous swimming at all chlorpyrifos concentrations tested (ANOVA,  $df = 4$ , Dunnett's test,  $p < 0.01$ ) in a concentration-dependent manner (BMC linear regression,  $p < 0.01$ , see BMC analysis below). The mean spontaneous swimming rates for each exposure group are plotted together in Figure 3B. For control fish, the spontaneous swimming rate averaged (mean  $\pm$  SE)  $4.9 \pm 0.3$  cm/s. Compared to controls, the basal swimming rate of chlorpyrifos-exposed fish at the lowest concentration ( $0.6 \mu\text{g/L}$ ) was reduced by  $27 \pm 5\%$  ( $3.6 \pm 0.2$  cm/s; Table 2). At the highest chlorpyrifos concentration ( $2.5 \mu\text{g/L}$ ), the fish were more lethargic, with swimming activity averaging only  $1.1 \pm 0.2$  cm/s. The baseline swimming speed of control fish did not significantly change when brine shrimp were added to the tank

Table 2. Acetylcholinesterase (AChE) activity and behavioral measures from juvenile coho exposed to chlorpyrifos ( $n = 15\text{--}17$  fish per exposure concentration). Response indices are mean  $\pm$  one standard error

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

<sup>a</sup> Nominal chlorpyrifos concentrations are in  $\mu\text{g/L}$ .

<sup>b</sup> Acetylcholinesterase activities are in  $\mu\text{mol/min/g}$  tissue.

<sup>c</sup> Swimming rates are in cm/s.

<sup>d</sup> First strikes are in s.

<sup>e</sup> Total strikes at brine shrimp are after a 1-min interval.

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

(4.9  $\pm$  0.3 vs 4.6  $\pm$  0.3 cm/s, respectively). Chlorpyrifos-exposed groups showed an increase in swimming rate when food was added to the tank, but at speeds lower than controls (ANOVA,  $df = 4$ , Dunnett's test,  $p < 0.05$ , except 0.6  $\mu\text{g/L}$ ). After the feeding swimming interval, swimming rate returned to the prefeeding rate seen during the spontaneous swimming interval.

The latency to strike and total strikes during the feeding interval also were significantly affected by chlorpyrifos exposures (Table 2). When brine shrimp were introduced into the observation tank ( $t = 0$ ), the time to first strike for control fish averaged 2.9 s. The latency to first strike increased with increasing chlorpyrifos exposures (linear regression,  $p < 0.01$ ). Figure 4A shows each cumulative strike made (as points connected by dashed lines) by individual fish over the observation period. On average, control fish made (mean  $\pm$  SE) 19.6  $\pm$  0.6 strikes at brine shrimp in the first minute, which decreased with increasing chlorpyrifos concentrations (BMC linear regression,  $p < 0.01$ ; see BMC analysis below). The mean total strikes made at the end of 1 min for each exposure group are plotted together in Figure 4B.

For the purpose of BMC estimations, linear regressions (with the  $y$ -intercepts fixed at the respective control means) were used to fit the data for spontaneous swimming rate (Fig. 3B) and total food strikes (Fig. 4B). The 95% lower-confidence intervals of the respective control means were used as the BMC statistical departure values. These departures corresponded to an 11% reduction (or  $-0.5$  cm/s) in spontaneous swimming rate, and an 8% reduction in total food strikes (or  $-1.8$  strikes). The relative departure for spontaneous swimming rate and total

strikes was chosen to be a 10% reduction, relative to the respective control means (Fig. 3A and B). These relative BMC values are listed in Table 3. Overall, in a direct comparison of the effects of chlorpyrifos on AChE activity and fish behavior, the statistical departures for brain AChE inhibition (0.3  $\mu\text{g/L}$  chlorpyrifos), spontaneous swimming rate (chlorpyrifos at 0.4  $\mu\text{g/L}$ ), and food strikes (chlorpyrifos at 0.3  $\mu\text{g/L}$ ) were found to be very similar.

#### *Brain AChE inhibition and reductions in spontaneous swimming and feeding are correlated in chlorpyrifos-exposed fish*

Coho fry exposed to chlorpyrifos showed significant, concentration-dependent inhibitions of brain AChE activity (Fig. 1B), spontaneous swimming rate (Fig. 3B), and total food strikes (Fig. 4B). For each individual fish, the two behavioral measures are plotted as a function of brain AChE activity in Figure 5. Reductions in spontaneous swimming rate and total food strikes were significantly correlated with reductions in AChE activity (slope = 0.21,  $r^2 = 0.58$ ,  $p < 0.01$ ; and slope = 0.85,  $r^2 = 0.53$ ,  $p < 0.01$ , respectively). Thus, reductions in brain AChE activity were a good indicator of behavioral impairments, as determined by a change (reduction) in spontaneous swimming rate and total food strikes.

## DISCUSSION

Some of the best examples of insecticide-induced AChE inhibition in fish are from large-scale spray programs [31], accidental chemical spills [3], and lakes that are receiving waters for complex mixtures of organophosphates and car-

Table 3. Benchmark concentration (BMC) estimates for inhibitions in brain and muscle acetylcholinesterase (AChE) activity, reductions in spontaneous swimming rate, and reductions in strike rate after chlorpyrifos exposures in coho fry. The benchmark response indicates the degree of adverse response for determination of the BMC estimate. Below, benchmark responses include the statistical departures and relative departures. For example, a BMC10 for chlorpyrifos (0.4  $\mu\text{g/L}$ ) is estimated to cause a 10% inhibition (relative departure) in the brain AChE activity of exposed fish

Benchmark response	Biochemical measures		Behavioral measures	
	Brain AChE	Muscle AChE	Spontaneous swimming rate	Total strikes
Statistical departure <sup>a</sup>	4% inhibition	8% inhibition	11% reduction	6% reduction
BMC <sup>b</sup>	0.3	0.5	0.3	0.3
Relative departure <sup>c</sup>	10% inhibition	10% inhibition	10% reduction	10% reduction
BMC <sup>b</sup>	0.4	0.6	0.3	0.4

<sup>a</sup> The statistical departure is the 95% lower confidence interval of the control mean.

<sup>b</sup> The BMC estimates are expressed in  $\mu\text{g/L}$ .

<sup>c</sup> A relative departure of 10% inhibition was selected for comparison with other studies.

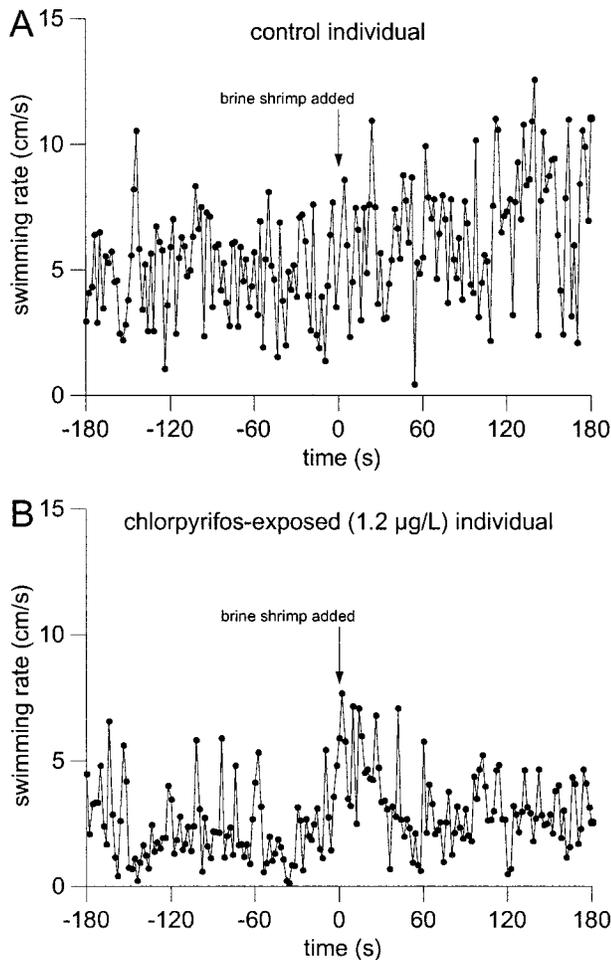


Fig. 2. Typical activity-grams obtained from (A) individual control and (B) chlorpyrifos-exposed (at 1.2 µg/L) fish. The three-dimensional position of each animal was captured at 2-s intervals. Swimming rate was monitored before and after the addition of food to the tank (arrows).

bamates via return flows [4]. However, Pacific salmon are typically exposed to lower levels of insecticides via spray drift, atmospheric deposition, non-point-source runoff, agricultural return flows, municipal discharges, and other common transport pathways. Under these ecological conditions, it can be difficult to link a one-time measure of cholinesterase activity to an individual pesticide exposure, particularly at concentrations lower than a few micrograms per liter. This is due, in part, to natural variability in AChE or BuChE gene expression as a function of the species, age, or tissue of the fish being monitored [5]. Organophosphates and carbamates also have different inhibitory potencies, and because they commonly occur in surface waters as mixtures, accounting for the possibility of cumulative toxicity can be problematic. Collectively, it can be difficult to control for these intrinsic and extrinsic factors during field investigations and this, in turn, may constrain estimates of exposure [26] as well as estimates of toxicological effect for fish collected from natural systems.

These limitations highlight the need for controlled laboratory experiments to determine the relationships between low-level pesticide exposure, cholinesterase inhibition, and behavioral impairment in fish. In the present study, we show that AChE inhibition is closely correlated with changes in juvenile coho behavior after chlorpyrifos exposures that approximate

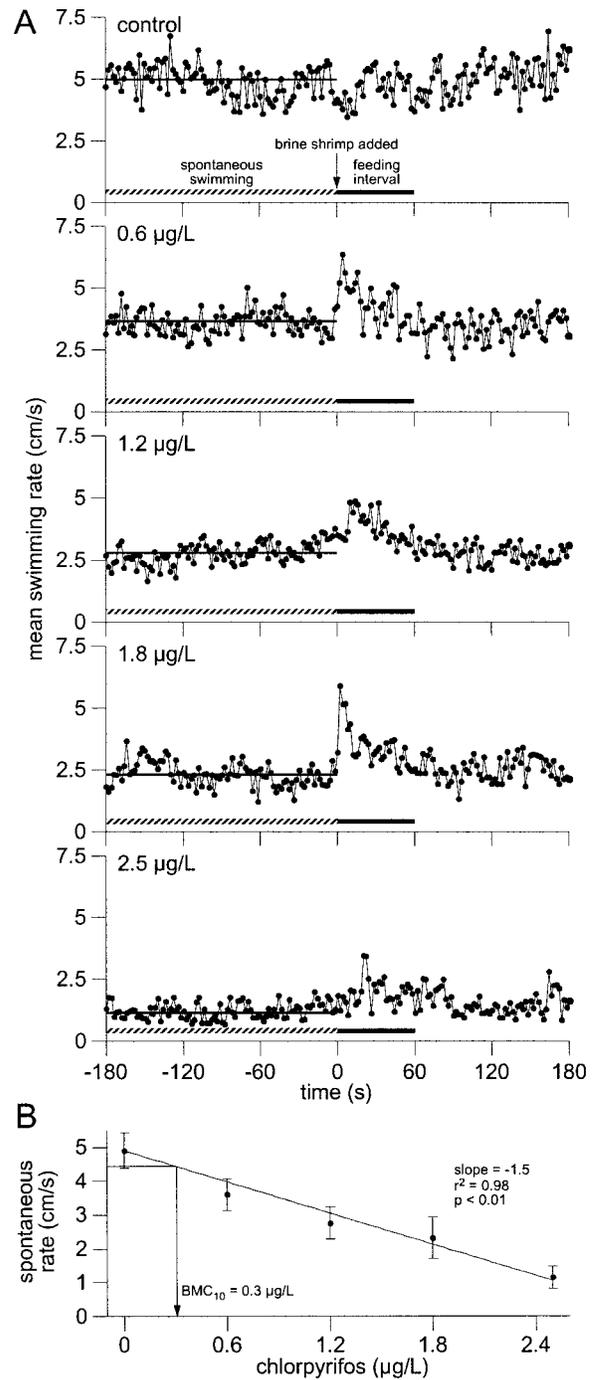


Fig. 3. Spontaneous and feeding swimming rates were reduced in juvenile coho after a 96-h exposure to chlorpyrifos. (A) Data collected from individual fish ( $n = 15-17$  fish per exposure concentration, obtained at 2-s intervals) were combined to produce averaged swimming rates for each chlorpyrifos exposure concentration. The hatched and solid bars at the bottom of graphs indicate the period in which spontaneous and feeding swimming rates were measured, respectively. The solid bars through the data points indicate the mean swimming speed during the spontaneous swimming intervals. (B) Mean spontaneous swimming rates of control and chlorpyrifos-exposed fish are plotted together and fit by linear regression for benchmark concentration analysis (y-intercept fixed at the control mean, 4.9 cm/s). The mean spontaneous swimming rates were significantly reduced in a concentration-dependent manner ( $p < 0.01$ ). As an example of benchmark concentration analysis, the horizontal line represents a 10% reduction in spontaneous swimming relative to the control mean benchmark concentration ( $BMC_{10}$ ), which corresponds to an estimated chlorpyrifos exposure concentration of 0.3 µg/L. Error bars represent 95% confidence intervals of group means.

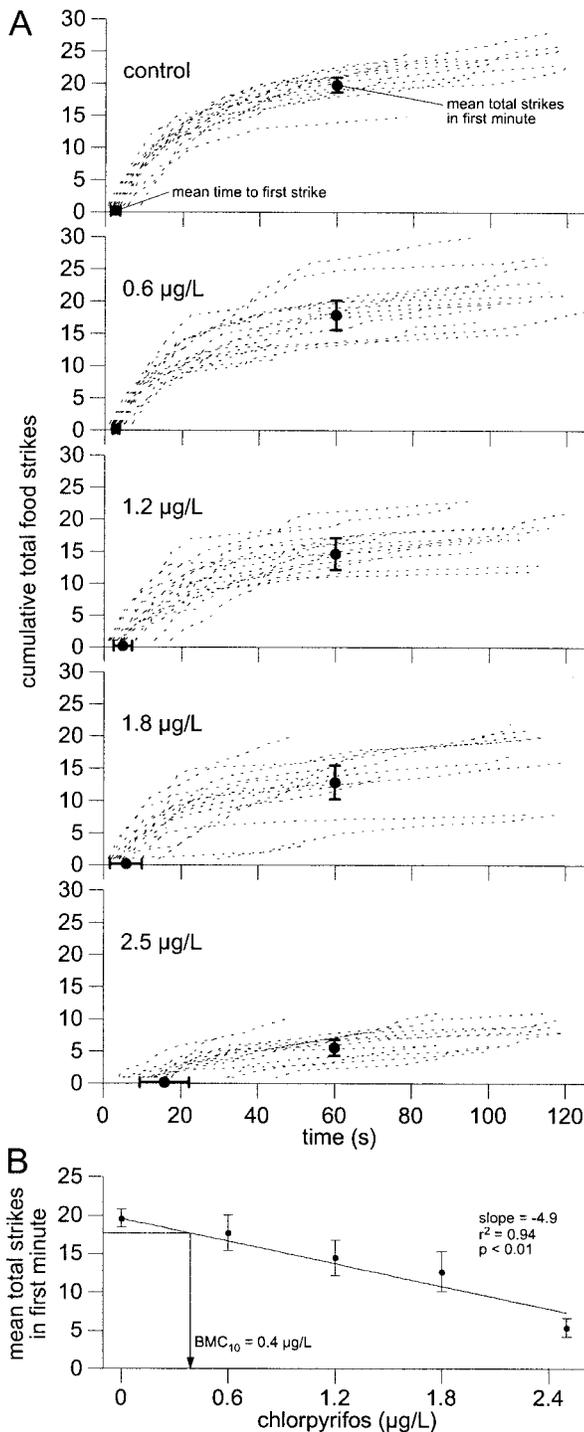


Fig. 4. Food strikes by juvenile coho were reduced after a 96-h exposure to chlorpyrifos. (A) Strikes were recorded over a 120-s interval after introducing 30 brine shrimp into the observation tank. Each dashed line indicates the cumulative strikes made by an individual fish ( $n = 15\text{--}17$  fish per exposure concentration). Closed points with  $x$ -error bars indicate the mean latency to first strike, and closed points with  $y$ -error bars indicate the mean total strikes made at the end of 1 min. (B) Mean total strikes (at the end of the 1-min feeding interval) for control and chlorpyrifos-exposed fish are plotted together and fit by linear regression for benchmark concentration (BMC) analysis ( $y$ -intercept fixed at the control mean, 19.6 strikes). The mean total strikes were significantly reduced in a concentration-dependent manner ( $p < 0.01$ ). When using a benchmark concentration analysis, the horizontal line represents a 10% reduction in strike rate relative to the control mean (BMC<sub>10</sub>), which corresponds to an estimated 0.4 µg/L chlorpyrifos exposure concentration. All error bars represent 95% confidence intervals of group means.

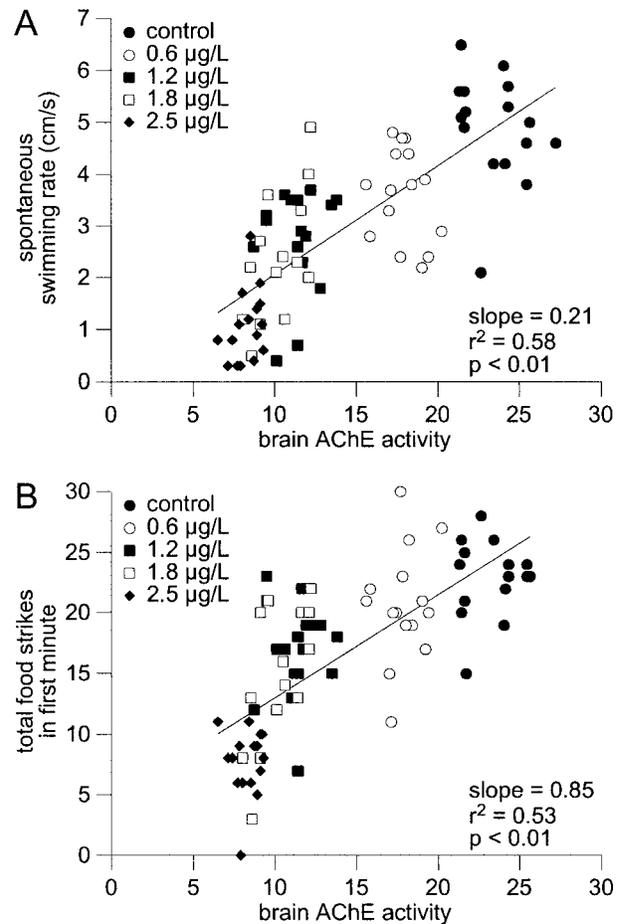


Fig. 5. Chlorpyrifos-induced reductions in acetylcholinesterase (AChE) activity were correlated with reductions in swimming and feeding behaviors. Fish were exposed for 96 h to a range of chlorpyrifos concentrations between 0 and 2.5 µg/L ( $n = 15\text{--}17$  fish per exposure concentration). After exposures, brain AChE activity, swimming behavior, and feeding behavior were measured for each individual fish. (A) Spontaneous swimming rate and (B) total strikes were significantly correlated with changes in brain AChE activity (Pearson correlation,  $p < 0.01$ ). Each point represents the dual measurements from an individual fish, and different symbols are used to distinguish the different exposure groups.

the upper range of surface water detections in urban and agricultural watersheds of California and the Pacific Northwest. Thus, sublethal behavioral effects can be reasonably predicted from laboratory measures of chlorpyrifos-induced AChE inhibition in the salmon nervous system. This provides an important link between in situ surface water monitoring data in salmon habitats [24], previous measures of pesticide-induced cholinesterase inhibition from laboratory studies [20], and salmon behavior, an endpoint with greater relevance for natural resource management than in vivo or in vitro rates of brain enzyme activity.

#### *Behavior as a sensitive and integrative measure of sublethal neurotoxicity*

For chlorpyrifos and other insecticides that share the anticholinesterase mechanism of action, a primary toxicological concern is for effects on the normal function of the fish nervous system, because these chemicals (or their metabolites) have the potential to interfere with cholinergic signaling. Measuring whole-brain changes in AChE activity, as we have done here,

is at best a rough indicator of potentially subtle changes in the regulation of acetylcholine concentrations in the clefts of muscarinic and nicotinic synapses across an array of cholinergic brain nuclei. Given that acetylcholine is one of the most widely distributed transmitter systems in the central and peripheral nervous systems of vertebrates [6], a general disruption of acetylcholine metabolism could impair many different neuroendocrine or neurobehavioral processes in fish.

Ideally, in terms of potential toxicological effects, a pesticide-induced inhibition of AChE would be considered at the next scale of biological organization; namely, as a change in the electrical properties of postsynaptic neurons in cholinergic networks. Depending on the neural networks involved, sublethal neurophysiological effects could ultimately be related to discrete changes in higher-order processes, including animal behavior. For example, the cellular and behavioral consequences of AChE inhibition have recently been evaluated in terms of egg-laying in the nematode *Caenorhabditis elegans* [32] and the respiratory motor patterns generated by the brainstem of AChE knockout mice [33]. In fish, AChE activity is required for the development of motor behavior, as indicated by abnormal muscle fiber formation, motorneuron innervation, and paralysis in zebrafish (*Danio rerio*) embryos that are homozygous for a mutation in the AChE gene that abolishes the catalytic activity of the enzyme [34]. Unlike *C. elegans*, mice, or zebrafish, however, cold-water fish species such as salmon are not major model systems for studying the neurochemical or neurophysiological mechanisms that give rise to discrete behaviors, including those that might be important for growth, survival, migration, or reproductive success. For example, it would be technically challenging to study the functional consequences of AChE inhibition at the cellular or network level in the coho salmon brain. By comparison, behavior is relatively easy to monitor, and behavioral endpoints are advantageous in that they reflect the coordinated activity of many areas of the fish nervous system. For example, swimming and feeding behaviors are the end product of sensorimotor integration, as characterized by the detection and transmission of environmental cues from peripheral sensory systems (e.g., visual, olfactory, or mechanosensory) to central brain networks, and from there to the hindbrain, spinal cord, and, ultimately, individual muscle groups (reviewed by Drapeau et al. [35]). Accordingly, behavioral observations are a practical and potentially sensitive way to detect distributed changes in neurotransmitter homeostasis [30].

Despite the advantages of behavioral endpoints, ethological research has not been particularly common in the field of aquatic toxicology. As noted more than a decade ago by Døving [36], this is because many behavioral endpoints have not been standardized; behaviors are often species-specific; interanimal variability can be high due to differences in age, appetite, and other state-specific conditions; and data collection can be labor intensive, particularly if fish are tested individually. Moreover, behavioral endpoints that are inherently subjective have the potential for observer bias.

In the present study, we controlled for the state-dependent condition of juvenile coho (e.g., the fish were the same age, from the same stock, and maintained with the same feeding regimen). Also, the computer-assisted data acquisition system allowed for quantitative, observer-independent monitoring of fundamental behaviors such as spontaneous swimming and feeding. When using this approach, we find that behavior is a sensitive indicator of sublethal chlorpyrifos exposure at nom-

inal concentrations as low as 0.3 to 0.4  $\mu\text{g/L}$ . This extends the results of recent studies on the sublethal effects of pesticides on fish behavior [8,13,14,18,19,37]. Also, in terms of anticholinesterase insecticides, our observation that spontaneous swimming speed is negatively correlated with salmonid brain AChE activity is consistent with similar, semiautomated behavioral studies of the effects of carbaryl [10] and malathion and diazinon [9] in rainbow trout.

#### *Chlorpyrifos exposures and the health of Pacific salmon*

The aim of the present study was to compare relative thresholds for AChE inhibition and behavioral impairment in juvenile coho under controlled chlorpyrifos exposure conditions. We monitored swimming and feeding behaviors because of their potential significance for the growth and survival of juvenile salmonids, and because these behaviors have been shown previously to be inhibited by cholinesterase-inhibiting insecticides [9,10,13]. In terms of nominal concentrations, our exposures were based on recent chlorpyrifos detections in salmon habitats in California and the Pacific Northwest [22–25]. The duration of each exposure was based on a previous study in steelhead [20] to allow for an interspecies comparison based on the relative departure values (BMC10) for AChE inhibition. Under natural conditions, however, there are many reasons why the stressor–response relationship for chlorpyrifos and wild salmon is likely to be more complex.

First, the loading of chlorpyrifos to salmon habitats in the western United States varies considerably across basins with different land uses and different climatic and hydrologic conditions. Because many common transport pathways are non-point source (e.g., surface runoff), chlorpyrifos loading to streams, rivers, and estuaries will often be determined by the intensity, duration, and relative frequency of storm events. A single insecticide pulse may last only a few hours [38]. Alternatively, resident fish may be exposed to repeated pesticide pulses from multiple spray events, storms, or return flows over the course of weeks or months. Recent evidence also suggests that chlorpyrifos toxicity can persist in the aquatic environment for days to weeks [24]. Thus, although the 96-h exposures used in this study are ecologically relevant, the actual duration of surface water contamination in different salmon habitats can be highly variable and site-specific.

Second, the individual life-history characteristics of Pacific salmon and steelhead will determine, in part, the extent of ecological exposure to chlorpyrifos and other pollutants. Given the similarities between the relative departure (BMC10) values for brain AChE inhibition in steelhead (0.6  $\mu\text{g/L}$  [20]) and coho (0.4  $\mu\text{g/L}$  this study), it is reasonable to assume similar inhibitory responses in other species of the genus *Oncorhynchus*. Pacific salmon vary in their spatial and temporal use of freshwater and estuarine habitats, including small streams in agricultural and urban drainages that are most likely to be contaminated with chlorpyrifos. For example, species such as chum (*O. keta*), pink (*O. gorbuscha*), and ocean-type chinook (*O. tshawytscha*) salmon that migrate to the estuary within a few weeks or months of hatching are less likely to be exposed to chlorpyrifos than coho, steelhead, sockeye (*O. nerka*), and stream-type chinook that may spend a year or more as juveniles in freshwater lakes and river systems [39].

The third consideration is that current-use pesticides typically occur in aquatic systems as mixtures. For example, a recent analysis of environmental monitoring data collected by the U.S. Geological Survey National Water Quality Assess-

ment Program found that >80% of surface water samples from urban streams contained three or more pesticides [40]. The issue of mixtures is particularly important for chemicals such as the organophosphates (e.g., chlorpyrifos, diazinon, fonofos, malathion, and azinphos-methyl) and carbamates (e.g., carbaryl and carbofuran), which are known to inhibit AChE activity in the vertebrate nervous system. Mixtures of these pesticides are likely to produce cumulative toxicity via AChE inhibition [41]. Consistent with this expectation, we have recently found that AChE extracted from the brains of adult chinook salmon is inhibited in a strictly additive fashion by binary combinations of carbaryl, chlorpyrifos, and the oxon metabolites of chlorpyrifos, diazinon, and malathion (N.L. Scholz and D.H. Baldwin, NOAA Fisheries, Northwest Fisheries Science Center, Seattle, WA, USA, unpublished results). In the present study, we observed significant inhibitory effects of chlorpyrifos at concentrations that approximate the upper range of documented detections in salmon habitats. Lower concentrations of chlorpyrifos might also elicit neurobehavioral toxicity in situations where the chemical co-occurs with other organophosphate or carbamate insecticides. In certain drainages in northern California, for example, chlorpyrifos frequently co-occurs with diazinon and other anticholinesterase insecticides [24,42].

In terms of toxicological response, we measured spontaneous swimming and feeding behaviors under controlled (and therefore relatively artificial) laboratory conditions. We did not determine the time course for recovery, and this may be an important ecological consideration, given the seasonal and intermittent transport of pesticides to salmon habitats. Evidence from other fish species suggests that recovery may be relatively protracted. For example, it takes mosquitofish (*Gambusia affinis*) more than six weeks to recover brain AChE activity after a relatively brief (8-h) exposure to chlorpyrifos (100 µg/L [5]). For coho salmon, the relative rate of recovery for biochemical and behavioral endpoints has not been investigated. Thus, the potential for longer-term behavioral impairments after acute exposures to chlorpyrifos remains to be determined.

It is interesting to note that Beauvais et al. [9,10] also found a close association between sublethal AChE inhibition and decreases in spontaneous locomotory activity in rainbow trout. Several consequences of lethargy and reduced feeding are possible in chlorpyrifos-exposed salmonids. These include the ability to avoid predators, defend territories, seek shelter, and form schooling aggregations. Reductions in feeding are a particular concern because rates of prey capture determine, in part, rates of energy assimilation and juvenile growth. The question of prey capture is further complicated by the fact that chlorpyrifos is an insecticide, and juvenile salmon predominantly feed on terrestrial and aquatic insects [39]. Chlorpyrifos could therefore inhibit growth via direct effects on feeding behavior as well as indirect effects [43] on the abundance of prey. The importance of juvenile growth rates for the survival and reproductive success of Pacific salmon is well documented. For example, smaller salmon experience a relatively higher rate of predation mortality when they transition from freshwater to the ocean [44,45].

In summary, large scale and long-term ecotoxicological investigations are highlighting the importance of sublethal effects in terms of the impacts of pollution on fish and other wildlife [46]. At the same time, acute lethality (median lethal concentrations) and other relatively insensitive toxicological endpoints are being critically evaluated with respect to their

use in ecological risk assessment [47]. In the future, toxicity studies can be expected to move away from traditional methods for standard toxicity tests, in part because of the need to tailor sublethal endpoints to the particular life-history requirements of the species of concern [48]. In this context, we have shown that simple behaviors are robust and sensitive indicators of sublethal neurotoxicity in juvenile coho exposed to chlorpyrifos. Although the regulation of cholinergic signaling in salmon is not well understood, examination of our data suggests a close functional relationship between transmitter hydrolysis (via AChE activity) and several discrete motor behaviors. Importantly, computer-assisted data acquisition techniques and accurate three-dimensional digital imaging have served to minimize observer bias and sampling noise. These improvements in sensitivity and resolution have allowed us to reexamine an old question [2] in a new light.

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