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Acute Metal Toxicology of Olfaction in Coho Salmon: Behavior, Receptors, and Odor-Metal Complexation

Bradley C. Rehnberg and Carl B. Schreck

Oregon Cooperative Fishery Research Unit, Department of Fisheries & Wildlife, Oregon State University, Corvallis, OR 97331

Chemoreception in fish is believed to play a mediating role in reproductive migration and pairing, schooling, feeding, parental recognition, and predator avoidance (Hara 1982). Adult salmonids orient to stream-specific odors during their freshwater reproductive migration. Fish olfaction is known to be inhibited by several common waterborne pollutants (Brown et al. 1982) although mechanisms of inhibition remain obscure. Metals from large mining operations in the Pacific Northwest continue to be a water pollution problem (Johnson et al. 1975).

One objective of our research was to determine the acute toxicities of mercury (Hg), copper (Cu), and zinc (Zn) to coho salmon olfaction. We used a behavioral assay of olfaction based on an avoidance reaction to L-serine in a two-choice Y-trough. Amino acids are potent odors for fish and are proving to be important components of complex natural odors (Hara 1982). A second objective was to gain some understanding of the mechanism of metal-induced olfactory inhibition by observing how metals affect the binding of L-serine to its olfactory cell membrane receptor. We have also taken the novel approach of addressing olfactory toxicology from the perspective of the odor molecule by considering metal speciation and metal-serine complexation chemistry on the basis of chemical equilibrium computations.

MATERIALS AND METHODS

Zero-age coho salmon Oncorhynchus kisutch (mean weight = 2.9 g) were trucked from the Oregon Department of Fish and Wildlife's Fall Creek Hatchery to the U.S. Environmental Protection Agency's Western Fish Toxicology Station (WFTS) 1 month prior to our experiments. The fish were kept under a natural photoperiod in circular fiberglass tanks, 1.5 m in diameter, supplied with running well water (12.5 -

17.4 C). Fish were fed Oregon Moist Pellets *ad libitum* twice daily.

Design of the two-choice Y-trough and the conditions of the behavioral assay were described in Rehnberg et al. (1985). Water flowed down the arms of the 2.4-m Y-trough at 6.6 L/min. Solutions of L-serine (Sigma) were introduced into one arm or the other from a calibrated Mariotte bottle. The arm not receiving serine received well water from a control Mariotte bottle. Stock metal solutions made from $ZnCl_2$, $CuSO_4$, or $HgCl_2$ contained concentrated HNO_3 (0.1 mL/L stock) to prevent precipitation. Stock metal solution was pumped to a constant-head delivery box which drained into the headbox supplying the two arms and separating channel of the Y-trough.

The behavioral assay began by placing 10 fish in the leg of the Y-trough downstream of a gate that blocked access to the fork area. Following a 1 hr 45 min exposure to a metal, the leg gate was raised and the fish were crowded into the fork area. The leg gate was then lowered and, when the fish became visibly calm (after about 5 min), serine was introduced into one arm of the Y-trough. A serine concentration of 10^{-8} M was used to evaluate the effects of Zn and Cu whereas 10^{-7} M was used in the Hg experiment. After 10 min of serine addition, the arm gates were raised giving the fish an opportunity to swim through one-way traps into either arm. After 30 min the arm gates were dropped and the number of fish in the arms and fork were counted. Using fresh fish each time, the assay was repeated at least six times for each treatment ($N \geq 60$). Arm choice was random when serine was absent from the Y-trough, indicating no intrinsic arm preference (Rehnberg et al. 1985). The arm receiving serine was changed after each test to counter any unexpected bias that might arise. Avoidance of Y-trough arms containing serine was evaluated by using a two-class X^2 test with a correction for continuity (Sokal and Rohlf 1981). The potential for metals to alter upstream orientation was tested by comparing the fractions of fish not entering a Y-trough arm in control assays to those in treatment assays (difference in proportions test---Dixon and Massey 1969).

Olfactory tissue for the receptor assay was obtained from adult coho salmon within 48 hr of their return from the ocean. Snouts were removed anterior to the eyes and stored frozen. To begin an assay, pairs of olfactory rosettes were dissected from 10 snouts and processed, with few modifications, according to the method of Cagan and Zeiger (1978). The homogenizing, washing, filtering, and centrifugation steps resulted in a sedimentable fraction (P2) enriched in plasma membranes. In preparation for a binding assay, P2 was suspended in cold 0.05 M Aces buffer (pH 7.0) at a concentration of 150 - 200 ug protein/mL. Total binding was found by incubating 1.00 mL P2 with 0.15 mL distilled H_2O and 0.15 mL L-[G-^{3}H]serine (ICN, 4 or 15 Ci/mmol). Nonspecific binding was found by incubating 1.00 mL P2 with 0.05 mL H_2O, 0.10 mL unlabeled L-serine (10^2 to 10^3-fold more than ^{3}Hserine), and 0.15 mL ^{3}Hserine. After incubation, duplicate 0.5 mL-aliquots from each assay volume were filtered through 25-mm Millipore filters (Type HAWP, 0.45 u) under vacuum. A 10-mL Aces buffer wash was pulled through the filter to reduce ligand binding to the filter. The filters were dissolved in 10 mL scintillation fluid and radioactivity was measured in a Packard Model 2002 liquid scintillation counter. The effects of metals on serine binding were shown by substituting 0.05 mL of a metal solution for 0.05 mL H_2O in both total binding and nonspecific binding assay mixtures.

Chemical equilibrium calculations were made using the MICROQL program (Westall 1979) on a Hewlett Packard HP85. MICROQL inputs were pH, $C_{T,metal}$ (= total metal concentration [M]), $C_{T,serine}$, and the C_T for other measured component ions of interest. $C_{T,metal}$ was determined by atomic absorption spectrophotometry. The difference between $C_{T,metal}$ and the calculated free metal ion concentration is referred to as complexed metal. Total organic carbon in WFTS well water was always below detection limits (2 mg/L), thereby eliminating consideration of humic substances in the calculations. Zinc species of interest and their stability constants uncorrected for temperature and ionic strength were Zn^{++}, $ZnOH^+$ ($10^{-9.0}$), $Zn(OH)_2^0$ ($10^{-16.9}$), $ZnSer^+$ ($10^{4.7}$), $Zn(Ser)_2^0$ ($10^{8.7}$), and $ZnSO_4$ ($10^{2.4}$) (Smith and Martell 1974, 1976). Copper species of interest were Cu^{++}, $CuOH^+$ ($10^{-7.9}$, Sillen and Martell 1971), $Cu(OH)_2^0$ (aq) ($10^{-13.7}$, Vuceta and Morgan 1977), $CuCO_3^0$ ($10^{6.8}$, Silman 1958), $CuSer^+$ ($10^{7.9}$, Smith and Martell 1974), and $Cu(Ser)_2^0$ ($10^{14.5}$, Smith and Martell 1974). Mercury species considered were Hg^{++}, $HgOH^+$ ($10^{-3.4}$, Smith and Martell 1976), $Hg(OH)_2^0$ ($10^{-6.2}$, Smith and Martell 1976) and $Hg(Ser)_2^0$ ($10^{17.5}$, Perkins 1953).

RESULTS AND DISCUSSION

Calculated means (mg/L) and standard deviations (in parentheses) for chemical parameters measured daily were dissolved oxygen, 9.3 (0.69); alkalinity as $CaCO_3$, 25.5 (2.83); hardness as $CaCO_3$, 30.5 (4.69);

Table 1. Fish not choosing a Y-trough arm as a fraction of the total number tested. Z values (in parentheses) from a difference in proportions test were all significant at $p < 0.01$ except at 10^{-5} Zn (ns).

Conc.	Hg	Cu	Zn
No metal	3/120	26/180	25/180
10^{-7}	19/60 (5.63)	31/60 (5.87)	40/60 (6.04)
10^{-6}	18/60 (5.42)	43/60 (8.48)	34/60 (4.83)
10^{-5}	17/60 (5.20)	27/60 (4.94)	19/60 (1.59)

calcium hardness as $CaCO_3$, 22.0 (3.94); magnesium hardness as $CaCO_3$, 8.2 (1.80); and pH, 6.72 (0.29). Means (mg/L) and standard deviations (in parentheses) for parameters measured weekly were NO_3, 0.85 (0.09); NH_3, < 0.005; PO_4, 0.135 (0.00); SiO_2, 21.2 (1.48); and total organic carbon, < 2.

The likelihood of a fish swimming upcurrent into either Y-trough arm was depressed by the presence of Zn, Cu, or Hg (Table 1). This depression of swimming behavior seems to be related only to the presence of a metal and not to its concentration since the smallest effects were seen at the highest metal concentrations. All fish appeared normal at 10^{-7} M metal, yet significant declines in upstream orientation was observed for all metals (Table 1). From these observations we cannot resolve whether the metals acted by reducing motivation, confusing the perception of current, or inducing general malaise. In any case, we conclude that the appropriate behavior in the context of this experiment, swimming against the current and selecting an arm, was depressed by the presence of these metals.

The effect of Hg, Cu, or Zn on the ability of juvenile coho salmon to detect and avoid L-serine is shown in Fig. 1. Zn had no observable effect on olfaction. The apparent decline in serine avoidance at 10^{-7} M Zn was probably a result of the small sample size, since higher Zn concentrations had no effect. Thus, although fewer fish swam upstream into Y-trough arms in the presence of Zn, those that did enter arms avoided serine to about the same degree as control fish. The avoidance of serine was inhibited at all test concentrations of Cu and Hg. The reason for the apparent preference for the serine arm at 10^{-6} M Hg is unknown. Increased skin mucus was evident at 10^{-5} M Hg, but the mucus content of the nasal sac appeared normal. Blood vessels on the surface of the olfactory rosette were more visible in fish tested at 10^{-5} M Hg, and the rosette tended to hemorrhage when disturbed by

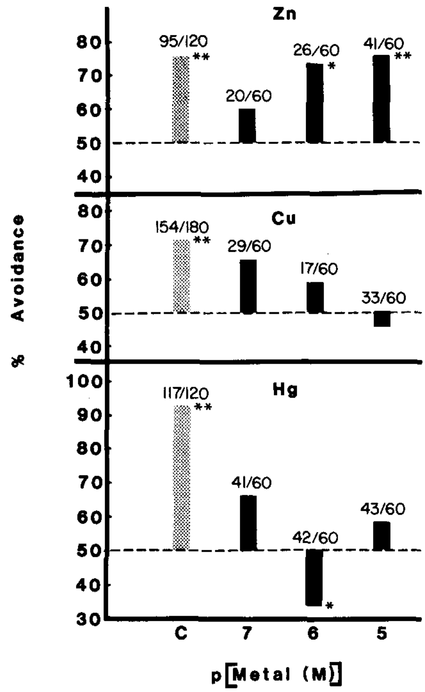

Figure 1. Avoidance of L-serine in the presence (solid bars) and absence (stippled bars) of metals. Fish entering either arm of the Y-trough as a fraction of the total number tested is shown above each bar. * = $p < 0.05$ and ** = $p < 0.01$.

a probe. The acute inhibitory effects of Hg and Cu on L-serine detection in rainbow trout have been demonstrated electrophysiologically (Hara et al. 1976). In that study, depression of the olfactory bulb response to 10^{-5} M L-serine was observed during 2-hr exposures to 0.10 mg/L $HgCl_2$ ($10^{-6.43}$ M) and 0.008 mg/L $CuSO_4$ ($10^{-7.30}$ M). To our knowledge, the present study is the first behavioral research on the olfactory performance of fish during brief exposures to low concentrations of Zn. Bloom et al. (1978) showed that pheromone detection by zebrafish Brachydanio rerio was lost after a 9-day exposure to 5 mg/L Zn.

The stimulatory effectiveness of an odor, when complexed with a metal, would seem improbable due to both steric and charge considerations. Of the three metals considered, only Cu showed a tendency to form complexes with serine (Table 2). Chemical equilibrium computations indicate that the proportion of serine complexed with Cu was a function of $C_{T,Cu}$ with serine

Table 2. Calculated distribution of chemical species during behavioral assays of olfaction in the presence of Cu. All concentrations expressed as p[M]. $C_{T,Cu}$ values in parentheses are the analytical values used in the calculations. Free Cu values in parentheses represent cupric ion activity measurements.

$C_{T,Cu}$	Free Cu	Complexed Cu	Free Ser	Complexed Ser
7 (7.20)	7.55	7.46	8.01	10.06
6 (6.05)	6.24 (6.09)	6.49	8.05	8.99
5 (5.05)	5.19 (5.11)	5.61	8.31	8.30

being approximately 50 percent bound at $C_{T,Cu} = 10^{-5}$ M. At the environmentally more realistic Cu concentrations of 10^{-6} and 10^{-7} M, however, MICROQL predictions indicate that most of $C_{T,Ser}$ was free and presumably available to stimulate an olfactory response. Cupric ion measurements at $C_{T,Cu} = 10^{-5}$ and 10^{-6} M show reasonably good agreement with calculated values (Table 2), thereby supporting the assumption of chemical equilibrium and the validity of the formation constants. Calculations for behavioral assays containing Hg predict that Hg was present primarily as $Hg(OH)_2^0$ and that Hg-serine complexes were negligible. From the results with Cu and Hg, we conclude that the observed inhibitory effects of these metals on serine detection cannot be explained by the formation of nonstimulatory metal-serine complexes. Calculations for behavioral assays with Zn indicate that both Zn and serine existed predominantly as free ions. Zn^{++} apparently does not interact with the serine olfactory receptor. The existance of free serine is consistant with our observation that serine was detected by the fish during exposures to Zn.

The inhibitory effects of Hg, Zn, and Cu on the binding of serine to its olfactory receptor are shown in Fig. 2. The small effects of Cu and Zn were essentially concentration-independent at metal concentrations of 10^{-7} to 10^{-5} M. Thus, the inhibitory effects of Cu-exposure seen in the behavioral assays cannot be explained by interactions at the serine receptor. In contrast, Hg clearly inhibits serine binding, showing a steep threshold between 10^{-5} and 10^{-6} M. It appears that the inhibition of olfaction measured behaviorally was more sensitive to low $C_{T,Hg}$ than the inhibition of serine binding measured _in vitro_. Perhaps the sensitivity of the binding assay would have been greater had we used a purified plasma membrane fraction. The fraction we used probably provided Hg^{++} with an abundance of surface area and reactive sites for nonspecific

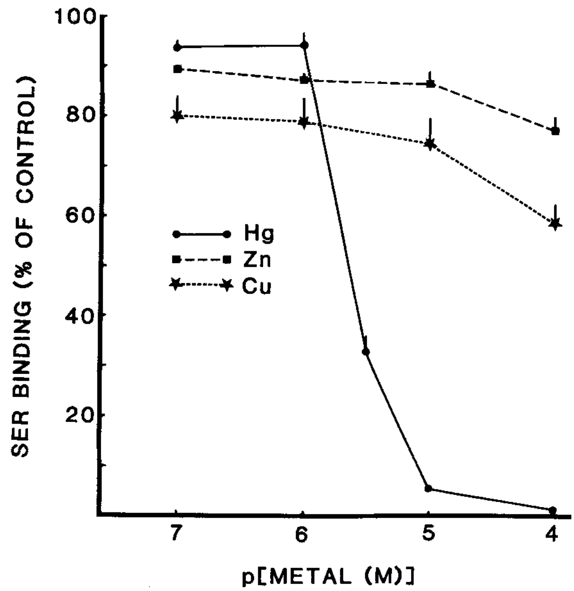

Figure 2. Binding of L-serine in the presence of metals as a % of binding in controls (no metals). Serine concentrations were 0.26 to 9.26 uM. Means and standard errors from three preparations are shown.

binding, which effectively lowered Hg++ activity in the incubation media. Cagan and Zeiger (1978) found that the binding of L-alanine to olfactory receptors of rainbow trout was severely inhibited by low concentrations of Hg (10^{-6} M), but not by high concentrations (10^{-3} M) of Zn or Cu.

In summary, the detection of L-serine by juvenile coho salmon was inhibited by Hg and Cu but not by Zn. None of these metals interfered with serine detection by forming nonstimulatory metal-serine complexes. The mechanism of action of Cu remains unclear whereas Hg appears to act at the level of the olfactory receptor. All three metals, however, were found to disrupt simple upstream movements in our experimental apparatus.

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REFERENCES

Bloom HD, Perlmutter A, Seeley RJ (1978) Effect of a sublethal concentration of zinc on an aggregating pheromone system in the zebrafish, Brachydanio rerio (Hamilton-Buchanon). Environ Poll 17:127-131

Brown SB, Evans RE, Thompson BE, Hara TJ (1982) Chemoreception and aquatic pollutants. In: Hara TJ (ed) Chemoreception in fishes. Elsevier, New York, p 376

Cagan RH, Zeiger WN (1978) Biochemical studies of olfaction: Binding specificity of radioactively labeled stimuli to an isolated olfactory preparation from rainbow trout (Salmo gairdneri). Proc Nat Acad Sci USA 75(10):4679-4683

Dixon WJ, Massey FJ (1969) Introduction to statistical analysis. McGraw-Hill, New York

Hara TJ (ed) (1982) Chemoreception in fishes. Elsevier, New York

Hara TJ, Law YMC, MacDonald S (1976) Effects of mercury and copper on the olfactory response in rainbow trout, Salmo gairdneri. J Fish Res Board Can 33:1568-1573

Johnson RD, Miller RJ, Williams RE, Wai CM, Wiese AC, Mitchell JE (1975) The heavy metal problem of Silver Valley, northern Idaho. Internat Conf on Heavy Metals in the Environment Vol 2 Part 2, Toronto

Perkins DJ (1953) A study of the effect of amino acid structure on the stabilities of the complexes formed with metals of group II of the periodic classification. Biochem J 55:649-652

Rehnberg BG, Jonasson BJ, Schreck CB (1985) Olfactory sensitivity during parr and smolt developmental stages of coho salmon. Trans Amer Fish Soc. In press

Sillen LG, Martell AE (1971) Stability constants of metal-ion complexes. Chem Soc (Lond) Spec Pub 25, Supp 1

Silman JFB (1958) The stabilities of some oxidized copper minerals in aqueous solutions at 25 C and 1 atmosphere total pressure. Doct Diss, Harvard University, Cambridge

Smith RM, Martell AE (1974) Critical stability constants. Vol 1, Amino acids. Plenum Press, New York

Smith RM, Martell AE (1976) Critical stability constants. Vol 4, Inorganic complexes. Plenum Press, New York

Sokal RR, Rohlf FJ (1981) Biometry. W.H. Freeman, San Francisco

Vuceta J, Morgan JJ (1977) Hydrolysis of Cu (II). Limn Oceanog 22:742-746

Westall J (1979) MICROQL I. A chemical equilibrium program in BASIC. Swiss Fed Inst Tech, Duebendorf

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