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Detection of L-serine and D,L-alanine by juvenile coho salmon (Oncorhynchus kisutch) was measured in a two-choice Y-trough. Threshold concentration for olfactory detection of L-serine was 10^{-8} M for zero-age parr, 10^{-7} M for yearling parr, 10^{-5} M for smolts in late April-early May, and 10^{-6} M for smolts in June. Threshold for the detection of D,L-alanine was 10^{-7} M for yearling parr, 10^{-6} M for smolts in late April-early May, and 10^{-5} M for smolts in June. Zero-age fish adapted to either freshwater or seawater were able to detect L-serine at 10^{-9} M.

In vitro olfactory receptor assays and behavioral experiments were used to examine the specificity of L-serine detection by coho salmon. Of the 10 amino acids tested, L-serine, L-alanine and L-threonine were similar

in their ability to compete for the serine receptor site. All three of these amino acids as well as L-histidine elicited behavioral avoidance responses in a 2-choice Y-trough. Behavioral cross-adaptation experiments indicated that the detection of L-serine was inhibited by the adapting amino acids L-serine, L-alanine, and glycine but not by L-threonine, L-aspartic acid, or L-histidine. Classical conditioning experiments showed that serine and alanine were not discriminated from each other although either could be discriminated from L-histidine. These results, taken together, indicate that coho salmon perceive L-serine and L-alanine as identical odors.

The olfactory detection of L-serine in a 2-choice Y-trough was inhibited by 2 hr exposures to mercury (Hg) or copper (Cu) but not by zinc (Zn). Upstream swimming behavior, however, was depressed by the presence of Zn as well as Hg or Cu. Of these metals, only Hg strongly inhibited the binding of L-serine to its olfactory receptor. The inability to detect L-serine in the presence of Cu did not result from Cu masking the presence of serine. Chemical speciation calculations suggest that serine-metal complexation was not extensive enough to compromise olfactory detection. Thus, the mechanism of action of Cu is unclear whereas Hg appears to act at the level of the olfactory receptor. The ability to detect and avoid L-serine was not inhibited by a continuous exposure to Zn at 100 or 500 ug/L for 21 days.

CHEMORECEPTION IN COHO SALMON (*ONCORHYNCHUS KISUTCH*): ASPECTS OF BEHAVIOR, PHYSIOLOGY, AND TOXICOLOGY

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CONTRIBUTION OF AUTHORS

Brian Jonasson provided the data on the detection of amino acids by yearling fish (Fig. 2). For this contribution as well as his ideas on salmonid olfaction, Brian is a co-author of Chapter II.

Lyle Curtis assisted in planning the logistics of the olfactory imprinting project at the Salmon River Hatchery. He and his staff were responsible for rearing and releasing the juvenile fish and tallying returns of the adult fish. Lyle is a co-author of Appendix II.

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I. GENERAL INTRODUCTION

The biology of olfaction has been a source of fascination for scientists since antiquity. The great physiologist Galen (199 AD) described a theory of olfaction for humans based on his dissections of animals (Siegel 1970). Observing perforations in the ethmoid bone, he reasoned that odor particles passed through these openings directly into the ventricle of the olfactory bulb. For Galen, odors arose from nearly all substances as invisible vapors mixed with air, and were small enough to enter the olfactory ventricles and produce stimulation. He saw expansion and contraction of the olfactory bulb as a mechanism for pulling odors into the ventricles by vacuum and expelling them by pressure.

Since Galen's time, the field of olfactory research has been notable for a great preponderance of theories. "Olfactory theories are as numerous as pebbles on the beach" wrote J. T. Davies (1971). This exaggeration was a comment on the absence of any generally agreed-upon paradigms in olfactory research. The interaction between odors and sensory cells, the generation of the electrical impulse, and the nature of sensory coding have frustrated

olfactory investigators to this day. In 1978, Cagan and Zeiger wrote "Despite a florid history of hypotheses as to the underlying mechanisms, virtually nothing is known about the biochemical basis of the specificity of interaction between an olfactory stimulus molecule and an olfactory receptor." Much of the theorizing has centered around the interaction between odor molecules and sensory cells as well as the process of sensory transduction. A brief description of eight such theories published between 1940 and 1960 is presented by Kleerekoper (1969).

Fish have been important experimental models in the development of contemporary ideas about the physiology of vertebrate olfaction. Using macrosmatic species such as rainbow trout (Salmo gairdneri) and channel catfish (Ictalurus punctatus), physiologists have recorded electrical activity from points along the olfactory axis to shed light on the nature of odor molecules, the dynamic range of responses to steps in odor concentration, and the nature of habituation. The power of the electrophysiological approach became apparent in one of the first studies done on vertebrates. Recording from the olfactory tract of catfish (Ictalurus), Adrian and Ludwig (1938) made three discoveries that have since been verified many times and in other vertebrates: 1) sensory cells show prominent resting discharges that are asynchronous, 2) after a period of prolonged stimulation, the response

becomes habituated and 3) the latency between the time of odor-arrival at the olfactory organ and time of electrical response is explained by the necessity of odor molecules to diffuse through a mucus layer to reach sensory cells. Fish have also been leading models for biochemical receptor research due, in part, to the soluble nature of natural odors. Cagan and collaborators (Cagan and Zeiger 1978; Rhein and Cagan 1980) have pioneered the use of a plasma membrane-enriched preparation derived from olfactory epithelium of rainbow trout to investigate odor-receptor interactions. They have extended this approach by showing that the receptors responsible for binding physiologically relevant odors are localized on cilia that extend from the sensory cell.

The role that olfaction plays in the behavior of fishes is still poorly understood. The difficulties inherent in studying sensory biology in the field are amplified in the aquatic environment. The environmental conditions in which olfaction would be expected to be most important to fish---at night, at poorly-lit depths, and in water with poor visiblilty---are precisely the conditions most difficult for making observations (Liley 1982). Consequently, there have been very few attempts to study olfaction in the field. von Frisch discovered the alarm reaction of ostariophysan fish by observing the behavior of schools of minnows in a lake. Acknowledging the

difficulties of these experiments, he soon moved his work to the laboratory (von Frisch 1941). The homing of fish is probably the best-studied phenomena from a sensory perspective because it is relatively easy to make observations in the field. The general technique has been to displace fish away from their home range or migratory goal, deprive them of one of their senses, and then observe their ability to accurately home (Smith 1985).

Perhaps the greatest obstacle to both ethologists and physiologists has been the lack of information on the chemistry of natural odors for fish. Most biologists have only rudimentary skills in analytical chemistry and are therefore not in a position to identify active substances. To date, not one semiochemical for fish has been unequivocally established and its chemical structure identified. The chemistry of the alarm substance in ostariophysan fishes has probably come closest to elucidation (Pfeiffer 1982). A great deal of olfactory research has been done using body fluids or complex and poorly-defined "extracts" or "rinses" of tissues or whole fish. The biologically active components in such mixtures are unknown and the possibility always exists that such a mixture contains a great deal of other information unrelated to the behavior under study. In studying olfactory receptors of the cockroach (Periplaneta), Sass (1978) noted the potential complexity of common fruit

odors. Bananas, for example, contain 60 different esters, 40 alcohols, 12 ketones, and 38 fatty acids. Until some analytical chemists become interested in the nature of odors for fish, the situation will not improve. In the meantime, investigators interested in fish olfaction will continue working with complex, undefined odors or with pure compounds of unknown relevance. Successful analytical chemistry, however, may ultimately reveal that most odors for fish are complex mixtures of stimulatory substances. In contrast to terrestrial animals, fish generally do not possess specialized glands that synthesize specific semiochemicals (Liley 1982). Prevailing evolutionary theory holds that semiochemicals originated from non-signal precursors (eg. metabolic byproducts) that were released into the environment where they secondarily became associated with a chemical message (Liley 1982). An example of this is the use of urine as a territorial marker by ungulates (Stoddart 1980). This hypothesis is especially appealing for aquatic animals because of the permeability of external tissues to biochemicals and the excretion of wastes directly into the medium in which they live. Complexity was clearly illustrated by Derby and Ache (1984) in their study of the stimulatory and interactive components of a natural food odor for the spiny lobster (Panulirus argus). Of the 31 major chemical components of "crab odor", a mixture of 15

of the components accounted for the full activity of the odor. Twelve of the components were stimulatory and three contributed to the mixture by suppressing the activity of the stimulatory components.

This dissertation is a study of olfaction in coho salmon (Oncorhynchus kisutch). The body of this work is divided into three chapters, each dealing with specific topics. The remainder of the introduction will be devoted to introducing each chapter with the intent of placing this research in a scientific and historical context.

Chapter one is a study of the olfactory capability of juvenile coho salmon at different ages and different physiological states. Should the olfactory abilities of juvenile coho salmon be expected to change with age? Yes, perhaps. The life cycle of coho salmon includes a brief period in which the fish undergoes a major transformation immediately before its oceanic phase. Changes in behavior, morphology, biochemistry, and physiology prepare the fish for its new environment (Hoar 1976). A great deal of work stimulated by the innovative experiments of Hasler et al. (1978) indicates that smoltification is also the period in which fish imprint to the odor of their home-stream. It is therefore reasonable to suggest that this period of endocrinological flux (Folmar and Dickhoff 1980) may include modifications of olfactory sensitivity. Electrical responses in the teleostean olfactory bulb and

telencephalon are known to be sensitive to elevations in circulating thyroxine (Oshima and Gorbman 1966a, b).

This chapter, then, asks whether olfactory sensitivity to amino acid odors change as the fish passes through parr and smolt stages of development. I also ask whether any observed change in olfactory performance can be attributed to the age of the fish or to its physiological capacity to adapt to seawater.

The topic of chapter two is related to one of the great mysteries of olfaction: How are animals able to be sensitive to so many different odors? From our own everyday experiences, we know that humans can sense a huge array of odors, many of which are distinguishable by the smallest of nuance. It is now evident that fish are also sensitive to a great variety of substances from a broad spectrum of chemical classes. Fish have been shown to be sensitive to amino acids (Tucker 1983), bile acids and their conjugates (Doving et al. 1980), conjugated steroids (Colombo et al. 1979), morpholine (Hirsch 1977), sodium chloride, vitamin B_{12}, sucrose (Rottiers and Lemm 1985), phenol and derivatives (Hasler and Wisby 1950; Tarrant 1966), inorganic ions (Bodznick 1978), and alcohols (Teichmann 1959). One wonders whether fish have specific olfactory receptor molecules for all or most of these odor molecules or whether there exist receptor types specific for broad functional groups, molecular size, and/or

electrical charges. The answer to this is currently unknown. Electrophysiological studies consistantly find that fish show differential responses (i.e., more or less electrical activity) to closely-related odors that are presented singly. Most of these electrophysiological data are from recordings at single sites in the olfactory bulb or tract, making difficult any inferences about the origin of these differences.

Early circumstantial evidence for a lock and key olfactory mechanism was provided by what may be called the stereochemical theory of olfaction. First proposed by Moncrieff (1951), it postulated the existance of a small number of primary odors and a corresponding number of receptor sites. Using this idea, Amoore (1970) identified, from the literature, approximately 600 organic compounds for which odors had been described. By noting the frequencies associated with the various odor descriptions, he was able to idenfify seven candidates for primary odors: camphoraceous, musky, floral, pepperminty, ethereal, pungent, and putrid. The idea was that primary odors were analagous to primary colors and primary tastes, i.e., any odor represents some combination of the seven primaries. Amoore then began analyzing the three-dimensional structure of the molecules in each primary odor class. He found that all camphoraceous molecules, for example, were approximately spherical with a diameter

of about 7 A. Similarly, the other primaries were unified by characteristic shapes, sizes, or charges. Drawing upon the lock and key model, he was able to make inferences about the structure of the primary receptor sites. The theory has been tested by synthesizing compounds of known structure but unknown odor. Predicted odors based on chemical structure have been verified by panels of "trained smellers" (Amoore 1964). More recently, Amoore (1970) has indicated that there are probably more primary odors than the seven originally postulated.

The notion that olfactory receptors are proteins associated with the external plasma membrane of sensory cells has undoubtedly been fueled by the success and intuitive appeal of similar notions in endocrinology, neurobiology, immunology, and pharmacology. Receptors for hormones, neurotransmitters, antigens, drugs, odors and taste molecules share at least one common attribute: all are chemoreceptors. Receptors most studied are ones having biomedical importance. The acetylcholine receptor isolated from the electric organ of fish (Electrophorus or Torpedo) is the only receptor for which the complete primary structure is known (Changeux et al. 1984). It is a transmembrane protein pentamer consisting of four different subunits and an ion channel. Progress in studying olfactory receptors in vertebrates has been retarded due to a lack of adequate experimental models.

A reliable receptor assay using fish olfactory epithelium has recently been developed by Cagan and Zeiger (1978) by adapting procedures used by Cuatrecasas (1971) to measure the binding of insulin to adipose tissue cells and cell membranes.

Chapter two focuses on amino acids as odors for coho salmon and more specifically on the specificity of the olfactory receptor site of L-serine. The first chapter shows that L-serine is detected by olfaction in coho salmon. In Chapter two we attempt to determine if the receptor site for L-serine is unique or if it is shared with other amino acids. This problem is addressed by using the in vitro biochemical technique of Cagan (Cagan and Zeiger 1978) with in vivo supporting evidence from observations of fish behavior.

Chapter three is an investigation of the effects that toxic materials may have on olfaction. Little is understood about the role that olfaction plays in the life of salmonids with the exception of two periods in its life cycle. There is good evidence that salmon smolts imprint to chemical characteristics of their home-steam. As adults, salmon use their recognition of home-stream odor(s) during their freshwater migration to accurately return to their stream of origin (Smith 1985). In theory, toxicants could affect the imprinting and/or adult-return process by 1) changing the overall chemical "bouquet" of a

river, 2) inducing avoidance reactions or 3) functionally disrupting olfactory processes. Avoidance reactions have been documented for several species of fish in response to a wide variety of toxicants (Giattina and Garton 1983). Only recently has the susceptibility of olfaction to waterborne pollutants been investigated (Brown et al. 1982). Olfaction would appear to be uniquely susceptible to dissolved toxicants because the sensory cells are in direct contact with the environment. In most fish the olfactory rosette rests in a pit that remains open to outside water. The sensory cell is a neuron with olfactory receptors in its external plasma membrane. Thus, toxicants have direct access to receptor areas and to regions of the plasma membrane that may function in cell excitability. Lastly, a toxicant with irritating effects could stimulate goblet cells in the sensory epithelium to overproduce mucus.

In this study behavioral observations were used to investigate the effects that metals have on the detection of L-serine. Mechanisms of action were considered by observing how metals affect odor-receptor binding. Sutterlin (1974) suggested that certain toxicants may interfere with olfaction by chemically combining with odor molecules before they ever reach the recipient. That possibility seemed real in these experiments because of the strong affinity between metals and amino acids (Smith

and Martell 1974). I therefore employed a chemical equilibrium model to obtain a quantitative indication of whether odor-metal interactions could help explain results.

II. Olfactory Sensitivity during Parr and Smolt Developmental Stages of Coho Salmon (*Oncorhynchus kisutch*)[1]

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INTRODUCTION

Olfaction is believed to be the primary sense guiding the freshwater migration of adult anadromous salmonids. Although the source of the olfactory cue is debatable (Nordeng 1977; Hasler et al. 1978), it is agreed that home stream odors flow toward the estuary and are used by migrants in orientation. Before their seaward migration, some anadroumous salmon (Oncorhynchus) and trout (Salmo) undergo a parr-to-smolt transformation (smoltification) that involves profound changes in morphology, physiology, and behavior (Hoar 1976; Folmar and Dickhoff 1980). Transplantation experiments have suggested that an ability to imprint to a discrete section of stream may be associated with a relatively brief period during smoltification (Cooper and Hirsch 1982). The relative importance of heightened olfactory sensitivity or increased long-term memory to the imprinting process is unknown. To our knowledge, olfactory sensitivity during the ontogeny of premigratory salmonids has not been documented. We measured the ability of coho salmon (Oncorhynchus kisutch) to detect and respond to serine and alanine in freshwater during their development from zero-age parr through the period commonly associated with

smoltification. In addition, we determined the olfactory sensitivity of zero-age fish in both freshwater and seawater to learn whether changes in olfaction are among the many physiological changes involved in adaptation to seawater. Although the chemical identity of odors used by coho salmon in ecological contexts is unknown, there is agreement that amino acids are chemostimulatory components of complex natural odors for fish (Hara 1982). L-serine has been given credit as the active salmon repellent in mammalian skin extract (Idler et al. 1956), but L-alanine may also contribute to the avoidance response.

METHODS

Parr-smolt Tests in Freshwater

Juvenile coho salmon were obtained from the Oregon Department of Fish and Wildlife's Fall Creek Hatchery at least 1 month before our experiments. Fish were held in flow-through fiberglass tanks, 1 or 1.5 m in diameter, and fed to repletion daily with Oregon Moist Pellets. Experiments with zero-age parr took place at the U.S. Environmental Protection Agency's Western Fish Toxicology Station (WFTS), Corvallis, Oregon, and those with yearling fish at Oregon State University's Smith Farm Laboratory, Corvallis, Oregon.

We assessed olfactory sensitivity by observing the

avoidance behavior of fish toward dilute concentrations of L-serine and D,L-alanine in a two-choice Y-trough. The 2.42-m trough was constructed with high-density overlay plywood and caulked with a silicone sealant (Fig. 1). Well water entered the head of the two Y-trough arms and the head of the separating channel between them. To determine if the design of the Y-trough provided an adequate separation of flow, rhodamine 6G dye was added to one arm and fluorescence was measured at several locations in the fork area (Table 1). Dye at the entrance of the arm receiving the dye was about 4.7×10^3 times more concentrated than at the entrance to the other arm. The separating channel was screened so that fish could not enter it. Flows down both arms averaged 8.0 L/min at Smith Farm and 6.6 L/min at WFTS. To encourage fish to swim into the arms, the fork area was illuminated and the arms and separating channel were covered with black plastic to exclude light. The leg gate blocked entrance to the leg; the arm gates could be rapidly raised and lowered without disturbing the fish. Curtains were placed around the trough to prevent the fish from seeing the observer. A funnel-type trap in the opening of each arm allowed the fish to enter, but generally prevented them from exiting.

An assay began when 10 fish were placed in the Y-trough fork area with all gates down. Assay conditions

differed slightly for zero-age and yearling fish. Zero-age fish were given about 5 min to calm down and begin exploratory swimming before odor presentation. An odorant was then introduced from a calibrated Mariotte bottle into the head of one arm for 10 min before the gates to both arms were raised. The fish were allowed 30 min during which they were free to enter an arm or remain in the fork area. The arm gates were then lowered and the numbers of fish in the scented and unscented arms and the fork area were recorded. Yearling fish were given a 30-min calming period before 10 min of odor presentation and then a 5-min arm selection period. At least six trials were conducted for each odor concentration. After each trial, the trough was drained and the odor source was moved so that the arms were alternately scented. Test solutions of L-serine and D,L-alanine (Sigma) dissolved in well-water were made daily. Control trials were conducted in the same way, except that well water containing no added amino acids was used instead of scented well water. Zero-age fish (mean weight = 10.8 g), used only once, were tested June through August. Yearling fish were tested at both the parr (January-February, mean weight = 22.0 g) and smolt stages (late April-early May and in June, mean weight = 45.0 g). Yearling fish considered to be smolts had lost parr marks and become silvery.

Avoidance of an amino acid concentration in the Y-

trough was assumed to be a measure of the fish's ability to detect the amino acid at that concentration. The arm selection behavior of the fish in the Y-trough was analyzed by a two-class chi-square test with a correction for continuity (Sokal and Rohlf 1981). Observations of the fish during trials indicated that social interactions did not prevent most fish from swimming freely and independently throughout the fork area of the Y-trough. The numbers of fish entering the arms of the trough were compared with the 1:1 ratio expected by random choice. The lowest nominal concentration of odorant that the fish avoided at a statistically significant level was considered to be the threshold concentration.

A preliminary experiment was run to determine whether serine is detected by olfaction or by some other mode of chemoreception. Fifty coho salmon smolts were anesthetized with ethyl m-aminobenzoate methanesulfonate (MS-222) and made anosmic by injections of liquid petroleum jelly (55 C) into the olfactory chamber with a syringe and blunted needle. A control group of 50 fish were similarly handled but did not receive the injection. Both groups were allowed at least 5 days of recovery before being tested with 10^{-6} M serine, as described above.

Seawater Tests

Oregon Aqua-Foods, Inc. rears juvenile coho salmon

in Springfield Oregon and trucks them, as 0-age "smolts", to their release facility in Newport Oregon. The fish are held in Yaquina Bay seawater for approximately 2 weeks for purposes of imprinting and osmoregulatory acclimation and are then released into the estuary. Experimental fish were obtained directly from the transport trucks at the Newport site. The process of obtaining fish, transporting them to Oregon State University's Hatfield Marine Science Center (HMSC), and placing them in flow-through fiberglass tanks required less than 20 minutes. Fish were held for 2 weeks in tanks supplied with either de-chlorinated Newport tap water or Yaquina Bay seawater and then tested for odor detection. A comparison of physical and chemical characteristics of the freshwater and seawater at the HMSC as well as the water at Smith Farm and WFTS is shown in Table 2. A Y-trough was installed at the HMSC and connected to freshwater and seawater lines. The behavioral assay as described above was used to establish thresholds for detection of L-serine. Additional behavioral information was recorded by observing swimming movements of the fish during three 5-minute periods: 0-5, 10-15, and 20-25 minutes after the arm gates were raised. Each time an individual fish crossed a scoring line at the mouth of an arm, a score was recorded for that arm. The data are presented using the graphical technique of Selset and Doving (1980). The observed scoring data for each

assay is added cumulatively to the data for the assays preceding it. Plotting percent avoidance against cumulative numbers of scores indicates whether or not a final percent avoidance value is being converged upon. Experimental trajectories are compared to a 1 % probability line generated from a cumulative binomial distribution function using an assumption of a random selection of arms ($p = q = 0.5$).

RESULTS

Fish in control trials showed no intrinsic preference for one trough arm or the other; ratios of the numbers of fish in the two arms did not differ significantly from 1:1 in any experiment (Figs. 2, 3, 4). The threshold concentration for the avoidance of serine was 10^{-8} M for zero-age parr, 10^{-7} M for yearling parr, 10^{-5} M for smolts in late April-early May, and 10^{-6} M for smolts in June (Fig. 2). The threshold concentration for the avoidance of alanine was 10^{-7} M for yearling parr, 10^{-6} M for smolts in late April-early May, and 10^{-5} M for smolts in June (Fig. 2).

The threshold concentration for the detection of serine in both freshwater- and seawater-adapted zero-age fish was 10^{-9} M (Figs. 3 and 4). Avoidance was very pronounced at serine concentrations of 10^{-5} to 10^{-8} M.

Identical detection thresholds were established by observing either the numbers of fish trapped in arms of the Y-trough or by counting the numbers of fish swimming past the scoring lines at the entrances to the arms.

Olfaction was responsible for mediating the avoidance response to serine. Fish made anosmic did not avoid the arm scented with serine in contrast to the clear avoidance by the intact fish (Table 3).

DISCUSSION

Our results conflict with the notion of heightened olfactory sensitivity during salmon smoltification. Rather, we found a difference of 2 to 3 orders of magnitude in sensitivity to serine between zero-age parr and smolts. For alanine, we observed a decrease of 1 to 2 orders of magnitude in sensitivity as the fish progressed through smoltification. The high sensitivity to serine observed in zero-age parr was also seen in zero-age fish adapted to freshwater or seawater. The lowest detection thresholds recorded (10^{-9} M serine) were with the Ore-aqua fish adapted to either freshwater or seawater. Although these were 0-age fish, they were fully seawater-ready as shown by their 100 % survival following abrupt transfer from freshwater to seawater. Whether they experienced the endocrinological trends usually seen in yearling fish passing through smoltification is unknown. In any case

their 2-week adaptation to seawater did not alter their ability to detect serine. The decline in sensitivity observed in yearling smolts, therefore, is not easily explained by physiological changes preparing the fish for seawater. Rather, it appears that the decline in sensitivity in yearling smolts was related more to age of fish than to seawater readiness. Bertmar (1983) followed the structural development of the olfactory organs of Baltic salmon (Salmo salar) during parr and smolt stages. Smolts had more primary lamellae, more developed secondary lamellae, more sensory epithelium, and relatively more receptor cells and these differences were a function of age, not size. Similar observations have been made for Pacific salmon (Oncorhynchus) (Pfeiffer 1963). Although decreasing olfactory sensitivity during a period when the olfactory organ is increasing in size and complexity may seem counterintuitive, there are no compelling reasons why macroscopic or cellular development should be correlated with olfactory sensitivity to all odors. Possibly more important to olfactory detection of specific odors are the abundance of odor receptors, the excitability of nervous tissue, and the sensory coding of electrical input at central locations.

The specific cation(s) that carrys current during the depolarization of olfactory sensory cells is unknown (Caprio 1984). The location of the electrogenic region of

the cell is also unknown. Single-cell recordings from the olfactory epithelium of lamprey (Entosphenus japonicus) indicated that the response to L-arginine was not affected by the sodium and potassium concentrations of the solution bathing the mucosa. But bathing the mucosa in a calcium-free solution containing the chelator EGTA blocked the olfactory response. Similarly, Yoshii and Kurihara (1983) found that EDTA-treated olfactory epithelium of carp (Cyprinus carpio) was not responsive to odors. The addition of a wide variety of inorganic cations as well as larger organic cations (tris (hydroxymethyl)-amino-methane chloride; 1,3-bis-[tris(hydroxymethyl)methylamino]propane; and choline chloride) reversibly restored the response. They suggested that cations do not generate the receptor potential by crossing the apical olfactory cell membrane. Cations do appear to play some supportive role in the excitation process. In our study, the abundance of cations in seawater did not enhance the detection of serine. Perhaps the requirement of cations for olfactory excitation is not severe and can be adequately met by any natural freshwater. It is also possible that the source of the cation(s) that supports excitation is dietary.

Change in chemoreceptive abilities during the life of animals is likely the rule rather than the exception. During the ontogeny of the minnow Phoxinus phoxinus, olfactory sensitivities increased as the structure of the

olfactory organ became more complex (Pashchenko and Kasumyan 1983). Mistretta and Bradley (1983) hypothesized that the changing gustatory responses to various salts during fetal-to-adult development in sheep result from changing proportions of receptors in the membranes of receptor cells. It is well documented that olfactory and gustatory sensitivities deteriorate with age in humans (Corso 1981). The correlation between olfactory sensitivity and estrus cycle in mammals suggests that hormonal modulation of olfaction occurs (Schmidt and Schmidt 1980). Oshima and Gorbman (1966) found that injections of thyroxine at pharmacological concentrations elicited a large increase in spontaneous unitary activity in the olfactory bulb of goldfish (Carassius auratus). The relationship between levels of spontaneous electrical activity in central locations and olfactory sensitivity to specific odors is unknown.

The concept of a discrete imprinting period for salmon remains unestablished, since exposure to release-site water sources for periods ranging from hours to months have been reported as sufficient to ensure accurate homing (Little 1983). In any case, olfactory imprinting does occur sometime during smoltification and a decrease in olfactory sensitivity would therefore not be an expected physiological adaptation. Absolute levels of olfactory sensitivity, however, may not play an important

part in home-steam imprinting. Instead, long-term memory of whatever odors are present above threshold concentrations during the period of imprinting may be the essential requirement. Although amino acids are considered to be important natural odors for fish (Caprio 1984; Hara 1982; Tucker 1983), it may be that serine and alanine in pure form are biologically irrelevant and that the detection of natural, more complex odors relevant to imprinting does become more acute during smoltification. It is also possible that what we measured was not a decrease in olfactory sensitivity but only a diminished avoidance response. One may speculate that sensitive avoidance reactions would not be adaptive if smoltification is a period during which the fish needs to maintain its position in a stream for purposes of imprinting (Jonasson 1983). Arguments against the existence of olfactory imprinting are summarized in Stabell (1984).

The value of using a behavioral assay is that it represents a composite measure of olfaction beginning with water entering the naris and ending with behavior directed by the central nervous system. A problem unique to behavioral assays, however, is uncertainty regarding the motivation of the fish to perform according to the criteria of the assay. For olfactory thresholds established behaviorally, perception of odor quality is an

important factor that may change with changes in odor concentration. In human taste tests, for example, NaCl at near threshold concentrations has been reported to have a sweet taste (Kroeze 1979). Because of uncertainties about the motivation of our experimental fish to avoid extremely low concentrations of amino acids, the thresholds we report may underestimate the true limits of detection. Nevertheless, the thresholds we found are comparable to those determined by electrophysiological methods. On the basis of recordings from the olfactory bulb, Hara (1972) estimated that the threshold concentration for the detection of L-serine and L-alanine by yearling coho salmon was between 10^{-6} and 10^{-7} M. This estimate supports our assumption that the avoidance of these amino acids in a Y-trough is a measure of detection. The inability of anosmic fish to avoid serine indicates that the avoidance response begins with odor detection. This is consistent with Sutterlin and Sutterlin's finding (1970, 1971) that chemoreception of D,L-serine by pre-adult Atlantic salmon Salmo salar is due to sensitivity of olfactory receptors and not gustatory receptors.

Lastly, threshold determinations may not merit the preoccupation given to them by olfactory researchers. It is easy but incorrect to visualize concentration-response functions as fixed shapes changing only in position along the abscissa as thresholds change. Thresholds are

statistical concepts that say nothing about the sensitivity within the dynamic range of responses (Bartoshuk 1978). The relative importance of absolute sensitivity will become apparent only as information accrues on the identity and concentration of odors in ecological settings.

Table 1. Separation of water flow in the fork area of the Y-trough. Station numbers refer to the sites at which dye samples were removed from the trough as shown in Figure 1.

Station	Dye concentration in fluorescence units[a]	Dye concentration as a percent of station 1
6	81	0.02
5	192	0.05
4	270	0.07
3	270,000	71.05
2	290,000	76.32
1	380,000	100.00

[a]Rhodamine 6G dye was introduced into one arm and allowed to reach a steady state concentration in the Y-trough. Fifteen mL composite samples consisting of 5 mL from the bottom, mid-depth, and surface were read for fluorescence on a Model 111 Turner Fluorometer.

Table 2. Physical and chemical characteristics of water from Smith Farm, Western Fish Toxicology Station (WFTS), and the Hatfield Marine Science Center (HMSC). Both freshwater (FW) and seawater (SW) data are shown for HMSC. Units are mg/L unless indicated otherwise. Characteristics not measured are shown by a dash.

Measurement	Smith Farm	WFTS	HMSC	
			FW	SW
Temperature (C)	12	12.5-15.1	14.0-17.0	10.5-15.5
pH (-log10[H+[M]])	7.31	6.72	6.85	7.61
Salinity	-	-	-	19.0-30.0
Alkalinity as $CaCO_3$	77	26	-	-
Hardness as $CaCO_3$	116	31	-	-
Ca as $CaCO_3$	59	22	-	-
NO_2/NO_3-N	4.83	0.19	0.08	0.04
NH_3-N	.007	< 0.005	< 0.005	< 0.005
PO_4-P	0.068	0.044	0.019	0.050
SiO_2-Si	21.5	9.9	6.8	1.1
Total organic carbon	2.7	< 2	1.6	1.7
Zn (ug/L)	< 25	< 10	-	-
Cu (ug/L)	< 5	< 5	-	-
Hg (ug/L)	< 2	< 2	-	-

Table 3. Selection of arms of a Y-trough by normal and anosmic coho salmon smolts exposed, in one arm, to 10^{-6} M L-serine. ** denotes $p < 0.01$.

	Arm Selection			
Fish	Serine present	Serine absent	No choice	X2
Control	10	33	7	11.3 **
Anosmic	23	21	6	0.02

Figure 1. Top view of Y-trough used in behavioral assay of olfaction. Measurements are in meters. Numerals show the sampling sites for dye used in Table 1.

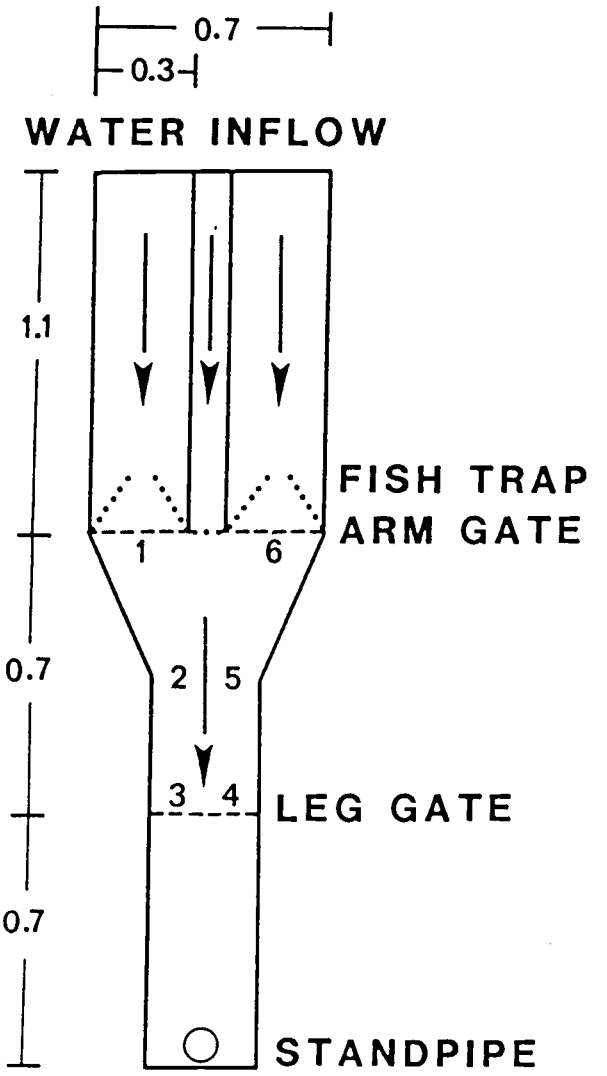
0.7
0.3
WATER INFLOW
1.1
FISH TRAP
ARM GATE
1
6
0.7
2
5
3
4
LEG GATE
0.7
STANDPIPE

Fig. 1

Figure 2. Percent avoidance of amino acids by juvenile coho salmon in a 2-choice Y-trough. "C" represents control trials in which no added amino acids were present. Numerals below the bars represent -log (amino acid concentration [M]) and numerals above the bars indicate the total number of fish that left the fork area of the Y-trough and entered either of the arms. Asterisks refer to statistical significance from a Chi-square test at $p < 0.05$* and 0.01**.

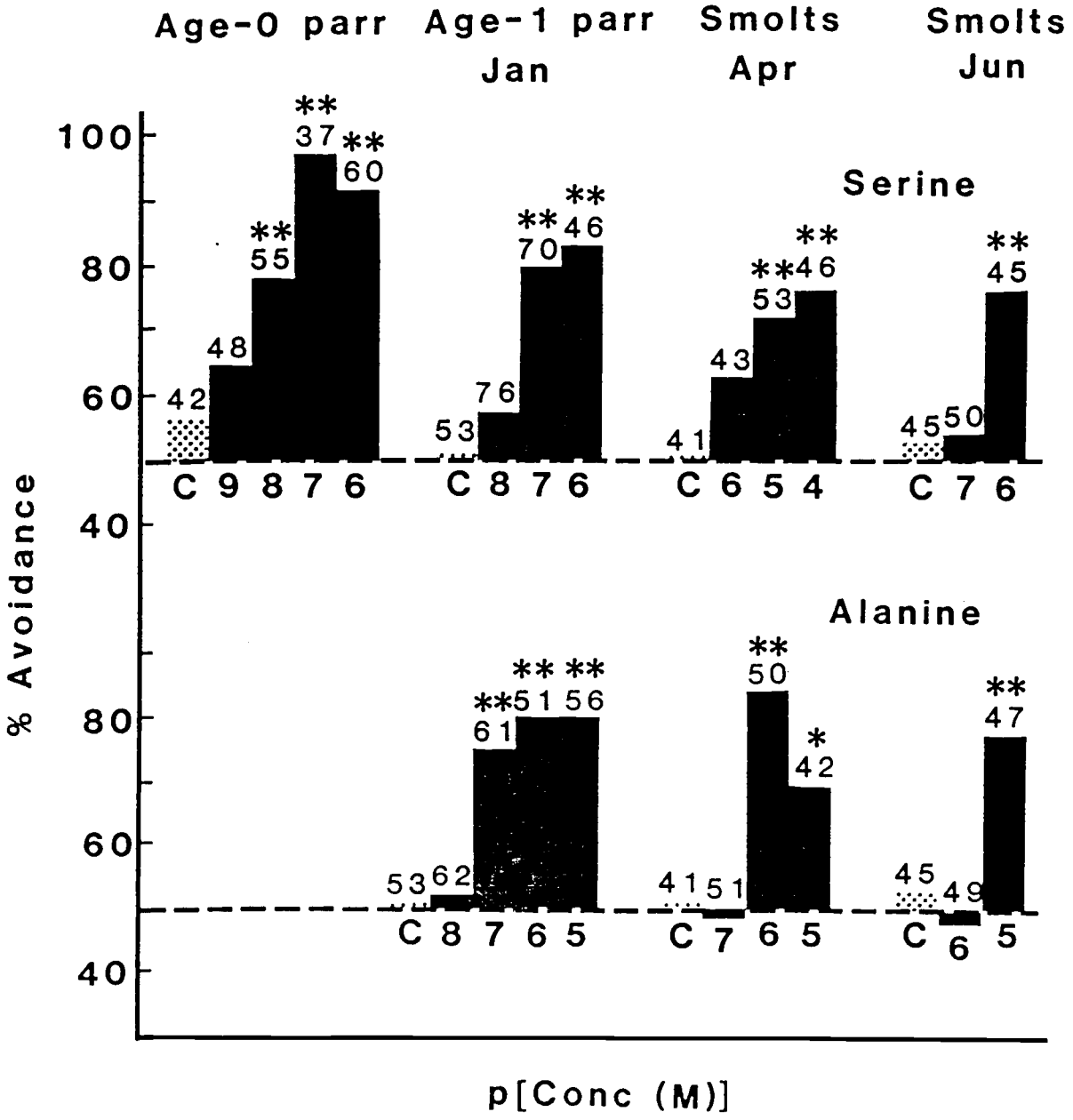
Age-0 parr
Age-1 parr
Jan
Smolts
Apr
Smolts
Jun
Serine
Alanine
% Avoidance
p[Conc (M)]
100
80
60
40
C 9 8 7 6
C 8 7 6
C 6 5 4
C 7 6
C 8 7 6 5
C 7 6 5
C 6 5

Fig. 2

Figure 3. Percent avoidance of L-serine by juvenile coho salmon in a 2-choice Y-trough. Tests in freshwater and seawater are shown by solid and hatched bars, respectively. "C" represents control trials in which no added amino acids were present. Numerals below the bars represent -log(amino acid concentration [M]) and fractions above the bars indicate the number of fish that entered either of the Y-trough arms out of the total number tested. Asterisks refer to statistical significance from a chi-square test at $p < 0.05$* and 0.01**.

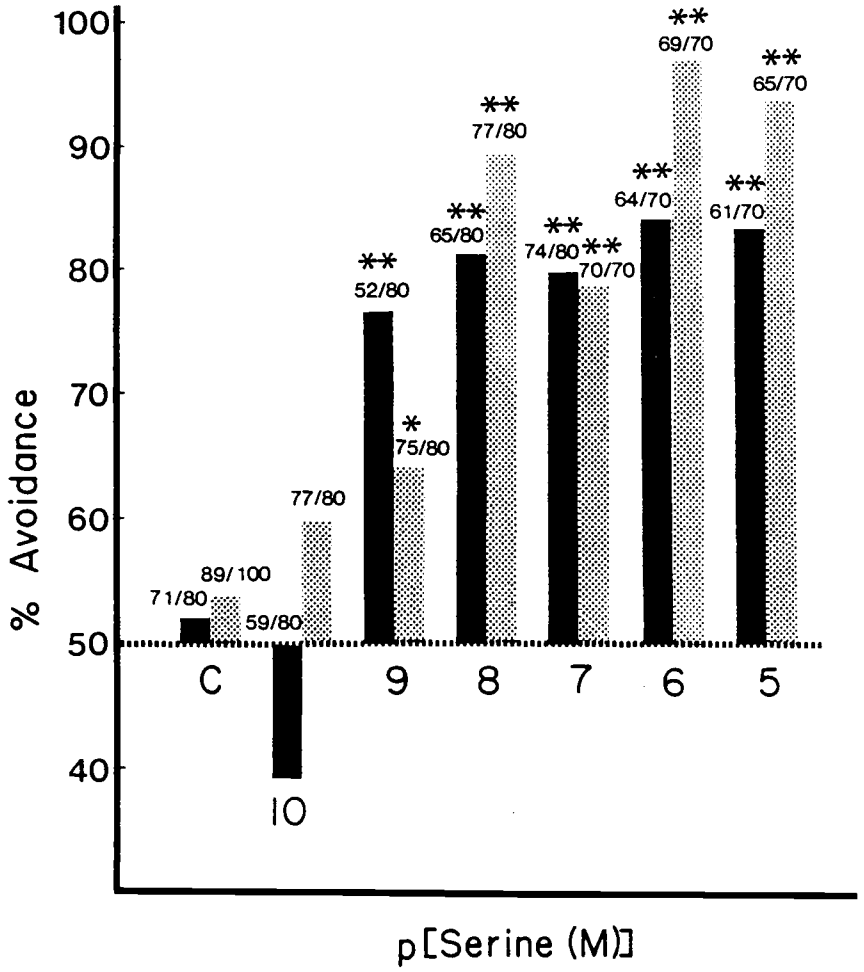
100
90
80
70
60
50
40
% Avoidance
71/80
89/100
59/80
77/80
**
52/80
*
75/80
**
65/80
**
77/80
**
74/80
**
70/70
**
64/70
**
69/70
**
61/70
**
65/70
C
10
9
8
7
6
5
p[Serine (M)]

Fig. 3

Figure 4. Behavioral avoidance of L-serine by juvenile coho salmon in freshwater (FW) and seawater (SW). The cumulative score is the observed number of behavioral scores for a test added cumulatively to similar data from preceding tests. Numerals next to solid lines represent -log(serine concentration [M]). "C" refers to control tests in which serine was not introduced. The broken line represents a 1 % probability boundary generated from a cumulative binomial distribution function using $p = q = 0.5$.

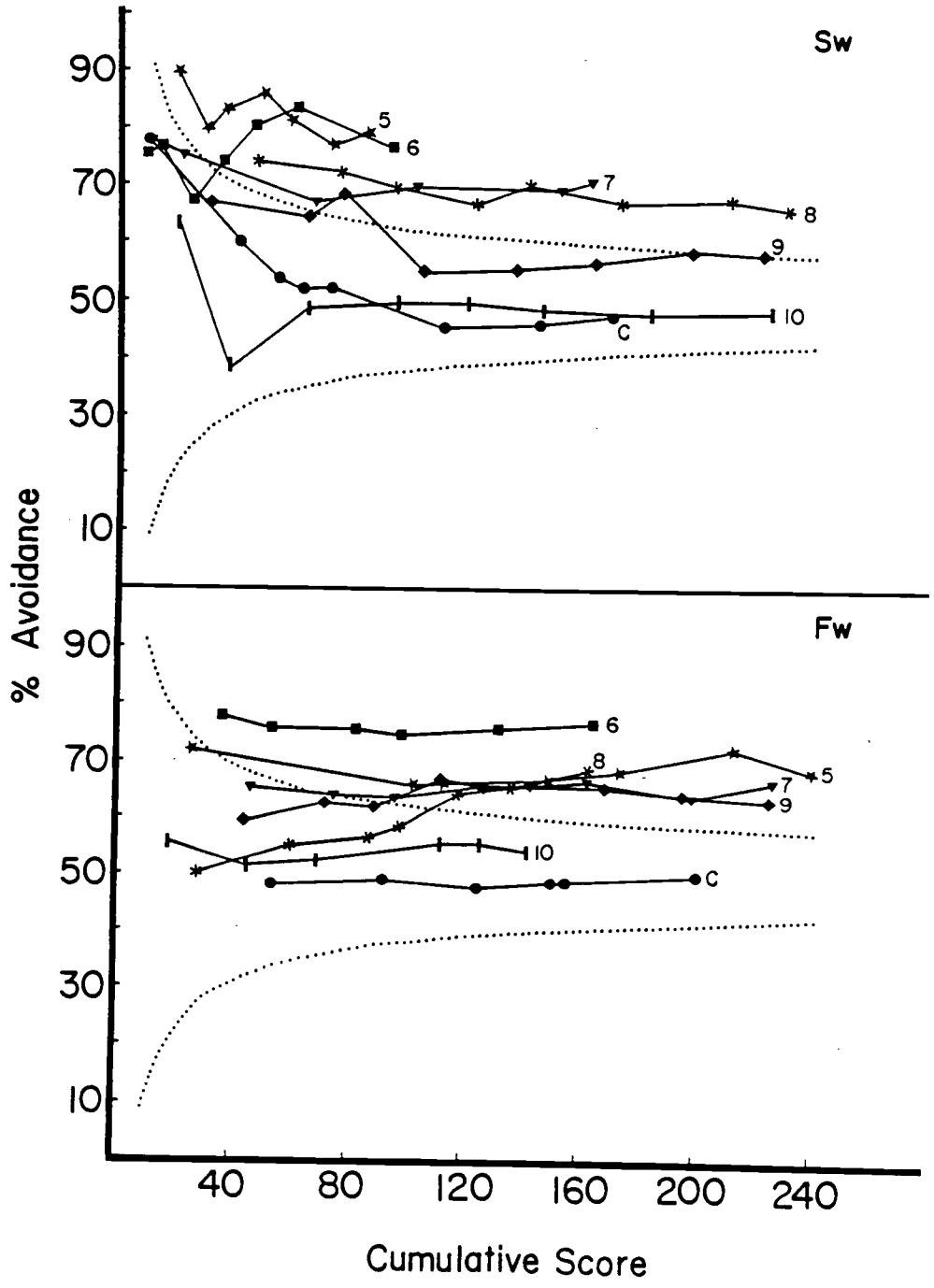
Sw
Fw
% Avoidance
90
70
50
30
10
40
80
120
160
200
240
Cumulative Score
5
6
7
8
9
10
C

Fig. 4

III. The Olfactory L-serine Receptor in Coho Salmon: Biochemical Specificity and Behavioral Response[1]

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INTRODUCTION

A great deal of behavioral and physiological research has shown that amino acids are potent odorants for many species of fish (Caprio 1984; Tucker 1983). Although it is unclear whether the research effort on single amino acids is proportional to their importance as natural odorants, they have been valuable model compounds for analyzing structure-activity relationships (Caprio 1982; Hara 1982; Tucker 1983). For this reason alone it is worthwhile to work with single amino acids despite the possibility that their primary olfactory significance resides in their contribution as components of complex natural odors (Carr et al. 1977; Pawson 1977).

Interest in the specificity of amino acid receptor sites in fish olfactory epithelium increased after the report by Cagan and Zeiger (1978). Using a plasma membrane enriched fraction derived from the olfactory rosettes of rainbow trout Salmo gairdneri, they provided biochemical evidence for a multiplicity of amino acid

binding sites. Brown and Hara (1981), using a similar rainbow trout preparation, supported the findings of Cagan and Zeiger but noted that the binding site for L-threonine, L-serine, and L-alanine showed some specificity for several neutral amino acids. The relevance of amino acid binding data to olfactory detection is supported by the high correlation observed between binding effectiveness and electrophysiological response magnitude for catfish (Ictalurus punctatus) (Cancalon 1978), rainbow trout (Cagan and Zeiger 1978; Brown and Hara 1981), and brook trout (Salvelinus fontinalis) (Brown and Hara 1982). Furthermore, most criteria for receptor status have been satisfied, eg., binding saturability and reversibility, relevant tissue distribution of binding activity, and an affinity roughly in keeping with concentrations of ligand known to elicit biological responses (Cagan and Zeiger 1978; Cancalon 1978; Rhein and Cagan 1980; Brown and Hara 1981).

The focus of this research is on the olfactory receptor site for L-serine in coho salmon Oncorhynchus kisutch. Previous behavioral experiments indicated that olfaction is used by juvenile coho salmon to detect dilute concentrations of L-serine (Rehnberg et al. 1985; Rehnberg and Schreck 1985). This report provides biochemical evidence indicating that olfactory properties of L-serine are mimicked by L-alanine and L-threonine. In addition to

overlapping binding specificity at the L-serine site, we show that coho salmon perceive L-serine, L-alanine, and L-threonine as aversive odors and, given the opportunity, avoid them behaviorally. These results along with data from behavioral cross-adaptation and odor discrimination experiments show that coho salmon perceive L-alanine and L-serine as identical odors.

METHODS

Receptor Assay

The olfactory receptor assay is similar to that used by Cagan and Zeiger (1978). Olfactory rosettes were removed from adult coho salmon heads and frozen at -30 C for later use. After thawing, the rosettes were homogenized, and the homogenate was fractionated by several differential centrifugation, filtering, and washing steps to obtain a plasma membrane-enriched fraction referred to as P2 (Cagan and Zeiger 1978). The word "receptor" is used in this paper to refer to the biochemical component(s) of P2 responsible for specifically binding L-serine. P2 was suspended in cold 0.05 M Aces Buffer (N-(2-acetamido)-2-aminoethanesulfonic acid---Sigma) (pH 7) to a final protein concentration of 100-200 ug/mL. Protein was determined by the method of

Bradford (1976). Competition for the L-serine receptor site was evaluated by incubating 1.00 mL P2 with 0.01 mL L-[G-^{3}H]serine (ICN, 15 Ci/mmol), 0.10 mL non-radioactive amino acid, and 0.19 mL distilled H_2O. The final concentrations in the assay tubes were 0.51 uM L-[^{3}H]serine and 100 uM unlabeled competing amino acid. Total binding was found by incubating 1.00 mL P2 with 0.01 mL L-[^{3}H]serine and 0.29 mL distilled water. Following the incubation, duplicate 0.5 mL aliquots from each assay test tube were filtered through 25 mm Millipore filters (Type HAWP, 0.45 u) under vacuum. Each filter was quickly washed with 10 mL cold Aces buffer to minimize nonspecific binding to the filter. The filters were dissolved in 10 mL scintillation fluid and radioactivity was determined in a Packard Model 2002 liquid scintillation counter. Preliminary incubations sampled periodically for up to 3 hrs indicated that a 60 min incubation was adequate for the binding reaction to approach equilibrium (Fig. 5). The binding of L-[^{3}H]serine in the presence of another amino acid, as a percentage of total binding, is taken as a measure of the competing amino acid's ability to occupy the L-serine receptor. Competing amino acids tested were L-serine, L-alanine, L-threonine, L-cysteine, glycine, L-histidine, β-alanine, L-glutamic acid, L-aspartic acid, and L-lysine (Sigma). Mean values for each amino acid from four different preparations were compared using one-

way analysis of variance and the Newman-Keuls Multiple Range Test. Data transformation before analysis of variance was unnecessary as indicated by the Hartley Test for homogeneity of variance (Neter and Wasserman 1974). Ionization fractions for amino acids at pH 7 were calculated using the equations given in Snoeyink and Jenkins (1980).

Behavioral Assay

The behavioral response of juvenile coho salmon to single amino acids was evaluated using a 2-choice Y-trough. A detailed description of the Y-trough and the conditions of the behavioral assay were given in Rehnberg et al. (1985). Juvenile coho salmon (mean weight = 20 g) were obtained from Oregon Aqua-Foods, Inc. and acclimated to de-chlorinated freshwater (16.5-18.5 C) in flowthrough 1.5 m circular tanks at Oregon State University's Hatfield Marine Science Center, Newport Oregon. Fish were fed Oregon Moist Pellets ad libitum once or twice a day.

To begin an assay, 10 fish were placed in the fork area of the Y-trough with the gates that control access to the trough's leg and two arms in the down position. Introduction of an odor from a calibrated Mariotte bottle was begun immediately. One arm was supplied with solution from a Mariotte bottle containing a single added amino

acid (L-serine, L-alanine, L-threonine, L-cysteine, β-alanine, L-aspartic acid, glycine, L-lysine, L-histidine; Sigma) dissolved in freshwater. The other arm received input from a Mariotte bottle containing only freshwater. All amino acids were tested at a concentration calculated to be 10^{-7} M in the arm receiving the odorant. The arm receiving the amino acid was changed after each test to counter any unexpected arm bias that might arise. Following the 10-min odor presentation, the gates blocking access to the arms were simultaneously raised and the fish were given 25 min to choose either arm or remain in the fork area. One-way funnel-type traps positioned just upcurrent from the mouth of the arms prevented fish from leaving an arm after entering it. Following the period of arm selection, the arm gates were lowered and the numbers of fish in the arms and fork area were recorded. Using fresh fish each time, the assay was repeated 6 to 8 times (N = 60-80 fish). The data on fish trapped in the arms of the Y-trough were compared to a random arm selection model (1:1) using a two-class chi square test with a correction for continuity (Sokal and Rohlf 1981).

A behavioral cross-adaptation experiment was used to determine if L-serine is detectable when presented in the context of other amino acids. Juvenile coho salmon were obtained from the Oregon Department of Fish and Wildlife's Fall Creek Hatchery (mean weight = 3 g) and acclimated to

well-water at the U.S. Environmental Protection Agency's Western Fish Toxicology Station (WFTS). A Y-trough was installed in a laboratory at WFTS using experimental conditions as described above. The fish were tested for the ability to detect and avoid 10^{-7} M L-serine in the presence of a higher concentration (35-100 x 10^{-7} M) of an adapting amino acid (L-serine, L-alanine, L-threonine, glycine, L-histidine, L-aspartic acid; Sigma). Stock solutions of the adapting amino acid were made in a 60 L nalgene carboy using well-water as the diluent. Stock solution was pumped from the carboy into a constant-head delivery box which drained into the headbox supplying the two arms and separating channel of the Y-trough. The assay was run as described above except that the adapting amino acid was present throughout the test. The fish showed no discernable reaction to the presence of the adapting amino acid.

Results from the above experiments suggested that L-serine and L-alanine were perceived as identical odors by juvenile coho salmon. Classical conditioning techniques were used to address this possibility. Single fish were placed in linear flow-through troughs (0.3 x 1.1 m) that received 6.0 L water/min. Fish were provided with a shelter by darkening the last 20 cm of the outflow end of the trough with black plastic. The troughs were surrounded by curtains fitted with one-way windows which

permitted observing the fish. Fish were initially conditioned to either L-serine, L-alanine, or L-histidine (10-6 M) by pairing the odor with food dropped from a remotely-operated feeding tube. Five mLs of concentrated amino acid solution were injected with a syringe into the water line supplying each trough. Each day a trough would receive 0, 1, or 2 injections of control water followed by an injection of amino acid. Fish were observed for 30 sec before an injection and for 30 sec after the injection was expected to arrive at the shelter. An incorrect response was recorded if the fish left its shelter during the 30 sec period before control or treatment injections or the 30 sec period after the control injection. When this occurred, fish were given a negative reinforcement by chasing them into the shelter with a PVC rod. A correct response was recorded if a fish left its shelter during the 30 sec period after the treatment injection. Food pellets were dropped at the end of the 30 sec period following treatment injection. A fish was considered conditioned (at criteria) if it acheived $\geq$ 80 % correct scores and $\leq$ 20 % incorrect scores during 10 consecutive trials. After criteria were reached, we began training the fish to behaviorally discriminate between two amino acids. The objective of the experimental design was to determine if serine could be discriminated from alanine. Training with histidine was included to verify that the

experimental fish were capable of discriminating between odors known to be qualitatively different. If a fish were conditioned to serine, for example, it may be exposed daily to 0, 1, or 2 control injections and an alanine and serine injection arranged in random order. A correct response of leaving the shelter after a serine injection was positively reinforced with food pellets. An incorrect response of leaving the shelter after an alanine injection was negatively reinforced. A fish was considered able to discriminate between two amino acids if it made $\geq$ 80 % correct scores and $\leq$ 20 % incorrect scores during 10 consecutive trials.

RESULTS

The high-affinity specific binding sites for L-serine were saturable (Fig. 6). In contrast, low-affinity nonspecific binding was linear up to 10-4 M serine. Because these data are incomplete and the Scatchard plot is curvilinear, the estimate of the dissociation constant for the high-affinity site of 2 x 10-6 M must be considered tentative. Also, the relation of bound serine to the logarithm of free serine concentration was not sigmoidal, indicating that the number of receptor sites could not be reliably estimated (Klotz 1982).

The binding of serine to its olfactory receptor was highly pH-dependent (Fig. 7). At pH 4, high-affinity

specific binding sites did not exist. Specific binding increased sharply above pH 6 and a maximum occurred at pH 7.8 - 8.0. Nonspecific binding did not respond to changes in pH. Superimposed on the binding data is a serine distribution diagram which shows that serine exists primarily as a zwitterion from pH 4 - 8 (Fig. 7).

The amino acids tested in the competitive binding assays showed varying ability to interfere with serine binding (Fig. 8). Serine itself was best able to compete for serine binding sites, but not to a statistically greater degree than alanine or threonine. The correct ranking of cysteine in this array is open to question due to the variability of the data. Generally, the neutral amino acids showed greater overlapping specificity for the serine site than the acidic or basic amino acids. According to ionization calculations, the neutral amino acids existed almost exclusively as zwitterions whereas glutamic acid, aspartic acid, and lysine existed as triply charged species. Two of the triprotic acids, cysteine and histidine, were predicted to exist predominantly as zwitterions.

Threonine, alanine, serine, and histidine were behaviorally avoided in the Y-trough at 10^{-7} M (Fig. 9). Percent avoidance scores for control tests and the other amino acids can be accounted for by the random selection of Y-trough arms.

Cross-adaptation experiments indicated that the detection and behavioral avoidance of serine was inhibited by the adapting amino acids serine (self-adaptation), alanine, and glycine but not by threonine, aspartic acid, or histidine (Fig. 10).

Successful conditioning of fish to single amino acids has been difficult. To date, conditioning criteria have been reached for two fish using serine as the stimulus and one fish using alanine (Table 4). One of the serine-conditioned fish has been able to discriminate between serine and histidine but not serine and alanine. Similarly, the fish conditioned to alanine has been able to discriminate between alanine and histidine but not alanine and serine. The other fish conditioned to serine has been successfully trained to discriminate between serine and histidine.

DISCUSSION

From results using L-threonine, L-serine, and L-alanine as both [^{3}H]ligand and as competing amino acid, Cagan and Zeiger (1978) postulated the existance of site TSA (threonine-serine-alanine) in rainbow trout. Repeating this work using a preparation derived from olfactory cilia confirmed the existance of site TSA as well as a site L which recognizes the basic amino acids L-lysine and L-arginine (Rhein and Cagan 1983). Our

demonstration that threonine, serine, and alanine were able to strongly compete for the serine binding site in coho salmon olfactory tissue suggests that site TSA exists in this fish also. Using labeled alanine and several competing amino acids, Brown and Hara (1981) presented evidence for rainbow trout which supports the notion of similar binding requirements for L-threonine, L-serine, and L-alanine. Novoselov et al. (1980) found that serine did not effectively compete for the L-alanine binding site in a membrane preparation from skate olfactory epithelium. Valine and β-alanine were somewhat effective as competitors, but the relative inability of L-alanine to compete for its own site makes interpretation difficult. A repeat of the study on skate showed that the serine and alanine binding sites were not used by any of the 9 competing amino acids tested (Fesenko et al. 1983). A rough generalization from our binding data is that large amino acids or those carrying a third charge (positive or negative) are not highly competitive for the serine binding site. As competing ligands, the triprotic amino acids showed binding characteristics related to their ionization fractions. The triprotic amino acids existing primarily as zwitterions at pH 7 (cysteine and histidine) were better able to compete for the serine binding site than the triply charged glutamic acid, aspartic acid, and lysine.

An important question when discussing olfactory specificity is whether different odor molecules that occupy the same receptor site are perceived as the same or similar odors. The binding of an odor molecule to an olfactory receptor is far removed from the perception of odor quality by a living fish. Of all the amino acids tested, only L-alanine appeared to share the same receptor, evoke the same behavioral response, and cross-adapt with serine. Thus, serine and alanine appear to be very similar or identical odors. The conditioning experiment showed that the fish were able to discriminate serine or alanine from a qualitatively different odor such as histidine. Inability to discriminate serine from alanine and vice versa confirms that these molecules are functionally identical odors to coho salmon. The consequences of shifting the position of the alanine amino group is seen in comparing the data for α-alanine and β-alanine. Unlike α-alanine, β-alanine was not highly competitive for the serine site and was not avoided behaviorally. Similarly, β-alanine competed weakly for α-alanine olfactory binding sites in rainbow trout and was only 6.2 percent as stimulatory as α-alanine based on electrophysiological measurements (Brown and Hara 1982).

Threonine effectively competed with serine for binding sites and was avoided behaviorally, but showed no cross-adaptation with serine in behavioral tests. Thus,

although serine and threonine may share the same receptor site, they are perceived as two different aversive odors. Brown and Hara (1982) noted that L-threonine behaved similar to L-alanine in binding competition experiments, but its dose-response curve measured electrophysiologically lay to the right of L-alanine suggesting a lower relative affinity for the receptor. The situation for glycine is unclear. There appeared to be some overlapping specificity with the serine receptor and considerable behavioral cross-adaptation, yet glycine was not perceived as aversive at 10^{-7} M. Histidine was aversive, yet was unlike serine in quality as indicated by the cross-adaptation experiments. The triply charged aspartic acid showed minimal competitiveness for the serine receptor, was not perceived as aversive, and was unable to disrupt serine detection by cross-adaptation.

The serine receptor, _in vitro_, was highly sensitive to changes in pH between pH 5 and 7. It is unknown whether whole-animal olfactory sensitivity to serine responds to changes in ambient pH in a similar fashion. Hara (1976) reported that the electrical response of the olfactory bulb in rainbow trout was maximal when the pH of amino acid solutions used to perfuse the nares was adjusted to near the amino acid isoelectric point. Because serine is largely dipolar in the pH 4 - 8 range, their observed maxima in bulbar response to serine at pH

5.7 cannot be attributed to a change in the stimulus molecule. Thus, the pH sensitivity observed in both electrophysiological and ligand binding research indicates that the receptor itself responds to pH conditions. Novoselov et al. (1980) found that alanine-binding to membrane fractions from skate olfactory epithelium also was biphasic with an optimum at pH 8.2 - 8.4.

Avoidance of serine, alanine, threonine, and histidine by coho salmon did not result from these substances being generally noxious or toxic since similar reactions are not seen in other fishes. Whiting (Merlangius merlangus) and cod (Gadus morhua) (Pawson 1977) and yellow bullhead (Ictaluris natalis) (Atema 1980) were attracted to L-serine. Carp (Cyprinus carpio) were indifferent to these four amino acids at a concentration as high as 5×10^{-2} M (anonymous 1967). In a field study, Sutterlin (1975) found L-alanine to attract winter flounder (Pseudopleuronectes americanus) and L-alanine or L-threonine to attract mummichog (Fundulus heteroclitus) and Atlantic silversides (Menidia menidia). L-histidine was found to be neutral to winter flounder and attractive to mummichog.

Free amino acids have been found in the skin mucus (Hara et al. 1984; Stabell and Selset 1980) and urine (Ogata et al. 1983) of fish, but the adaptive significance of avoiding dilute solutions of single amino

acids remains unknown. In the life of a salmon there are no obvious needs for a sensitive avoidance response other than the need to keep away from predators. Brett and MacKinnon (1954) observed that solutions derived from rinses of tissue from several mammalian species (including bear and sea lion), when added to a fish ladder, temporarily disrupted the upriver migration of adult coho and chinook salmon (Oncorhynchus tshawytscha). Later, Idler et al. (1956) reported that L-serine was the active salmon repellent in extracts of human skin. To attribute all the repellent activity of human skin extract to L-serine is premature since other amino acids, including L-alanine, L-threonine, and L-histidine are also present (Hamilton 1965; Oro and Skewes 1965). It is likely that skin extracts from other mammalian predators also contain a variety of amino acids. The report by Shparkovskiy et al. (1981) casts some doubt on the evolutionary significance of salmonid fishes avoiding serine. They presented eleven amino acids to adult pink salmon (Oncorhynchus gorbuscha) and Atlantic salmon (Salmo salar) at about 10^{-5} M and found that only D,L-alanine was avoided by both species. The only other avoidance reaction observed was by Atlantic salmon in response to D,L-valine. All other amino acids elicited either an attraction response or no response. D,L-serine was attractive to pink salmon and neutral to Atlantic salmon.

Perhaps the most prudent conclusion at this time is that single amino acids are avoided because they are simply unpleasant odors when presented in concentrations above those normally encountered in nature.

Table 4. Behavioral discrimination between pairs of amino acids by juvenile coho salmon. Fish initially conditioned to one amino acid were trained to discriminate between that odor and another amino acid.

Fish	Initially conditioned to	Discrimination Conditioning	
		Odor	Discrimination
1-2	Ala	His	yes
1-2	Ala	Ser	no
2-1	Ser	His	yes
3-2	Ser	His	yes
3-2	Ser	Ala	no

Fig. 5. Kinetics of the binding of L-serine to olfactory receptors from coho salmon. The three symbols represent data points from three preparations. Closed and open symbols represent specific and nonspecific binding, respectively. ^{3}Hserine concentrations were 1.25 uM (squares, circles) or 2.45 uM (triangles).

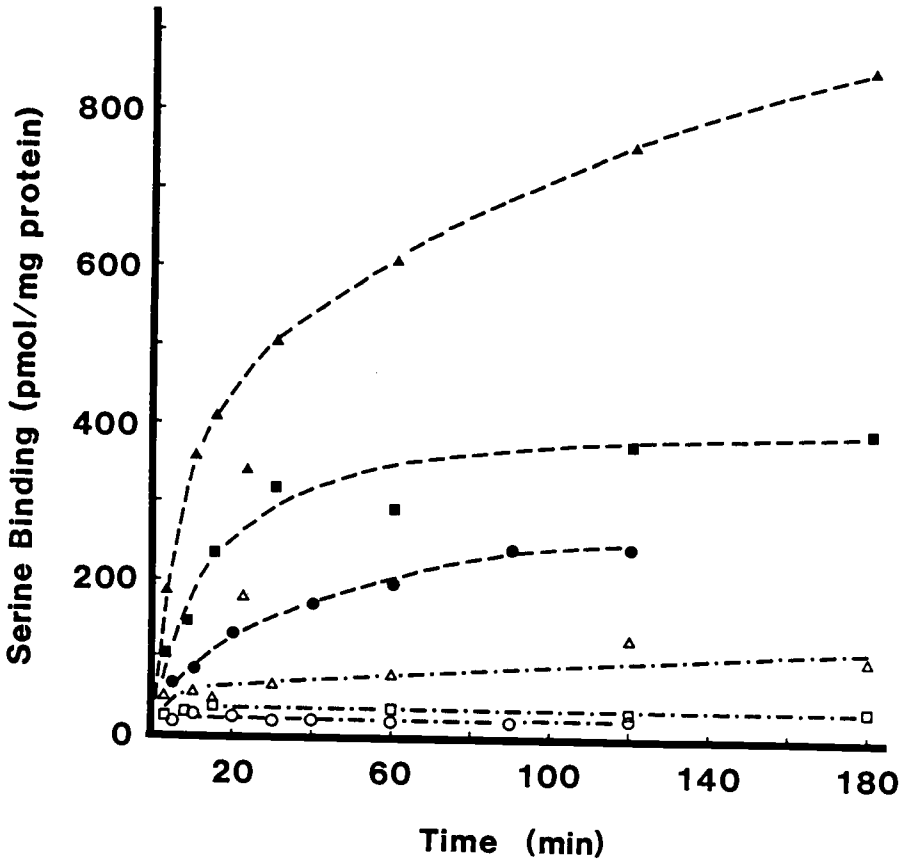
Serine Binding (pmol/mg protein)
800
600
400
200
0
20
60
100
140
180
Time (min)

Fig. 5

Fig. 6. Binding of L-serine to olfactory receptors from coho salmon. Means and standard errors from six preparations are shown.

Fig. 6

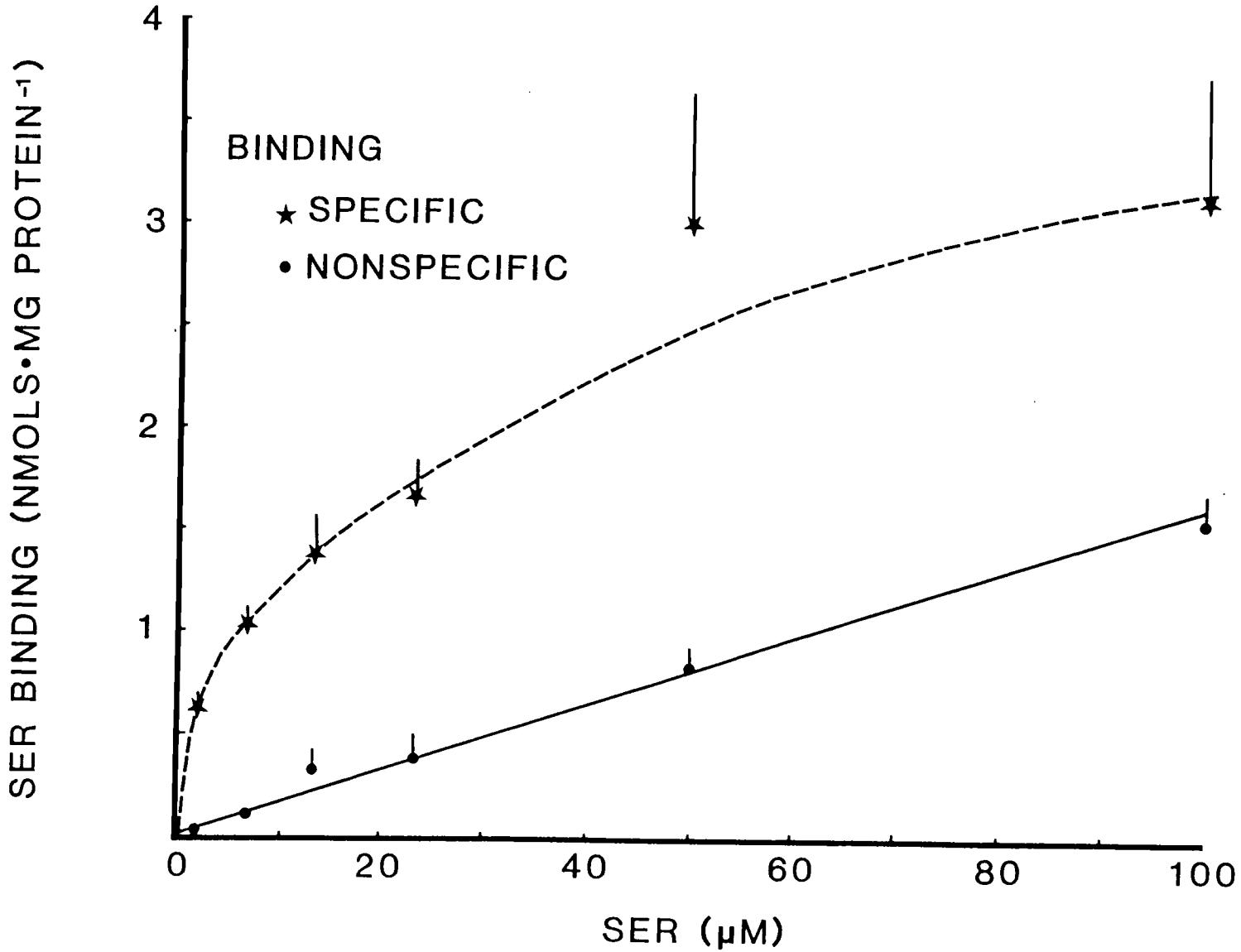
SER BINDING (NMOLS•MG PROTEIN-1)
4
3
2
1
BINDING
★ SPECIFIC
• NONSPECIFIC
0
20
40
60
80
100
SER (μM)

Fig. 7. Binding of L-serine (0.51 uM) to olfactory receptors as a function of pH. The three symbols represent data points from three preparations. Closed and open symbols represent specific and nonspecific binding, respectively. Also shown are the ionization fractions of L-serine (pK_{a1} = 2.21, pK_{a2} = 9.15) as a function of pH. Symbols α_0, α_1, and α_2 refer to fully protonated, monoprotonated, and deprotonated forms of L-serine, respectively.

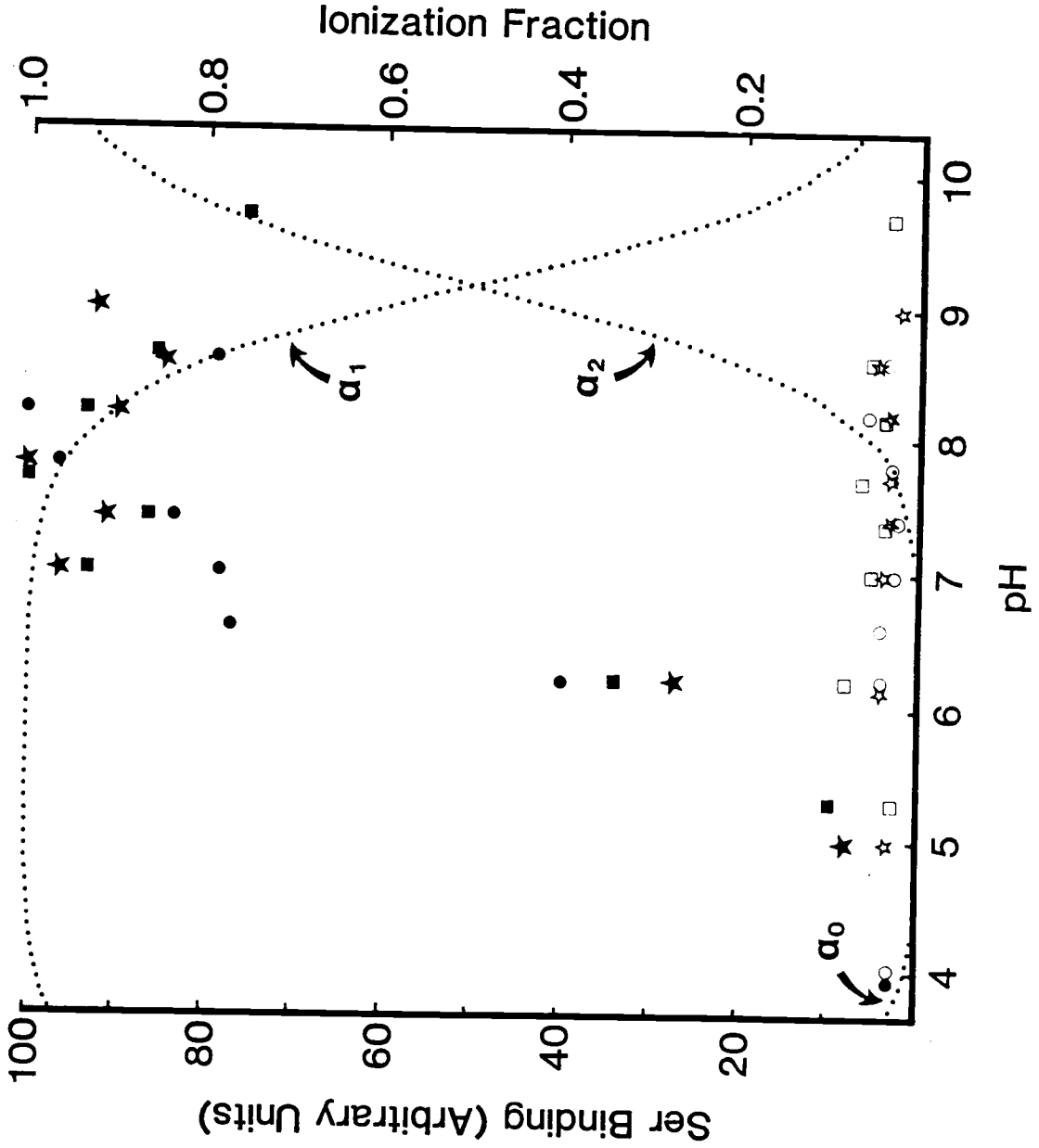
Ionization Fraction
1.0
0.8
0.6
0.4
0.2
α_1
α_2
α_0
100
80
60
40
20
Ser Binding (Arbitrary Units)
4
5
6
7
8
9
10
pH

Fig. 7

Fig. 8. Binding of L-[3H]serine to coho salmon olfactory receptors in the presence of competing amino acids, expressed as a percent of total binding. Means and standard errors were calculated from four preparations (n = 4). Amino acids sharing a common underline are not significantly different from one another ($p < 0.05$). Ionization fractions at pH 7 are given above the bar for each amino acid. Single values refer to the fraction of diprotic amino acids predicted to be doubly charged. Dual values refer to the fraction of triprotic amino acids predicted to be doubly or triply charged, respectively.

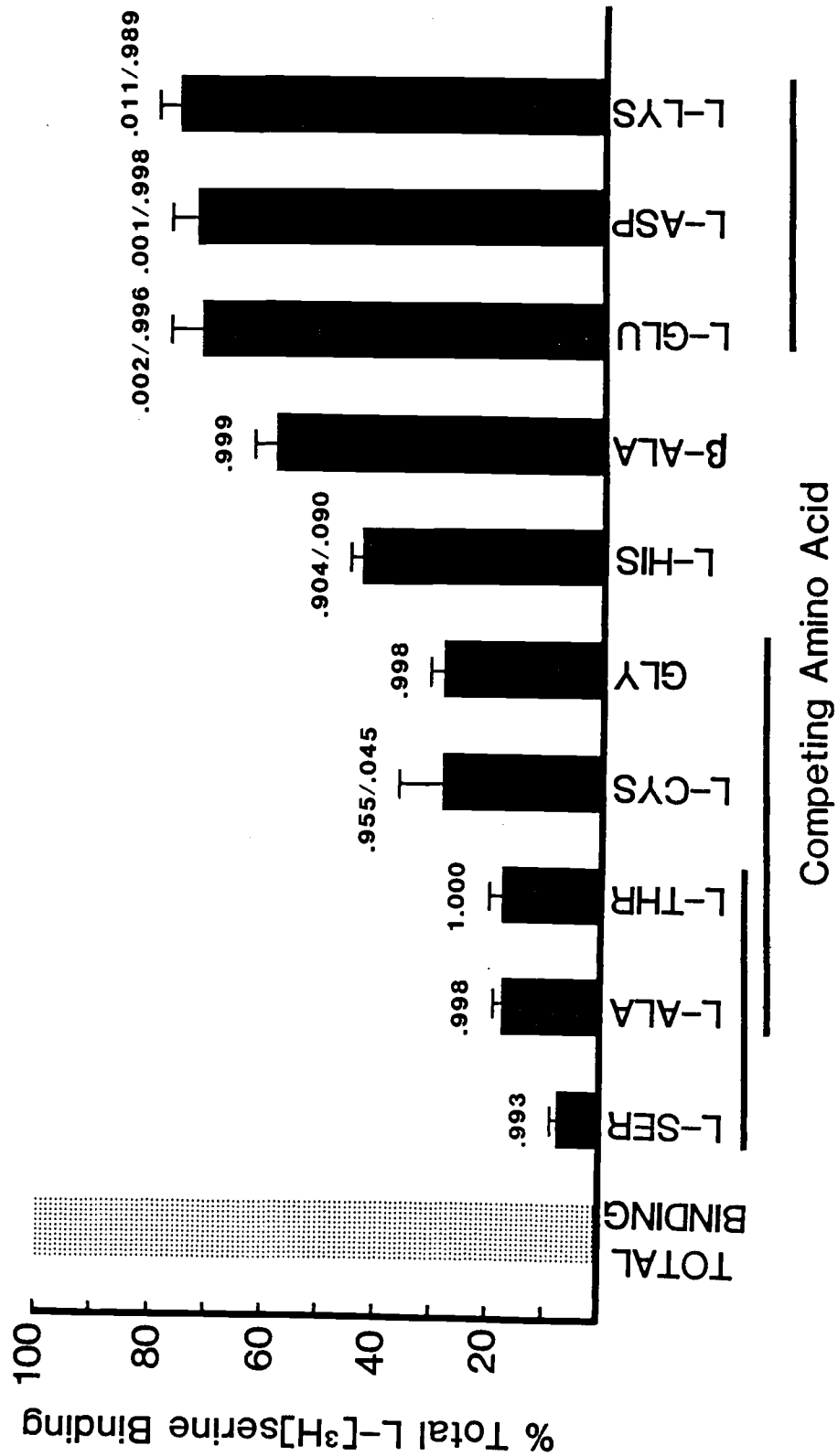
% Total L-[3H]serine Binding
100
80
60
40
20
TOTAL BINDING
L-SER
L-ALA
L-THR
L-CYS
GLY
L-HIS
β-ALA
L-GLU
L-ASP
L-LYS
.993
.998
1.000
.955/.045
.998
.904/.090
.999
.002/.996
.001/.998
.011/.989
Competing Amino Acid

Fig. 8

Fig. 9. Avoidance of single amino acids by juvenile coho salmon in a 2-choice Y-trough. All amino acids were tested at 10^{-7} M. Control assays in which no amino acids were added are designated "C". Ratio above each bar indicates the number of fish that entered a Y-trough arm out of the total number of fish tested. * and ** refer to $p < 0.05$ and $p < 0.01$, respectively.

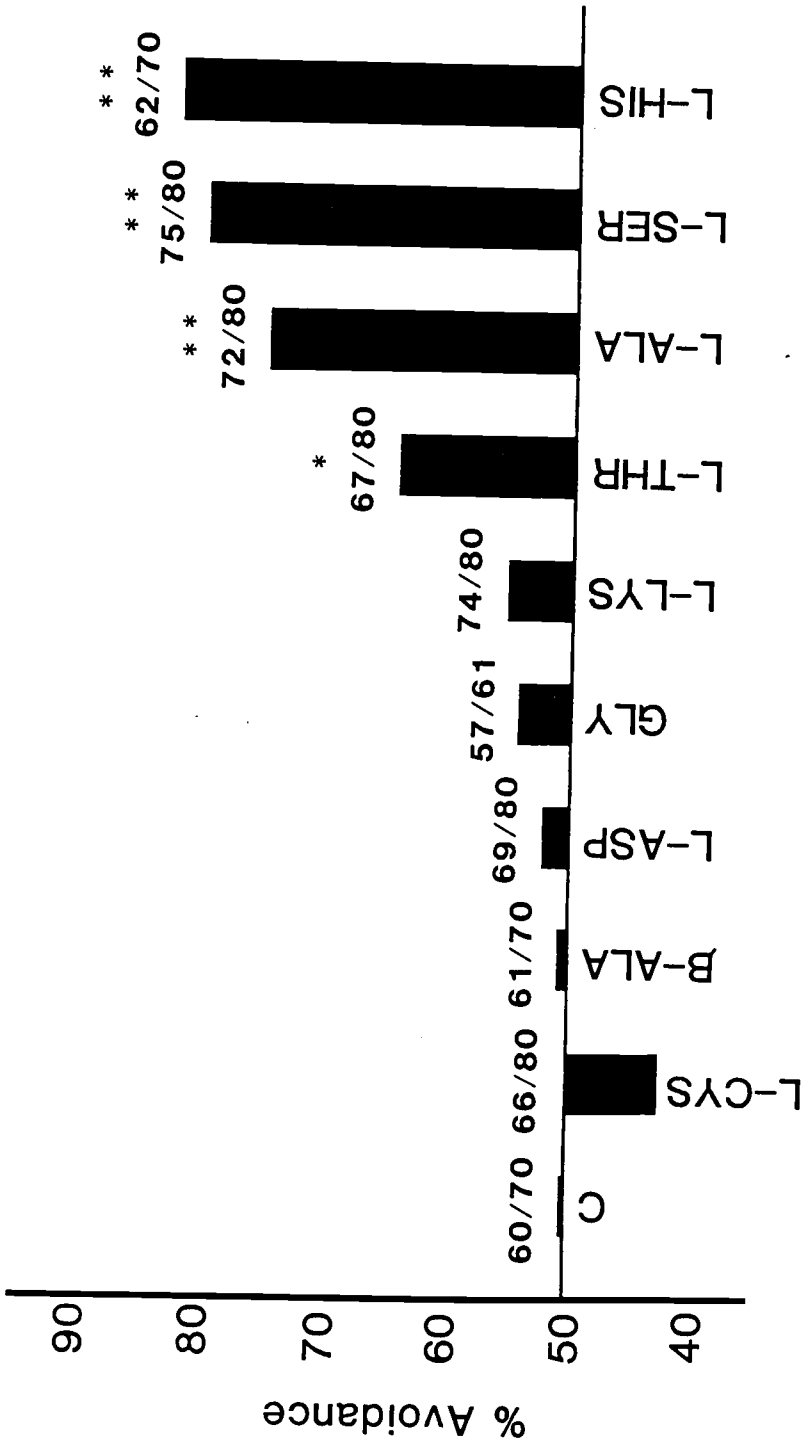
% Avoidance
90
80
70
60
50
40
C
60/70
L-CYS
66/80
B-ALA
61/70
L-ASP
69/80
GLY
57/61
L-LYS
74/80
L-THR
*
67/80
L-ALA
**
72/80
L-SER
**
75/80
L-HIS
**
62/70

Fig. 9

Fig. 10. Avoidance of L-serine (10-7 M) by juvenile coho salmon in the absence (C) and presence of an adapting amino acid. Concentration of adapting amino acid was 10-5 M except for L-aspartic acid (7 x 10-6 M) and L-threonine (3.5 x 10-6 M). Ratio above each bar indicates the number of fish that entered a Y-trough arm out of the total number of fish tested. ** refers to $p < 0.01$.

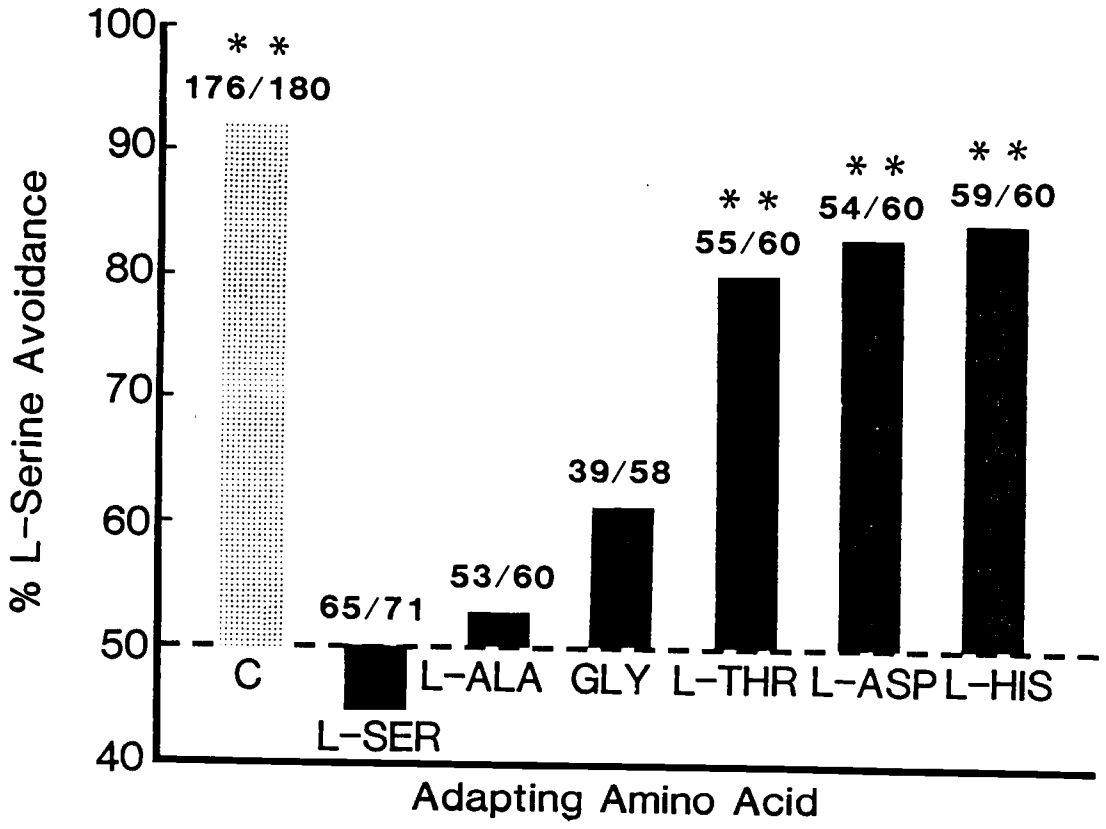
100
90
80
70
60
50
40
% L-Serine Avoidance
* *
176/180
65/71
53/60
39/58
* *
55/60
* *
54/60
* *
59/60
C
L-SER
L-ALA
GLY
L-THR
L-ASP
L-HIS
Adapting Amino Acid

Fig. 10

IV. Acute and Chronic Effects of Metals on the Olfactory Detection of L-serine by Coho Salmon: Behavior, Receptors, and Serine-Metal Complexation[1]

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INTRODUCTION

Chemoreception in fishes is believed to play a mediating role in such complex behaviors as reproductive migration and pairing, schooling, feeding, parental recognition, and predator avoidance (Hara 1982). Although the role of chemoreception in the daily life of salmonid fishes is poorly understood, it is known that olfaction plays a central part in reproductive homing. Stream-specific odors of unknown composition are used by returning adults as guideposts in freshwater navigation (Hasler et al. 1978). Fish olfaction is strongly inhibited by several common waterborne pollutants (Brown et al. 1982; Lemly and Smith 1985; Olsen and Hoglund 1985; Sutterlin 1974) although mechanisms of inhibition remain obscure. Anthropogenic input of metals to rivers in the Pacific northwest continues to be a problem, especially

near large mining operations (Johnson et al. 1975).

One objective of our research was to determine the toxicities of mercury (Hg), copper (Cu), and zinc (Zn) to coho salmon olfaction. To achieve this using unrestrained fish, we adapted 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 prominent components of complex natural odors (Hara 1982). A second objective was to gain some understanding of the mechanism of metal-induced olfactory inhibition. This was addressed by observing how metals affect the binding of L-serine to its olfactory cell membrane receptor. Sutterlin (1974) raised the possibility that certain pollutants may render odor molecules non-stimulatory by chemically combining with them. This possibility was addressed 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 were obtained from the Oregon Department of Fish and Wildlife's Fall Creek Hatchery (mean weight = 2.9 g) and from the Eagle Creek National Fish Hatchery (mean weight = 2.5 g). All fish were transported to the U.S. Environmental Protection Agency's Western Fish Toxicology Station (WFTS)

at least 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.

Behavioral Assay

Design of the two-choice Y-trough and the conditions of the behavioral assay were described in Rehnberg et al. (1985). The basic behavioral assay began by placing 10 fish in the fork area of the Y-trough. After the fish became visibly calm (after about 5 min), L-serine (Sigma) was 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. After 10 min of exposure to the contents of the Mariotte bottles, the arm gates of the Y-trough were raised simultaneously, 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 arm bias that might arise. Avoidance of Y-trough arms containing serine was

evaluated by using a two-class chi square test with a correction for continuity (Sokal and Rohlf 1981). The potential for metals to reduce upstream orientation was evaluated by comparing the fractions of fish in control assays not entering either arm to those in treatment assays by using a difference in proportions test (Dixon and Massey 1969).

Fish from the Eagle Creek stock would not swim through the fish traps in the arms of the Y-trough as described above. Consequently, use of the traps was abandoned when using those fish and the protocol was modified to resemble the assay used by Selset and Doving (1980). The assay was run as described above until the arm gates were raised. The fish were observed for three 5-minute periods: 0-5, 10-15, and 20-25 min after the arm gates were raised. Each time a fish crossed a scoring line at the mouth of an arm, a score was recorded for that arm. The data are presented using the graphical technique of Selset and Doving (1980). The observed scoring data for each assay are added cumulatively to the data for the assays preceding it. Plotting percent avoidance against cumulative numbers of scores indicates whether or not a final percent avoidance value is being converged upon. Experimental trajectories are compared to a 1 % probability line generated from a cumulative binomial distribution function using an assumption of a random

selection of arms ($p = q = 0.5$).

Acute Exposure to Zn, Cu, and Hg

Acute exposures to Zn, Cu, or Hg took place in the Y-trough immediately before behavioral tests. Ten fish were placed in the leg of the Y-trough downstream of a gate that blocked access to the fork area. 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. 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 the behavioral assay was begun as described above. Thus, fish were exposed to a metal for about 2 hr before being given access to the arms of the Y-trough. Stock metal solutions made from $ZnCl_2$, $CuSO_4$, or $HgCl_2$ contained concentrated HNO_3 (0.1 mL/L stock) to prevent precipitation. Acidification of stock solutions did not measurably change the pH of test water in the Y-trough. All metal solutions were aged overnight in a nalgene carboy prior to use in an assay. 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 experiment with Hg.

Chronic Exposure to Zn

To evaluate how prolonged exposure to Zn affects olfaction, three exposure treatments in flow-through (17

L/min) 570 L tanks were used as follows: a control tank receiving no Zn and two treatment tanks receiving input from a stock $ZnCl_2$ solution resulting in nominal total Zn concentrations of 100 and 500 ug/L. Mean values (ug/L) and standard errors from daily measurements of total Zn concentration were: control tank--- < 25, 0.000; 100 ug/L tank---100, 1.711; 500 ug/L tank---477, 7.186. The exposure to Zn was continuous and lasted for 21 days. Fish were removed from the exposure tanks at 24 hr, 96 hr, and 21 days and tested for the ability to detect and avoid serine. Tested fish were used once.

Olfactory Receptor Assay

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. The word "receptor" in this paper is used to denote the biochemical entities in P2 that are responsible for specific binding of L-serine. In preparation for a binding assay, P2 was suspended in cold 0.05 M Aces buffer (N-(2-acetamido)-2-aminoethanesulfonic acid---Sigma) (pH

7.0) at a concentration of 150 - 200 ug protein/ml. Protein was determined by the method of Bradford (1976). 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 a 2 hr 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.

Owing to copper's participation in complexation reactions (Chapman and McCrady 1977; McCrady and Chapman 1979; Wagemann and Barica 1979), further receptor assays were run using a pair of more natural carbonate buffers as the incubation media. The buffers chosen were the "very soft water" and "hard water" reconstituted freshwater solutions described by the U.S. Environmental Protection Agency (1975). Fraction P2 and serine incubated in the

soft water solution ($10^{-3.85}$ M $NaHCO_3$, $10^{-4.36}$ M $CaSO_4$, $10^{-4.21}$ M $MgSO_4$, $10^{-5.17}$ M KCl, pH = 7) were expected to be exposed to greater cupric ion activity and less total complexed Cu than incubations in the hard water solution ($10^{-2.64}$ M $NaHCO_3$, $10^{-3.16}$ M $CaSO_4$, $10^{-3.00}$ M $MgSO_4$, $10^{-3.97}$ M KCl, pH = 8) (Chapman and McCrady 1977).

Water Chemistry

Daily measurements of dissolved oxygen, pH, alkalinity, hardness, and dissolved calcium and magnesium in the well water were made according to the American Public Health Association (1980). Well water samples were analyzed weekly for NO_2 plus NO_3, NH_3, PO_4, SiO_2 and total organic carbon by U.S. Environmental Protection Agency chemists at the Corvallis Environmental Research Laboratory. Total metal concentrations and cupric ion activities were determined by WFTS chemists. Chemical equilibrium calculations were made by using the MICROQL program (Westall 1979) on a Hewlett Packard HP 85. Decisions to consider specific reactions were based on the literature, trial and error, and previous computations using the chemical equilibrium program MINEQL (Westall et al. 1976) on a CDC CYBER 170/720 computer. 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. It is known that amino acids adsorb to humic substances (Wetzel 1983) and that humic acid at concentrations less than 2 mg/L can reduce the toxicity of Cu to aquatic organisms (Winner 1985). However, McCrady and Chapman (1979) showed that WFTS well-water had less complexing capacity for Cu than a reconstituted soft water made from inorganic salts and carbon-filtered deionized water. 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 mean (mg/L) and standard error (in

parentheses) for chemical parameters measured daily were dissolved oxygen, 9.3 (0.11); alkalinity as $CaCO_3$, 25.5 (0.49); hardness as $CaCO_3$, 30.5 (0.76); calcium hardness as $CaCO_3$, 22.0 (0.70); magnesium hardness as $CaCO_3$, 8.2 (0.32); and pH, 6.72 (0.05). Mean (mg/L) and standard error (in parentheses) for parameters measured weekly were NO_3, 0.85 (0.029); NH_3, < 0.005; PO_4, 0.135 (0.001); SiO_2, 21.2 (0.49); 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 5). This depression of swimming behavior was related to the presence of a metal and not necessarily to its concentration since the smallest effects were seen at the highest metal concentrations. At metal concentrations of 10^{-5} M, test fish exposed to Cu or Hg showed signs of being stressed, whereas Zn caused no observable effects. Approximate 96-hr LC50 values (concentrations causing 50 % mortality in 96 hr) for coho salmon parr at WFTS are 10^{-5} M Zn and 5×10^{-7} M Cu (Chapman, personal communication). Lorz et al. (1978) reported a 96-hr LC50 of about 10^{-6} M Hg for yearling coho salmon in water with total hardness ranging from 85 - 93 mg/L as $CaCO_3$. All fish appeared normal at 10^{-7} M metal, yet significant declines in upstream orientation was observed for all metals (Table 5). 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, i.e., 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. 11. 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. To our knowledge, this is the first behavioral research on the olfactory performance of fish during brief exposures to environmental concentrations of Zn. 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 a probe. The acute inhibitory effects of Hg and Cu on the detection of L-serine in rainbow trout have been demonstrated electrophysiologically (Hara et al. 1976). In that study, depression of the response of the olfactory

bulb 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).

The stimulatory effectiveness of serine, 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 6). Chemical equilibrium computations indicate that the proportion of serine complexed with Cu was a function of $C_{T,Cu}$ with serine 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, 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 6), thereby supporting the assumption of chemical equilibrium and the validity of the formation constants. Speciation calculations for behavioral assays containing Hg predict that Hg was present primarily in complexed forms and that serine existed as a free ion (Table 6). Of the Hg complexes, $Hg(OH)_2^0$ predominated, whereas Hg-serine complexes were negligible. If Hg^{++} is the toxic form of Hg and our calculations of speciation are even remotely accurate, we can conclude that the observed decline in behavioral avoidance of serine was induced by

extraordinarily low concentrations of Hg^{++} (< 10^{-11} M, Table 6). 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. For the behavioral assays conducted in the presence of Zn, MICROQL computations indicate that both Zn and serine existed predominantly as free ions (Table 6). Zn^{++} apparently does not interact with the serine receptor nor does it seriously irritate olfactory epithelium. 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. 12. 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 exposure to Cu 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 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.

The binding of serine and the calculated chemical speciation in the presence of Cu under conditions intended to simulate soft- and hard-water are shown in Fig. 13. The inhibition of binding in soft-water buffer at 10^{-5} M and 10^{-4} M Cu was greater than that seen in Aces buffer (Fig. 12), but the effects were nevertheless relatively mild. The inhibition of serine binding in soft water buffer can be accounted for by the loss of free serine as $C_{T,Cu}$ increased (Fig. 13). Serine was predicted to be about 98 % free at $C_{T,Cu} = 10^{-7}$ M but only about 6 % free at $C_{T,Cu} = 10^{-4}$ M. Loss of free serine to copper complexes in the hard-water buffer was less severe due to an increased prevalence of $CuCO_3^0$ and $Cu(OH)_2^0$ species. The inhibition of binding in the hard-water buffer was peculiar in that inhibition was about 70 % at 10^{-5} M Cu, but only 40 % at 10^{-4} M Cu (Fig. 13). These data also indicate that the binding of serine to its receptor was not sensitive to cupric ion activity. At all copper concentrations, the cupric ion concentration in the soft

water experiments was calculated to be nearly two orders of magnitude greater than that in the hard water experiments. These differences in cupric ion concentration were not reflected in comparable differences in the inhibition of serine binding (Fig. 13).

The inability of Cu to complex with serine to a quantitatively significant degree or to interfere with the binding of serine to its receptor suggested that it might inhibit olfaction by masking the presence of odors. To address this possibility, three sets of serine avoidance assays were run: (1) control assays in which no Cu was added, (2) assays in which added Cu was present throughout the test and (3) assays in which the addition of Cu continued for 2 hr and then was stopped. Following a 15-min period in which the trough was allowed to clear of Cu, fish were tested for the ability to detect and avoid serine in the usual manner. The inhibition of serine detection by a 2-hr exposure to 10^{-6} M Cu was repeatable (Fig. 14). Clearance of Cu from the Y-trough did not result in improved serine recognition, indicating that the exposure to Cu had a residual effect. Thus, the inability of coho salmon to detect serine in the presence of Cu did not result from Cu masking the presence of serine. Atlantic salmon exposed to a filtrate of aluminum smelter waste (cryolite recovery sludge) for 30 - 60 min were found to be less chemosensitive to glycine if tested

within 24 hr of the exposure (Johnstone et al. 1982). The residual inhibitory effect of the waste disappeared between 24 and 48 hr after the exposure. The conclusion reached earlier that the presence of an added metal in the Y-trough depressed upstream swimming behavior was contradicted in these experiments. Fish exposed to Cu throughout the duration of the assay were behaviorally active and readily entered both arms of the trough (Fig. 14).

During the 21-day exposure to Zn, mean values (mg/L) and standard errors for WFTS well water characteristics measured daily were, dissolved oxygen---9.2, 0.05; alkalinity as $CaCO_3$---27.0, 0.36; hardness as $CaCO_3$---26.8, 0.64; calcium hardness as $CaCO_3$---19.2, 0.39; magnesium hardness as $CaCO_3$---7.6, 0.36; and pH---6.8, 0.03. Mean values (mg/L) and standard errors for parameters measured weekly were NO_3---0.593, 0.029; NH_3---0.028, 0.015; PO_4---0.134, 0.005; SiO_2---18.05, 2.213; and total organic carbon---2.5, 0.20.

Exposure to 100 or 500 ug/L Zn did not have a measurable effect on serine detection even after 21 days (Fig. 15). A plot of results from tests when serine was not added to the Y-trough shows that fish did not preferentially select either arm. All mortality observed in the experiment occurred during the first 96 hr of exposure in the tank containing 500 ug/L Zn. Despite this

toxicity, fish were able to detect and avoid serine when tested after 24- or 96-hr of exposure to 500 ug/L Zn. After 21 days of exposure, fish from control and treatment groups avoided serine, and their avoidance trajectories converged to similar final values (Fig. 15). There was no indication that longer exposures to Zn would have caused observable deleterious effects.

The no-effect findings following acute or chronic exposures to Zn should not be taken to indicate that Zn cannot inhibit olfaction in fishes. On the contrary, application of concentrated solutions of $ZnSO_4$ to olfactory epithelia is one of the techniques commonly used to selectively destroy sensory cells. Degenerative effects, confined to sensory cells, have been observed in channel catfish after application of $ZnSO_4$ at concentrations ranging from 10^{-2} to 1.7×10^{-1} M (Cancalon 1980). Bloom et al. (1978) showed that pheromone detection by zebrafish Brachydanio rerio was lost after a 9-day exposure to 5000 ug/L Zn. Nevertheless, in light of our findings with environmental concentrations of Zn, the sensitive avoidance response shown by salmonids towards Zn (Giattina and Garton 1983) probably represents a greater threat to the life cycle of anadromous salmonids than any inhibitory effects that Zn might have on olfaction.

In summary, the detection of L-serine by juvenile coho salmon was inhibited by brief exposure to Hg or Cu.

Neither of these metals interfered with serine detection by forming nonstimulatory metal-serine complexes. Cu did not inhibit the binding of serine to its olfactory receptor nor did it interfere with serine recognition by masking its presence. The mechanism of action of Cu remains unclear whereas Hg appears to act at the level of the olfactory receptor. Behavioral tests following acute or chronic exposure to Zn indicated that olfaction was unaffected by environmental concentrations of Zn. All three metals, however, were found to disrupt simple upstream movements in our experimental apparatus.

Table 5. 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} M Zn (ns).

	Metals in Y-trough		
Conc. (M)	Hg	Cu	Zn
No Metal	3/120	26/180	25/120
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)

Table 6. Calculated distribution of chemical species during behavioral assays of olfaction in the presence of Zn, Cu, or Hg. All concentrations are 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. $C_{T,serine}$ was 10^{-8} M in experiments with Zn and Cu and 10^{-7} M in the experiment with Hg.

Metal	$C_{T,metal}$	Free Metal	Complexed Metal	Free Serine	Complexed Serine
	7 (6.82)	6.82	8.58	8.00	12.52
Zn	6 (6.00)	6.01	7.77	8.00	11.71
	5 (4.96)	4.97	6.73	8.00	10.67
	7 (7.20)	7.55	7.46	8.01	10.06
Cu	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
	7 (6.94)	13.74	6.94	7.00	15.34
Hg	6 (5.82)	12.82	5.82	7.00	14.22
	5 (4.94)	11.94	4.97	7.00	13.34

Figure 11. Avoidance of L-serine by juvenile coho salmon 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 ** = $p < 0.01$.

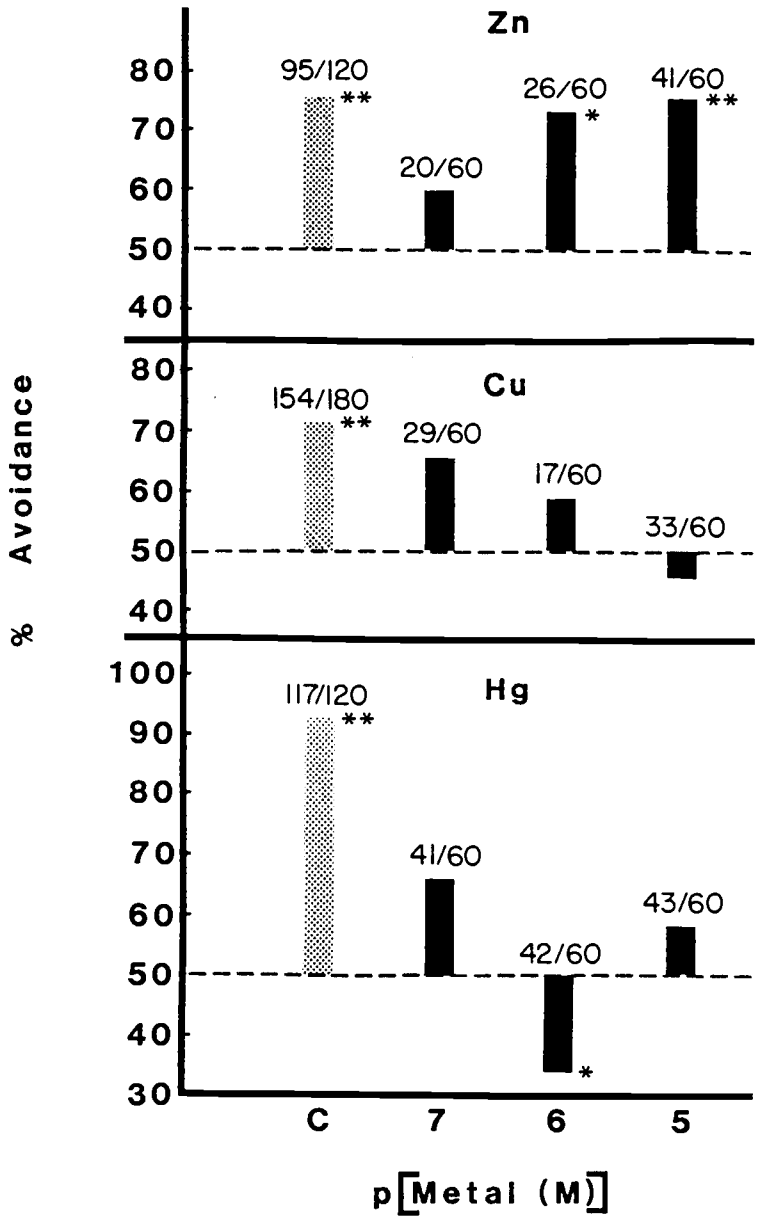
Zn
95/120
**
20/60
26/60
*
41/60
**
Cu
154/180
**
29/60
17/60
33/60
Hg
117/120
**
41/60
42/60
*
43/60
% Avoidance
C
7
6
5
p[Metal (M)]

Fig. 11

Figure 12. Binding of L-serine to its olfactory receptor in the presence of metals as a % of binding in controls (no metals). Serine concentrations were 0.26 to 9.26 uM. Means plus one standard error were calculated from three preparations.

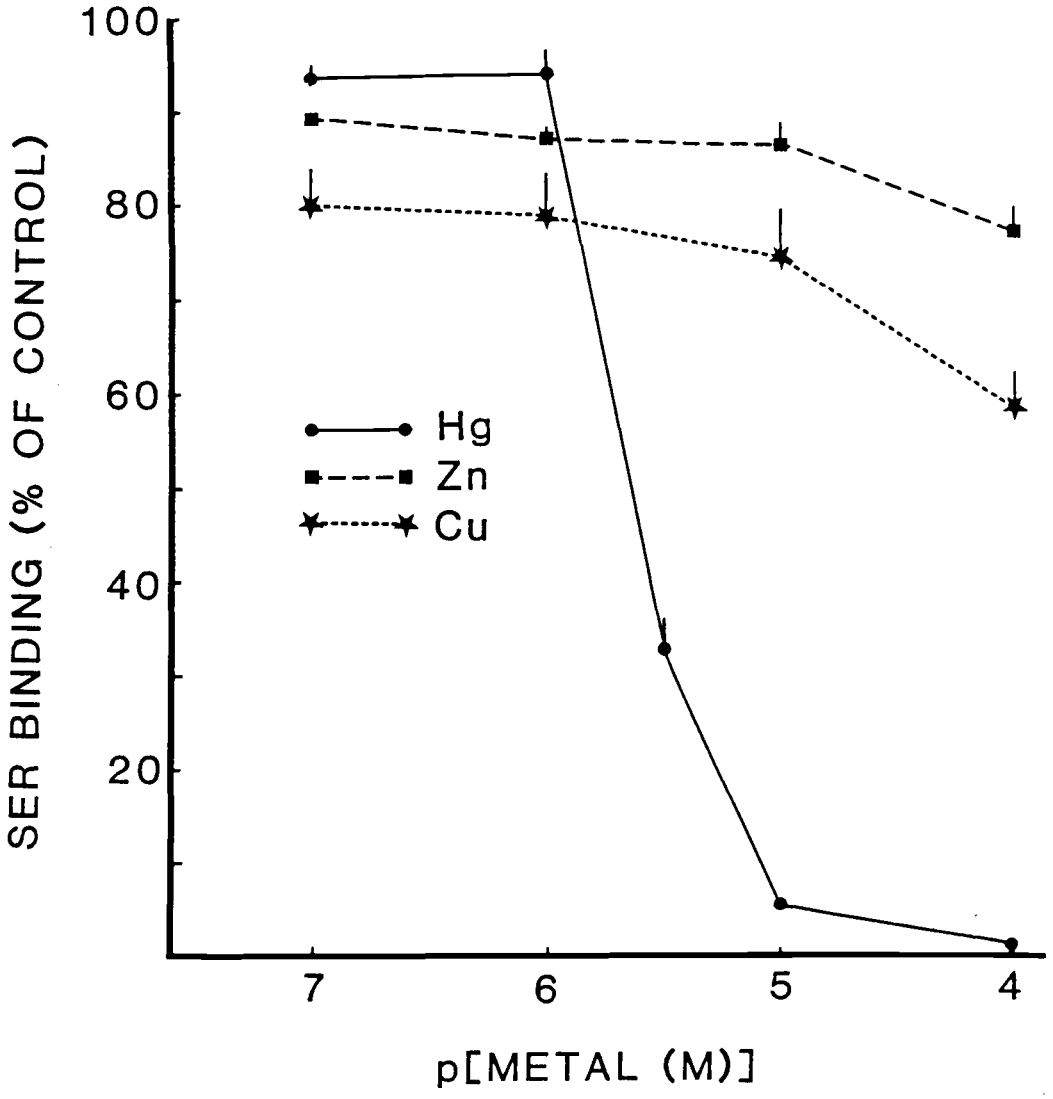

Fig. 12

Figure 13. Effect of Cu on the binding of L-serine to its olfactory receptor in (a) soft-water buffer and (b) hard-water buffer. Serine concentrations were 0.26 or 0.51 uM. Means plus one standard error were calculated from four preparations using soft-water buffer and five preparations using hard-water buffer. Predictions of chemical species present in the incubation mixture are shown for each Cu concentration.

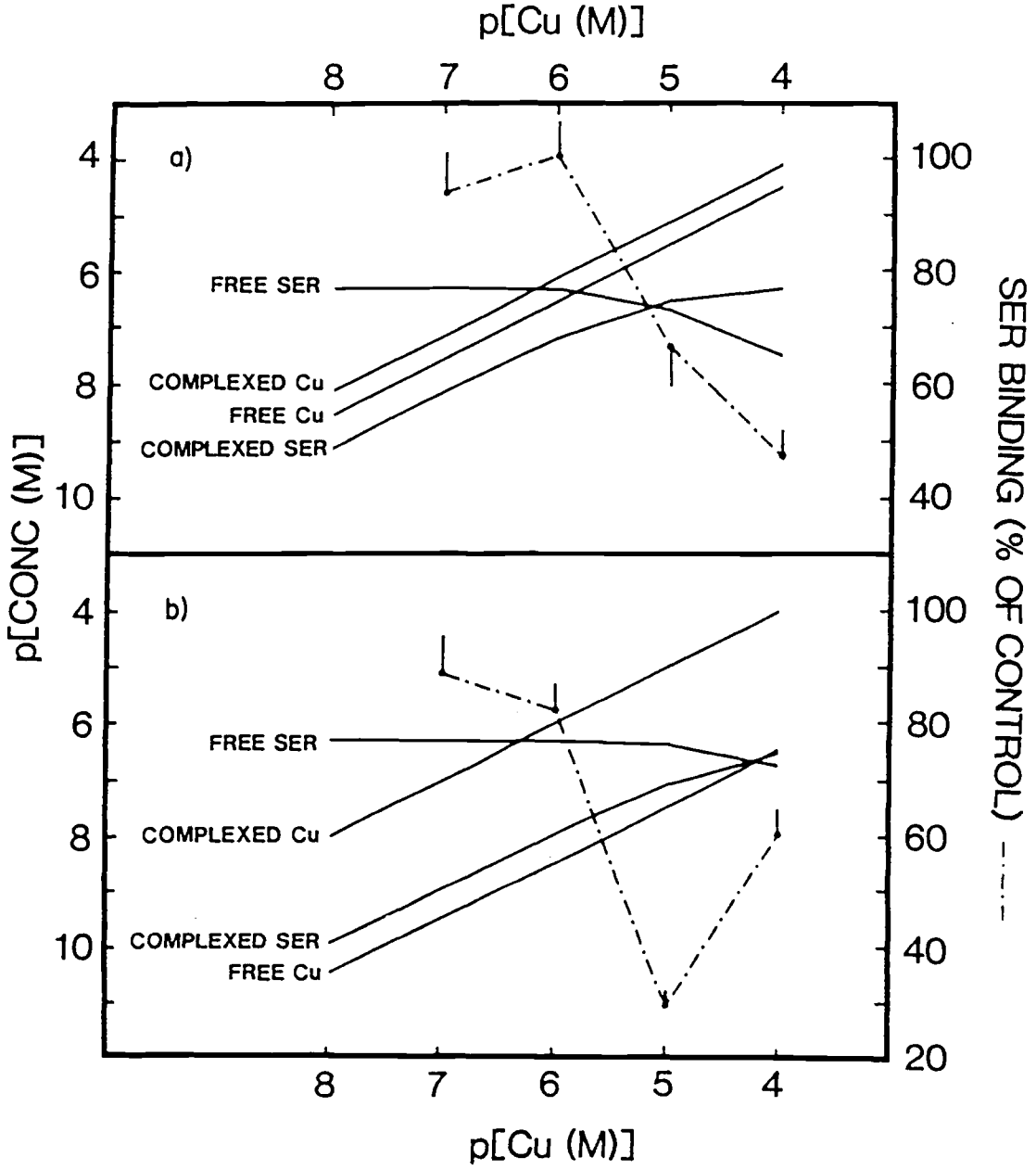
p[Cu (M)]
8
7
6
5
4
a)
FREE SER
COMPLEXED Cu
FREE Cu
COMPLEXED SER
b)
FREE SER
COMPLEXED Cu
COMPLEXED SER
FREE Cu
p[CONC (M)]
SER BINDING (% OF CONTROL) ----
100
80
60
40
20

Fig. 13

Figure 14. Avoidance of L-serine (10^{-7} M) by juvenile coho salmon in the presence and absence of Cu. Solid lines represent the avoidance response of fish following exposure to no added Cu (C), 10^{-6} M Cu (Cu), and 10^{-6} M Cu followed by a clearance period (Cln). The broken line represents a 1 % probability boundary generated from a cumulative binomial distribution function using $p = q = 0.5$.

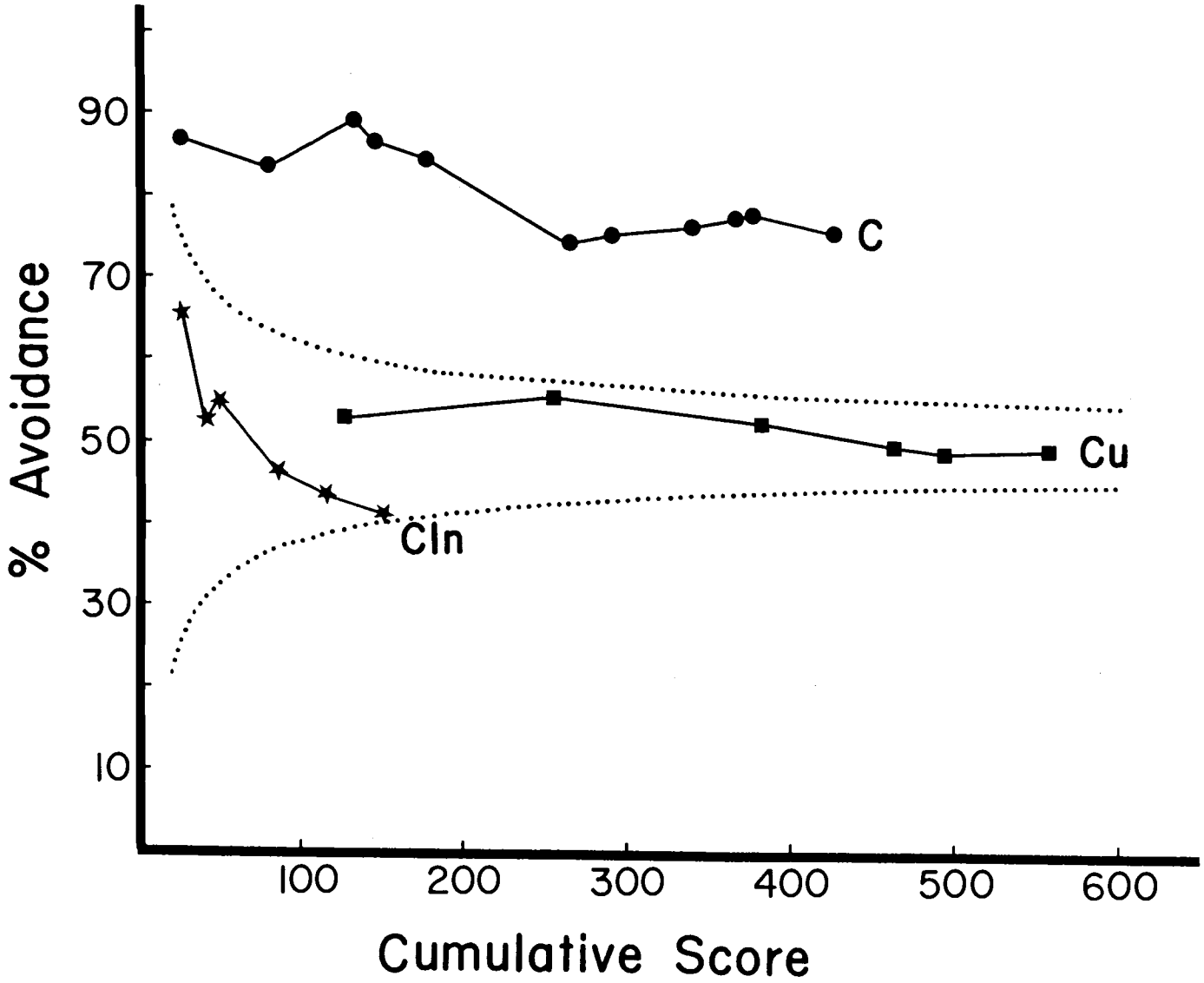
90
70
50
30
10
% Avoidance
C
Cu
Cln
100
200
300
400
500
600
Cumulative Score

Fig. 14

Figure 15. Avoidance of L-serine (5×10^{-7} M) by juvenile coho salmon following exposure to no Zn (C), 100 ug/L Zn (100), or 500 ug/L Zn (500). Results are from tests after (a) 24 hr, (b) 96 hr, and (c) 21 days of exposure to Zn. A behavioral test without added serine and using fish not exposed to Zn is shown by line B in (a). The broken line represents a 1 % probability boundary generated from a cumulative binomial distribution function using $p = q = 0.5$.

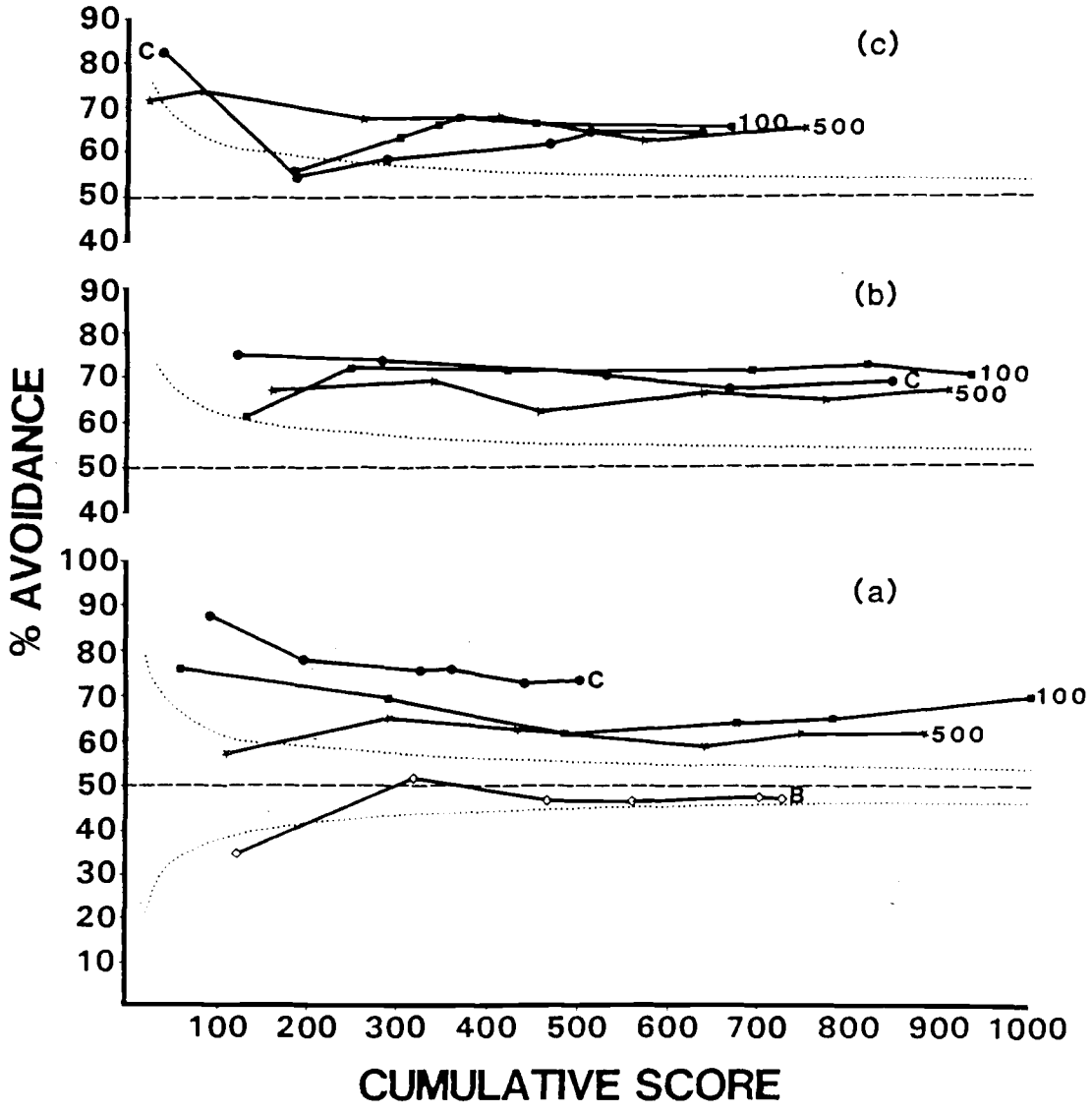
(c)
(b)
(a)
C
100
500
B
% AVOIDANCE
CUMULATIVE SCORE
90
80
70
60
50
40
30
20
10
100
200
300
400
500
600
700
800
900
1000

Fig. 15

V. CONCLUDING REMARKS

The findings from this dissertation can be placed in a conceptual framework by modifying Schreck's (1981) concept of physiological performance capacity. Physiological performance can be viewed as an N-dimensional geometric capacity "delimited ultimately by the organism's genotype and proximately by environmental influences..." (Schreck 1981). The "sensory performance capacity" of an animal at any given time can be defined by vectors describing the performance of all individual senses, eg. vision, olfaction, etc. (Fig. 16). As an animal ages, its sensory capacity changes due to underlying development or deterioration in each individual sense. In this way we may visualize the sensory capacity of an animal changing through time in response to its genetic endowment and environmental constraints. Environmental constraints that could, in theory, reduce sensory capacities below genetic potentials are disease, poor diet, stress, and toxic substances.

Representing olfaction by a single vector is, of course, overly simplistic. The ability of an animal to detect single odor molecules during its ontogeny can be

better represented by its own, narrower, performance capacity (Fig. 17). The "olfactory performance capacity" at any given time can be defined by vectors describing the ability of olfaction to detect distinct chemical stimuli. This "ability" could be represented by absolute detection thresholds, the magnitude of the dynamic range of response, or some other measure. The olfactory performance capacity of an animal could, in principle, be experimentally determined but it would require a monumental research effort.

Olfaction viewed in this way means that the ontogenetic conclusions reached using alanine and serine as odors cannot be extended to other odors or to olfactory performance as a whole. This is especially true for alanine and serine because the results in chapter III indicate that they are functionally the same odor. Had we measured the olfactory sensitivity of juvenile coho salmon to histidine, for example, an increase, decrease, or no change in sensitivity might have been observed. Sensitivity to an odor could change due to changing responsivity at one or more central locations or to changes in peripheral olfactory receptors. Hormone receptors in the plasma membrane of target cells are known to increase (up regulation) or decrease (down regulation) in response to local hormone concentrations (Hadley 1984). The consequence of receptor regulation is a change in the

sensitivity of target cells to the hormone. My work supports the assertion that the process of olfaction begins with an odor molecule binding to a receptor. In the case of serine, this initial interaction is followed by neural events that lead to recognition and ultimately to the avoidance response. This work also adds to the growing body of evidence that a specificity of interaction exists between an olfactory receptor and an odor. Thus, olfactory sensory input could vary due to processes analagous to up and down regulation. A change in the density of specific receptors in olfactory sensory cells would not necessarily require new receptors to be inserted into the cell membrane or old receptors to be deleted. The cell cycle for olfactory sensory cells is very short (Moulton 1974). A change in olfactory sensitivity could be realized if newly developing cells contained proportions of specific receptors different from those in past populations of cells.

The manner in which toxic substances reduce a fish's potential olfactory performance capacity to a lower realized capacity is open to speculation. Elevated concentrations of certain toxicants may have a pervasive deleterious effect and inhibit the detection of all odors by all species of fish. Hg and Cu may belong to this category of toxicant. Other, less generally reactive or irritating toxicants may inhibit the detection of only a

small number of odors. The results presented and the literature cited in chapter III indicate that olfactory receptors have a biochemical specificity for certain physical and chemical features of odor molecules. By analagy, olfactory receptors having odor specificities may be vulnerable to certain toxicants that have specific physical or chemical attributes. Although Zn++ did not inhibit the receptor for L-serine in coho salmon, it may interact with other olfactory receptors structurally dissimilar to the serine receptor.

Fig. 16. Conceptual sensory performance capacity of an animal illustrated using two example performance vectors. Both potential and realized performance capacities are shown to be a function of ontogeny.

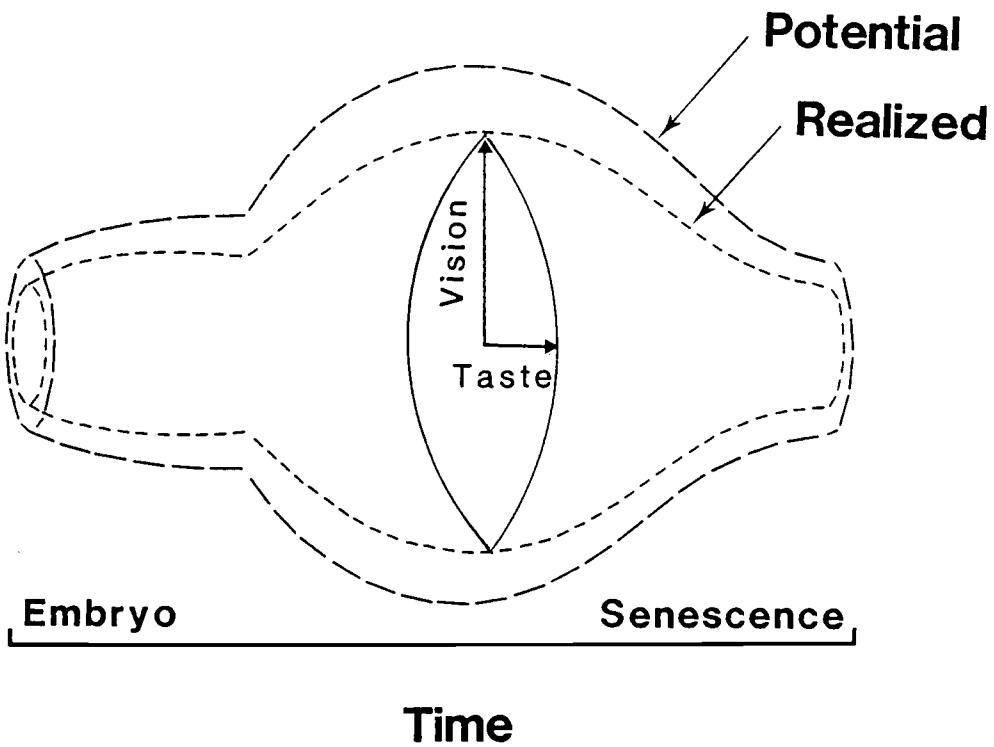

Fig. 16

Fig. 17. Conceptual olfactory performance capacity of an animal using two example performance vectors. The vectors illustrated represent the ability to detect histidine and serine.

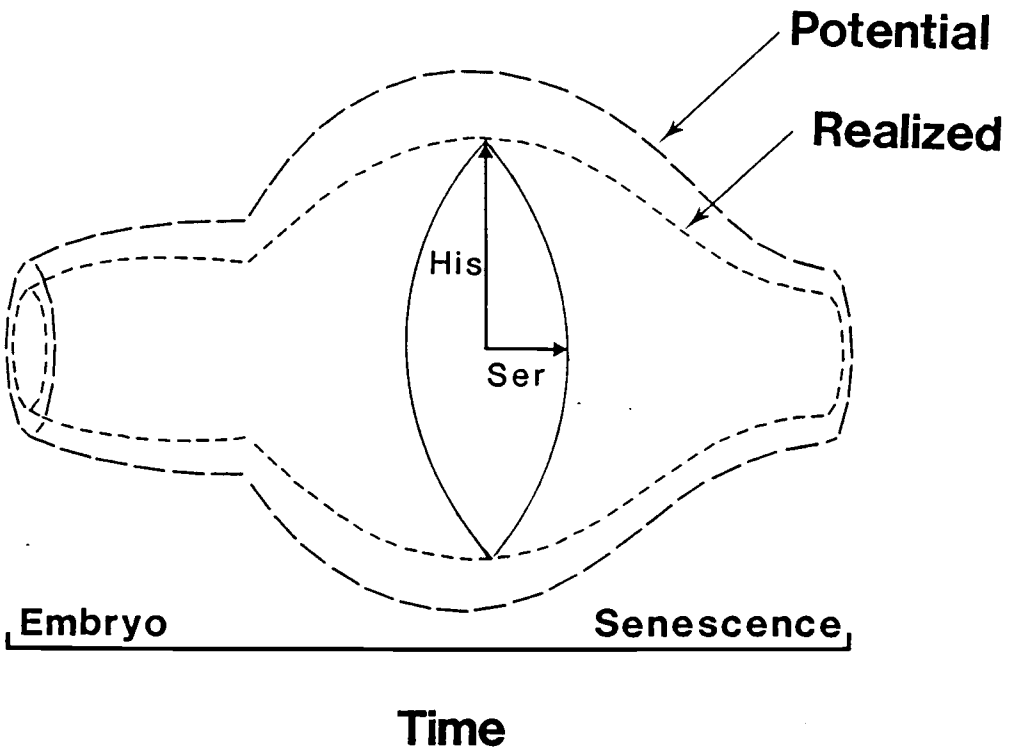

Fig. 17

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Appendices

Appendix I. Chemosensory Detection of Predators and the Physiological Stress Response by Coho Salmon[1]

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INTRODUCTION

Chemoreception provides fishes with a unique environmental perspective that can be used to direct or modify behavior. Predatory species of fish use chemoreception to detect prey (Little 1983) and it seems likely that some prey species reciprocate by using chemoreception to detect and avoid predators. The experience of chemically detecting a predator might be expected to induce a feeling of fright in a fish. "Fright" is a word commonly used to describe behavioral reactions of fish (Laming 1981; Smith 1982; von Frisch 1941) even though it is impossible to know if fish experience emotions as we know them. Although psychological aspects of odor perception by fish are difficult to address experimentally, the presence or absence of stress can be assessed by making relevant physiological measurements. The chemoreception of biochemicals emanating from a predator could be perceived as frightening to a prey species, and as Schreck and Lorz

(1978) suggest, frightening experiences could trigger a General Adaptation Syndrome (GAS) stress response. Physiological evidence for psychological stress in fish has been found in subordinate individuals within social dominance hierarchies (Schreck 1981). Although stress research on fish has focused primarily on physical stresses (Mazeaud and Mazeaud 1981; Schreck 1981), it is known that fish show a stress response after exposure to certain dissolved chemicals, eg., copper (Donaldson and Dye 1975; Schreck and Lorz 1978), ammonia or nitrite (Tomasso et al. 1981), endrin (Grant and Mehrle 1973), kanamycin (McBride et al. 1975), phenol or ammonia (Swift 1981), and hydrogen ion (Brown et al. 1984; Barton et al. 1985). Toxic chemical stress, however, may be closely related to physical stress provided that the response to the chemical is initiated by an irritation of sensitive external tissues (eg. gill epithelium). Detection of certain biochemicals, on the other hand, may be stressful because they encode information having survival value such as the presence of a predator or a territorial boundary.

The objective of this research was to look for a relationship between the behavioral avoidance response and the physiological stress response in coho salmon following detection of predator and nonpredator chemical stimuli. Chemical stimuli tested were L-serine, human skin extract, and whole-body and broken-skin extracts from northern

squawfish (Ptychocheilus oregonensis) and largescale sucker (Catastomus macrocheilus). The fright reaction and avoidance response of coho salmon to L-serine and human skin extract is well known (Brett and MacKinnon 1954; Idler et al. 1956). Mammalian skin extracts seem to be generally repellent to Pacific salmon and L-serine is responsible for much of its activity. It may be that L-serine is a kairomone originating in the skin of mammalian predators (Weldon 1980). Squawfish and sucker were chosen to test for effects of predator and nonpredator chemical stimuli, respectively. In addition, both species are ostariophysan fishes and have an alarm substance (Schreck-stoff) in their skin capable of inducing alarm reactions in conspecifics (Pfeiffer 1963). We tested broken-skin extracts of these fish to determine if alarm substance(s) could induce behavioral avoidance or stress responses in an unrelated fish.

MATERIALS AND METHODS

Behavioral Assay

The avoidance response to chemical cues was evaluated using a 2-choice Y-trough. Juvenile coho salmon (mean weight = 20 g) were obtained from Oregon Aqua-Foods, Inc. and acclimated to de-chlorinated freshwater (10.0 - 18.5 C) in flowthrough 1.5 m circular tanks at Oregon State University's Hatfield Marine Science Center,

Newport, Oregon. Fish were fed Oregon Moist Pellets once or twice daily. The design of the Y-trough and the conditions of the behavioral assay were described in Rehnberg et al. (1985). Briefly, an assay began when 10 fish were placed in the fork area of the Y-trough with access to the trough arms blocked. A test substance was then introduced from a calibrated Mariotte bottle into the water flowing down one of the trough arms. The other arm received a similar input from a Mariotte bottle containing only unscented water. Following a 10 min input from the bottles, the gates blocking access to the arms were simultaneously raised and the fish were allowed 25 min to choose either arm or remain in the fork area. One-way funnel-type traps located in the entrance of the arms prevented fish from leaving an arm after entering it. At the end of the test period, the arm gates were dropped and the numbers of fish in the arms and fork area were recorded. Using fresh fish each time, the assay was repeated 7 - 9 times (N = 70 - 90 fish) for each treatment. Control tests were conducted in an identical manner except that both of the Mariotte bottles contained only well-water. The data on fish trapped in the Y-trough arms were compared to a random arm selection model (1:1) using a two-class chi square test with a correction for continuity (Sokal and Rohlf 1981).

Substances tested were L-serine (Sigma) (10^{-5} M) and

extracts made from human skin (HSE), whole northern squawfish (SQE), squawfish broken-skin (SQBSE), and whole largescale sucker (SUE). HSE was prepared by submerging a human hand in 1 L of freshwater for 2 min, followed by the other hand for 2 min. This solution was then brought to a final test volume of 15 L. Squawfish and suckers were obtained by electroshocking in the Willamette River near Corvallis OR. These fish were transported to tanks at our Smith Farm laboratory and held for at least 6 days before being used as a source of extract. To obtain whole-fish extract, three adult fish were placed in a flowthrough fiberglass tank containing about 140 L of water. Mean weights for donor fish ranged from 531 - 1152 g for squawfish and 752 - 1076 g for suckers. Twelve hr before sampling the extract, the water inflow was stopped in order to ensure a concentration of stimuli. Aerated water was circulated around the fish with a submersible pump. After 12 hr, water was removed from the tank and added directly to the Mariotte bottle immediately before the behavioral tests. SQBSE was prepared by killing an adult squawfish (445 - 636 g) with blows to the head and making 25 superficial punctures of the skin on both sides of the fish. The fish was placed in a 20 L bucket containing 10 L water, and the water was circulated around the fish with a magnetic stirring bar for 10 min. The water was filtered through 8 layers of cheesecloth directly into a

Mariotte bottle and brought to 15 L. The ratio of Mariotte bottle drip-rate to Y-trough arm flow-rate was either 1:16 or 1:177.

Stress Response

The possibility of a stress response being induced by biochemical stimuli was assessed by determining the kinetics of plasma cortisol, thyroxine, and glucose in juvenile coho salmon after an exposure to extracts similar to those used in the behavioral tests. Sixty juvenile coho salmon obtained from the Willard National Fish Hatchery were added to each of 6 fiberglass tanks containing about 140 L of flowing well-water (4.1 L/min). During the 30-day acclimation, the fish were fed OMP, ad libitum, twice daily. Feeding was discontinued 24 hr before all experiments. The first experiment began with a 1 hr introduction of control water (well-water), L-serine (10^{-5} M), or HSE into duplicate tanks at a 1:16 dilution. At 1, 2, and 6 hr after beginning the exposure, 10 fish from each tank were netted and quickly killed by immersion in a solution of ethyl m-aminobenzoate methanesulfonate (MS222 ---200 mg/L). Blood was sampled from the severed caudal artery with a heparinized capillary tube, and, after centrifugation, the plasma was stored at -20 C. Immediately after taking the sample at Hr 6, all remaining fish in each tank were given a severe physical stress in

order to verify that our experimental fish were capable of a stress response. This was done by netting the fish and placing them in a 20 L bucket of water that contained an inner bucket penetrated with holes. The inner bucket was raised and the fish were held out of water for 30 sec and then returned to their tank. One hr after the physical stress, plasma was sampled from 10 fish from each tank. The experiment was repeated substituting SQE and SQBSE or SUE and SUBSE as the test substances. The latter experiment used fish obtained from the Eagle Creek National Fish Hatchery. All extracts were obtained as described above.

Plasma cortisol was measured by radioimmunoassay as described by Redding et al. (1984). Plasma thyroxine was measured by the method of Dickhoff et al. (1978). Plasma glucose was measured by the method of Wedemeyer and Yasutake (1977). Data were analyzed using 2-way analysis of variance (ANOVA). Experiments with significant treatment effects were further analyzed at each sampling time using 1-way ANOVA and Duncan's Multiple Range Test. Data not conforming to the homogeneity of variance assumption were $\log_{10}$-transformed before analysis.

RESULTS

SQBSE and SQE tested at 1:177 were not avoided in behavioral assays (Fig. 18). In tests at 1:16, however,

SQE was avoided whereas SUE was not. As anticipated, both HSE and serine were strongly avoided.

One hr after beginning an exposure (i.e. "Hr 1") to either HSE or serine, a very small but statistically significant elevation in plasma cortisol was observed (Fig. 19). At Hrs 2 and 6, control and treatment values had converged to similar levels. The fish were capable of being acutely stressed as indicated by the predictably large cortisol response detected 1 hr after the imposition of the physical stress (Hr 7) (Fig. 19). The general trend in cortisol dynamics observed when using squawfish-based extracts was a very significant elevation following the exposure and was nearly identical to results using sucker-extracts (Fig. 19). In both cases, broken-skin extracts triggered responses intermediate between those induced by control water and whole-fish extracts. The differences between treatment and control levels of plasma cortisol at Hr 1 were statistically significant in both experiments. Cortisol levels in treatment fish decreased at rates that made them similar to control values by Hr 6.

Plasma glucose concentrations found in fish exposed to serine and HSE were not statistically different than control values at all sampling times (Fig. 20). In the experiments using squawfish or sucker whole-fish and broken-skin extracts, treatment levels of glucose were significantly higher than control levels at Hr 1.

Treatment levels at Hrs 2 and 6 were also greater than control levels, but differences were not significant. Physical stress induced a very significant elevation in plasma glucose levels at Hr 7 in all three experiments (Fig. 20).

Thyroxine in fish exposed to serine was not different from titres found in control fish (Table 7). Fish exposed to SQE showed very small but statistically significant elevations in thyroxine levels compared to controls.

DISCUSSION

These results show that prey species of fish can detect and make avoidance reactions to chemical stimuli emanating from predators. Juvenile coho salmon avoided predator substances such as serine, HSE, and SQE but not an extract from the nonpredatory sucker. In addition, we demonstrate that a chemoreceptive experience can induce a large stress response. Plasma cortisol, however, does not follow a clear predator-nonpredator line of interpretation. Although serine and HSE were strongly avoided, they induced only a very small elevation in plasma cortisol. Although this elevation was statistically significant, we have reservations about its biological significance because the mean cortisol titres were well within the range commonly seen in undisturbed

fish. The titres also appeared very small in comparison to those observed in physically stressed fish. In contrast, fish exposed to squawfish and sucker extracts showed a large cortisol response. SQE and SUE induced cortisol responses that were 87 % and 92 % of the response seen in physically stressed fish, respectively. The observation that SUE induced a large cortisol response but was not behaviorally avoided, coupled with the results that serine and HSE were strongly avoided but triggered only a very small cortisol response, casts further doubt on the general applicability of the GAS to fish. In anthropomorphic terms, Schreck and Lorz suggested that stimuli that produce fright, discomfort, or pain in fish elicit GAS-type reactions. If fright was something felt by the fish as they avoided serine and HSE in the Y-trough, then the lack of a clearcut cortisol response after exposure to these odors weakens the case for fright as being a cornerstone of the GAS for fish. The most prudent interpretation of these data is that levels of plasma cortisol respond to the perception of some, but not all, novel chemical stimuli.

Sucker or squawfish extracts derived from broken skin induced cortisol values intermediate between control values and those in fish exposed to whole-fish extracts. The effectiveness observed for broken skin extracts invites speculation on the possible utilization of

ostariophysan alarm substances by non-ostariophysan fishes. Both largescale sucker and northern squawfish have alarm substance in their skin (Pfeiffer 1963). Alarm substance is a signal to ostariophysan fishes that a predator has attacked a conspecific or a member of some other related species (Smith 1982). It is possible that non-ostariophysan fishes could evolve the behavioral mechanism of "eavesdropping" on the alarm substance signal to use to enhance their own chances of survival. Our only behavioral test of broken skin extract was of SQBSE at 1:177 which may have been too dilute to be effective.

Conclusions drawn from the glucose data generally parallel those based on plasma cortisol. Fish exposed to HSE or serine showed no stress response, as indicated by plasma glucose, until they were physically stressed. Fish exposed to squawfish and sucker odors, however, did show small but significant elevations in plasma glucose. But because plasma glucose is tightly regulated by a variety of physiological mechanisms, its use as a sensitive indicator of subtle or non-physical stresses may be limited. Barton et al. (1985) reported that juvenile rainbow trout had significantly elevated levels of plasma cortisol but normal concentrations of plasma glucose after exposure to low pH for 2, 4, or 6 hr.

Detection of SQE was followed by large increases in plasma cortisol and very small increases in glucose and

thyroxine. Thyroxine is not considered to be an indicator of stress in the manner of corticosteroids and catecholamines. Nevertheless, circulating levels of thyroxine and 3,5,3'-triiodothyronine (T_3) are known to respond to certain physical and chemical stresses. Brown et al. (1978) found a transitory increase (100 - 200 %) in plasma thyroxine within 2 hr of either blood sampling from caudal vessels or saline injection in coelomic or cardiac regions of rainbow trout. Variable changes in plasma thyroxine and T_3 were seen in rainbow trout in response to elevated hydrogen ion activity (Brown et al. 1984). In all cases including the data reported here, the magnitude of the thyroxine response to stress is relatively small. It is generally accepted that alertness, mental acuity, spontaneous electrical activity of nerve tissue, sensitivity to external stimuli, and motor activity are affected by the activity of the thyroid gland in vertebrates (Gorbman et al. 1983). Whether adaptive changes in any of these states occur in fish in response to small and transitory elevations in circulating thyroxine is currently unknown.

Most of what is known about physiological states following chemoreceptive experiences come from studies in which ostariophysan fishes have been exposed to alarm substances. Within 10 min of being exposed to its own alarm substance, the ide (Leuciscus idus) showed an

approximately 200 % increase in muscle lactate, pyruvate, glucose-6-phosphate, and glucose levels (Gronow 1974). Abramis brama showed increases in plasma potassium concentration and decreases in sodium for as long as 24 hr after detecting conspecific alarm substance (Malyukina et al. 1982). Minnows (Phoxinus phoxinus) responded to skin extracts from conspecifics with cardiac deceleration (Pfeiffer and Lamour 1976). Based on measurements of the dorsal light response of the black tetra (Gymnocorymbus ternetzi), Pfeiffer and Riegelbauer (1978) suggested that the enhanced optical alertness in fish exposed to alarm substance indicated a change in central nervous system excitation.

This research marks a limited first attempt to understand the physiological reactions of fish in response to predator and nonpredator chemical stimuli. In summary, piscine chemical stimuli induced a brief but well-developed stress response in juvenile coho salmon. Chemical stimuli from the predatory squawfish induced an avoidance response in salmon whereas stimuli from the nonpiscivorous sucker did not. In contrast to the effects of piscine chemical stimuli, L-serine and human skin extract each induced strong avoidance responses without inducing a stress response. We conclude that a causal link between fright and the stress response is not supported and that GAS-type stress responses in fish are

not a necessary outcome of subtle or nonphysical stresses.

Fig. 18. Percent avoidance of predator and nonpredator chemical stimuli by juvenile coho salmon in a 2-choice Y-trough. Fractions above each bar indicate the number of fish choosing an arm out of the total number tested. 1:16 and 1:177 refer to the ratio of Mariotte bottle drip-rate to Y-trough arm flow-rate. Acronyms refer to control (C), 10^{-5} M L-serine (SER), and extracts of human skin (HSE), squawfish broken-skin (SQBSE), squawfish (SQE), and sucker (SUE). ** denotes $p < 0.01$ from a chi square test.

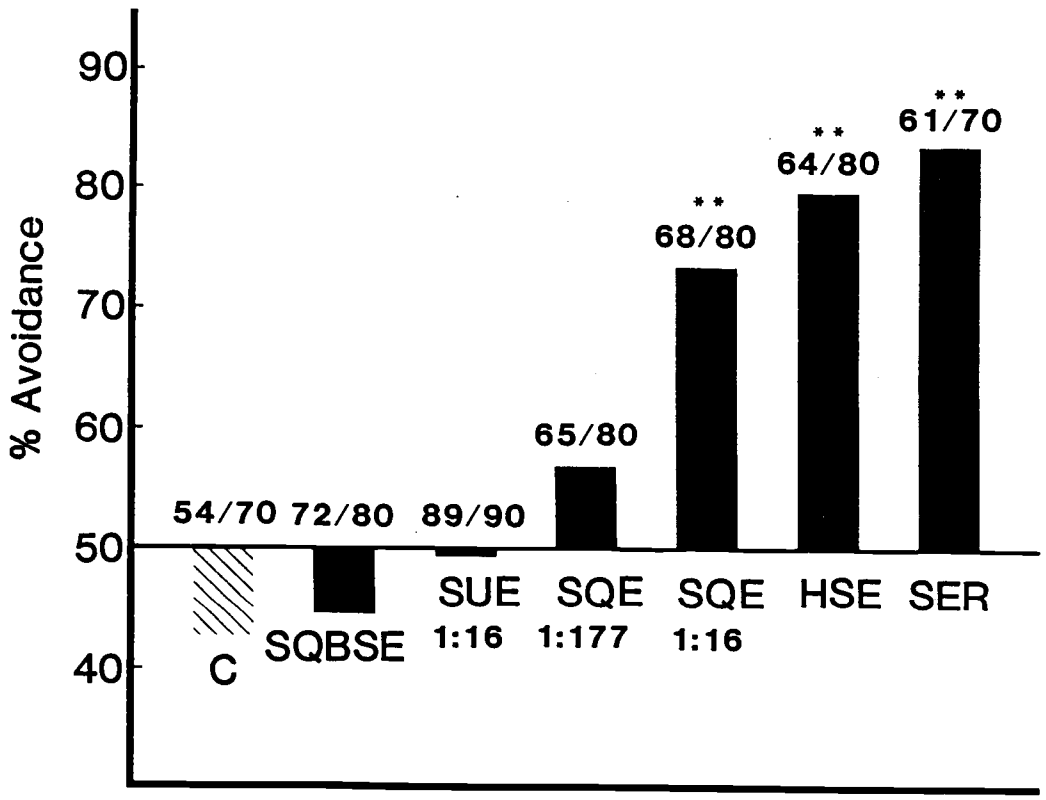
% Avoidance
90
80
70
60
50
40
54/70
72/80
89/90
65/80
**
68/80
**
64/80
**
61/70
C
SQBSE
SUE
1:16
SQE
1:177
SQE
1:16
HSE
SER

Fig. 18

Fig. 19. Plasma cortisol levels of juvenile coho salmon after exposure to the following substances: control (C), 10^{-5} M L-serine (SER), and extracts of human skin (HSE), squawfish (SQE), squawfish broken skin (SQBSE), sucker (SUE), and sucker broken skin (SUBSE). A 1-hr introduction of test substance began at Hr 0 and plasma was sampled at Hrs 1, 2, and 6. After the Hr 6 sample, remaining fish were physically stressed and then sampled 1 hr later (Hr 7). Means and standard errors were calculated from 16 - 20 fish.

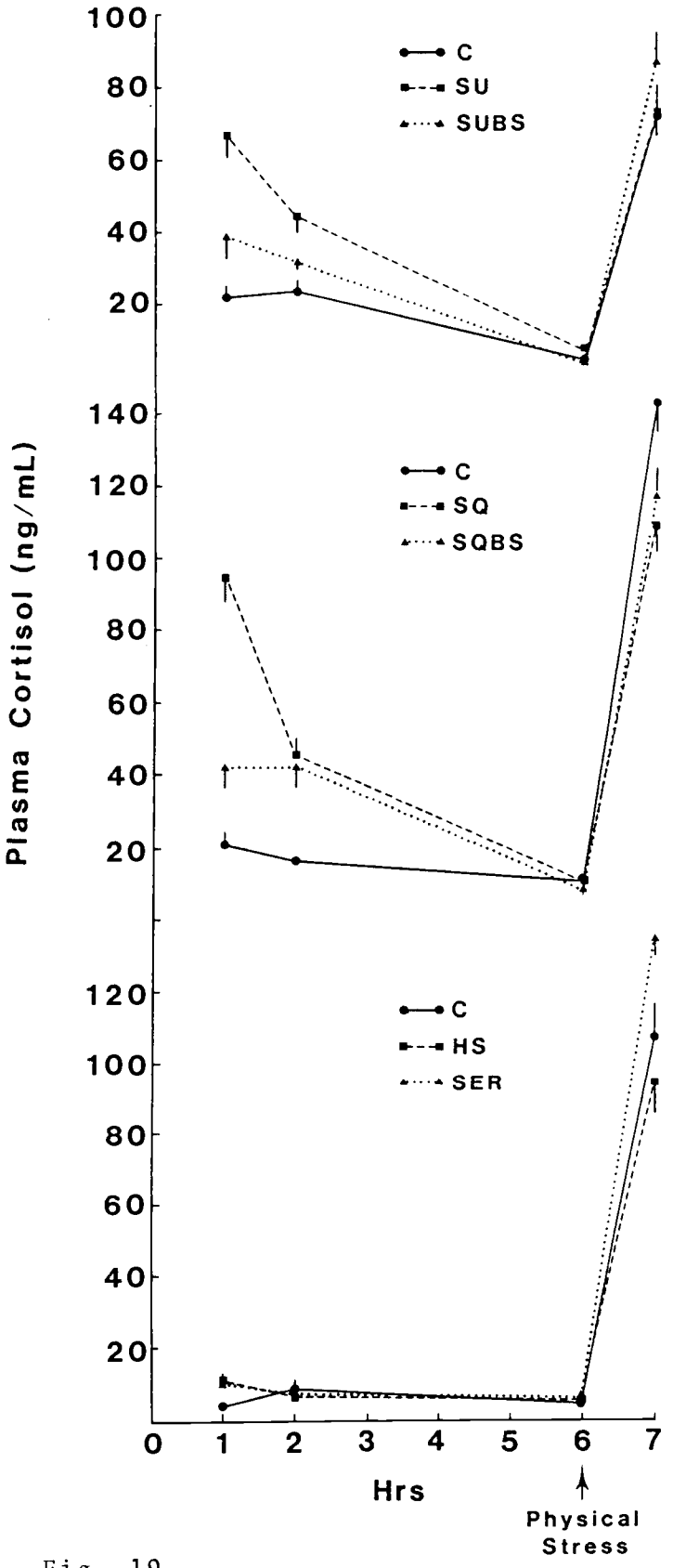
Plasma Cortisol (ng/mL)
100
80
60
40
20
C
SU
SUBS
140
120
100
80
60
40
20
C
SQ
SQBS
120
100
80
60
40
20
C
HS
SER
0
1
2
3
4
5
6
7
Hrs
Physical
Stress

Fig. 19

Fig. 20. Plasma glucose levels of juvenile coho salmon after exposure to the the following substances: control (C), 10^{-5} M L-serine (SER), and extracts of human skin (HSE), squawfish (SQE), squawfish broken skin (SQBSE), sucker (SUE), and sucker broken skin (SUBSE). A 1 hr introduction of test substance began at Hr 0 and plasma was sampled at Hrs 1, 2, and 6. After the Hr 6 sample, remaining fish were physically stressed and then sampled 1 hr later (Hr 7). Means and standard errors were calculated from 10 fish.

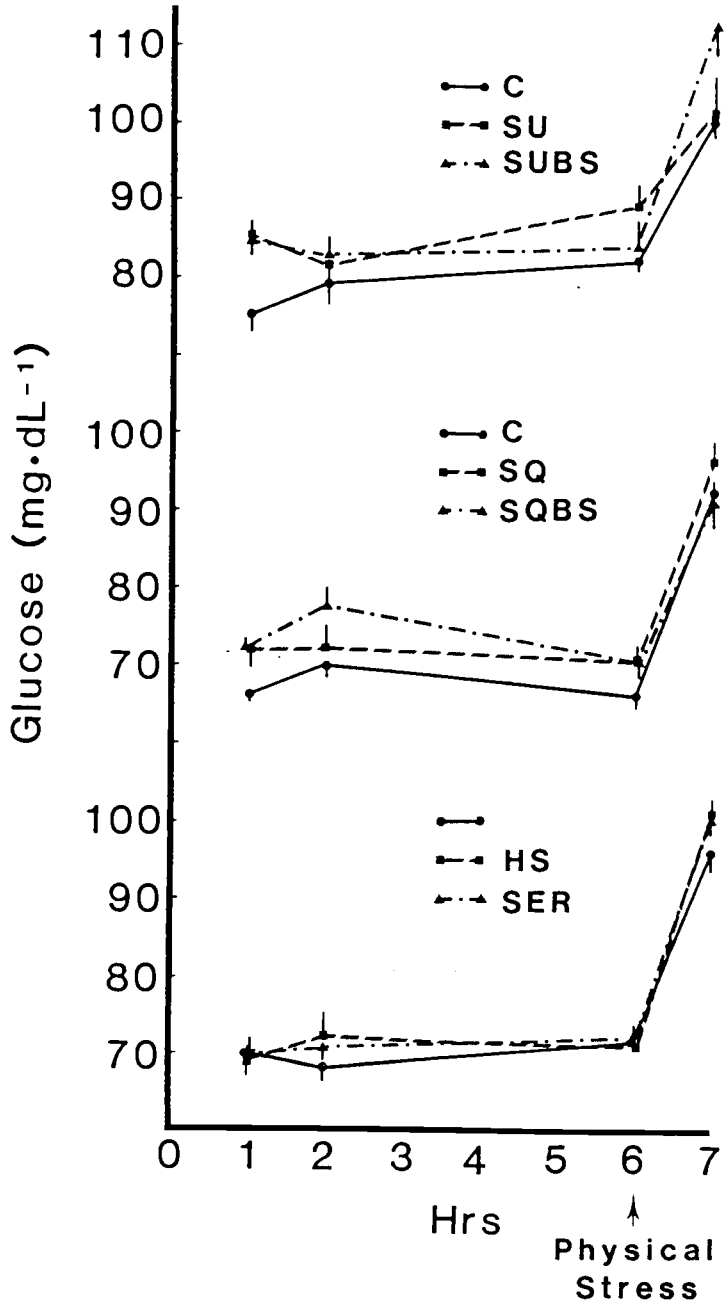
110
100
90
80
C
SU
SUBS
100
90
80
70
C
SQ
SQBS
Glucose (mg·dL⁻¹)
100
90
80
70
HS
SER
0 1 2 3 4 5 6 7
Hrs
Physical
Stress

Fig. 20

Table 7. Plasma thyroxine levels in juvenile coho salmon after exposure to control water, L-serine (10^{-5} M), or squawfish extract (SQE). A 1-hr introduction of test substance began at Hr 0 and plasma was sampled at Hrs 1, 2, and 6. Means (ng/mL) and standard errors (in parentheses) were calculated from 14 fish.

	Hr		
Substance	1	2	6
Control	3.2 (0.2)	3.7 (0.3)	4.6 (0.5)
Serine	4.3 (0.4)	4.5 (0.4)	4.4 (0.6)
Control	6.4 (0.9)	8.2 (0.5)	6.7 (0.7)
SQE	8.5 (0.7)	9.1 (0.6)	9.3 (1.3)

Appendix II. Homing of Coho Salmon (*Oncorhynchus kisutch*) Exposed to Morpholine[1]

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Olfactory imprinting to artificial stimuli has been shown to be a potential tool in managing stocks of anadromous salmonids (Hasler et al. 1978). Smolts of coho salmon (Oncorhynchus kisutch) exposed to morpholine or phenethyl alcohol before release into Lake Michigan were found to home, as adults, to tributaries scented with these odors (Cooper et al. 1976; Scholz et al. 1976; Johnsen and Hasler 1980). The management potential of artificial olfactory imprinting has not been realized partly because of the lack of field testing at hatcheries. We evaluated the efficacy of artificial olfactory imprinting at an Oregon coastal salmon hatchery. A specific objective was to determine if imprinted fish preferentially entered the hatchery fish ladder when the imprinting chemical was present. Secondly, we wanted to know if the presence of the imprinting chemical in the fish ladder would deter homing of fish with no previous exposure to the chemical. The latter eventuality is of concern if artificial imprinting chemicals are to be used in stream systems having runs of wild salmon or trout.

The experiment was conducted at the Oregon Department of Fish and Wildlife's Salmon River Hatchery, located on the Salmon River about 5 km upstream from the Pacific Ocean. There are no migratory barriers between the estuary and the hatchery and no major sources of water pollution upstream from the hatchery. A total of 21,735 smolt-size coho salmon marked with a right ventral fin-clip and placed in a flow-through pond were exposed to morpholine at a calculated concentration of 5×10^{-5} mg/L for 15 days. After allowing the pond to clear of morpholine for 6 days, a control group of 20,434 fish were given a left ventral fin-clip and placed in the same pond. Thirteen days later, on April 13, 1982, all fish were released into the Salmon River. During the autumn of 1982 and 1983, morpholine was added on various days to the hatchery's fish ladder (5×10^{-5} mg/L) and returns when morpholine was present or absent were recorded.

Of 42,169 fish released, only 62 returned (0.15 %) ---33 jacks in 1982 and 29 adults in 1983. The lower than expected return was likely a result of poor ocean conditions (El nino), impaired swimming ability due to the loss of a ventral fin, and possibly the effects of handling during marking. The number of imprinted fish returning (27) was not statistically different from that of the controls (35) (Table 8, chi square corrected for continuity = 0.790). The failure of morpholine to

increase the return of imprinted fish may have been a by-product of small sample size and the unusual weather during autumn 1983. Between September 27 and October 21, a period when returns to the hatchery are normally greatest, there was no significant precipitation and only four adult fish returned. When freshets finally occurred, fish moved into the hatchery in large numbers, resulting in a very uneven distribution of returns. Thus, the migratory readiness of the fish waiting for appropriate flow conditions may have obscured any attractive effects that morpholine might have had. Including both years, morpholine-exposed and control fish returned on 10 days when morpholine was present in the fish ladder, and on 17 days when it was not. Nevertheless, these data indicate that the control fish were not repelled by the presence of morpholine in the fish ladder (Table 8). Also, with morpholine absent from the ladder, imprinted fish returned as readily as control fish. Our data do not support the report by Mazeaud (1981) that morpholine is a nonspecific attractant to salmonids.

It remains possible that exposing the fish to a higher concentration of morpholine for longer than 15 days would have resulted in enhanced returns of morpholine-exposed fish. More important, the presence of morpholine did not confound the orientation of unexposed fish, indicating that they could still recognize other relevant

cues in the hatchery water supply. This observation suggests that the migration of wild stocks would not be hindered by morpholine-scented waters.

Table 8. Returns of adult coho salmon to the Salmon River Hatchery when morpholine was present or absent in the fish ladder. Adults were exposed to morpholine or not exposed (controls) as smolts.

	Fish ladder water		
Treatment as smolts	Morpholine present	Morpholine absent	Totals
Morpholine-exposed	9	18	27
Control	17	18	35
Totals	26	36	62