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Title: Chemodetection of Threatening Chemical Stimuli by Juvenile Coho Salmon (*Oncorhynchus kisutch*)

Abstract approved: Dr. Carl B. Schreck

The chemotactic behavior of juvenile coho salmon to various chemical stimuli was assessed using a two-choice Y-maze. It was hypothesized that juvenile salmon would consistently avoid chemical stimuli emanating from sources indicative of a threat, namely: stressed conspecifics, predatory fishes, and industrial (pulp mill) effluents. Initial tests demonstrated that juvenile salmon were capable of detecting all three sources and would avoid each if simultaneously given a choice of control (blank) water. Further testing revealed that salmon could use odors (i.e., body rinses) to discriminate between stressed and unstressed conspecifics, preferring the odor of unstressed conspecifics over that of stressed conspecifics. Also, parr could discriminate between odors of four species of Pacific Northwest fish [largescale sucker (*Catostomus macrocheilus*), northern squawfish (*Ptychocheilus oregonensis*), carp (*Cyprinus carpio*), and walleye (*Stizostedion vitreum*)] representing a native nonpredator, native predator, exotic nonpredator, and exotic predator, respectively. In paired-odorant trials with these species, parr preferred the odor of the native

nonpredator (sucker) over all of the other species' odors, and parr were always repulsed by carp odor. Tests with effluents from two pulp mills in Grays Harbor, Washington, demonstrated that salmon would not avoid a 0.03% solution of Weyerhaeuser Company effluent or a 0.3% solution of ITT Rayonier effluent, but were strongly repulsed by 30% solutions of each effluent. In addition, neither effluent appeared to impair the olfactory ability of juvenile salmon since they could detect L-serine (10^{-4} M), a generally repellent amino acid present in skin odor from mammalian predators, in the presence of either effluent.

Chemodetection of Threatening Chemical Stimuli by
Juvenile Coho Salmon (*Oncorhynchus kisutch*)

by

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Chemodetection of Threatening Chemical Stimuli by
Juvenile Coho Salmon (*Oncorhynchus kisutch*)

CHAPTER 1. Introduction

The aquatic environment is filled with chemicals emitted from biotic and abiotic sources, and it is not surprising that many fishes have highly developed chemosensory systems with which to discern useful information about their surroundings (Liley 1982). Salmonids in particular are noted for their ability to detect and identify chemical stimuli from a variety of biologically important sources, including: natal streams (Harden-Jones 1968; Hasler and Scholz 1983; Stabell 1984), conspecifics (Stabell 1984; Olsén and Hoglund 1985; Quinn and Busack 1985; Olsén 1985; Olsén 1986; Stabell 1987), predators (Brett and MacKinnon 1954; Rehnberg and Schreck 1987), and pollutants (Sutterlin 1974; Brown et al. 1982). While numerous studies have described the salmon's chemosensory-mediated migration, we have an imperfect understanding of the role chemoreception plays in modifying other aspects of salmonid behavior.

Like other aquatic organisms, salmonids encounter chemical cues that could be uniquely characterized as threatening (emanating from or indicative of life-threatening sources) or non-threatening (e.g., food). Detecting and escaping from threatening chemostimuli has obvious survival value, since responding animals should have an adaptive advantage over non-responding animals (Grant and Mackie 1974). Therefore, the goal of this research was to determine if juvenile salmon exhibit consistent chemotaxic behavior when confronted with threatening chemostimuli from three sources likely to be encountered by

salmonids in the Pacific Northwest: injured conspecifics, predators, and industrial (pulp mill) effluents. The experiments reported here were designed to answer the following questions:

- 1) Can juvenile salmon detect and discriminate between chemical cues from stressed and unstressed conspecifics?
- 2) Can juvenile salmon detect and discriminate between chemical cues from native predatory and nonpredatory fishes?
- 3) Can juvenile salmon detect and discriminate between chemical cues from exotic predatory and nonpredatory fishes?
- 4) Will juvenile salmon avoid waters containing pulp mill effluents, and if so, what concentrations are not repulsive to fish?
- 5) Will nonrepulsive concentrations of pulp mill effluents disrupt the salmon's ability to detect the chemical cue of a predator?

CHAPTER 2. Chemosensory Discrimination of Stressed and
Unstressed Conspecifics by Juvenile Coho Salmon
(*Oncorhynchus kisutch*)

Abstract

Coho salmon parr (*Oncorhynchus kisutch*) can discriminate between stressed and unstressed conspecifics solely on the basis of chemical stimuli. In a two-choice Y-maze, parr preferred water conditioned by unconfined conspecifics over water in which conspecifics had been severely confined for 12 h. The behavior of test fish corresponded to the physiological stress response (indicated by plasma cortisol titers) of confined and unconfined fish used as odor sources. The results suggest that intraspecific chemical cues could allow salmonids to recognize and avoid threatening conditions, and demonstrate the need to consider the effects of repellent odors in chemo-orientation studies.

Introduction

The ability to detect chemical cues from threatened fish may help conspecifics avoid harmful conditions. A well-documented example of a chemically-mediated avoidance behavior is the fright reaction of some fishes, whereby skin secretions of an injured fish elicit a fright response from nearby conspecifics (Pfeiffer 1963a). While the response is common in ostariophysian fishes, members of the family Salmonidae reportedly lack a typical fright reaction (Schutz 1956; Marusov 1975). However, it has not been demonstrated that salmonids are unable to detect chemicals (hereafter referred to as "odors") from threatened conspecifics. Detection, if present, could prompt fish to have a heightened awareness of a potential threat and take whatever action is appropriate, not necessarily a stereotyped behavioral reaction.

Numerous studies have shown that salmonids can recognize odors from conspecifics (Stabell 1984; Olsén and Hoglund 1985; Quinn and Busack 1985;

Olsén 1985; Olsén 1986; Stabell 1987) and avoid odors which signify an imminent threat, e.g., pollutants (Sutterlin 1974; Brown et al. 1982) and predator rinses (Brett and MacKinnon 1954; Rehnberg and Schreck 1987). There is also evidence that they are repelled by odors from conspecifics that have been crowded but not injured (Stabell 1982). In light of the potential that whole-body rinses have for communicating an imminent danger, the present study was conducted to determine if juvenile coho salmon (*Oncorhynchus kisutch*) are capable of detecting and discriminating between odors from stressed and unstressed conspecifics.

Materials and Methods

I performed experiments designed to assess both odor detection and odor discrimination by salmon parr. The odor detection experiment measured the parr's ability to discern blank (control) water from odors of confined or unconfined conspecifics. The odor discrimination experiment measured the avoidance/preference behavior of parr to a pairwise combination of odors from confined and unconfined conspecifics.

Both experiments were conducted using coho salmon parr from the Oregon Department of Fish and Wildlife's Eagle Creek Hatchery (ECH; mean weight 25 g). I repeated the odor discrimination experiment using parr from Oregon Aqua Foods (OAF; mean weight 27 g) to determine if results were reproducible with a different salmon stock. All fish were transported to Oregon State University's Smith Farm Laboratory at least two weeks before being tested, placed in stock tanks receiving flow-through well water (11°C), and fed to repletion daily with Oregon Moist Pellets. Fish were removed to a separate

holding tank after being tested or used as an odor source.

Avoidance/preference behavior was assessed using a flow-through Y-maze described by Rehnberg et al. (1985) (Figure 2.1). Well water flowed into the head of each arm and the separating channel (which served to reduce mixing between the arms) at a rate of 3.8 L/min. Test solutions (odors or control water) were introduced from calibrated Mariotte bottles via flexible tubing into the head of each arm at a rate of 240 mL/min (1:15, Mariotte bottle drip rate:arm flow rate). One-way funnel traps at the arm entrances helped to keep parr within an arm after entering it, and fixed screens prevented fish from entering the separating channel and leaving the fork area. To encourage parr to swim into the arms, the fork area was illuminated, whereas the arms and separating channel were provided with shade.

Test solutions were obtained as follows. In the odor detection experiment, 120 parr were netted from their stock tank and distributed equally into six perforated plastic buckets placed in two identical fiberglass "conditioning" tanks (Figure 2.1); a third "control" tank contained well water but no fish. For the odor discrimination experiment, 120 parr were similarly distributed (i.e., three buckets/tank, 20 parr/bucket) but no control tank was used. All tanks received flow-through well water @ 13 L/min and were aerated with an airstone. Water in each tank was kept at a volume of 120 L using standpipes which served to maintain parr at a density of 1 fish/600 mL in conditioning tanks. Parr were left undisturbed and unfed to adjust to this density for 7-12 days. At the end of the acclimation period, water inflows were removed, and fish in one conditioning tank were subjected to a 12-h confinement stress by raising the perforated buckets so that the water level just covered the fishes' backs (a confinement

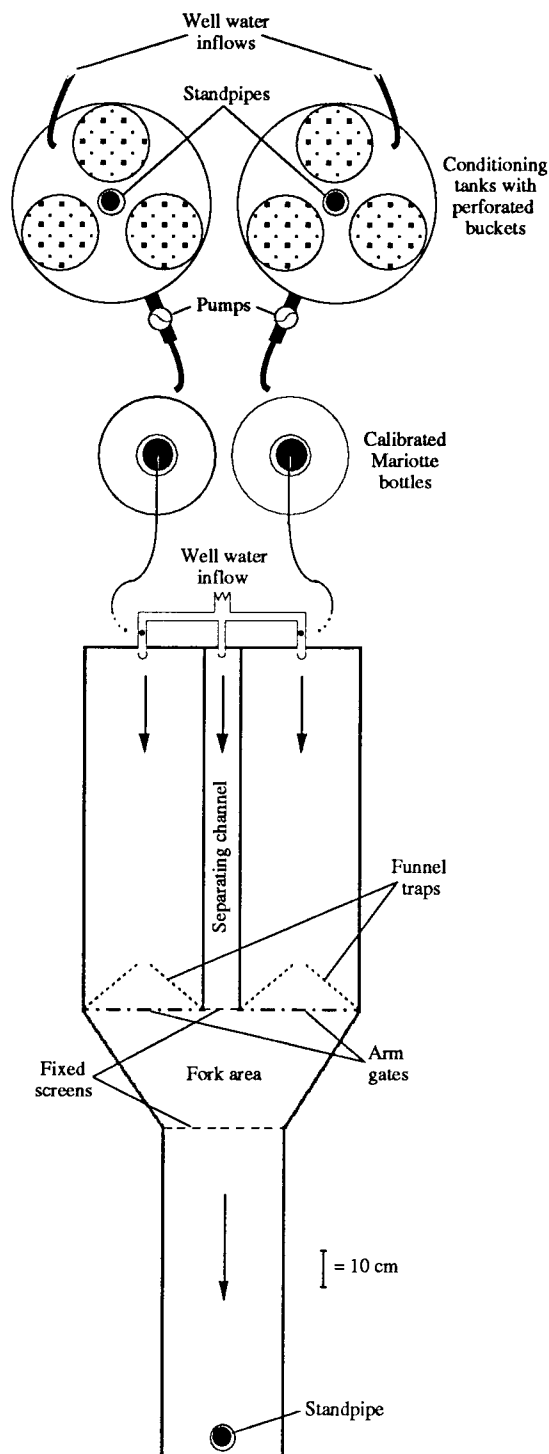


Figure 2.1. Top view of flow-through Y-maze, conditioning tanks (not to scale), and Mariotte bottle delivery system.

density = 1 fish/68 mL); “unconfined” coho in the adjacent tank were left undisturbed during this confinement period. Periodic observations were made of fish in both tanks to evaluate any behavioral differences, with special care taken to not disturb the unconfined parr. After 12 h, parr were quickly removed from both tanks by pulling all buckets. Also, airstones were withdrawn so as not to drive off any odors in the water, and lids were placed over each tank. After completing blood sampling (described subsequently), behavioral tests were begun using solutions pumped from the tanks to the Mariotte bottles; all tests were completed 8-12 h after removing fish from the conditioning tanks.

In the odor discrimination experiment, blood samples were taken to verify that stress-related physiological differences existed between confined and unconfined groups. Immediately after removing all parr from conditioning tanks, twelve fish from each group were quickly transferred to separate buckets containing lethal solutions of neutralized ethyl *m*-aminobenzoate methanesulfonate (MS 222, 200 mg/L). In addition, 12 conspecifics were netted from the stock tank and similarly killed; the “resting” cortisol titers of these fish made it possible to determine if unconfined parr had sufficient time to recover from the handling stress incurred during transfer to their conditioning tank. Parr were bled by severing the caudal peduncle with a razor blade and blood was collected in ammonium-heparinized capillary tubes. Plasma was separated by centrifugation and stored at -20°C for later analysis. Plasma cortisol titers were measured using a radioimmunoassay described by Redding et al. (1984). Plasma cortisol data from each test date were subjected to ANOVA to determine if significant differences existed between confined, unconfined, and stock tank parr. Differences between these groups were examined with Tukey’s honestly

significant difference test at the $P < 0.05$ level.

A behavioral trial began when 10 parr were netted from the stock tank and placed into the maze fork area with arm gates down. After fish were in the maze, test solutions were introduced from the Mariotte bottles into each arm. In the odor detection experiment, one arm received control water while the other received odor from confined or unconfined fish, whereas in the odor discrimination experiment, one arm received odor from confined coho while the other received odor from unconfined fish. After a 10-min "calming" period, both arm gates were lifted and parr were allowed 25 min to enter an arm or remain in the fork area. Observations through a one-way window indicated that most parr swam singly and in groups throughout the fork area, hence they were exposed to both test solutions. Also, fish appeared to choose maze arms independent of other fish (i.e., "following behavior" or fighting was uncommon). Following this 25-min choice period, arm gates were quickly lowered and the numbers of parr in each arm and the fork area recorded. After removing all fish, the maze was drained, rinsed, and filled with well water for the next trial. Test solutions were alternated between the arms after each trial by switching the Mariotte bottle inflow lines. Using naive parr for each trial, six trials were conducted for each test ($N=60$ fish/test). The numbers of fish choosing each arm were compared to a random arm selection model (1:1) using a two-class chi-square test with the Yates correction for continuity (Zar 1984). Preference of one test solution over the other was defined as significant departure from this 1:1 ratio using a $\chi^2 > 3.8$, $P < 0.05$.

Results

In the odor detection experiment, parr significantly preferred control water over the odor of either confined or unconfined fish (Table 2.1). In the odor discrimination experiment, both ECH and OAF parr significantly preferred the odor of unconfined conspecifics over that of confined fish (Table 2.2). In this experiment, the number of parr choosing to not enter either arm varied between tests (17 and 52% for ECH and OAF parr, respectively), but arm choice was consistent; in 11 of 12 trials, more fish were recorded in the arm with water from unconfined parr than in the arm with water from confined parr.

In both experiments, parr confined for 12 h in the conditioning tank displayed behavioral signs of stress (e.g., initial excited swimming, disequilibrium, and excessive opercular movements) that were absent in unconfined fish. Physiological differences were also apparent; plasma cortisol titers were significantly elevated in the confined parr as compared to unconfined parr and fish from the stock tank (Figure 2.2). Although the cortisol concentrations in the unconfined ECH parr was significantly higher than in fish from the stock tank, it was similar to that of both groups of unstressed OAF fish and well within the range found for rested, unstressed coho salmon (Fagerlund et al. 1983; Patiño et al. 1986).

Discussion

The results from the first experiment demonstrate that parr can detect the odor of either confined or unconfined conspecifics, as evidenced by a strong avoidance of both odors. Moreover, the odor discrimination experiment revealed that parr preferred the odor of unstressed fish over that of stressed fish.

Table 2.1. Selection of odors by coho salmon parr from Eagle Creek Hatchery in paired choice tests using control water (C) and odors from stressed (S) or unstressed (U) hatchery conspecifics; na = not applicable. Asterisks denote significant departure from a 1:1 ratio at $P < 0.001$.

Treatment	Odor selection			No Choice	$\chi^2_{\text{corr.}}$
	C	U	S		
Control vs. Unstressed parr odor	32	5	na	23	18.3***
Control vs. Stressed parr odor	38	na	8	14	18.3***

Table 2.2. Selection of odors by coho salmon parr from Eagle Creek Hatchery (ECH) and Oregon Aqua Foods (OAF) in paired choice tests using odors from stressed and unstressed hatchery conspecifics. Asterisks denote significant departure from a 1:1 ratio at $P < 0.05^*$ and $P < 0.001^{***}$.

Test fish	Odor selection		No Choice	$\chi^2_{\text{corr.}}$
	Unstressed parr odor	Stressed parr odor		
ECH	33	17	10	4.5 *
OAF	26	3	31	16.7 ***

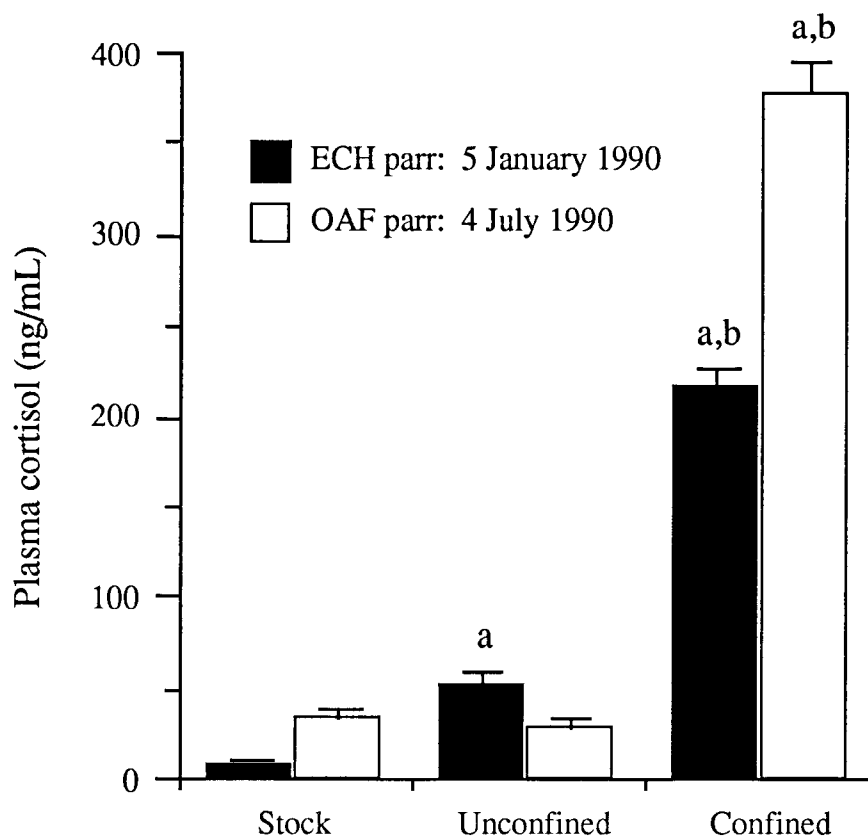


Figure 2.2. Mean plasma cortisol levels of coho salmon parr acclimated to a stock tank (Stock), unconfined in an odor conditioning tank (Unconfined), and confined for 12 hours in an odor conditioning tank (Confined). Fish were sampled on dates indicated; Eagle Creek Hatchery (ECH) parr were given 7 days to adjust to conditioning tanks, while Oregon Aqua Foods (OAF) parr were given 12 days. All means and standard error of means are based on a sample of 12 fish. Letters above bars denote groups with cortisol titers significantly different ($P < 0.05$) than (a) hatchery conspecifics kept in a stock tank or (b) conspecifics unconfined in the odor conditioning tank.

This finding was consistent using fish from both ECH and OAF salmon stocks, and reveals that odor of stressed fish is unquestionably the more repulsive of the two since parr in this experiment swam toward an odor (unstressed fish) which they avoided in the odor detection experiment. As expected, confined parr had significantly higher cortisol titers than unconfined fish, suggesting that the observed avoidance behavior is correlated with the physiological stress response of confined fish used as odor sources. In addition, the ratio of fish choosing water from unstressed parr over that from stressed parr was nearly five times greater in the second test, corresponding to the higher cortisol titers of confined OAF parr (1.7 times greater than titers of confined ECH parr in the first test).

Confined fish in the present experiment were undoubtedly distressed and the significant and consistent avoidance of their odor suggests that young salmon may be capable of responding in an adaptive manner to chemical cues from injured conspecifics (e.g. hiding in a shaded area away from a distressed fish's odor). This finding contrasts with negative responses observed using skin extracts (Brett and MacKinnon 1954; Schutz 1956; Marusov 1975), suggesting that a salmonid avoidance behavior may require the recognition of a mixture of odors or at least an odor different than skin extract alone. However, I can only speculate as to the difference between odors from stressed and unstressed parr. When subjected to severe physical disturbances, salmonids exhibit predictable physiological responses, such as diuresis and elevated levels of plasma cortisol and glucose (Strange et al. 1978; Barton et al. 1980; Schreck 1982). Since amino acids, bile acids, intestinal contents, and skin mucus can be detected by salmonids (Hara 1972; Døving et al. 1974; Fisknes and Døving 1982; Hara et al. 1984; Rehnberg et al. 1985), it is possible that confined parr in the present study

responded to a severe crowding stress by secreting body fluids into their holding water at levels that were repellent to fish in the maze. Also, it cannot be ruled out that stressed fish released different chemicals than unstressed fish, and not just copious amounts of body fluids.

Alarm substances have been defined as chemicals which “communicate the presence of danger in a given species provided that they are produced by members of the same species” (Pfeiffer 1977). Alerted fish (receivers) displaying a fright reaction presumably reduce their chance of suffering the injury that has befallen the sender (Smith 1982). The strongest fright reaction is sudden, rapid swimming and avoidance of the secretion, while the weakest response is characterized by temporary crowding and uneasiness (Pfeiffer 1963b). The significant avoidance identified in my study suggests that salmon parr may release an alarm substance, but whatever the chemical(s), it does not induce a classical fright reaction as described by Pfeiffer (1963b). While avoidance behavior in an experimental situation does not indicate that such a response occurs in nature, my results do suggest that salmonids can discriminate between intraspecific chemical cues and that chemoreception may play an important role in the recognition and avoidance of life-threatening conditions.

While numerous orientation studies have demonstrated that salmonids are generally attracted to odors from familiar conspecifics, there is also evidence that they may be repelled by the odor of threatened fish. Stabell (1982) observed that Atlantic salmon parr avoided water conditioned by conspecifics in favor of unconditioned (blank) water. He suspected that the avoidance response was evoked by a “distress signal” released by parr that had been unintentionally crowded in the conditioning tank. Similarly, Colley (1981) hypothesized that a

“fright substance” was responsible for unexpected avoidance responses observed during orientation experiments with adult chum (*O. keta*) salmon. In his experiments, test fish were slightly attracted to the odor of a single female, but the attraction was eliminated when odor from a group of females (i.e., a higher density of fish) was used. In addition, test fish were strongly repelled by the odor of a particular male chum apparently stressed by confinement in the conditioning tank. Hence, my results appear to augment similar findings by Colley (1981) and Stabell (1982). Furthermore, my results demonstrate that salmonids that have been stressed clearly impart something into the water that can be recognized and avoided by conspecifics.

Based on my findings, it seems prudent to exercise caution when conducting experiments using “natural” fish odors. Laboratory studies with other animals demonstrate that stressful conditions can alter an organism’s odor and the subsequent behavior of test animals. For example, stressed earthworms (*Lumbricus terrestris*) secrete a mucus substance that repels conspecifics as well as the stressed individual (Ressler et al. 1968). Similarly, rats and mice can discriminate between the odors of stressed and unstressed conspecifics (Valenta and Rigby 1968; Carr et al. 1970), and rats will increase their running time in a straight-way maze upon encountering the odor of a conspecific which had previously been stressed and removed from the maze (Courtney et al. 1968). Also, blacktip reef sharks (*Carcharhinus melanopterus*) display a “violent hunting reaction” when exposed to water from an unseen tank containing either a stressed conspecific or stressed prey species (Tester 1963). Hence, while the source of the repellent odor in the present study is not known, my results corroborate findings from other studies and underscore the need to consider the

effects of repellents when using odors from live animals in chemo-orientation studies.

CHAPTER 3. Chemosensory Discrimination of Predaceous and
Nonpredaceous Fishes by Juvenile Coho Salmon
(*Oncorhynchus kisutch*)

Abstract

In avoidance/preference tests, naive coho salmon (*Oncorhynchus kisutch*) can discriminate between four freshwater fish species solely on the basis of chemical stimuli. When exposed to pairwise combinations of odors, salmon preferred the odor of a native nonpredator (largescale sucker, *Catostomus macrocheilus*) over that of a native predator (squawfish, *Ptychocheilus oregonensis*), exotic predator (walleye, *Stizostedion vitreum*), and exotic nonpredator (common carp, *Cyprinus carpio*). The responses appear to be species-specific, suggesting that juvenile salmon may use interspecific odors to monitor nearby predators and nonpredators.

Prey species have evolved elaborate morphological and behavioral mechanisms to avoid or escape from predators; early detection of a predator would greatly reduce the risk of being eaten (Edmunds 1974; Endler 1986). While considerable research has been given to visually-mediated predator detection, evidence from numerous studies with invertebrates (Snyder and Snyder 1970; Feder 1972; Ayer et al. 1973; Hoffman 1980; Williams and Moore 1985; Martinez 1986; Dodson 1988) and vertebrates (Reed 1969; Pfeiffer 1977; Petranksa et al. 1987; Kats 1988; Magurran 1989) suggests that chemical cues (hereafter referred to as "odors") may play an important role in the recognition and surveillance of aquatic predators.

Pacific salmon (genus *Oncorhynchus*) have a keen chemosensory system, able to discern an array of odors and direct both macro- and microhabitat shifts that may serve to protect an individual as well as maintain discrete populations or stocks (Hasler and Wisby 1951; Ricker 1972; Leggett

1977; Nordeng 1977). While in fresh water, juvenile salmon (parr) are preyed upon by numerous piscine predators that may emit species-specific odors that are detectable by parr (Rehnberg and Schreck 1987). Surprisingly, few studies have assessed a salmonid reaction to predator odors. In one, dilute rinses of mammalian skin (e.g., seal skin, bear paw, and human hand) were found to elicit a strong avoidance reaction from adult salmon ascending a fish ladder en route to their spawning grounds (Brett and MacKinnon 1954). Subsequent testing resulted in the identification of the amino acid l-serine as an active repellent in mammalian skin and sweat (Idler et al. 1961). In a second study, coho salmon (*O. kisutch*) parr demonstrated an innate avoidance reaction to odor from the predaceous northern squawfish (*Ptychocheilus oregonensis*), but not odor from the nonpredaceous largescale sucker (*Catostomus macrocheilus*) (Rehnberg and Schreck 1987).

While it is known that salmon can detect the odors of predators and nonpredators, it is not known whether or not the fish can discriminate between them. Such an ability may allow salmon parr to survey odors from local fishes and take evasive action upon detecting a predator that is nearby but out of sight. I hypothesized that natural selection may have favored fish that could use chemical cues to discriminate between a predator and nonpredator, and that this could result in an innate avoidance of native predators. Hence, I tested the specificity of the salmon's chemotaxic response to odors of a native predator (squawfish) and nonpredator (largescale sucker), both species native to waters where juvenile coho salmon would have an evolutionary history. As a logical extension to this hypothesis, I further theorized that parr may not be able to discriminate between odors from exotic species, i.e., those with which they have

not coevolved. Therefore, I also evaluated the salmon's responses to odors from an exotic predator (walleye, *Stizostedion vitreum*) and nonpredator (common carp, *Cyprinus carpio*), both species which have recently been introduced into rivers of the Pacific Northwest (Scott and Crossman 1973; Lee et al. 1980).

Avoidance/preference behavior was assessed in two experiments using a flow-through Y-maze apparatus (Figure 3.1). Experiment I consisted of a control test and four single-donor tests. In the control test, parr were presented with a choice between two solutions of blank well water (control water) to verify random choice of maze arms, while in single-donor tests, they were presented with a choice between control water and water in which one of the four donor species had been kept (conditioned water). In experiment II, I conducted six paired-donor tests using water conditioned by each donor species in opposite arms. These tests were designed to determine if parr would discriminate between pairwise combinations of the odors from the four donor species. Tests in experiment II were repeated 1 month later and demonstrated that parr from different populations exhibit similar avoidance/preference behavior. All tests were performed between 17 August and 28 December 1990.

Coho salmon parr from the Eagle Creek National Fish Hatchery (mean weight 23 g) and Oregon's Fall Creek fish hatchery (mean weight 38 g) were moved to Oregon State University's Smith Farm Experimental Facility in Corvallis, Oregon. Parr were fed to repletion daily and acclimated to well water (12-13° C) in flow-through tanks for at least one month prior to testing. Fall Creek parr were used only in the second of experiment II's tests. Since all parr were reared in hatcheries they did not have contact with other fish species. Furthermore, the four donor species (squawfish, largescale sucker, walleye, and

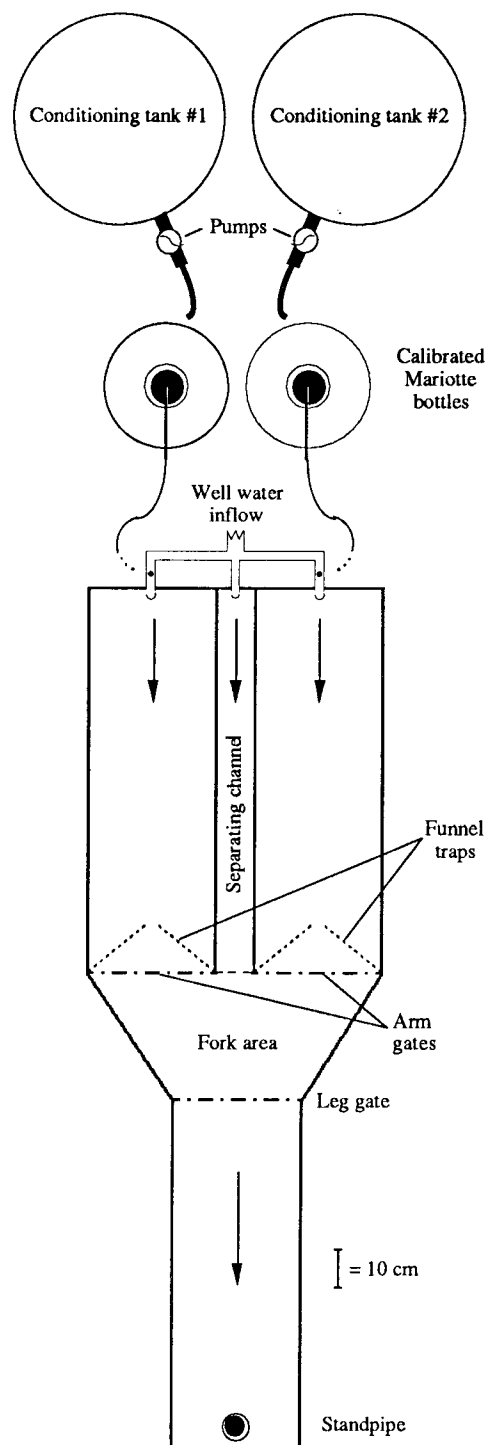


Figure 3.1. Top view of flow-through Y-maze, conditioning tanks (not to scale), and Mariotte bottle delivery system.

carp) do not inhabit waters supplying either hatchery, therefore parr had no prior exposure to chemical cues associated with these predators and nonpredators. However, some or all of these species may be encountered by other populations of salmon while residing in fresh water. Adult squawfish, carp, and suckers were electrofished from the Willamette River, Oregon, and adult walleye were caught by hook and line from the Columbia River. Fish of each donor species were transported to the laboratory and held in separate holding tanks receiving flow-through well water. All donor species were fasted for at least 20 days prior to being used as an odor source in order to reduce the amount of fecal matter in test solutions. I used only donor fish that were in good physical condition, and left them undisturbed for one week before using them in subsequent tests.

To obtain test solutions of odorants, two or three fish (approximately 2 kg total weight) of each donor species were netted and placed into one of two identical fiberglass tanks (1 m diameter) with 140 l of aerated, static well water. Control tanks in experiment I contained only aerated, static well water. After 12 hours, donor fish and airstones were removed. To control for any odor associated with netting the donor fish, several sweeps with a clean net were also made in the control tanks. Test solutions were mixed for 5 seconds and pumped to their respective Mariotte bottles just prior to each trial.

Well water was pumped into the head of each arm and the separating channel at a rate of 3.8 l/min. Test solutions were added via constant-head Mariotte bottles situated on a shelf over each arm. Flexible tubing allowed test solutions to be easily alternated between maze arms. In both experiments, test solutions were introduced into the arms of the maze at a rate of 240 ml/min (1:15, Mariotte bottle drip rate:maze arm flow rate). A flowing channel served

to reduce mixing between the arms and was screened to prevent fish from entering. One-way funnel traps at the arm entrances helped to keep parr within an arm after entering it, and a fixed screen prevented them from leaving the fork area of the maze. To encourage parr to swim into the arms, the fork area was illuminated, whereas the arms and separating channel were shaded.

A trial began when 10 parr were placed into the fork area of the maze (with arm gates down) and test solutions were introduced into each arm from the Mariotte bottles. After a 10-minute "calming" period, both arm gates were lifted and parr were allowed 25 minutes to enter an arm or remain in the fork area. Observations through a one-way window revealed that parr swam singly and in groups throughout the fork area and thus were able to sample both test solutions. Also, fish appeared to make arm choices independent of other fish (i.e., "following behavior" or fighting was uncommon). Following this choice period, the arm gates were quickly lowered and the numbers of parr in each arm and the fork area recorded. After removing all parr, the maze was drained, thoroughly rinsed, and filled for the next trial; test solutions were alternated between the arms by switching the Mariotte bottle inflow lines. Using naive parr for each trial, I conducted six or twelve trials for each test. The numbers of fish choosing each arm were compared to a random arm selection model (1:1) using a two-class chi-square test with the Yates correction for continuity (Zar 1984). Preference or avoidance of one test solution over another was defined as a significant departure from this 1:1 ratio.

Parr in the control test had no preference for solutions from either Mariotte bottle (i.e., parr apparently did not perceive differences in odors from the two conditioning tanks or Mariotte bottles), but did demonstrate a slight but

significant ($P=0.04$) preference for the left arm of the maze. However, I feel that this preference is anomalous because fish had no preference ($P=0.71$) for either side of the maze during single- and paired-donor tests (292:301, left arm choice:right arm choice). Parr strongly avoided rinses of all donor species in single-donor tests (Figure 3.2), and consistently preferred to enter arms receiving only control water. Their strong avoidance of sucker rinse in these single-donor tests differs from the “no response” observed by Rehnberg and Schreck (1987). The reason for this difference is unclear, but may be related to the fact that parr in their study were much more likely to leave the fork area and enter either arm (86% of fish tested) than parr in my experiments (47%). Parr swam normally in the maze and fed actively soon after being tested, hence they did not appear to be severely stressed by test protocol. Also, in an ancillary test, parr strongly avoided odor from suckers left undisturbed for six days in their odor conditioning tank (i.e., not netted as in the present experiments). Therefore, I do not believe that the avoidance observed was due to stress-related odors from donor species elicited by a brief handling (netting). Another possible reason my results differ is due to dietary-related odor differences between the suckers used in my experiments and those used by Rehnberg and Schreck (1987). Keefe (1990) has shown that the diet of a donor fish may alter the fish's odor and the subsequent orientation behavior of test fish. Unfortunately, I do not know the dietary history of donor fish in the present study, except that all were fasted for at least 20 days prior to being used as an odor source.

In the paired-donor tests of experiment II, both Eagle Creek and Fall Creek parr demonstrated similar preferences (Table 3.1). Although the number

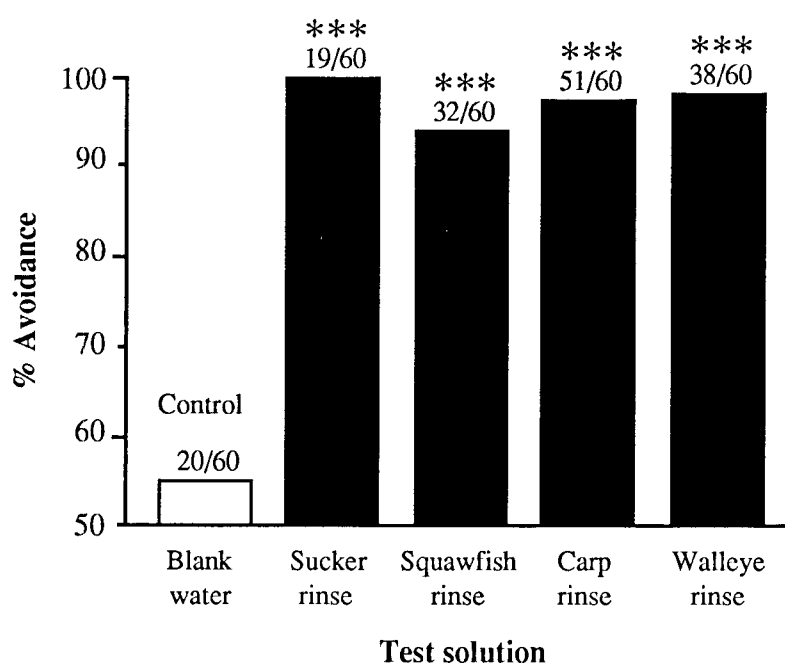


Figure 3.2. Percent avoidance of various test solutions by coho salmon parr. Fractions above bars denote the number of parr making a choice and the total number of parr tested. Asterisks indicate tests significantly different ($P < 0.001$ ***) than random arm choice (i.e. 50%) based on a χ^2 test with the Yates correction for continuity.

Table 3.1. Avoidance/preference of paired test solutions by two stocks of coho salmon parr. D1, number of parr choosing arm of donor species #1; D2, number of parr choosing arm of donor species #2; nc, number of fish choosing neither arm. Asterisks denote nonrandom arm selection ($P < 0.05^*$ and $P < 0.001^{***}$) based on a χ^2 test with the Yates correction for continuity.

Paired test solutions (donor #1 vs. donor #2)	Eagle Creek parr					Fall Creek parr				
	D1	D2	nc	N	$\chi^2_{\text{corr.}}$	D1	D2	nc	N	$\chi^2_{\text{corr.}}$
Sucker vs. Squawfish	16	4	100	120 ^a	6.1 *	44	6	10	60	27.4 ***
Sucker vs. Carp	24	5	31	60	11.2 ***	36	12	12	60	11.0 ***
Sucker vs. Walleye	38	1	21	60	33.2 ***	43	10	7	60	19.3 ***
Walleye vs. Carp	55	3	2	60	44.8 ***	44	7	9	60	25.4 ***
Walleye vs. Squawfish	18	2	40	60	11.3 ***	46	9	5	60	23.6 ***
Squawfish vs. Carp	34	3	23	60	24.3 ***	30	13	17	60	6.0 *

^a Twelve trials were conducted and their data pooled since only five parr chose an arm (sucker) in the first six trials and expected values would have biased the χ^2 analysis.

of parr making a choice varied considerably (17-97% of parr in each test), the direction of avoidance/preference was consistent between trials; in nearly every trial more fish chose the odorant that was significantly preferred overall. Of the six paired-donor comparisons, sucker rinse was always preferred (over the opposing species' rinse), whereas carp rinse was always avoided. Walleye rinse was preferred when tested against rinses of squawfish and carp, and squawfish rinse was always avoided except when carp rinse was present in the opposite arm. Test results for this experiment were repeatable using odorant solutions from new squawfish, suckers, and carp, therefore I believe that the conditioned water was indeed a species-specific solution and not based solely on qualities of individual donor fish.

Based on these results, it is apparent that parr can detect body rinses of these four donor species, and can discriminate between odors of a native predator and nonpredator. In addition, this discrimination may break down with exotic predators and nonpredators, since parr overwhelmingly avoided the odor of carp in favor of that of walleye. I can only speculate as to the motivation behind these avoidance/preference responses. For example, some of my results can be interpreted as risk assessment by parr. It has recently been demonstrated in the laboratory that salmonids subjected to a predation risk from a native predator will alter their foraging tactics (e.g., shorten attack distance and reduce movements) to minimize their chances of being eaten or injured (Dill and Fraser 1984; Metcalfe et al. 1987a; Metcalfe et al. 1987b). Field studies also suggest that salmonids will alter their use of stream habitats to minimize predation by squawfish (Eggers 1978; Brown and Moyle 1991). If the hatchery coho salmon parr used in my experiments (fish which have never been in a natural

environment nor encountered any of these donor species) represent a lineage of salmon that were selected for their ability to discriminate between the native sucker and squawfish, but not the exotic carp or walleye, then they may respond to the odor of these latter species in a cautious manner. This might elicit from parr a generalized “fear of the unknown”, whereby they would avoid an exotic fish’s odor even if it meant swimming into an area with a “known” predator’s odor. In other words, if parr have evolved effective antipredator responses towards the native squawfish, they may find themselves at a dangerous disadvantage if they draw near to a totally unfamiliar species. Indeed, when presented with choices between odors from native species and odors from exotic species, parr chose the native species’ odor in four out of five tests. However, since coho parr were repelled more strongly by squawfish odor when tested against walleye odor, my results also suggest that odors from some exotic fishes may in fact be less threatening than those from a known predator. In light of this, the parr’s behavior appears to be complex and not simple avoidance of all predators and non-avoidance of nonpredators.

Another possible explanation is that the avoidance or preference of a particular species’ odor is governed by the quality and quantity of the odor itself, and not coupled with any instinctive assessment of risk. Although all test solutions were presented at the same rate, it is possible that the concentration of odor perceived by parr may have differed due to species-specific release rates. For example, the complete avoidance of the nonpredaceous carp’s odor may actually be due to parr perceiving this species as having an overpowering or offensive smell/taste, and not associated with any perception of threat.

In summary, my results demonstrate that coho salmon parr readily detect

and discriminate between the odors of different fish species, including odors from a native predator and nonpredator. Furthermore, parr in my experiment had no prior exposure to odors of any of the donor species, hence the avoidance/preference responses appear to be independent of any conditioning. Such an ability suggests that interspecific chemical cues may provide salmon with an innate means to monitor other fishes and reduce their chance of encountering predators, especially in conditions where visibility is poor.

CHAPTER 4. Avoidance/Preference Behavior of Juvenile Coho
Salmon (*Oncorhynchus kisutch*) Exposed to Pulp Mill Effluent

Introduction

Coho salmon (*Oncorhynchus kisutch*) smolts encounter large discharges ($>1.7 \times 10^8$ liters/day) of pulp mill effluent during their downstream migration through Grays Harbor, Washington. Smolt survival is believed to be reduced by the impaired water quality resulting from effluent releases (Seiler 1989), and exposure to even sublethal concentrations of these effluents could disrupt the orientation behavior of young salmon.

The purpose of my study was to assess behavioral responses of young coho salmon to acute exposures of effluent from two Grays Harbor pulp mills. The first objective was to determine the avoidance/preference behavior of smolts to several effluent dilutions. Assays were designed to determine effluent concentrations that are neither attractive nor repulsive (i.e., nondisruptive) to smolts. A second objective was to determine if the effluents could mask perception or alter behavior to another odorant. I did this by testing whether the effluents masked the response to L-serine, an amino acid found in mammalian skin and known to elicit strong avoidance behavior in salmonids (Brett and MacKinnon 1954; Rehnberg and Schreck 1986; Rehnberg and Schreck 1987). This objective was to evaluate the possibility that effluent could confound recognition of a biologically significant odorant. The idea was to test the ability of the salmon to avoid the amino acid in the presence of effluent at concentrations found to be unavoided when no amino acid was present. Rehnberg and Schreck (1986) found that toxicants such as heavy metals can eliminate recognition of other odorants. During smolt migration, such a masking effect by effluent could have deleterious consequences for processes such as predator avoidance, foraging, and imprinting.

Materials and Methods

Coho salmon smolts (mean weight = 24 g) were transported from Washington's Humptulips Hatchery to the testing site at the Aberdeen Sewage Treatment Plant in Aberdeen, Washington. Fish were held outdoors in 750L flow-through circular tanks supplied with water from a nearby tributary [Wishka River water (WRW)], and given at least six days to adjust to these conditions before testing.

I tested effluent routinely discharged by the Weyerhaeuser Company (WeyCo) and ITT Rayonier (ITT) pulp mills. Since WeyCo effluent is acidified to a pH = 3.0 prior to release (to retard growth of fecal bacteria), this effluent was neutralized with NaOH prior to testing. Wishka River water (13-15°C, pH = 7.1) was used in control trials and served as the diluent for effluent solutions. For each effluent, I tested 3-4 concentrations likely to be encountered by smolts in the harbor: 30%, 3%, 0.3%, or 0.03% (v:v). The effluent concentration that did not elicit significant avoidance was subsequently mixed with 10^{-4} M L-serine (an L-serine concentration avoided by smolts in a preliminary positive control test) then tested to determine if L-serine avoidance is diminished by the presence of pulp mill effluent. All tests were conducted <24h after collecting effluent from each mill and allowing it to reach temperatures equivalent to WRW.

The avoidance/preference behavior of smolts was assessed using two identical Y-mazes, each illuminated with one 100 watt incandescent light fixture (Rehnberg et al. 1985). All tests were conducted between 0600 -1800 hr. Test solutions were supplied via a constant-head delivery system capable of delivering test solution to one arm of the maze and WRW to the other arm; flow rates in each arm were 3.6 L/min. The mazes and the delivery system were drained and

thoroughly rinsed with WRW between trials. To begin a trial, a single naive fish was placed in the downstream end of the maze. After a 12 minute adjustment period, a gate was lifted and the fish was allowed 5 minutes to choose between entering either the control or effluent-treated arm, or remaining in the fork area. After this 5 minute choice period, arm gates were dropped and fish choice recorded. Mazes were then drained, rinsed thoroughly, and refilled with WRW. Test solutions were alternated between the two arms of each maze after each trial.

In most tests, the numbers of fish choosing each arm were compared to a random arm selection model (1:1) using a chi-square goodness of fit test with the Yates correction for continuity (Zar 1984). Avoidance or preference was defined as significant departure from this 1:1 ratio using a $\chi^2 > 3.8$, ($P < 0.05$). In tests where L-serine was mixed with unavoided concentrations of each mill's effluent, arm selection frequencies were compared to those obtained in the positive control test using Cochran's corrected chi-square statistic for a 2x2 contingency table (Zar 1984).

Results and Discussion

Both WeyCo and ITT effluents at 30% were significantly avoided (Figure 4.1). Further testing revealed that effluent concentrations had to be reduced to 0.03% WeyCo and 0.3% ITT before they were no longer avoided. For each test, the number of fish not choosing either arm was variable (2-8 fish) for each effluent (Figure 4.1). Fish in the control test selected each arm with equal propensity (i.e., no significant difference from a 1:1 ratio). These results suggest that coho smolts are sensitive to minute amounts of both pulp mill effluents and, given the choice, generally prefer to avoid such waters.

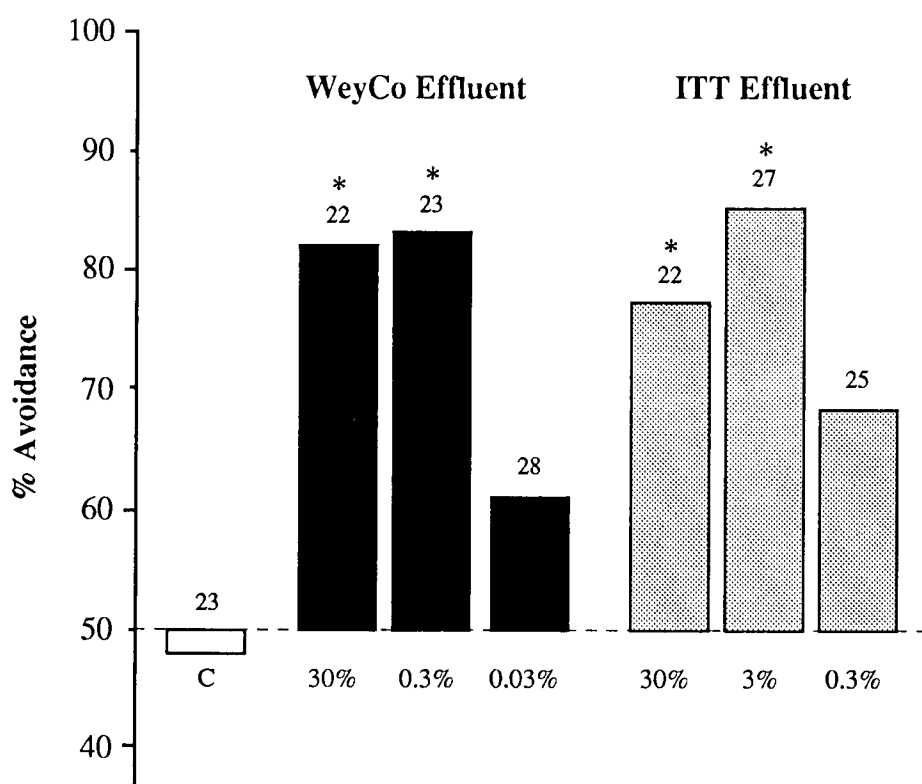


Figure 4.1. Avoidance of various dilutions of pulp mill effluents (percentages below bars) by juvenile coho salmon. "C" represents control test. Numbers above the bars indicate the number of fish entering either arm of the Y-maze. Asterisks refer to significant differences from a χ^2 test ($P < 0.05$).

Previously unavoided concentrations of effluent (0.03% WeyCo and 0.3% ITT) did not appear to inhibit the detection of 10^{-4} M L-serine; arm selection behavior was not significantly different than that in the positive control test (Figure 4.2). Therefore, it can be concluded that low concentrations of effluent probably do not interfere with the detection of a generally repulsive constituent of mammalian (i.e., predator) skin odor.

Effluent concentrations avoided by smolts in the present study are comparable to (or lower than) those reported as either repulsive or toxic in other studies with salmonids (Jones et al. 1956; Sprague and Drury 1969; Walden 1976; Fisher 1982). Ancillary experiments (Stone and Schreck unpub.) revealed that an acute exposure (3-4h) to 30% WeyCo resulted in a significant increase in plasma cortisol titers in coho salmon, indicating a stress-related physiological correlate to the acute behavioral reactions in the present study. Other studies have demonstrated that acute effluent exposures can elicit secondary stress responses from salmonids (McLeay and Brown 1975; McLeay 1977). Thus, while the actual effluent concentrations encountered by emigrating coho salmon in the inner harbor are not known, it appears that smolts perceive at least higher effluent concentrations as noxious and respond accordingly.

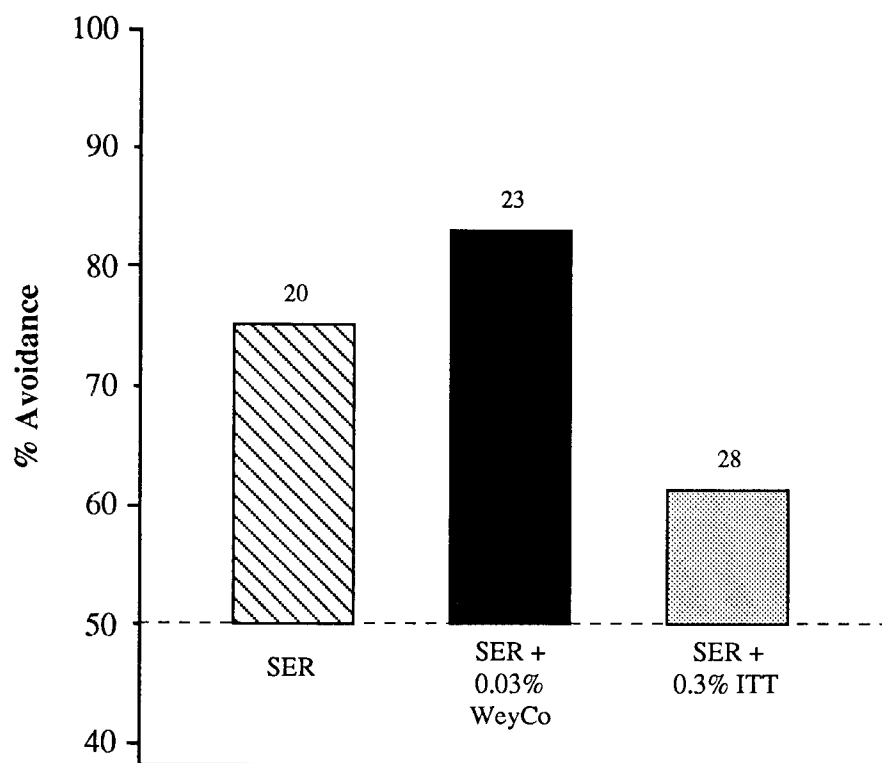


Figure 4.2. Avoidance of 10^{-4} M L-serine (SER) in control water only and when mixed with two previously unavaoided effluents (percentages below bars).

Numerals above the bars indicate the number of fish entering either arm of the Y-maze. Arm selection in the effluent tests was not significantly different from the test with SER only (χ^2 test, $P > 0.05$).

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