

Facilitative Effect of Preexposure on Heart-Rate Conditioning to an Olfactory Cue in Atlantic Salmon (*Salmo salar*)

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To examine the general principle of latent inhibition in the olfactory modality of Atlantic salmon (*Salmo salar*), we measured levels of heart-rate conditioning after a nonreinforced exposure to L-cysteine during the postsmoltification period for smolts and desmolts (juvenile salmon retained in freshwater). Odor preexposure occurred after the sensitive period for olfactory imprinting. A context-related hypothesis predicted that latent inhibition would retard conditioning in both smolts and desmolts. However, preexposure to L-cysteine facilitated the acquisition of the conditioned response in both groups as compared with nonexposed fish. This suggests that the capacity of juvenile salmon to learn and store odor information persists beyond the sensitive period of olfactory imprinting. Heart-rate reduction was also more accentuated in smolts than in desmolts and may possibly be related to greater activity of the thyroid gland in smolts.

Recognition of a home site in a variety of fish species has often been attributed to learning mechanisms that involve olfaction (see review by Dodson, 1988). The olfactory hypothesis of home stream recognition by returning adult salmon (see review by Hasler & Scholz, 1983) proposes that the olfactory identity of the natal stream is imprinted and registered in long-term memory during the crucial developmental period known as *smoltification*. Morphological, physiological, and behavioral changes that preadapt young salmon to life in sea water occur during this period. The odor memory is subsequently recalled during the spawning migration. In juvenile Atlantic salmon (*Salmo salar*), experiments that have demonstrated a long-term memory, which resulted from a nonreinforced experience with an odor during smoltification (Morin, Dodson, & Doré, 1989a), provide evidence of a sensitive period for olfactory imprinting. Fish were exposed to the amino acid L-cysteine during one of six 8-day intervals of smoltification, and their unconditioned heart-rate reduction to L-cysteine was measured after smoltification. It was found that the magnitude of such reduction was greater in

fish preexposed to L-cysteine from the 21st to the 28th day of smoltification as compared with fish preexposed to L-cysteine during any other interval of smoltification. In these experiments, an amino acid was chosen as a substitute for natal stream odor principally for ease of quantification of the olfactory stimulus. Free amino acids are found everywhere in the natural environment and in the mucus of fish and are likely candidates as biologically meaningful chemical signals (reviewed by Hara, MacDonald, Evans, Marui, & Arai, 1984). Compared with other amino acids, L-cysteine is highly effective in evoking olfactory receptor responses in fishes and is an effective conditional stimulus (Morin, Dodson, & Doré, 1987).

A nonreinforced experience with a stimulus may either inhibit or facilitate subsequent conditioning to that stimulus depending on the context in which stimulus preexposure occurs in relation to that of the conditioning situation (Lubow, Rifkin, & Alek, 1976). In a conditioned stimulus (CS) preexposure paradigm, latent inhibition (Lubow & Moore, 1959) generally results when conditioning occurs in the same environment as that experienced during preexposure, provided that the conditional stimulus is identical to that of the preexposure experience. Thus, latent inhibition typically occurs when an animal is conditioned to a familiar stimulus in a familiar environment as compared with a novel stimulus in a familiar environment (Channel & Hall, 1981, 1983; Doré, 1981, 1984). Latent inhibition has been found in a variety of testing situations and species (Lubow, 1973). However, it has not been clearly demonstrated in fish, and species other than goldfish (*Carassius auratus*) have not been tested (Braud, 1971; Kitagawa, 1976; Shishimi, 1985).

The objective of this study was to examine the general principle of latent inhibition in the olfactory modality of Atlantic salmon. Except for one study (Lubow et al., 1976), this important sensory system in the life of many animal

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species has not been used to demonstrate latent inhibition in a CS preexposure paradigm. In this study, we measured heart-rate conditioning in smolts in the presence of L-cysteine after nonreinforced exposures to that odorant during the post-smoltification period. A critical element of this procedure is that odor preexposure occurred after the sensitive period for olfactory imprinting, at a time when the presence of environmental odors is expected to be less important for imprinting and subsequent home stream recognition. In the natural life cycle of Atlantic salmon, freshwater odor cues become unavailable to smolts that after smoltification migrate to the sea. We also measured heart-rate conditioning in desmolts after nonreinforced exposures to L-cysteine during the postsmoltification period. Desmolts are fish that have undergone a process of desmoltification because they have been prevented from migrating to sea after the smoltification period (Evroytseva, 1963). They are behaviorally and physiologically different from smolts. Desmolts of Atlantic salmon migrate upstream and lose their capacity to tolerate seawater, whereas smolts move downstream and exhibit a marked hypoosmoregulatory ability in seawater (Lundqvist & Eriksson, 1985). The use of both desmolts and smolts allows us to compare olfactory learning in preexposed fish of approximately the same age but belonging to different developmental stages. Because we examine heart-rate conditioning to a familiar stimulus in a familiar environment rather than a novel stimulus in a familiar environment, a context-related hypothesis predicts the occurrence of latent inhibition to retard conditioning in both smolts and desmolts.

Method

Subjects

The subjects were 24 two-year-old juvenile Atlantic salmon (*Salmo salar*). They were hatchery-raised offspring of a wild stock obtained from the Sainte-Anne River, located on the Québec North Shore, 370 km to the northeast of Québec City, Canada. Fish were transported to the Aquarium du Québec and housed in a 300-L rectangular home tank (157.5 cm wide \times 50.8 cm high \times 35.6 cm deep). A second 300-L recovery tank was used to house the fish between training sessions. The home tank was supplied with dechlorinated water (4.0 °C \pm 0.5) and maintained under a constant 8:16-hr light-dark photoperiod regime. From December 23 to 29, 1984, after 30 days of holding, smoltification was induced in all fish by acclimating them to 11.0 °C \pm 0.5 by 1 °C daily increments and to a long 16:8-hr light-dark photoperiod by 1-hr daily increments. In the laboratory, smoltification began on December 30, 1984, and was completed on February 25, 1985, for a total smoltification period of 58 days. Fish were fed daily with a mixture of shrimp, crabmeat, and scallops.

Apparatus

Heart-rate responses of fish to an odoriferous stimulus were assessed in an experimental chamber connected to different water lines and to computer-assisted electronic systems. This procedure is fully described in Morin, Verrette, Dodson, and Doré, (1987). The main water line contained dechlorinated water that flowed at a constant velocity (20.5 mm/s) from a head tank to the experimental chamber before its evacuation through an outlet pipe. The stimulus-input line

to the experimental chamber delivered either water from the head tank or a chemical stimulus from an Erlenmeyer flask that contained the stimulus solution and had been placed in the head tank. Water temperature in the head tank as well as in the experimental chamber remained constant (11 °C \pm 0.5) for the entire study. A peristaltic pump, which was controlled by a pair of solenoid valves, was used for stimulus injection. A gill-perfusion line brought water from the head tank directly to the gills of fish (300 ml/min). The experimental chamber was equipped with immobilizing devices to hold the fish in position for adequate monitoring and stimulation. Electrodes used for delivering conditioning shocks to the fish or for monitoring cardiac rhythm were connected to a modular instrument system, and those used for chemodetection were connected to a chemical calibration system. Both electronic systems interacted with a microcomputer that controlled all events of the experimental sessions. The experimental chamber was located in a black box to eliminate any external visual disturbance.

Procedure

Four groups of fish were conditioned to L-cysteine. Immediately before conditioning, the fish designated as smolts by their silvery appearance were either exposed to L-cysteine (*preexposed smolts*) or to distilled water (*nonexposed smolts*) 31 days after the end of smoltification. Similarly, fish designated as desmolts by the reappearance of freshwater juvenile characteristics (e.g., contrasting dark back and yellowish belly and vertical parr marks) were either exposed to L-cysteine (*preexposed desmolts*) or distilled water (*nonexposed desmolts*) 152 days after the end of smoltification.

Each fish was initially anesthetized with a 1% methyl pentynol solution, then injected in the dorsal musculature with a neuromuscular relaxant, d-tubocurarine chloride (0.002 mg/g body weight), and placed in the experimental chamber. Injection of small quantities of tubocurarine does not interfere with learning or with odor sensitivity (Morin et al., 1987, 1988). In the two groups of preexposed smolts and desmolts, a session began with a 25-min acclimatization period followed by 10 preexposures to L-cysteine. Resting heart rate was measured during the last minute of each 5-min interval during the acclimatization period (5 measures/subject). A preexposure trial lasted for 8 s and began with 7.3 ml of L-cysteine (5.7×10^{-3} mol) injected into the experimental chamber. When the stimulus reached the fish, it was diluted to a concentration of 3.8×10^{-4} mol. Such preexposure to L-cysteine did not elicit any subsequent unconditioned cardiac responses to L-cysteine during conditioning. The 10 preexposure trials were separated by short intertrial intervals of random duration (45–135 s) and were without shocks. Fish were conditioned to L-cysteine 5 min after the last odor-preexposure trial. Each fish was presented repeatedly with the systematic pairing of an odorous stimulus shortly followed by an electric shock. A conditioning trial began with 7.3 ml of L-cysteine (5.7×10^{-3} mol) injected into the experimental chamber. A 2-s continuous shock stimulus was delivered to fish 8 s later. Intensity of stimulation (30–46 mA) was adjusted at the beginning of the conditioning session to produce strong bradycardia, which reduced heart rate to an average of 10 beats/min. Fish were trained over two sessions (intersession interval of 24 hr) of 20 trials each, separated by short intertrial intervals of random duration (45–135 s). Fish were removed from the apparatus between sessions and allowed to recover in aquaria. All the fish used in this experiment received the same treatment except that during preexposure trials L-cysteine was replaced by distilled water in the two groups of nonexposed smolts and desmolts.

In the presence of an odor cue, heart-rate reduction typically occurs in the context of a single interbeat interval (IBI), and the phenomenon is masked when IBIs measured during the entire odor-stimulation

Table 1
Resting Heart Rate (in Beats Per Minute) During Acclimatization Period and Heart Rate Reduction (in Percentages) During Preexposure Periods in Four Groups of Juvenile Atlantic Salmon

Group	n	Resting heart rate <i>M</i> ± <i>SE</i>	Heart rate reduction	
			Water <i>M</i> ± <i>SE</i>	L-cysteine <i>M</i> ± <i>SE</i>
Preexposed smolts	6	81.9 ± 1.9		0.6 ± 0.0
Preexposed desmolts	6	69.8 ± 2.9		0.2 ± 0.1
Nonexposed smolts	6	84.1 ± 2.5	0.4 ± 0.1	
Nonexposed desmolts	6	68.2 ± 3.2	0.5 ± 0.1	

interval are averaged. For this reason, the largest IBI recorded 8 s before odor presentation was compared with the largest IBI recorded 8 s into odor stimulation. The percentage of heart-rate reduction ($100 - [\text{prepresentation IBI} \times 100 \div \text{presentation IBI}]$) was averaged for each block of five conditioning trials. In addition, the percentage of heart-rate reduction was measured for each animal during each of the 10 preexposure trials.

We have previously demonstrated that 1- and 2-year-old Atlantic salmon learned to decelerate their heart rate in response to L-cysteine and that cardiac reduction by conditioned fish was significantly greater than that of fish under a random-pairing situation in which shock stimulation randomly preceded or followed olfactory stimulation and for which the time interval between both stimuli was also randomized (Morin et al., 1987). Random pairing is a widely accepted control procedure in classical conditioning studies (Mackintosh, 1983). In addition, six groups of 2-year-old Atlantic salmon smolts conditioned to L-cysteine during one of six 8-day intervals of smoltification all learned to decelerate their heart rate in relation to random-pairing control groups (Morin et al., 1989a). Using such a methodological approach, we have consistently demonstrated conditioning to olfactory cues that is clearly more related to the CS-US relation rather than to pseudoconditioning or sensitization. For this reason, we considered the inclusion of a random-pairing control group in this experiment unnecessary.

Results

Resting heart rate measured during the acclimatization periods was greater for smolts than desmolts but similar for the preexposed and nonexposed groups of each developmental stage (Table 1). Heart-rate reduction in response to either L-cysteine or distilled water during the preexposure trials was negligible in the case of both smolts and desmolts (Table 1). Group means of heart-rate reduction in response to L-cysteine in conditioned fish treated differently on the basis of odor preexposure are presented in Figure 1 for blocks of five trials. A two-way ANOVA for both training sessions revealed a significant Groups × Blocks interaction effect, $F(21, 140) = 5.82, p < 0.01$, during heart-rate conditioning. An analysis of simple effects emphasized overall differences between groups of fish conditioned during Block 5, $F(3, 140) = 29.27, p < 0.01$, Block 6, $F(3, 140) = 25.07, p < 0.01$, Block 7, $F(3, 140) = 24.70, p < 0.01$, and Block 8, $F(3, 140) = 11.92, p < 0.01$. For each block of the second training session, a Duncan's multiple range test further indicated significantly greater heart-rate reduction in any group of preexposed fish as compared with any group of nonexposed fish ($p < 0.01$). At Blocks

5, 6, or 7, heart-rate reduction was also greater in preexposed smolts than preexposed desmolts ($p < 0.01$), whereas no difference was found during the second training session between nonexposed smolts and nonexposed desmolts.

A two-way ANOVA was also used to compare block differences in heart-rate reduction during each training session. There was a significant Groups × Blocks interaction during Session 1, $F(9, 60) = 3.60, p < 0.01$. Heart-rate reduction increased significantly from Block 1 to 4 in both preexposed smolts, $F(3, 60) = 9.44, p < 0.01$, and preexposed desmolts, $F(3, 60) = 9.04, p < 0.01$, but not in either group of nonexposed fish. In both groups of preexposed fish, heart-rate reduction increased significantly from Blocks 1 to 4 ($p < 0.01$), Blocks 2 to 4 ($p < 0.01$), and from Blocks 3 to 4 ($p < 0.01$). A significant Blocks × Groups interaction was also found during Session 2, $F(9, 60) = 6.13, p < 0.01$. More specifically, heart-rate reduction in response to L-cysteine increased significantly from Blocks 5 to 8 in nonexposed smolts, $F(3, 60) = 20.68, p < 0.01$, nonexposed desmolts, $F(3, 60) = 7.18, p < 0.01$, and preexposed desmolts, $F(3, 60) = 9.69, p < 0.01$. In both groups of nonexposed fish as well as in preexposed desmolts, significant differences were found between Blocks 5 and 8 ($p < 0.01$), Blocks 6 and 8 ($p < 0.01$), and Blocks 7 and 8 ($p < 0.01$). A significant difference between

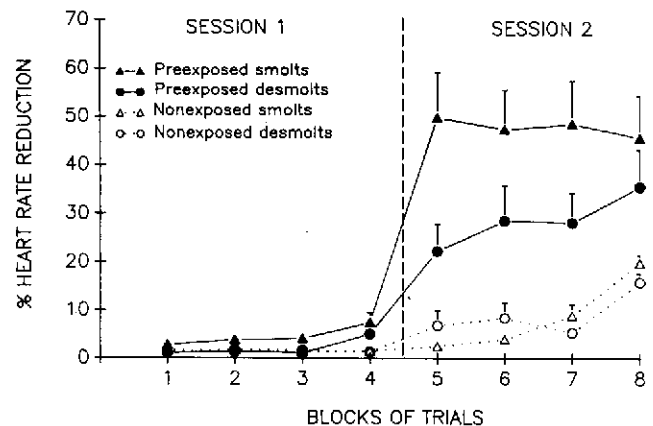


Figure 1. Percentages of heart-rate reduction (\pm SE) in response to L-cysteine per block of five trials in two groups (smolts and desmolts) of 2-year-old Atlantic salmon.

Blocks 5 and 7 ($p < 0.01$) was also found in nonexposed smolts.

Discussion

The results of this experiment demonstrate that an exposure to L-cysteine facilitates rather than retards the acquisition of a conditioned response to L-cysteine in juveniles of Atlantic salmon when conditioning occurs in the context of a CS preexposure paradigm. This facilitative effect was obtained after only 10 preexposure trials. Our results indicate that odor preexposure promotes a greater ability for olfactory conditioning in juvenile salmon after they have undergone smoltification, which suggests that smolts of Atlantic salmon can learn from an odor preexposure even if the preexposed odor is experienced after the sensitive period for olfactory imprinting. This may imply, as we suggested elsewhere (Morin et al., 1989a), that the capacity of juvenile salmon to learn and store odor information in memory persists beyond the sensitive period. Applied to the natural ecology of Atlantic salmon, our results that show a facilitative effect of preexposure on heart-rate conditioning to an olfactory cue suggest that the mere presence of an odor cue in freshwater may help smolts to learn something about the chemical characteristics of their natal river. Whether the odor information experienced after the sensitive period for olfactory imprinting is readily transformed into long-term memory and can be used by homing adults to identify their natal river remains to be demonstrated, however.

The acquisition of specific behavioral patterns other than those involved in homing ability may be facilitated in smolts as a result of being exposed to certain odors. Whereas predation is an important cause of mortality in juvenile salmon, smolts may use their odor experience for the learning of predator avoidance, which may optimize survival. Thus we hypothesize that an early odor experience improves learning if it increases survival. Evidence that such learning makes good ecological sense is found in several fish studies. For example, Goodey and Liley (1986) reported that young guppies (*Poecilia reticulata*), initially exposed to predator chemical cues, learn to escape real predators later in life, which suggests that fish may effectively use a CS preexposure learning mechanism to enhance survival. More recently, it has been shown that hatchery-raised juvenile coho salmon (*Oncorhynchus kisutch*) learned to escape predation if they had previously experienced live predators (Olla & Davis, 1989). Thus, besides suggesting some implication in homing behavior, facilitative learning effects that result from odor-preexposure may enhance survival in smolts.

Desmolts also exhibited a facilitation effect of a preexposure to L-cysteine on subsequent heart-rate conditioning. However, desmolts preexposed to L-cysteine had a significantly lower level of heart-rate conditioning than smolts. We propose that the influence of thyroid hormones on the central nervous system may account for this phenomenon. The magnitude of a conditioned response to L-cysteine changes dramatically in juveniles of different physiological states and the ability for olfactory learning during smoltification is positively correlated

with thyroid activity (Morin, Dodson, & Doré, 1989b). Maximum thyroid activity occurs during smoltification after which plasma thyroxine levels rapidly decline. A positive correlation between resting heart rate and plasma thyroxine in juvenile salmon has been documented (Morin et al., 1989b), and the higher resting heart rate of smolts as compared with desmolts in this experiment indicates that thyroid activity was greatest in smolts. Thyroid hormones are known to affect excitability by increasing the activity of adrenergic receptors (Hadley, 1988), which have been identified in the hearts of salmonids (Laurent, Holmgren, & Nilsson, 1983). It is important to note, however, that during smoltification, the magnitude of cardiac deceleration during conditioning experiments was not correlated with either resting heart rate or plasma-thyroxine levels but was maximal after surges in thyroid activity as revealed by histological measures (Morin et al., 1989b). Such surges in thyroid activity were characterized by a greater ability for cardiac conditioning to L-cysteine, and one such surge followed by apparently rapid incorporation of thyroxine by the brain and target organs was characteristic of the sensitive period for olfactory imprinting (Morin et al., 1989b). Thus, greater ability for olfactory conditioning is not simply a result of a more excitable heart under the influence of circulating thyroid hormones but appears to be the result of more specific mechanisms that involve thyroid hormones and the central nervous system.

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