Heart rate conditioning to L-cysteine and other chemical stimuli in young Atlantic salmon (Salmo salar)¹

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As a preliminary step towards the use of natural odorants in laboratory imprinting studies, olfactory learning by young Atlantic salmon, Salmo salar, was assessed by heart rate conditioning to L-cysteine (3.8 × 10⁻⁴ M). Fish were successfully conditioned and decelerated their heart rate to L-cysteine compared with that of a control group tested under random pairing sequences of L-cysteine and an electrical stimulation. The cardiac conditioning response and sensitivity of fish to L-cysteine was unaffected by a procedure of curarization. Cardiac performance of fish conditioned to L-cysteine was also compared with that of other groups of fish exposed to four different odorous substances. The conditioned response increased significantly across trials when morpholine, L-cysteine, or L-phenylalanine was used as the conditioning stimulus, but remained unchanged throughout the experiment in the presence of L-serine or L-threonine.


Comme étape préliminaire à l'utilisation de substances odorantes naturelles dans des études de laboratoire sur l'emprunte, l'apprentissage olfactif a été évalué chez de jeunes Saumons atlantiques, Salmo salar, par un conditionnement cardiaque à la l-cystéine (3.8 × 10⁻⁴ M). Les poissons ont été conditionnés avec succès et ont décéléré leur rythme cardiaque à la l-cystéine comparativement à un groupe témoin exposé à des séquences aléatoires de l-cystéine et d'une stimulation électrique. Le conditionnement cardiaque et la sensibilité à la l-cystéine n'ont pas été perturbés par la curarisation. La performance cardiaque de poissons conditionnés à la l-cystéine a été également comparée à celle d'autres groupes de poissons exposés à quatre substances odorantes différentes. La réponse conditionnée a augmenté de façon significative au cours d'essais successifs lorsque la morpholine, la l-cystéine ou la l-Phénylalanine était utilisée comme stimulus conditionnel mais est demeurée inchangée en cours d'expérience lorsque les poissons étaient en présence de l-serine ou de l-théronine.

Introduction

The precision of homing behavior in adult salmonids has often been attributed to early olfactory exposure to and long-term memory of natal river scents (see Hasler and Scholz 1983 for review) originating from aquatic plants and the soil (Hasler 1954; Hasler and Larsen 1955) composing the riverine drainage basin. Imprinting, a term derived from ethological research on precocial birds (Hess 1973), is currently used by fish biologists to describe this olfactory learning phenomenon in salmonids. However, the underlying learning process and mechanisms are poorly understood (Saglio 1986) and need further investigations (McCleave et al. 1984).

An essential criterion for the imprinting process is the existence of a sensitive period that normally occurs in the presence of a biologically meaningful stimulus (Immelmann and Suomi 1981). The major objective of our research project has been to verify in the laboratory the existence of such a sensitive period for olfactory imprinting in young Atlantic salmon, Salmo salar.

Morpholine and phenethyl alcohol, synthetic organic chemicals not normally found in the natural environment, are the only chemicals used to date in olfactory imprinting studies on homing salmonids (Stabell 1984), and morpholine has been used to investigate learning phenomena in salmonids (Wisby 1952; Walker 1967; Hirsch 1977; Sandoval 1980; Morin et al. 1987). Difficulties in obtaining normal electroencephalographic (EEG) responses to morpholine from the olfactory system of fishes (e.g., Sutterlin and Sutterlin 1971; Hara 1973; Hara and Macdonald 1975) suggest that this chemical may irritate the olfactory membrane (Hara and Brown 1979) in addition to exciting receptor cells of the epithelium. We were therefore concerned that the use of a stimulus suspected of being non-specific irritant could interfere with the demonstration of a sensitive period in the laboratory. To avoid such a possibility, we assessed the effectiveness of a natural odorant as a conditional stimulus in learning experiments.

Amino acids are potent olfactory stimulants in a number of fish species (e.g., Suzuki and Tucker 1971; Goh et al. 1979; Caprio 1980; Little 1981; Hara 1982) including salmonids (Hara 1972; Hara et al. 1973; Satou and Ueda 1975; Belghaia and Doving 1977; Bodnack 1978). Sutterlin and Sutterlin (1970, 1971) have examined multiunit neural responses in Atlantic salmon, Salmo salar, and reported a high sensitivity of olfactory receptors to amino acids, whereas taste receptors remained relatively unresponsive to that type of stimulation. L-cysteine was suggested as a stimulus for our experiments (T. J. Hara, personal communication). Compared with other amino acids, L-cysteine is highly effective in evoking olfactory receptor responses in fishes (e.g., Caprio 1978; Silver 1982) but may not be effective in evoking gustatory responses in salmonids (e.g., Marui et al. 1983).

The first objective of the present study was to measure heart rate responses of conditioned fish to L-cysteine and to compare their cardiac performance to that of a control group exposed to random pairing sequences of L-cysteine and shock stimuli (experiment 1). This truly random control procedure (Mackintosh...
1983) is necessary to demonstrate that fish acquire a conditioned response as a result of a systematic pairing of l-cysteine and shock and not simply because of a natural responsiveness to l-cysteine or pseudoconditioning (Rescorla 1967).

Before fish are tested in the presence of l-cysteine, they are immobilized with d-tubocurarine, a neuromuscular relaxant. Thus, our second objective was to compare (experiment 2) curare-injected and saline-injected fish trained for cardiac conditioning or tested under random odor-shock contingencies to determine if curarization interferes with olfactory learning or odor sensitivity.

As morpholine is commonly used as a conditional stimulus in salmonid olfactory learning experiments, our third objective was to compare (experiment 3) the effectiveness of l-cysteine as a conditional stimulus with that of morpholine in the same experimental context. In addition, we have evaluated the effectiveness of three other amino acids to test whether other naturally occurring chemosensory stimuli were equally effective as conditional stimuli.

**Materials and methods**

Twelve 1-year-old Atlantic salmon, *Salmo salar*, (fork length 8.1–9.2 cm) were used in experiment 1, while fifty-four 2-year-old fish of this species (fork length 14.8–17.4 cm) were used in experiments 2 and 3. Before testing, fish were kept in aerated aquaria (157.5 × 50.8 × 35.6 cm) under a photoperiod of 8 h light : 16 h dark, in water of constant temperature (10 ± 0.5°C). Fish were fed daily and their water renewed weekly.

The methodology and procedure used to study heart rate conditioning to odorous chemicals in young salmon is described in Morin et al. (1987). Briefly, experiments were conducted in a cylindrical test chamber receiving three different water and stimulus inputs (Fig. 1), in which fish could be electrically stimulated and their heart rate monitored. The main input line filled the test chamber with dechlorinated water flowing at a velocity of 20.5 mm/s. The second input line supplied small quantities of water alternating with an odorous solution used as the conditional stimulus. A peristaltic tubing pump and solenoid valves were used for delivering the stimulus to the test chamber. The third input line leading to a mouthpiece was used for gill perfusion (300 mL/min). Cardiac electrodes were positioned under pectoral fins, whereas those used to shock the fish were placed medially between adipose and caudal fins. Electrodes and solenoid valves were connected to computer-assisted electronic systems and all events of an experimental session, including data collection and analysis, were controlled by an Apple II microcomputer. Chemoelectrical detection electrodes (Morin et al. 1987) situated between the fish's nares and the injection tube were also used for chemical calibration and for adjusting the duration of chemical stimulation.

Each fish was anesthetized (1% methyl pentylen) and injected intramuscularly with d-tubocurarine chloride (0.002 mg/g body weight) before being placed in the test chamber. Fish were secured in a semi-cylindrical holder lined with neoprene pads. An experimental session began with a 35-min acclimatization period during which resting heart rate was measured (over an 8-s time interval) every 5 min.

A conditioning trial was initiated with a 5-s injection of 7.3 mL of l-cysteine (5.7 × 10⁻³ M) into the test chamber. Applying the dilution factor calculated during a chemical calibration test (see Morin et al. 1987 for details), we estimated that fish were exposed to a concentration of 3.8 × 10⁻⁴ M. Following closure of this chemical valve, 8 s elapsed before the stimulus reached the test chamber. Stimulus dispersion in the chamber lasted for another 8 s before the fish was stimulated with a 2-s continuous electric shock. The intensity of each shock (25–42 mA) was adjusted during the first five trials to induce consistent arrhythmicity averaging 10 beats/min.

In experiment 1, experimental fish were exposed to l-cysteine followed by an electric shock. Random pairing fish were presented with the shock stimulus randomly preceding or following exposure to l-cysteine, and the time interval separating the stimuli was also randomized. In experiment 2, we assessed the effect of curarization on cardiac conditioning and responsiveness to l-cysteine by comparing fish injected with d-tubocurarine (0.002 mg/g) with fish injected with an isotonic saline solution. Fish were injected once with either solution before their placement in the test chamber. In experiment 3, fish were conditioned to five different odorant chemicals (l-cysteine, morphine, l-phenylalanine, l-serine, and l-threonine) presented singly.

Each session consisted of 20 trials separated by short intertrial intervals of random duration (45–135 s). We found from previous observations that heart rate reduction to an odor cue typically occurs in the context of a single interbeat interval (IBI), and that the phenomenon is masked when IBIs measured during the entire odor-stimulation interval are averaged. For this reason, the largest IBI recorded during odor stimulation was used to describe cardiac deceleration to the conditioning stimulus (Holland and Teeter 1981). The largest IBI recorded 8 s before the odor presentation was compared with the largest IBI recorded during the 8-s odor stimulation, and the percentage of cardiac deceleration was calculated according to the following formula:

\[ \frac{100 - \left( \frac{\text{largest IBI during} \times 100}{\text{largest IBI before}} \right) \times 100}{\text{largest IBI before}} \]

Different criteria for conditioning are used in heart rate conditioning studies on fish chemosensory perception (e.g., Johnstone 1980; Holland and Teeter 1981). Learning criteria are usually inadequately defined (Anderson 1964) and may not be sensitive enough to detect small but significant changes occurring in the context of a learning situation. Holland and Teeter (1981) developed a working criterion of 15% cardiac deceleration during stimulus presentation as a positive response for conditioning. With this approach, success rate of conditioning, based on the number of fish reaching the working criterion, was relatively poor (28% of all conditioned fish). However, imposing a "yes or no" criterion of conditioning on each fish may neglect important cardiac changes occurring in a group of conditioned fish (McCleave et al. 1974).

We defined two different response criteria of cardiac conditioning in fish, which were based on statistical considerations and appropriate methodological controls (Erspamer and Meyes 1978, 1979). In our study, learning was judged to have occurred if (i) the magnitude of the cardiac reduction response to L-cysteine by conditioned fish increased significantly between blocks of trials and (ii) cardiac reduction by conditioned fish was significantly greater than that by fish tested under a random pairing situation, a widely accepted control procedure in Pavlovian conditioning studies (Mackintosh 1983). The use of a random control procedure is recommended in classical conditioning inves-
Fig. 2. Mean percentages of heart rate reduction in two groups of six 1-year-old Atlantic salmon over two training sessions (40 trials). Half the fish were stimulated with L-cysteine (3.8 × 10^{-4} M) followed by an electric shock (conditioning group, ●), while the other half received a random sequence presentation of both stimuli (random-pairing group, ○). Vertical bars represent the mean ± SEM.

Fig. 3. Mean percentages of heart rate reduction in four groups of six 2-year-old Atlantic salmon over two training sessions (40 trials). Groups were either injected with tubocurarine (circles) or with a saline solution (triangles). Half the fish were stimulated with L-cysteine (3.8 × 10^{-4} M) followed by an electric shock (conditioning groups, ●, ▲), while the other half received a random sequence presentation of both stimuli (random-pairing groups, ○, △). Vertical bars represent the mean ± SEM.

Results

In all three experiments, resting heart rates were regular, with moderate differences between fish (78–89 beats/min), while coefficients of variation within individuals were generally small (0.21–0.49%). Two fish with slow or irregular resting heart rates were not used in the study.

Learning responses to L-cysteine

The percentage of heart rate reduction in fish conditioned to L-cysteine was compared with that of a control group tested under a random pairing of odor and shock (Fig. 2). The average cardiac performance of the experimental group during the first 15 trials (6.1%) increased to 12.8% at the end of the first experimental session. The conditioned response subsequently stabilized over the second training session, although a slight decrease in performance was observed at blocks 6 (10.4%) and 7 (9.2%). A random pairing of stimuli in the control group resulted in small cardiac decelerations across all 40 trials.

An analysis of variance (ANOVA) covering both experimental sessions showed group differences (F = 11.4, df = 1,10, P < 0.01). To identify when fish began to acquire the conditioned response, a separate ANOVA was performed on each session. In the first session, the overall cardiac performance of conditioned fish did not differ from that of the random pairing group. However, some group differences were found across blocks of trials (F = 4.41, df = 3,30, P < 0.05), as the interaction effect of blocks × groups was also significant (F = 4.52, df = 3,30, P < 0.01). Analysis of simple effects confirmed that block differences occurred in conditioned fish (F = 9.47, df = 3,30 P < 0.01) but not in fish tested under the random pairing procedure. Paired comparisons between block means further showed significant differences between block 4 and any of the preceding blocks of trials (P < 0.01). In the second experimental session, both groups of fish differed systematically from each other (F = 8.96, df = 1,10, P < 0.05), and for either group, the performance from blocks 5 to 8 was similar to that found at block 4.

Curareization effects on olfactory learning

Injection of d-tubocurarine did not affect cardiac performance when fish were exposed to a random order of L-cysteine and shock stimulus or when fish were conditioned (Fig. 3). For both conditioned fish and fish tested under a random pairing situation, differences in heart rate reduction between saline-injected and curare-injected fish were not significant. Independently of the type of injection administered to fish, groups trained for heart rate conditioning to L-cysteine had a higher performance than random pairing groups (F = 10.85, df = 1,20, P < 0.01). Moreover, differences in cardiac performance between conditioning and random pairing groups appeared across blocks of trials (F = 8.32, df = 1,140, P < 0.01). A significant interaction effect of groups × blocks (F = 9.03, df = 7,140, P < 0.01) further suggested that differences in cardiac performance between conditioning and random pairing groups were not found at every block of trials. They were only significant at block 4 (F...
Fig. 4. Mean percentages of heart rate conditioned responses to five different chemicals \((3.8 \times 10^{-4} \text{ M})\) in 2-year-old Atlantic salmon. Each group of six fish was tested over two training sessions (40 trials). Vertical bars represent the mean ± SEM.

\[ F = 3.89, \text{df} = 1.160, P < 0.05, \text{block 6}(F = 4.86, \text{df} = 1.160, P < 0.05), \text{block 7}(F = 10.23, \text{df} = 1.160, P < 0.01), \text{and block 8}(F = 18.12, \text{df} = 1.160, P < 0.01). \] Cardiac performance in both conditioning groups increased regularly from blocks 1 to 4 and from blocks 5 to 8 without any indication that olfactory learning would soon reach a plateau.

Conditioning to L-cysteine compared with conditioning to other chemicals

Heart rate conditioning responses to L-cysteine, L-phenylalanine, L-serine, L-threonine, and morpholine are shown in Fig. 4. The magnitude of heart rate reduction was significantly related to the type of odor used for testing \((F = 2.97, \text{df} = 4, 25, P < 0.05)\). The degree of cardiac deceleration also differed between blocks of five trials \((F = 20.92, \text{df} = 7, 175, P < 0.01)\). However, differences between blocks were not applicable to every group of fish exposed to a given chemical, as suggested by a significant groups × blocks interaction effect \((F = 3.29, \text{df} = 28, 175, P < 0.01)\). Interblock variations appeared in fish conditioned to L-cysteine \((F = 5.61, \text{df} = 7, 175, P < 0.01)\), morpholine \((F = 20.0, \text{df} = 7, 175, P < 0.01)\), and L-phenylalanine \((F = 6.43, \text{df} = 7, 175, P < 0.01)\). The rate of cardiac deceleration remained unchanged throughout the experiment for fish tested with L-serine or L-threonine. In other words, fish failed to condition to L-serine and L-threonine.

Cardiac conditioning to L-cysteine resembled that observed in the presence of morpholine. For example, the percentage of heart rate reduction to L-cysteine increased across trials and learning was evident at the end of the experiment as multiple comparison analyses showed a significantly higher rate of responding at block 8 (trials 36–40) compared with any preceding block of trials \((P < 0.01)\). Similarly, heart rate reduction increased across trials in fish exposed to morpholine and a cumulative learning effect was observed near the end of the second training session, as results from blocks 7 and 8 differed significantly from those of preceding blocks \((P < 0.01)\).

In contrast to the results with L-cysteine and morpholine, the magnitude of heart rate reduction to L-phenylalanine increased dramatically near the end of the first experimental session \((P < 0.01)\), as the reduction observed at block 4 was higher than that at blocks 1, 2, or 3 \((P < 0.01)\). No subsequent block of trials showed a significantly higher level of heart rate reduction.

**Discussion**

We induced a response to L-cysteine that was consistent with learning when cardiac performance of conditioned fish was compared with that of a control group tested under random pairing of conditioning stimuli. Fish were not conditioned to cues other than the odor itself, since we have already shown that fish cannot be conditioned to distilled water followed by an electric shock (Morin et al. 1987). In conditioning young salmon to reduce heart rate to L-cysteine, we found no indication that the procedure inducing curare paralysis interfered with olfactory learning or with resting heart rate.

The correlation between a conditional stimulus (e.g., odor) and an unconditional stimulus (e.g., shock) is an important variable in classical conditioning and will influence the occurrence of a conditioned response (Dickinson 1980). In our first experiment, conditioned fish were presented with a systematic pairing of an odor followed by a shock. Since the odor “signals” the presence of shock, this perfect correlation between stimuli typically induces excitatory conditioning or an increase in the magnitude of the conditioned response (e.g., Weissman and Lister 1969). Conversely, learning did not occur when fish were tested under a random pairing situation where the presence of the odor was uncorrelated with that of the shock. This treatment represents a genuine control procedure with which to assess excitatory conditioning (see Mackintosh 1983 for details).

Because L-cysteine is as effective a conditional stimulus as morpholine but does not have the disadvantage of being a potential nonspecific irritant, we propose the use of L-cysteine as a conditional stimulus in olfactory learning experiments. We have also demonstrated that other amino acids are not equally effective as conditional stimuli. No conditioning occurred in fish exposed to L-serine and L-threonine. This is consistent with studies that have commonly reported greater responses of the olfactory system of fishes to L-cysteine than to L-serine at stimulus concentrations varying from \(10^{-7}\) to \(10^{-2}\) M (e.g., Caprio 1978; Silver 1982) or to L-threonine (e.g., Suzuki and Tucker 1971; Caprio 1977) at stimulus concentrations varying from \(10^{-5}\) to \(10^{-3}\) M.

Fish learned to reduce heart rate to L-phenylalanine across experimental trials but took roughly half the time (block 4) to acquire a significant conditioned response of that taken by fish tested with L-cysteine (trials 36–40). However, we suspected that fish detected L-phenylalanine through both olfaction and taste, thus allowing faster heart rate conditioning than if the stimulus was only detected by either chemical sense alone. Consistent gustatory (Marui et al. 1983) and olfactory (Hara 1973) responses to L-phenylalanine have been reported in rainbow trout. If both senses were used to perceive L-phenylalanine, our results could be interpreted in terms of associative strength developed simultaneously and additively by two sensory modalities (Bitterman 1979; Mazur 1986). Conditioning stimuli mediated by several sensory modalities produced faster or greater learning in invertebrates (e.g., Bouillon and Bitterman 1980, 1982) and vertebrates (e.g., Eninger 1952) than conditioning stimuli mediated by a single sensory modality.

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Erratum: Heart rate conditioning to L-cysteine and other chemical stimuli in young Atlantic salmon (*Salmo salar*)

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On p. 2711, the formula given in the right-hand column for calculating the percentage of cardiac deceleration should read as follows:

\[
100 - \left[ \frac{\text{largest IBI before}}{\text{largest IBI during}} \times 100 \right]
\]

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