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Compound uniqueness and the interactive role of morpholine in fish chemoreception

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SUMMARY

Compound uniqueness and the interactive role of morpholine in fish chemoreception.

The purpose of the present study was to examine the uniqueness of a chemosensory compound in the context of the olfactory hypothesis of home stream recognition by anadromous fish. Although juvenile salmonids imprinted to low concentrations of the artificial chemical morpholine are decoyed to streams scented with morpholine at the same concentrations during the homing migration, there is no electrophysiological evidence that fish can respond to morpholine at imprinting concentrations. We hypothesized that this long-standing anomaly may be due in part to the interaction of the components of a chemosensory compound stimulus producing new stimuli that acquire associative strength independently of the separate components. Support for the general principal of chemosensory compound uniqueness is provided by appetitive conditioning experiments of goldfishes' anticipatory target responses which show that; (1) morpholine contributes to the uniqueness of a compound stimulus at a concentration below the threshold level of morpholine presented as a single discriminative stimulus and (2), a compound of morpholine and L-serine is discriminable from the individual components in a negative patterning schedule. Ablation of the olfactory tract revealed that both morpholine and L-serine were also detected through sensory channels other than olfactory. Our results illustrate the principal that the stimulatory ineffectiveness of the individual components of a chemosensory compound cannot be taken to imply that they do not contribute to the stimulatory effectiveness of the whole.

Key words: Fish chemoreception. Morpholine. Discriminative conditioning. Compound uniqueness.

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RESUME

Originalité de la substance et rôle d'interaction de la morpholine avec les chemorécepteurs du poisson.

L'objet de la présente étude consiste en l'examen du caractère unique d'un composé chimiosensoriel dans le contexte de l'hypothèse de la reconnaissance olfactive de la rivière natale par les poissons anadromes. Les salmonidés juvéniles exposés à de faibles concentrations de morpholine sont attirés, durant la migration de retour, vers les cours d'eau aromatisés de morpholine à des mêmes concentrations. Malgré cela, il n'y a pas d'évidence électrophysiologique que les poissons puissent réagir aux concentrations d'exposition de morpholine. Nous suggérons l'hypothèse que cette anomalie est due en partie à l'interaction des composants d'un stimulus composé chimiosensoriel produisant un nouveau stimulus qui acquiert une force associative, indépendamment des composants pris séparément. Le principe général du caractère unique d'un composé chimiosensoriel est appuyé par des expériences de conditionnement appétitif du poisson rouge qui démontrent que : (1) la morpholine contribue au caractère unique d'un stimulus composé à une concentration au-dessous du seuil de concentration de la morpholine lorsqu'elle est présentée comme un simple stimulus discriminatif ; (2) un composé de morpholine et de L-serine se distingue de chacun des composants pris individuellement selon un programme de discrimination composants(S^+)/composé(S^-) ("negative patterning"). L'ablation du tractus olfactif a révélé que la morpholine est la L-serine sont aussi détectées à travers des voies sensorielles autres qu'olfactifs. Nos résultats illustrent le principe qui veut que l'inefficacité des composants individuels d'une substance chimiosensorielle n'implique pas qu'ils ne contribuent pas à l'efficacité du composé.

Mots-clés : Chimioréception. Morpholine. Conditionnement discriminatif. Caractère unique de l'activité chimiosensorielle d'un composé.

INTRODUCTION

There is considerable evidence that many migratory fish species use chemosensory cues to orient towards the home site (Kleerekoper, 1982). In the case of anadromous Pacific salmon, the olfactory hypothesis of Hasler and Wisby (1951) states that juvenile fish imprint to the distinctive odor of their natal stream and use this information, stored as long-term memory, to relocate the stream during the spawning migration. The hypothesis is supported by the results of experiments in which juvenile salmonids exposed to a low concentration (5.7×10^{-10}M) of the artificial chemical morpholine were decoyed to streams scented with morpholine at the same concentration during their homing

migrations (Hasler and Scholz, 1983). Further support is provided by electrophysiological studies in which the magnitude of the olfactory bulbar response to 1.1×10^{-1}M morpholine of adults exposed as fingerlings to 5.7×10^{-10}M morpholine was significantly greater than that of unexposed fish (Dizon et al., 1973; Cooper and Hasler, 1974; 1976).

Morpholine (C_4H_9NO), a strongly basic heterocyclic amine used in a variety of industrial applications, was originally chosen for artificial imprinting studies because of its high solubility in water, its chemical stability in the natural environment and because it is supposedly not found in natural waters. Morpholine can apparently be detected by unconditioned coho salmon (*Oncorhynchus kisutch*) at a concentration of 5.7×10^{-10}M . It is reported as neither attracting nor repelling fish at low concentrations (reviewed by Hasler and Scholz 1983).

A persistant criticism of the electrophysiological experiments has been that the high concentrations (10^{-1}, 10^{-2}M) of morpholine employed produce irritational responses which are not related to normal olfactory function (Hara 1974; Hara and Macdonald 1975; Hara and Brown 1979, 1982). Since electrophysiological work with a variety of salmonids shows no olfactory bulbar response to morpholine at imprinting concentrations, it has been suggested that returning migrants were responding, not to morpholine alone, but to products of an interaction of morpholine with stream odors (Hara et al., 1984). The latter suggestion is consistent with the widely-held but untested notion that stream odors are unique mixtures and that salmon learn the entire "bouquet" of their natal stream (Hasler and Scholz 1983; Hara et al., 1984).

The view that anadromous fish may imprint to unique mixtures of chemosensory stimuli is in agreement with the more general principle that biologically meaningful scents- releasing feeding and social behavior- in fish will often be due to mixtures rather than single substances (Bardach and Villars 1974). For example, combinations of amino acids and betaine chosen on the basis of bioassays of natural food extracts stimulate feeding behavior in fish even when the separate components do not (Carr 1982). Sandoval's (1980) demonstration that juvenile coho salmon conditioned to a compound of phenethyl alcohol and morpholine exhibited no conditioned response to either chemical alone also

provides support for the view that compound stimuli are unique relative to their components.

Animal learning theory provides several hypotheses to account for the development of learned responses to compound stimuli (reviewed by Bellingham et al., 1985). The principal of additive summation states that responding to a compound stimulus is due to the summation of the separate associative strengths of the components. Reinforced training with individual components followed by tests with the compound reveals a higher level of responding to the compound than to either component. On the other hand, experiments designed to produce differential conditioning of a compound and its components demonstrate that a compound can act as a functional unit. In experiments in which unreinforced compound presentations are mixed with reinforced component presentations (negative patterning), less responding to the non-reinforced compound simply cannot represent a summation of the high levels of responding to the reinforced components (Woodbury 1943). In such cases, the compound stimulus must be perceived as more than the sum of its components.

Several hypotheses have been proposed in an attempt to reconcile these two apparently opposing points of view. For example, the "unique stimulus" hypothesis of Rescorla (1972) retains the principle of additive summation, but proposes that interaction of the components of a compound produces new stimuli unique to the compound. Compound-unique stimuli acquire associative strength in the same way as the separate components (Bellingham et al., 1985).

Some evidence for compound uniqueness in fish has been obtained with conditional discriminations that involve differential reinforcement of symmetric combinations of colours and a tone (Bitterman 1984). Goldfish were appetitively trained with a target using four compounds, each composed of two of the following components; red, green, 200-Hz tone, no tone. Some animals were rewarded for responding to red-tone and green-no tone (but not to red-no tone or green tone), while the rest were rewarded for responding to red-no tone and green-tone (but not to red-tone and green-no tone). In neither case could food be predicted from colour alone nor the presence or absence of tone alone, but only from the two in combination. Fish responded more

to the reinforced compounds than to the non-reinforced compounds in all cases. The discriminative responding could not have been obtained by responding differentially to the individual components because each was included equally often in a reinforced and nonreinforced compound.

The purpose of the present study was to examine the general principal of compound uniqueness in the context of chemosensory stimuli. We examined discriminative response acquisition to morpholine presented as a single stimulus and as a component of a compound stimulus to assess the plausibility of an interactive interpretation of the role of morpholine in fish chemoreception. The subjects -selected for reasons of convenience- were common goldfish, and the additional component stimulus -selected for ease of quantification- was L-serine (an amino-acid often used as an olfactory stimulus in electrophysiological studies). The specific objectives of the study were; (a) to test the hypothesis that morpholine may contribute to the discrimination of a compound at a concentration that is not discriminable when morpholine is presented as a single stimulus, (b) to test the hypothesis that morpholine interacts with other chemical components to produce compounds with unique properties, and (c) to determine the importance of olfaction in the observed chemosensory discrimination.

MATERIAL AND METHODS

The subjects were 10-cm goldfish, experimentally naive but well-adapted to the laboratory. They were maintained on a 12-hr light-dark cycle and a 24-hr feeding schedule in a large tank, partitioned to form individual compartments, through which water was circulated at a temperature of 20°C. The water was in part returned after filtration and in part continuously replaced. The training aquarium, to which individual subjects were carried in turn from their living compartments, was a 38-L tank housed in a dark, acoustically-isolated enclosure. The water flowing through it was continuously replaced.

The training technique, a variant of a technique used extensively to study visual discrimination in fishes (Bitterman 1984), was to reward a target-striking response in the presence of one chemical stimulus (S^+) and not in the presence of another (S^-). Mounted at one end of the training aquarium behind a black plexiglas panel, and accessible to the animal only through a smaller, circular opening in the panel, was a circular target of light-diffusing plexiglas, 4 cm in diameter, that was illuminated

with red light. The target, diagramed in figure 1, was suspended on a thin metal rod, the upper end of which was connected to a strain gauge whose output, when the animal struck at the target, operated a response-relay. At the centre of the target was a small plexiglas cup into which an attractive liquid food (tetramin flakes blended with water and thickened with tragacanth) could be delivered through teflon tubing from a motor-driven syringe. Three smaller tubes, each connected to a separate pump, pierced the target in a triangular array 1 cm above the food cup as shown in the diagram. Through these tubes, the chemical solutions to be discriminated could be delivered at the rate of 0.03 $ml.s^{-1}$. The circulation of water in the training aquarium was so designed that the solutions did not diffuse widely but washed over the face of the target and were carried away in the outflow situated directly below and behind the target.

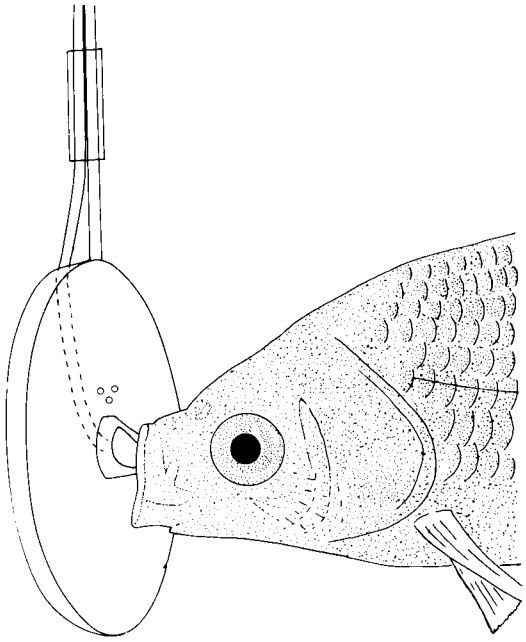


FIGURE 1: The circular target with food cup and tube for delivery of liquid food, three inlets for the delivery of chemosensory stimuli (delivery tubes not illustrated) and striking position of fish.

FIGURE 1 : Illustration de la cible circulaire avec le récipient et le tube par lequel la nourriture liquide est distribuée, les trois ouvertures par lesquelles les produits stimulants sont éjectés (leurs tubes d'alimentation ne sont pas illustrés) et la position du poisson lorsqu'il répond.

All chemical solutions were prepared using tap water and concentrations are expressed as moles per litre (M). Because of the dilution of solutions in the vicinity of the target, concentrations of chemosensory stimuli discriminated by fish are

unknown but must be considered to be less than prepared concentrations.

EXPERIMENT 1

PROCEDURE

To test the hypothesis that morpholine may contribute to the discrimination of a compound at a concentration that is not discriminable when morpholine is presented as a single stimulus, 10 naive fish were pretrained to take food from the food cup when the target was illuminated. A drop of food was delivered to the cup, and each contact of the animal with the target automatically produced another drop of food. When all the animals were feeding readily, they were divided into two matched groups of five (Group W and Group L) and discriminative training was begun. Each daily session consisted of a series of 40 trials separated by intervals of 15 sec in darkness. At the start of each trial the unbaited target was illuminated and one of two different solutions (S^+ and S^-) was pumped through it. For 15 sec, all responses to the target, now entirely without consequence, were recorded to provide a measure of the anticipation of food. Then the recording stopped, but the target remained illuminated and the flow of solution continued. On S^+ trials each response to the target in the next 10 sec was rewarded with a drop of food after which the trial ended. On S^- trials there was no food, but each response reset a 10-sec penalty clock, and the trial did not end until the animal permitted the clock to time out. The function of the penalty procedure was to discourage response to S^-. In each training session, 20 S^+ and 20 S^- trials were presented in quasi-random order (Gellermann 1933).

For Group W (to designate water as the solvent), S^+ was a 5×10^{-4}M solution of morpholine, and S^- was tap water. The concentration of morpholine was selected in pilot experiments with other goldfish that were trained first with higher concentrations and then with concentrations that were progressively reduced until discrimination failed at 5×10^{-4}M. For Group L (to designate L-serine as the solvent), S^+ was a 5×10^{-4}M solution of morpholine prepared with a 5×10^{-3}M solution of L-serine as the solvent. S^- was a 5×10^{-3}M solution of L-serine that pilot experiments showed to be readily discriminable by fish. Discrimination in either group W or L could be expected only on the basis of the morpholine, and the only question (given our pilot experiments) was whether discrimination would be possible in the context of L-serine.

RESULTS

Although fish did not discriminate between ambient water and morpholine, they readily discriminated between L-serine and a compound of morpholine and L-serine. In figure 2, the results of the experiment are plotted in terms of the mean rate of response

by each group to each stimulus in each of seven sessions of discriminative training. An overall analysis of variance shows a significant stimulus effect, $F(1,8) = 9.27$, $p=0.0159$. There is also a significant interaction of groups x sessions x stimuli, $F(6,48) = 3.24$, $p=0.0093$, which seems to reflect the fact that the animals of group L began to respond differentially to the stimuli only in the fourth session. Separate analyses show, for group W, no significant stimulus effect, $F(1,4) = 1.45$, $p=0,2948$, but, for group L, a significant stimulus effect, $F(1,4) = 7.93$, $p=0,0480$, and a highly significant interaction of sessions x stimuli, $F(6,24)=7.44$, $p=0,0001$.

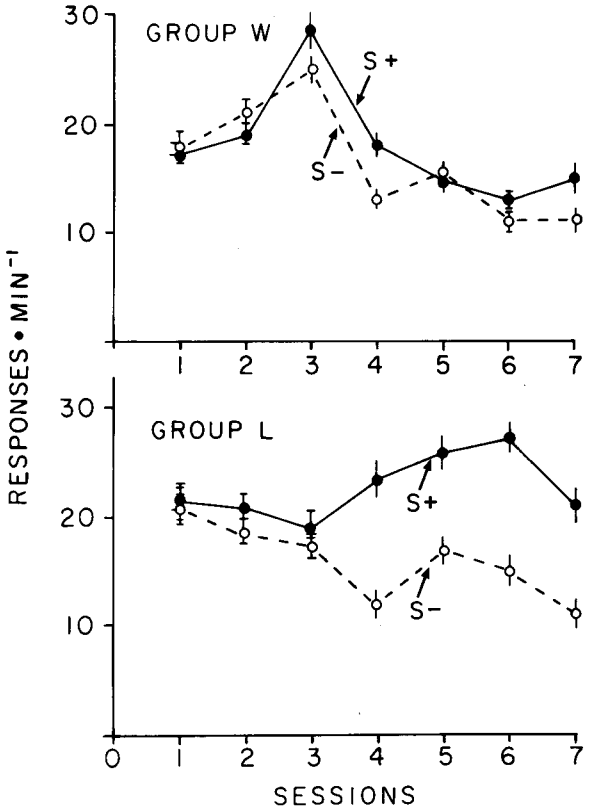


FIGURE 2: The discrimination of morpholine as a single stimulus (Group W; S^+- morpholine, S^-- water) and in compound with L-serine (Group L; S^+- morpholine + L-serine, S^-- L-serine). Each data point represents the mean conditioned response of 5 fish receiving 20 training trials per stimulus per session. Vertical lines represent standard errors of the mean. S^+- rewarded trials, S^-- unrewarded trials.

FIGURE 2 : Test de discrimination entre la morpholine pris comme seul stimulus (Groupe W; S^+- morpholine, S^-- eau) et d'un mélange avec la L-serine (Groupe L; S^+- morpholine + L-serine, S^-- L-serine). Chaque point représente la réponse conditionnée moyenne de 5 poissons ayant subi 20 essais par stimulus par session. Les lignes verticales représentent l'erreur-type. S^+- essai renforcé, S^-- essai non-renforcé.

EXPERIMENT 2

PROCEDURE

To test the hypothesis that morpholine may interact with other chemical components to produce compounds with unique properties, nine goldfish were trained using a negative patterning schedule to discriminate a 5×10^{-3} M compound of L-serine and morpholine from the same stimuli presented individually at the same molar concentration. The fish were pretrained as in experiment 1. In the discriminative training, there were 36 trials per day of which 24 were S^+ trials, half signalled by morpholine and half by L-serine. The remaining 12 trials were S^- trials signalled by the compound. S^+ and S^- trials were presented in quasi-random orders that were changed for each training session (Gellermann, 1933). Discriminative training was continued for 13 sessions to provide at least 4 sessions following response acquisition during which individual performance could be evaluated for a subsequent experiment (see experiment 3).

A critical feature of this procedure is that the components were reinforced while the compound was not. If the compound was perceived only as a sum of the components (additive summation), the fish should respond more to the compound (if there were any differences at all) than to the separate components. Lesser response to the compound would indicate new sensory properties generated by interaction of the components (Rescorla, 1972).

RESULTS

The results of the experiment are plotted in figure 3 in terms of the mean rate of response to each stimulus in each of 13 sessions of discriminative training. Analysis of variance shows a significant stimulus effect, $F(2,16) = 28.6$, $p<0.001$. The conditioned response to L-serine was significantly greater than the response to morpholine, $F(1,8) = 12.93$, $p=0.007$, which in turn was significantly greater than the response to the compound, $F(1,8) = 73.39$, $p<0.0001$.

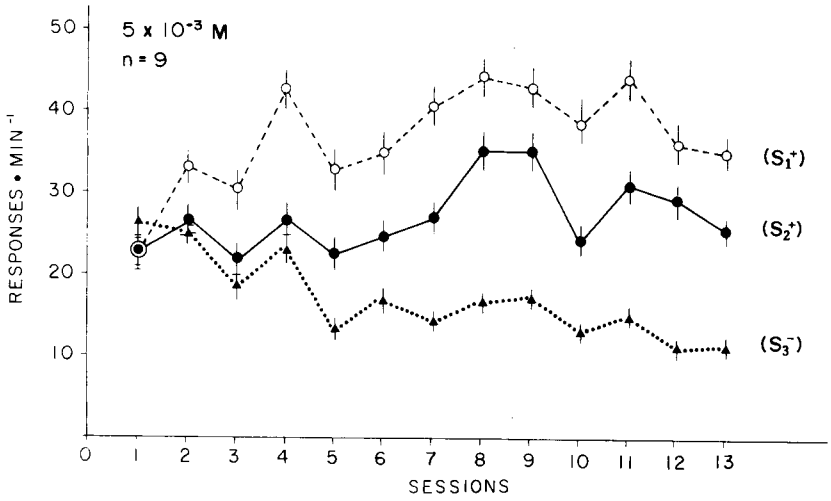


FIGURE 3: The discrimination of L-serine (S_1^+) and morpholine (S_2^+) from a compound of the 2 chemicals (S_3^-). Each data point represents the mean conditioned response of 9 fish receiving 12 training trials per stimulus per session. Vertical lines represent standard errors of the mean. S^+- rewarded trials, S^-- unrewarded trials.

FIGURE 3 : Test de discrimination entre la L-serine (S_1^+) et la morpholine (S_2^+) d'un mélange contenant ces 2 produits (S_3^-). Chaque point représente la réponse conditionnée moyenne de 9 poissons ayant subi 12 essais par stimulus par session. Les lignes verticales représentent l'erreur-type. S^+- essai renforcé, S^-- essai non-renforcé.

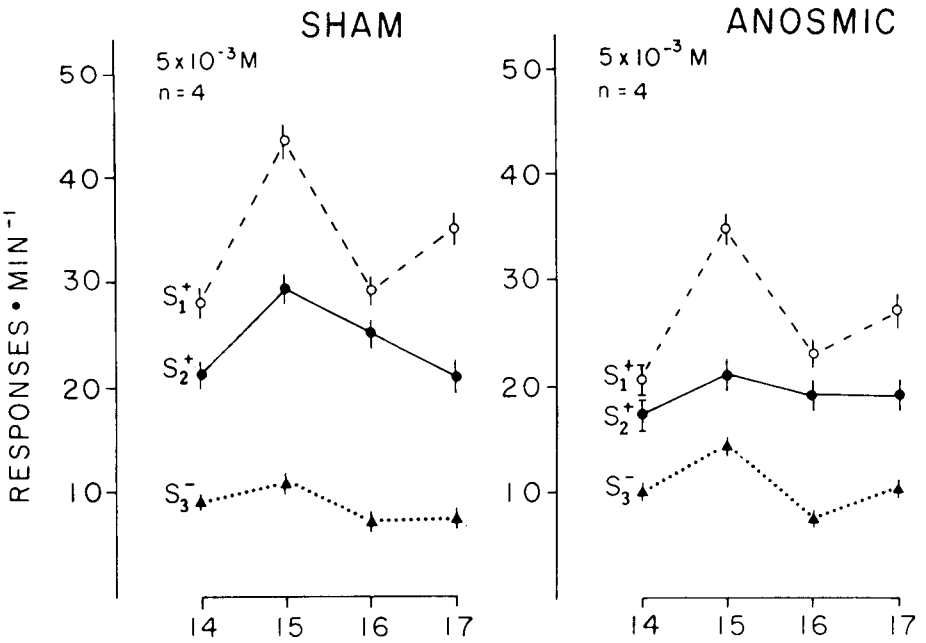
EXPERIMENT 3

PROCEDURE

The role of olfaction in the discrimination was evaluated in further work with the subjects of Experiment 2. One fish was dropped from the experiment due to disease and the remaining eight fish were divided into two groups that were matched for performance in the last four training sessions of Experiment 2. One group was rendered anosmic following anesthetization with MS222 by transecting the olfactory tract and removing a portion of it through a small hole in the skull posterior to the nares. The second group received the same treatment except that olfactory tracts were left intact. After four days of recovery, both the anosmic and the sham-operated group received four sessions of discriminative training as in experiment 2. It was expected that the anosmic group would fail to discriminate if olfaction was necessary.

RESULTS

As shown in figure 4, both the anosmic and the sham-operated group continued to discriminate the compound from its components.



FIGUR 4: Compound discrimination of L-serine and morpholine by sham operated and anosmic fish. Identification of stimuli as in Figure 2. Each data point represents the mean conditioned response of 4 fish receiving 12 training trials per stimulus per session. Vertical lines represent standard errors of the mean.

FIGURE 4 : Test de discrimination entre la L-serine (S_1^+) et la morpholine (S_2^+) d'un mélange contenant ces 2 produits (S_3^-) par des poissons anosmiques et des poissons témoins opérés sans ablation. Chaque point représente la réponse conditionnée moyenne de 4 poissons ayant subi 12 essais par stimulus par session. Les lignes verticales représentent l'erreur-type. S^+- essai renforcé, S^-- essai non-renforcé.

Overall analysis of variance shows a significant stimulus effect, $F(2,12) = 41.92$, $p<0.0001$, but no significant groups effect, $F(1,6) = 0.33$, $p=0.584$, and no interaction of groups x stimuli, $F(2,12) = 2.23$, $p=0.149$, or of groups x sessions x stimuli, $F(6,36) = 0.341$, $p=0.910$. A separate analysis of variance of the data for the anosmic group reveals a significant stimulus effect, $F(2,6) = 38,31$, $p=0.0004$. As in the preoperative training of Experiment 1, the anosmic fish responded

significantly more to L-serine than to morpholine, $F(1,3) = 16.59$, $p=0.026$, and significantly more to morpholine than to the compound, $F(1,3) = 55.26$, $p=0.005$.

DISCUSSION

The finding in experiment 1 that morpholine can interact with L-serine permitting the discrimination of the compound stimulus at a concentration of morpholine that is not discriminable when presented as a single stimulus indicates an interaction of the components producing a unique stimulus. In addition, the observation in experiment 2 that fish responded less to a nonreinforced compound of L-serine and morpholine than to either of its separately-reinforced components is contrary to the principal of additive summation and supports the view that the compound stimulus acts as a functional unit with the ability to acquire associative strength that is independent of the separate components. As such, these results provide support for an interactive role of morpholine in fish chemoreception.

The results of experiment 3 show that the discrimination of compound-unique properties generated by an interaction of components may be mediated by the gustatory or general chemical sense. This does not necessarily imply that olfaction was not involved in the successful discrimination exhibited by sham-operated fish. Although not significant, the responses of anosmic fish to morpholine and L-serine were somewhat fewer than those of sham-operated fish. Similar experiments to study the relative importance of taste in discrimination are not feasible because of the widespread distribution of taste buds over the fish body and the complexity of their innervation.

A speculative mechanism responsible for the compound uniqueness observed in this study may involve stereochemical interactions at the level of specific receptor sites. Hara (1982) has suggested that the stimulatory effectiveness of amino acids depends on their interaction with receptor membrane structures of definite shape, size and charge distribution and that a multiplicity of such receptor sites exists in the fish olfactory epithelium. Similar hypotheses have been proposed for the taste system (reviewed by Brown and Hara, 1982). Olfactory responses to amino acids in rainbow trout (*Salmo gairdneri*) are pH dependent because of ionization of both stimulant molecules and receptor sites (Hara, 1976). As a 5×10^{-4} solution of morpholine has a pH of 8.5, the successful compound discrimination exhibited by Group L of experiment 1 may have been due to the ionization of L-serine in compound with morpholine. However, the discrimination cannot be explained on the basis of pH alone as Group W was unable to discriminate a similar concentration (and pH) of morpholine from water.

In conclusion, these results clearly illustrate the general principal that the stimulatory ineffectiveness of the individual components of a chemosensory compound cannot be taken to imply that they do not contribute to the stimulatory effectiveness of the whole. Although our results do not constitute proof of an interactive role for morpholine in salmon homing studies because of differences in species and chemical context, they lend credence to an account, in terms of compound uniqueness, of the anomalous results of electrophysiological studies conducted as correlates of artificial olfactory imprinting studies. The subjects of these studies were not imprinted in distilled water scented with

morpholine alone but in fish-hatchery holding tanks where they were exposed to many chemosensory cues, including the mucus of conspecifics that is rich in amino acids and an effective olfactory stimulant (Hara et al., 1984). If we accept the proposal that imprinting is an associative process like that involved in conditioning (Bateson, 1979), the ineffectiveness of morpholine presented alone at imprinting concentrations in electrophysiological studies does not necessarily contradict the results of *in situ* decoy experiments. This hypothesis remains to be tested.

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