

# One-trial associative learning modifies food odor preferences of a terrestrial mollusc

(conditioning/slugs/*Limax*)

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**ABSTRACT** We present evidence of rapid and reliable associative learning by the terrestrial mollusc, *Limax maximus*. Slugs were exposed once to a pairing of a highly attractive food odor (potato or carrot) and a saturated solution of quinidine sulfate, a bitter-tasting plant substance. In comparison with control slugs, the exposed slugs subsequently displayed a markedly reduced preference for the odor paired with quinidine. This reduced odor preference was limited to the specific odor paired with quinidine and did not generalize to other food odors.

The insight into nervous system function obtained from the study of invertebrates has encouraged the belief that the neural mechanisms mediating the influence of associative learning on behavior might be fruitfully studied in such organisms. Consequently, searches for behavioral procedures that yield evidence of durable associative learning by invertebrates have been initiated in several laboratories (1–6).

In this paper, we report a behavior change in the terrestrial pulmonate mollusc, *Limax maximus*, that can be attributed to associative learning. Specifically, using a Pavlovian-like conditioning procedure, we have shown that the slug's normal preference for odors generated by edible food plants, such as potato or carrot, can be reduced by pairing a brief feeding experience on potato or carrot (conditioned stimulus) with exposure to a saturated solution of quinidine sulfate (unconditioned stimulus), a bitter-tasting plant extract.

## MATERIALS AND METHODS

The slugs in our experiments (3–5 g) included both field-collected and laboratory-bred animals. They were housed in groups of 10–15 in ventilated plastic containers (21 × 30 × 4 cm) and had continuous access to an enriched artificial diet consisting of Purina rat chow (500 g), sea sand (50 ml), calcium carbonate (10 g), and vitamins (1 teaspoon) moistened with Tegosept M (0.24%). One week before the initiation of training, the slugs were deprived of food and placed individually in ventilated plastic containers (15 cm diameter) lined with moistened filter paper. On the training day, each slug was placed individually for 2 min in a 10-cm-diameter plastic petri dish containing a substrate of Pillsbury's Hungry Jack instant potatoes/saline (40 g/250 ml) and allowed to feed. The saline contained 55.4 mM Na<sup>+</sup>, 4.2 mM K<sup>+</sup>, 7.0 mM Ca<sup>2+</sup>, 4.6 mM Mg<sup>2+</sup>, 80.1 mM Cl<sup>-</sup>, 0.2 mM H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 2.5 mM HCO<sub>3</sub><sup>-</sup>, and 5.0 mM dextrose. At the end of this 2-min period, the experimenter slowly infused 5 ml of a saturated solution of quinidine sulfate (1 g/90 ml of saline) into the dish of each experimental slug [group PQ (*n* = 4)] and 5 ml of saline into the dish of each control slug [group PS (*n* = 5)]. The slugs showed an aversive reaction to quinidine sulfate characterized by attempted withdrawal from it, copious mucus secre-

tion, and a writhing type behavior. Each animal remained in contact with the quinidine for 20 min, after which it was rinsed with saline and returned to its home cage.

Twenty-four hours later, an odor-preference task (7, 8) was used to evaluate the influence of these two treatments. The test apparatus, (Fig. 1) consisted of a circular plastic dish (14 cm in diameter) with a perforated floor. This dish sat directly over two food-odor sources that were separated by a 2-cm gap down the center of the apparatus (the 'neutral zone'). On the first test trial, the food odors were raw carrot (30 g/15 ml saline), pureed in a blender, and Purina rat chow mash (10 g/100 ml). On the second trial, potato odor was pitted against rat chow odor.

On each test trial, the slug was placed in the neutral zone and observed until it crossed the boundary line between the neutral zone and one of the odor sources. This choice was recorded as 'first choice'. During the next 4 min, the time spent by the slug over each odor source and over the neutral zone was recorded. A slug was judged to be over an odor source when the anterior third of its body (head area) was out of the neutral zone. The experimenter did not know whether the slug being tested was a member of group PQ or of group PS.

## RESULTS AND DISCUSSION

**Experiment 1.** We found that, when given a choice between potato odor versus rat chow odor, there were differences in the amount of time spent over potato [Fig. 2A; *t*(7) = 6.4, *P* < 0.001]. The slugs that had experienced the potato-quinidine pairing (group PQ) spent markedly less time over the potato odor than the slugs that had experienced potato paired with saline (group PS). There was no overlap in the distribution of scores for the two sets of animals on the potato versus rat chow test. The percentage of time spent over the potato odor by individual slugs in group PQ ranged from 10% to 30%, whereas the range for slugs in group PS was 60–100%. In addition, potato odor was the first choice of all (five) slugs in group PS, but was the first choice of only one of four slugs in group PQ. In contrast, when presented with a choice between carrot and rat chow odors (see Fig. 2B), slugs in groups PQ and PS did not differ in the percentage of time spent over carrot, *t*(7) = 0.06, *P* < 0.25. Likewise, there was no difference in their first choice. Carrot odor was the first choice for three of four slugs in group PQ and of four of five slugs in group PS.

The reduced potato-odor preference shown by group PQ, as compared with group PS, suggests that the slugs in group PQ associated potato odor with the aversive quinidine exposure. One cannot, however, dismiss the possibility that the reduced potato-odor preference displayed by the slugs in group PQ was a product of some nonspecific or nonassociative consequence of

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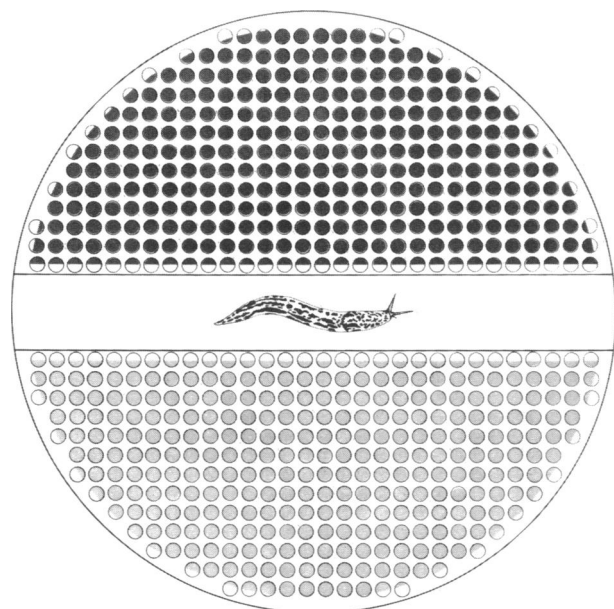


FIG. 1. Schematic representation of test apparatus. The slug is in a neutral zone that is bordered on each side by two different odor-generating food sources that are directly under the perforated floor of the chamber.

their quinidine experience. The conclusion that the reduced potato-odor preference shown by slugs in group PQ was determined by associative learning requires additional evidence that their reduced potato-odor preference depended on the potato-quinidine pairing and not just on exposure to quinidine.

**Experiment 2.** This experiment was designed to provide such evidence and to demonstrate that the slugs' preference for carrot odor also can be reduced by a carrot-quinidine pairing and thereby extend the generality of this phenomenon. Four treatment conditions ( $n_s = 4$ ) were compared. On the training day, slugs in groups PQ and PS were initially allowed to feed on carrot for 2 min and two other groups of slugs (Groups CQ and CS) were allowed to feed on potato for 2 min. Twelve hours later, the slugs in group PQ were exposed to a potato odor-quinidine pairing, the slugs in group CQ were exposed to a carrot

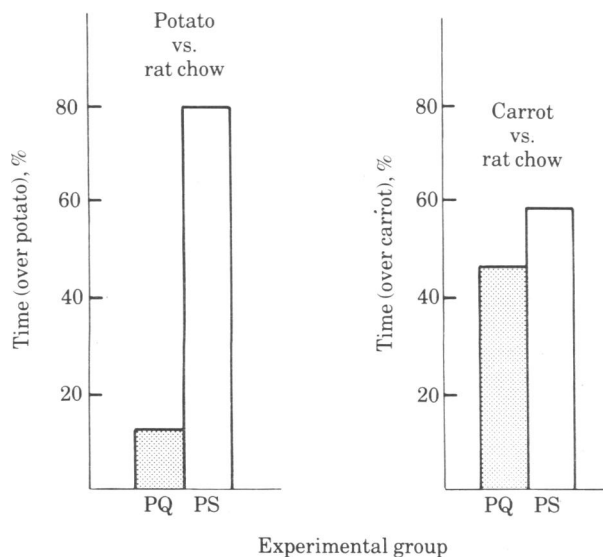


FIG. 2. Experiment 1: mean percentage of time spent over potato or carrot odor.

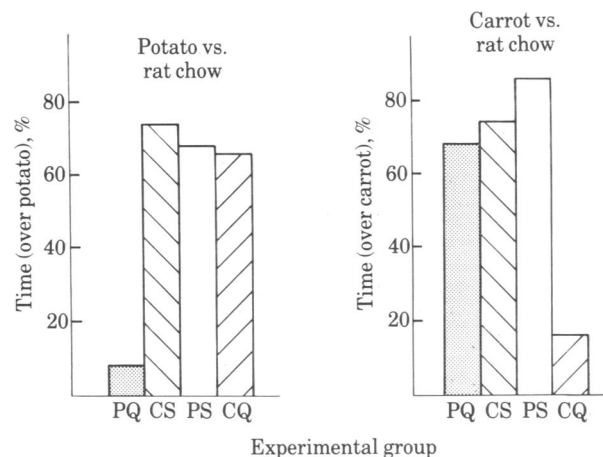


FIG. 3. Experiment 2: mean percentage of time spent over potato or carrot odor.

odor-quinidine pairing, and the slugs in groups PS and CS were exposed to a potato odor-saline pairing and a carrot odor-saline pairing, respectively.

Approximately 24 hr after training, each slug was tested three times for its potato-odor preference and three times for its carrot-odor preference. Half of the slugs in each group were first tested for their potato-odor versus rat-chow-odor preference and the other half were first tested for their carrot-odor versus potato-odor preference. Each of the six consecutive test trials was terminated 1 min after the slug made its first choice, and the time the animal spent over each odor and in the neutral zone during this 1-min period was recorded. The intertrial interval was approximately 20 sec; during this interval, the slug was placed in its 'home' container. The test environment was thoroughly washed between animals to remove any slime trail deposited by the preceding slug.

The mean percentages of the times the slugs spent over the potato and carrot odors cumulated over the three test trials on which these odors were present are shown in Fig. 3. From the results of the potato-odor versus rat-chow-odor preference test, it is evident that the slugs in group PQ spent markedly less time over the potato odor than the slugs in groups CS, PS, and CQ. Similarly, from the results of the carrot-odor versus rat-chow-odor preference test, it is evident that the slugs in group CQ spent less time over the carrot odor than the slugs in groups PQ, CS, and PS. Separate analyses of variance on the potato-odor preference versus rat-chow-odor preference test and on the carrot-odor versus rat-chow-odor test showed differences among the groups,  $F_s(3, 12) \geq 12.48$ ,  $P < 0.002$ . Post hoc individual comparisons (Newman-Keuls test) indicated that group PQ was reliably different ( $P < 0.02$ ) from groups PS, CS, and CQ on the potato-odor preference versus rat-chow-odor preference test and that group CQ differed ( $P < 0.02$ ) from groups PQ, PS, and CS on the carrot-odor preference versus rat-chow-odor preference test. There were no other reliable differences. The first choice data yielded a similar pattern of results. In the 12 potato-odor preference versus rat-chow-odor preference tests (four slugs, by three tests), potato odor was the first choice for slugs in group PQ on only 2 occasions but was the first choice in 11, 11, and 10 trials for slugs in groups CQ, PS, and CS, respectively. In the 12 carrot-odor preference versus rat-chow-odor preference test trials, carrot odor was never the first choice for slugs in group CQ but was the first choice in 11, 10, and 10 trials for slugs in groups PQ, PS, and CS, respectively.

Several important conclusions are implied by these data. First, exposure to quinidine *per se* is not a sufficient condition

to reduce the slugs' preference for either potato odor or carrot odor. If it were, then slugs in groups PQ and CQ should have displayed reduced preference for *both* of these odors in comparison with groups CS and PS. But the odor preferences of the slugs in groups PQ and CQ were *selectively* reduced. Slugs in group PQ displayed a reduced preference for potato odor (the odor paired with quinidine), but their preference for carrot odor was not reduced in comparison with that of slugs in groups PS and CS. The symmetrically opposite pattern of results was displayed by slugs in group CQ. They displayed a reduced preference for carrot odor (the odor paired with quinidine), but their preference for potato odor was not reduced in comparison with that of groups PS and CS. The reduced odor preferences that were observed in this experiment *depended* on the odor-quinidine pairing. Thus, we conclude that associative learning, resulting from the odor-quinidine pairing, altered the slugs' preference for that odor. Apart from suggesting that slugs have associative learning capabilities, the results of this experiment extend the generality of the phenomenon. Slugs can evidently associate at least two food-generated odors, potato and carrot, with quinidine. Moreover, a resultant conditioned aversion to potato did not detectably generalize to carrot or vice versa.

**Experiment 3.** Gustatory stimulation provided by the slug feeding on the conditioning trial might have potentiated the learned change in odor preference observed in previous experiments (9). Thus, in a third experiment, we exposed slugs to the food odor (potato) without allowing them to feed and followed this exposure with exposure to quinidine. The slugs that received this treatment [group PQ ( $n = 5$ )] displayed a reduced potato-odor preference in comparison with slugs [group PS ( $n = 5$ )] exposed to a potato odor-saline pairing,  $t(8) = 2.76$ ,  $P < 0.05$ , but the carrot-odor preferences of the two groups did not differ (Fig. 4). Comparison of the two PQ groups (see Figs. 3 and 4) shows that the odor-quinidine pairing (group PQ in Fig. 4) is as effective in reducing the slugs' preference for potato odor as is the odor-plus-taste-quinidine pairing (group PQ in Fig. 3). These results imply that the slugs do not need to taste the food-odor source to acquire an odor-quinidine association. Thus, there is no evidence that the slugs' learned change in odor preference was potentiated by gustatory stimulation provided by the food-odor source.

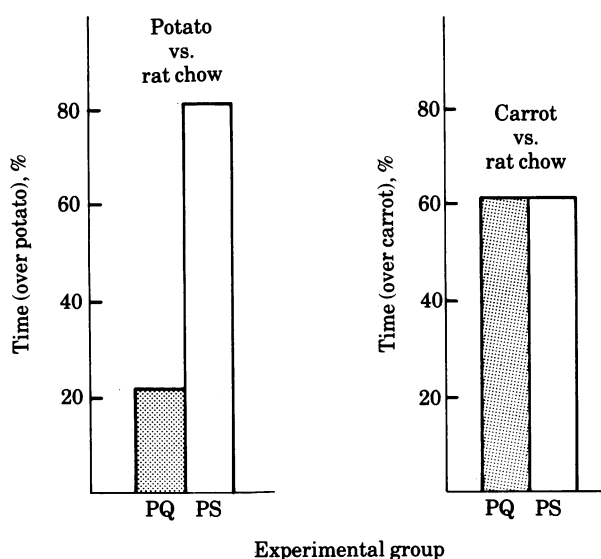


FIG. 4. Experiment 3: mean percentage of time spent over potato or carrot odor.

Associative learning has previously been demonstrated in *Limax* and in a number of other gastropod molluscs (1–6, 10). The aversive events used to produce learned changes in food selection ( $\text{CO}_2$  and electric shock), however, were events that the animal would not normally encounter in its natural environment. In contrast, a principal feature of our procedures (see also ref. 11) was that they allowed us to ask the slug to learn about the relations of two classes of stimuli (olfactory and gustatory) that normally regulate its food selection (12). Thus, our results not only suggest that the slug is capable of associative learning but also suggest that associative learning processes may play an important role in regulating the food selection of slugs in their natural environment.

A second feature of our procedures is that they permit systematic manipulation of a number of experimental variables (e.g., concentration and duration of the odor and quinidine experience, the interval separating them) so that we might characterize the necessary and sufficient conditions that produce and optimize learning in the slug and determine whether learning in the slug is influenced by the variables that influence learning by mammalian species. Moreover, the sensitivity of our procedures allowed us to demonstrate that *Limax* can learn the more complex stimulus-reinforcer relations characteristic of vertebrate learning (e.g., second-order conditioning and the Kamin blocking effect) (unpublished data; ref. 13).

The relatively simple nervous system of an invertebrate such as *Limax* potentially allows a physiological analysis of neural circuits involved in behavior, and some progress has been made in the analysis of the circuits involved in feeding (14, 15). Moreover, evidence of associative learning in the isolated brain of *Limax* has recently been obtained (16) by using a conditioning paradigm similar to the one used in our whole-animal experiments. Thus, by using behavioral procedures that allow a clearer definition of the variables that influence associative learning by the intact slug and an *in vitro* preparation that is amenable to cellular analysis, it may be possible to make progress in understanding the synaptic interactions that underlie associative learning.

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