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Olfactory Learning and Memory in the Cockroach *Periplaneta americana*

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ABSTRACT—The olfactory learning capability of the male cockroach *Periplaneta americana* was studied using an operant discriminatory conditioning paradigm. Cockroaches were highly motivated to search for a sucrose solution by giving them a diet that contained no sugar and by limiting their water supply, and they were individually trained to associate peppermint with sucrose solution and vanilla with NaCl solution. Their odor preference was tested before and after training by allowing them to choose between peppermint and vanilla sources. The time spent visiting each odor source served as a measure of odor preference. Cockroaches exhibited an innate preference for vanilla over peppermint but the preference for the latter increased after only one training session. The olfactory memory formed by a single training session remained for at least one week but significantly decayed from 2 to 24 hr after training. Memory formed by three training sessions was extremely robust, exhibiting no significant decay from one day to four weeks after training. The preference formed was easily altered by reversal training in which vanilla was associated with sucrose solution and peppermint with saline. This study shows that cockroaches have an excellent olfactory learning capability characterized by rapid acquisition, extremely long retention and easy re-writing of memory.

INTRODUCTION

Odors play important roles in searches by animals for food or for mates, especially for nocturnal animals such as cockroaches. The cockroach *Periplaneta americana* is one of the few insects whose olfactory systems have been studied at both the peripheral and central levels (Boeckh and Ernst, 1987; Smith and Getz, 1994; Lemon and Getz, 1999). A class of olfactory receptor neurons on the antennae responds to sex pheromone components (Sass, 1983) and the other class of neurons responds to general food odors (Selzer, 1984; Fujimura *et al*., 1991; Getz and Akers, 1997), and axons of these two classes of receptor neurons terminate in different glomeruli of the antennal lobe (Boeckh and Ernst, 1987). Neurons that originate from the antennal lobe project to the mushroom bodies (higher associative centers) and the lateral protocerebrum (Boeckh and Ernst, 1987; Malun *et al*., 1993; Nishikawa *et al*., 1998). The mushroom bodies have highly organized internal structures (Mizunami *et al*., 1998a, b; Iwasaki *et al*., 1999) and have neural connections to various protocerebral neuropils (Yamazaki *et al*., 1998; Nishino and Mizunami, 1998; Li and Strausfeld, 1997, 1999).

Most previous studies on olfactory behavior of cockroaches have focused on pheromone-induced orientation behavior of males. Components of female sex pheromone (periplanone-A and -B) induce males to face upwind (Seelinger and Gagel, 1985) and to walk a zigzag path upwind toward a pheromone source (Tobin, 1981). While many efforts have made to clarify neural mechanisms underlying pheromone-induced orientation behavior of males (Boeckh and Ernst, 1987), few efforts were made to clarify neural mechanisms underlying processing of general food odors (Smith and Getz, 1994; Lemon and Getz, 1999), mainly because no appropriate learning paradigms in which to study the capability of cockroaches to discriminate and memorize general odors have been established.

The olfactory learning capability of cockroaches was reported by Balderrama (1980) who studied the performance of female cockroaches in an operant discriminatory conditioning paradigm that was designed to associate menthol with sucrose solution and vanilla with NaCl solution. He reported that: 1) cockroaches could learn even in one training session, 2) the memory formed by one training session could be retained for one week, 3) memory formed by one training session was robust with no significant decay for one week after the training, and 4) increase in the number of training sessions did not enhance memory retention. However, we find no documentation of the olfactory learning behavior of cockroaches since

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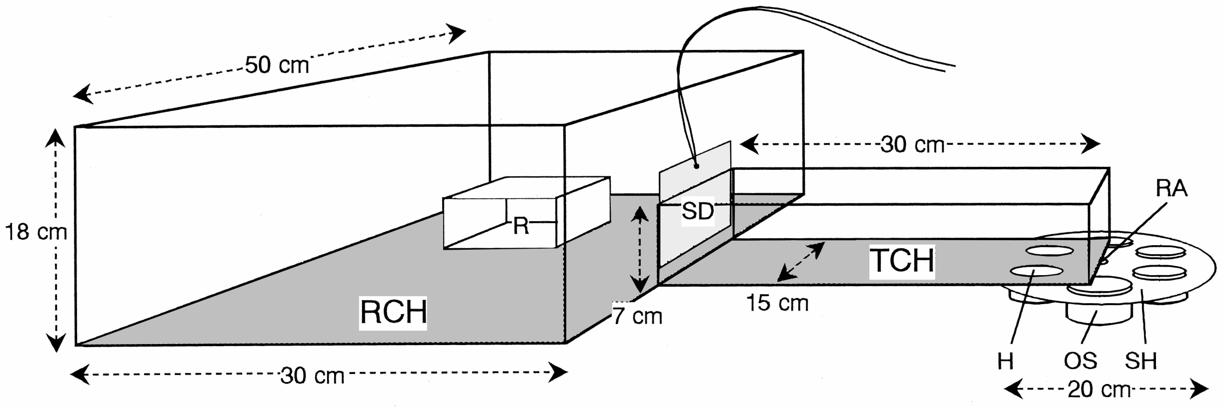

Fig. 1. A diagram of the experimental apparatus. **RCH**, resident chamber; **TCH**, training chamber; **R**, refuge; **SD**, sliding door; **SH**; odor source holder; **RA**, rotating axle; **OS**, odor source; **H**, holes connecting the chamber with two of the six odor sources.

the publication by Balderrama. Such being the case, we examined the olfactory learning capability of cockroaches in detail in order to confirm and extend the findings of Balderrama. We studied the performance of male cockroaches in a conditioning paradigm similar to that used by Balderrama (1980), with some improvements to his experimental procedure.

MATERIALS AND METHODS

Insect

The adult male cockroaches *Periplaneta americana* we used were obtained from a laboratory colony maintained under a 14hr:10hr light:dark photoperiod at 27–29°C. All experiments were done in a subjective night in a room with a reversed 12hr:12hr light:dark cycle (light, 1:00–13:00) at 26–30°C.

Experimental arrangement

The training paradigm was a modification of that used by Balderrama (1980). The apparatus (Fig. 1) consisted of two acrylic chambers, a “resident chamber” and a “training chamber”, connected by a sliding door that could be opened and closed manually. The wall of each chamber was smeared with Vaseline to prevent escape, and the floor was covered with black cardboard. There was a wooden refuge in the resident chamber.

At least one week before the start of the experiment, a group of 20–30 cockroaches was placed in the resident chamber. Each cockroach could be individually recognized by a paper label attached to the pronotum. In order to motivate the cockroaches to search for sucrose solution, they were provided yeast extract (which contained no sugar) *ad libitum* and water was available for drinking for only 1 hr per day.

On the floor of the training chamber, there were two circular holes (each 3.0 cm in diameter) that connected the chamber with two of the six sources of odors. Each odor source consisted of a plastic tube (4.0 cm in diameter, 3.5 cm in depth) covered with a fine gauze net. The six tubes were mounted on a rotatable holder, the axle of which was fixed to the front wall of the training chamber. Three of the six odor sources were used for testing, and the other three were used for training (Fig. 2). Just before starting the experiment, a filter paper (1.5 cm × 2.5 cm) soaked with odor solution was put into the tube. The filter paper was replaced with a fresh one every 2 hr during the experiment in order to prevent reduction in odor intensity. Two kinds of odor, vanilla (Meijiya, Tokyo, Japan) and peppermint (Kyoritsu Syokuhin, Tokyo, Japan), were used.

Odor preference test

The experiment consisted of two stages, a preference test and training, and was performed during the dark period under dim red light. During the experiment, the behavior of the cockroach was monitored by a CCD infrared camera (ELMO, Tokyo, Japan), and images were stored on an 8-mm video at a rate of 30 frames/s. Before start of

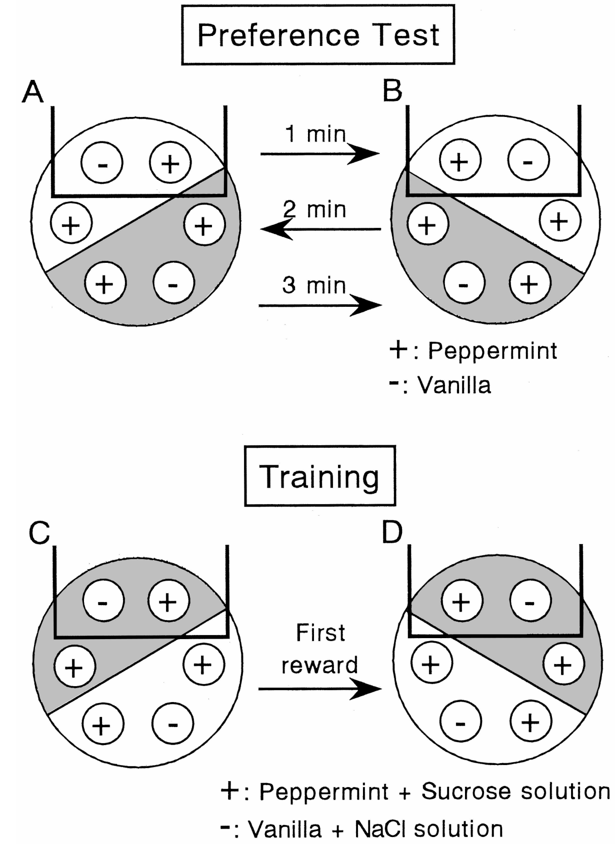

Fig. 2. Procedures for the preference test and training shown as viewed from above the odor source holder and a part of the training chamber. (+) represents the peppermint source and (–) represents the vanilla source. The three odor sources in the white half of the holder are for the preference test. Relative positions during the test are shown in (**A**) and (**B**). The three sources in the gray half of the holder are for training. Relative positions during training are shown in (**C**) and (**D**). The top net of the peppermint source contained drops of sucrose solution, and the top net of the vanilla source contained saline solution.

the preference test, vanilla and peppermint sources were placed on the floor of the training chamber (Fig. 2A), then, the sliding door was opened. Most cockroaches in the resident chamber approached the entrance of the training chamber while vigorously swinging their antennae. After a cockroach had entered the training chamber, the door was closed and the test started. During the test, the source holder was rotated every minute so that the relative positions of vanilla and peppermint odors were interchanged. Every test lasted four minutes (Figs. 2A and B). We defined a "visit" to an odor source as when the cockroach probed the top net with its mouth (labrum, labium, mandible and/or maxilla). The time spent visiting each odor source was measured based on video records. Relative odor preference was measured using a "peppermint preference index" (%), i.e., the total time spent visiting the peppermint source divided by the total time spent visiting either sources, multiplied by 100, and by a "learning index" (%), i.e., the preference index after training minus that before training. Wilcoxon's test was used to compare the odor preferences of a given cockroach group in different tests, the Mann-Whitney *U*-test was used to compare the performances of different cockroach groups, and the χ^2 test was used to evaluate initial preference.

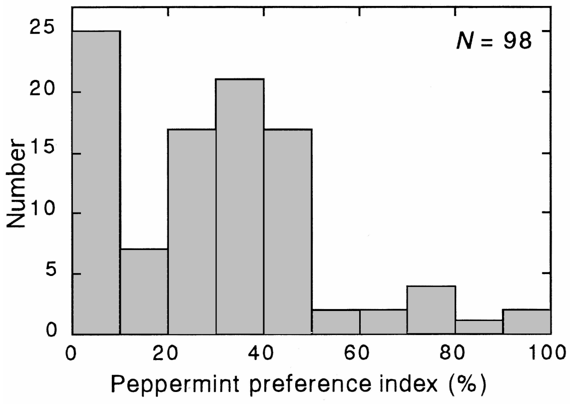

Fig. 3. Initial odor preference of cockroaches. The histogram shows distribution of the peppermint preference index (see Materials and methods) for each individual in a group of cockroaches before training. *N* is the number of cockroaches tested.

Training

Immediately after the end of a preference test, a training session was started. Because cockroaches had an innate preference for vanilla over peppermint (see Results), training was designed to associate peppermint with reward (30% sucrose solution) and vanilla with non-reward (20% NaCl solution) (Fig. 2C): 0.5 ml of sucrose solution was placed on the top net of the peppermint source and 0.5 ml of NaCl solution was placed on the top net of the vanilla source. When the cockroach visited the peppermint source, the insect was allowed to lick the sucrose solution for 15 seconds. Then, the relative odor

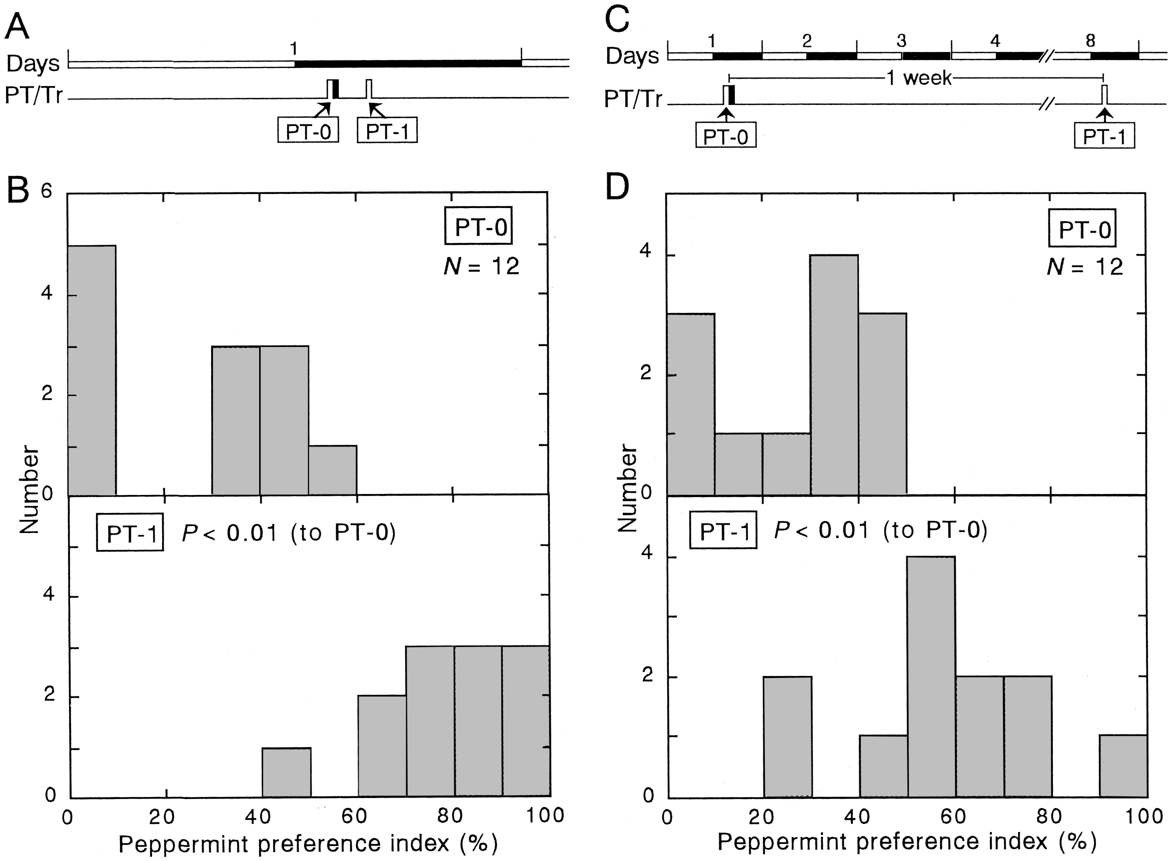

Fig. 4. The effects of a single training session. (**A**) and (**C**) are the time schedules for the preference test (PT, white bar) and for training (Tr, black bar), the results of the tests are shown as histograms in (**B**) and (**D**), respectively. The white and black parts of the time bar indicate photophase (12 hr) and scotophase (12 hr), respectively. In (**B**) and (**D**), distribution of the peppermint preference index (see Materials and methods) for each individual in a group of cockroaches in tests given before (PT-0 of (**B**) and (**D**)) and 2 hr (PT-1 of (**B**)) or 1 week (PT-1 of (**D**)) after training are shown. In each histogram, the results of statistical comparison with PT-0, using Wilcoxon's test, are shown with the significance level (*P*). *N* is the number of cockroaches tested.

positions were interchanged (Fig. 2D). After two rewarded visits, the training session was concluded by opening the sliding door and by gently pushing the cockroach into the resident chamber. At various times after training, cockroaches underwent preference tests, which we refer to as “retention” tests.

RESULTS

General behavior of cockroaches

The typical behavior of the cockroaches during the experiment was as follows: After the sliding door was opened at the start of a preference test, the cockroaches entered the training chamber very cautiously and walked straight toward either of the odor sources. At the odor source, the cockroach touched the top net with its mouth. After a while, the cockroach went away from the source and started to walk around in the training chamber. Typically, cockroaches visited odor sources repeatedly for the first two minutes from the start of the test and rarely visited thereafter. Therefore, a four-minute test was sufficient to evaluate the odor preference of cockroaches. Cockroaches that never visited odor sources during the test were not trained on that day and were tested again on another day.

During the training session, when the cockroach probed the saline solution at the vanilla source with its mouth, it retreated immediately. However, touching the saline with the antennae did not induce retreat. When the cockroach probed the sucrose solution at the peppermint source with its mouth, it started to intensively lick the solution. When positions of the odor sources were changed after the first rewarded visit (Fig. 2D), the cockroach rapidly retreated from the saline solution, and started to walk around the chamber.

Initial odor preference

In the initial preference test, the peppermint preference index was less than 50% in 87 of 98 cockroaches (Fig. 3), and the χ^2 test showed that they had a significantly greater preference for vanilla than for peppermint ($P < 0.005$). This appears to represent the innate preference of cockroaches, since they had never experienced vanilla or peppermint odor before the test.

Effects of a single training session

Two hr after a single training session (PT-1 of Fig. 4B), cockroaches exhibited a significantly greater preference for peppermint ($P < 0.01$, Wilcoxon’s test) than seen before the training (PT-0 of Fig. 4B), indicating that one training session is sufficient to establish olfactory learning. Cockroaches tested one week after a single training session (PT-1 of Fig. 4D) also exhibited a significantly greater preference for peppermint than seen before the training (PT-0 of Fig. 4D, $P < 0.01$, Wilcoxon’s test), indicating that memory formed by a single training session can be maintained for at least 1 week.

We compared odor preferences of three groups of cockroaches, each of which was given a retention test 2 hr (PT-1 of Fig. 4B), 24 hr (PT-1 of Fig. 5) or 1 week (PT-1 of Fig. 4D) after a single training session. The initial preference for peppermint did not differ among the three groups ($P > 0.05$, Mann-Whitney *U*-test). At 2 hr after a single training session, the cockroaches exhibited a significantly greater peppermint preference than seen at 24 hr ($P < 0.05$, Mann-Whitney *U*-test) or 1 week ($P < 0.01$, Mann-Whitney *U*-test) after training, indicating that there was a memory decay from 2 to 24 hr after training. Analysis using the learning index (difference between

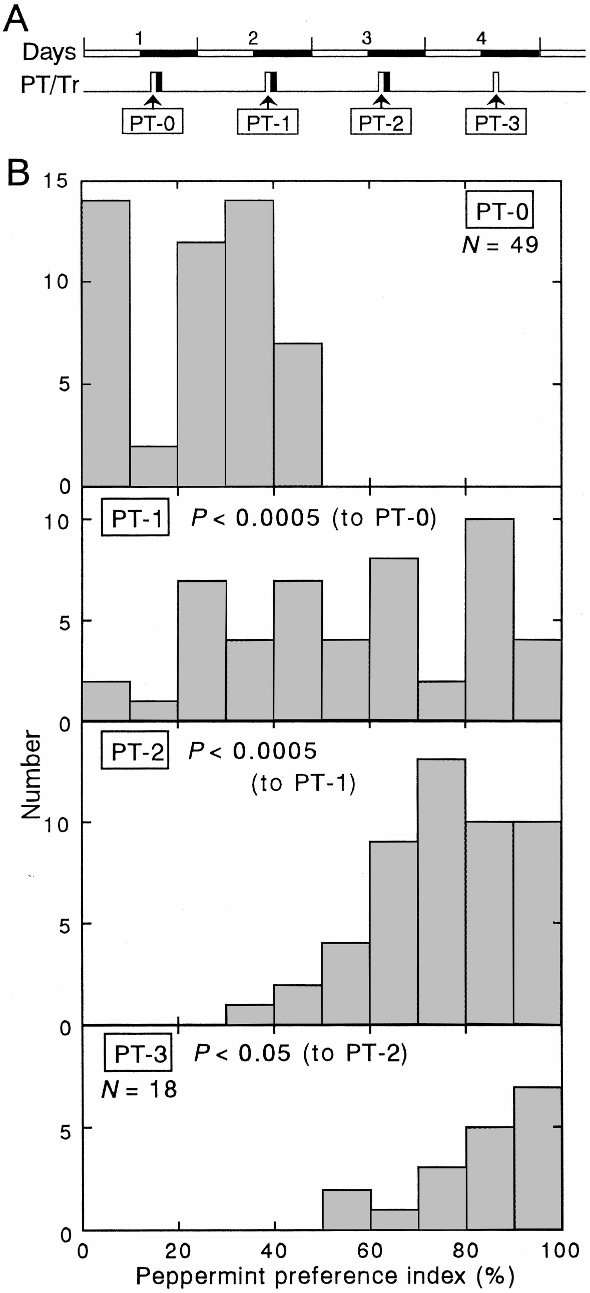

Fig. 5. The effects of three training sessions performed on three consecutive days. (**A**) shows the time schedule. (**B**) shows histograms of the distribution of the peppermint preference index for each individual in a group of cockroaches in tests made before (PT-0) and 1 day after the first (PT-1), second (PT-2) and third (PT-3) training sessions. In each histogram, the results of statistical comparison, using Wilcoxon’s test, are shown with the significance level (*P*). *N* is the number of cockroaches tested.

the peppermint preference before and that after the training) also showed a significant memory decay from 2 to 24 hr after training ($P < 0.05$, Mann-Whitney *U*-test). However, no significant memory decay was found from 24 hr to 1 week after a single training session, based on results of analysis using the peppermint preference index or learning index ($P > 0.5$, Mann-Whitney *U*-test).

Effects of three training sessions

Effects of three training sessions performed on three consecutive days are shown in Figs. 5–7. The preference for peppermint increased with increase in the number of training sessions. The preference for peppermint after the second training session (PT-2 of Fig. 5) was significantly greater than that after the first training session (PT-1, $P < 0.0005$, Wilcoxon's test). The preference for peppermint after the third training session (PT-3) was greater than that after the second session ($P < 0.05$, Wilcoxon's test).

We then compared odor preferences of three groups of cockroaches, each of which was given a retention test 1 day (PT-3 of Fig. 5), 1 week (PT-3 of Fig. 6), or 4 weeks (PT-3 of Fig. 7) after three training sessions. The initial preference for peppermint did not differ among the three groups ($P > 0.5$, Mann-Whitney *U*-test). Figs. 5–7 may give an impression that the peppermint preference after 4 weeks of the training was slightly less than that after 1 day or 7 days, but this difference was non-significant: The preference for peppermint did not significantly differ among cockroaches tested 1 day, 7 days and 4 weeks after three training sessions ($P > 0.25$ for 1 day vs. 1 week, $P > 0.05$ for 1 day vs. 4 weeks, Mann-Whitney *U*-test), indicating no significant memory decay from 1 day to 4 weeks after the training. Analysis using the learning index also showed no significant memory decay from 1 day to 4 weeks

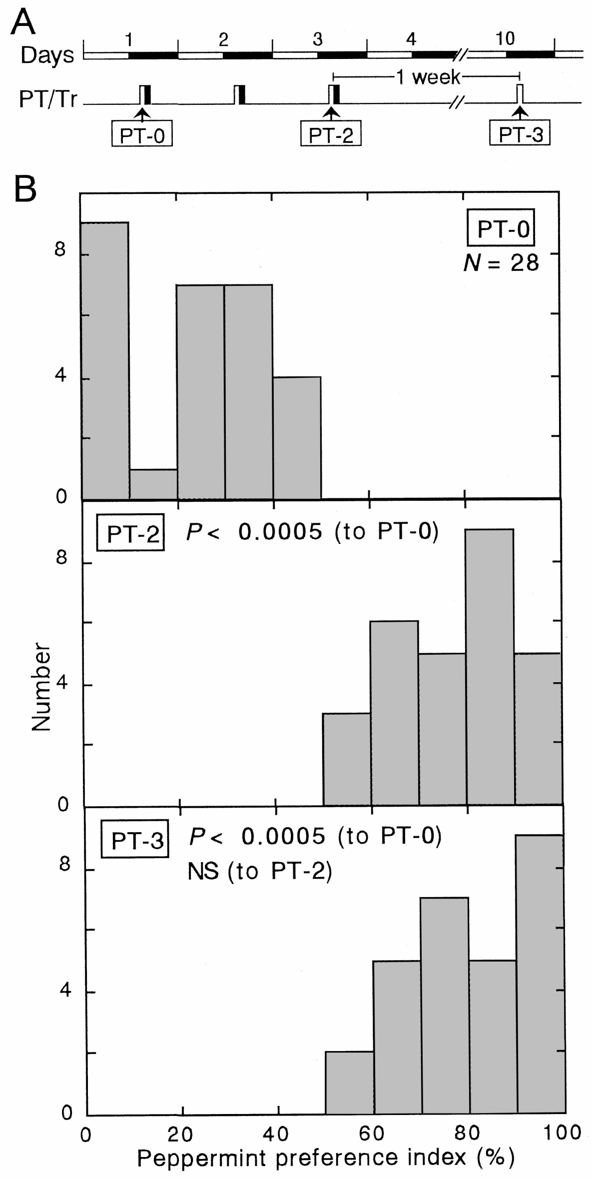

Fig. 6. Memory retention 1 week after three training sessions. (**A**) shows the time schedule. (**B**) shows histograms of the distribution of the peppermint preference index for each individual in a group of cockroaches in tests made before training (PT-0), 1 day after the second training session (PT-2) and 1 week after the third training session (PT-3). In each histogram, the results of statistical comparison, using Wilcoxon's test, are shown with the significance level (*P*). NS, not significant. *N* is the number of cockroaches tested.

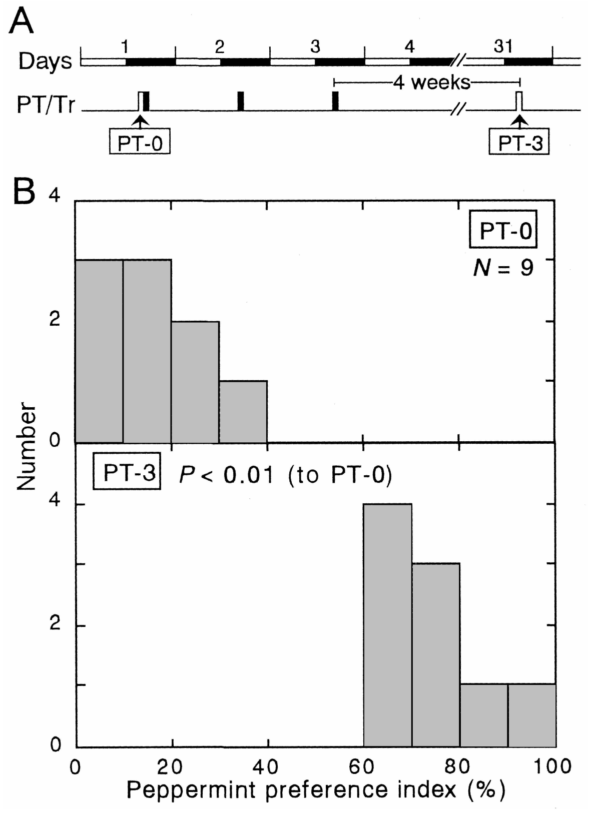

Fig. 7. Memory retention 4 weeks after three training sessions. (**A**) shows the time schedule. (**B**) shows histograms of the distribution of the peppermint preference index for each individual in a group of cockroaches in tests made before training (PT-0) and 4 weeks after the third training session (PT-3). In the PT-3 histogram, the results of statistical comparison, using Wilcoxon's test, are shown with the significance level (*P*). *N* is the number of cockroaches tested.

after three training sessions ($P > 0.25$ for 1 day vs. 1 week, $P > 0.05$ for 1 day vs. 4 weeks; Mann-Whitney *U*-test), indicating that memory formed by three training sessions was well retained.

Effects of reversal training

Cockroaches given three training sessions to associate peppermint with sucrose solution and vanilla with saline solution were re-trained to associate vanilla with sucrose solution and peppermint with saline (Fig. 8). After the first reversal training session (PT-4 of Fig. 8), the preference for peppermint was significantly less than that after the third training session (PT-3, $P < 0.01$, Wilcoxon's test). After the third reversal training session (PT-6), the preference for peppermint did not significantly differ from that of the initial preference (PT-0, $P > 0.05$, Wilcoxon's test). Odor memory, therefore, can be easily re-written by reversal training. Further re-training, however, did not have significant effects on odor preference: The preference for peppermint after the fourth and fifth (PT-8) reversal training sessions did not differ significantly from that after the third reversal training session (PT-6, $P > 0.05$, Wilcoxon's test).

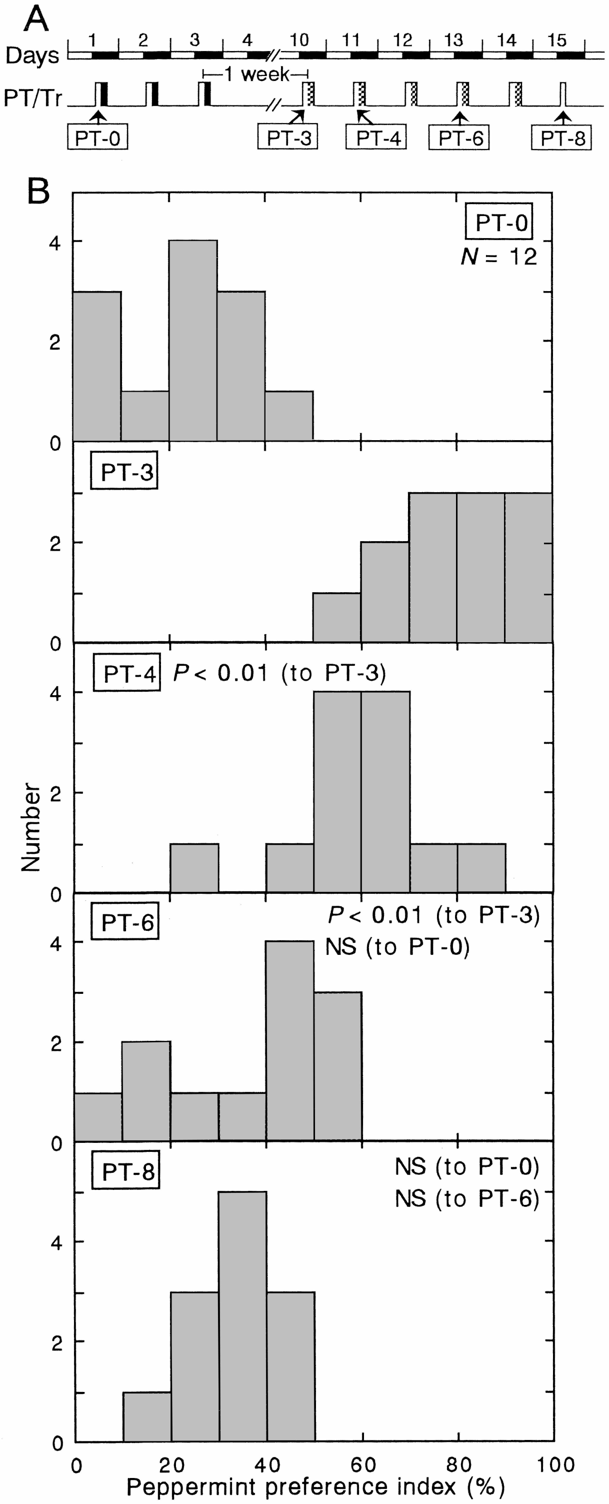

Fig. 8. Effects of reversal training, tested on some of the cockroaches used for the experiment shown in Fig. 6. (**A**) shows the time schedule. White and black bars indicate the preference test and training, respectively, and the gray bar indicates reversal training, in which vanilla was associated with sucrose solution and peppermint with saline solution. (**B**) shows histograms of the distribution of the peppermint preference index for each individual in a group of cockroaches in tests made before training (PT-0), 1 week after the third training session (PT-3), and 1 day after the first (PT-4), third (PT-6) and fifth (PT-8) reversal training sessions. In each histogram, the results of statistical comparison, using Wilcoxon's test, are shown with the significance level (*P*). NS, not significant. *N* is the number of cockroaches tested.

DISCUSSION

Major findings in the present study

The aim of the present study was to re-examine and extend the findings of Balderrama (1980), who studied olfactory learning capability of female cockroaches *Periplaneta americana*. The experimental paradigm used in the present study excluded the possibility that learning could be accomplished using cues other than olfactory ones, such as spatial, tactile or visual cues or their own odor marks. This paradigm was similar to that used by Balderrama (1980) but we updated experimental procedures. First, while he trained cockroaches to associate menthol with sucrose solution, we trained cockroaches to associate peppermint with sucrose solution, because we found that menthol was highly repellent for cockroaches and the training to associate it with sucrose solution took time. Second, while drinking water was provided *ad libitum* in his procedure, we limited the water supply to enhance the cockroaches' motivation to search for sugar water. Third, Balderrama measured the time spent visiting each odor source under dim red light, we recorded the behavior of cockroaches by infrared CCD camera and the times were later measured based on video recordings. As cockroaches move very quickly, real-time evaluation of cockroach behavior is difficult to perform accurately. Another difference between our study and that of Balderrama (1980) was that he studied the performance of female cockroaches and we studied the performance of male cockroaches. The majority of studies on olfactory systems of cockroaches have used males, and we considered that it was therefore important to examine the olfactory learning capability of males.

We were able to confirm three of the main conclusions made by Balderrama (1980): 1) one training session is sufficient to alter cockroaches' initial preference, 2) the altered performance is retained for at least 1 week and 3) the altered performance can easily be changed by re-training.

The present results, however, did not support two other conclusions made by Balderrama (1980). First, he concluded that memory formed by one training session is very robust, exhibiting no significant decay from just after (5 min – 5 hr) to 7 days after training ($P > 0.05$, *t*-test). However, we found that there was significant memory decay from 2 to 24 hr after one training session. Decay of memory formed by one or a small number of training sessions has been reported for olfactory learning of other insects, including the honey bee *Apis mellifera* (Menzel *et al*., 1993; Menzel and Müller 1996; Menzel, 1999), the fruit fly *Drosophila melanogaster* (Dudai, 1977; Dabnau and Tully, 1998), and the cricket *Gryllus bimaculatus* (Matsumoto and Mizunami, 2000). Second, although Balderrama (1980) reported that an increase in the number of training sessions did not improve memory retention, we found that memory retention was enhanced as the number of training sessions was increased. Our observation is, again, in accord with reported results on the honey bee (Menzel *et al*., 1993; Menzel and Müller 1996), the fruit fly (Dudai, 1977; Dabnau and Tully, 1998) and the cricket (Matsumoto and Mizunami, 2000). It is difficult to consider that the differences between the results of the present study and those of the study by Balderrama (1980) reflect sexual differences of the cockroaches tested, and it is more likely that the great dispersion of his data (see Discussion section in that paper) might have obscured real differences. The smaller variance of the data in the present study is obviously due to the use of improved experimental procedures.

We found that memory formed by three training sessions exhibited no significant decay from one day to four weeks, suggesting that this memory can be retained for an unusually long time, most likely for at least a few months. In conclusion, the results of this study show that cockroaches have a high capability of olfactory learning, characterized by rapid acquisition, unusually long retention, and easy rewriting of memory. These excellent olfactory learning capabilities of cockroaches may match their life-styles, that is, they are nocturnal insects relying heavily on olfaction and have omnivorous feeding habits, selecting what is edible or inedible after testing various organic materials.

Contact chemoreceptors on cockroach antennae

The antennae of *Periplaneta americana* are equipped with contact chemoreceptors that were reported to respond to sucrose or NaCl solution (Hansen-Delkeskamp, 1992). We found no behavioral evidence to suggest that cockroaches are capable of finding sucrose or NaCl solution using their antennae. During the training session, not all of the cockroaches that touched the sucrose solution with their antennae approached the sucrose solution to drink it, and not all of the cockroaches that touched the NaCl solution with their antennae retreated from the solution. The role of contact chemoreceptors on cockroach antennae as related to behavior remains to be elucidated.

Future perspective

Cockroaches have been used as a pertinent model to study neural mechanisms underlying odor-induced behavior, and there has been an accumulation of information on mechanisms for detecting and processing of female sex-pheromone by males (Boeckh and Ernst, 1987). However, neural mechanisms underlying the processing of general food odors remain largely unknown (Smith and Getz, 1994; Lemon and Getz, 1999), and analyses of odor discriminatory capability in an appropriate learning paradigm are urgently needed for a better understanding of neural coding of general odors. We are currently examining the capability of cockroaches to discriminate aliphatic alcohols with different lengths of carbon chain, using a learning paradigm modified from that used in the present study (M. Sakura and M. Mizunami, unpublished observations). The results of such experiments may provide valuable information for interpreting response characteristics of olfactory receptors to these substances described previously (Fujimura *et al*., 1991; Gets and Akers, 1997). Moreover, we have established a method for making extracellular recordings of activities of brain neurons from freely behaving cockroaches, using thin wires (Mizunami *et al*., 1998c; Okada *et al*., 1999) and a method for training cockroaches to associate odors with reward or non-reward while making extracellular recordings of the activities of brain neurons, by improving the present training procedure (Y. Kobayashi and M. Mizunami, unpublished observations). Thus, cockroaches may prove to be a pertinent model for studying neural correlates of olfactory learning in normally behaving insects. The basic features of olfactory learning of cockroaches described here may provide a basis for future study of neural mechanisms of olfactory learning.

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