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Classical Olfactory Conditioning in the Cockroach *Periplaneta americana*

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ABSTRACT—We established a classical conditioning procedure for the cockroach, *Periplaneta americana*, by which odors were associated with reward or punishment. Cockroaches underwent differential conditioning trials in which peppermint odor was associated with sucrose solution and vanilla odor was associated with saline solution. Odor preference of cockroaches was tested by allowing them to choose between peppermint and vanilla sources. Cockroaches that had undergone one set of differential conditioning trials exhibited a significantly greater preference for peppermint odor than did untrained cockroaches. Memory formed by three sets of differential conditioning trials, with an inter-trial interval of 5 min, was retained at least 4 days after conditioning. This conditioning procedure was effective even for cockroaches that had been harnessed in plastic tubes. This study shows, for the first time in hemimetaborous insects, that both freely moving and harnessed insects are capable of forming olfactory memory by classical conditioning procedure. This procedure may be useful for future electrophysiological and pharmacological studies aimed at elucidation of neural mechanisms underlying olfactory learning and memory.

Key words: classical conditioning, learning, memory, cockroach, olfaction

INTRODUCTION

The cockroach, *Periplaneta americana,* is one of insects whose olfactory system has been studied at both the peripheral and central levels by anatomical and physiological methods (Boeckh and Ernst, 1987; Fujimura *et al*., 1991; Li and Strausfeld, 1997, 1999). Responses of olfactory receptor neurons on the antennae to pheromonal and non-pheromonal odors have been well characterized (Fujimura *et al*., 1991; Gets and Akers, 1997), and their axon terminals have been shown to form glomeruli in the antennal lobe and make synaptic connections with interneurons (Boeckh and Ernst, 1987). Projection neurons that originate in the antennal lobe terminate in the mushroom body and the lateral protocerebrum (Boeckh and Ernst, 1987; Strausfeld and Li, 1999; Nishino *et al*., 2003). Mushroom bodies have highly organized internal structures and have neural connections to various protocerebral neuropils (Mizunami *et al*., 1997, 1998a, b; Li and Strausfeld, 1997, 1999; Okada *et al*., 2003).

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Olfactory learning in insects has proven to be an ideal model in which to study many aspects of learning and memory and their neural mechanisms. In honey bees, *Apis mellifera*, studies using local cooling of the brain have suggested that the antennal lobe and the mushroom body participate in olfactory memory processing (Erber *et al*., 1980; Menzel, 1999). In fruit-flies, *Drosophila melanogaster*, mutants with defects in mushroom body structure exhibited impairment in olfactory learning (Heisenberg *et al*., 1985; Heisenberg, 2003). However, the neural mechanisms underlying olfactory memory processing in insects are still not clear.

Studies using operant conditioning procedures have shown that cockroaches have excellent olfactory learning and memory capabilities (Balderrama, 1980; Sakura and Mizunami, 2001, Sakura *et al*., 2002). Sakura and Mizunami (2001), for example, showed that: 1) a single training session is sufficient to alter a cockroach's odor preference, 2) memory formed by three training sessions lasts at least 4 weeks and 3) the odor preference altered by training can easily be changed by re-training. In these studies, conditioning was designed to associate one odor with reward and another odor with punishment by allowing cockroaches to

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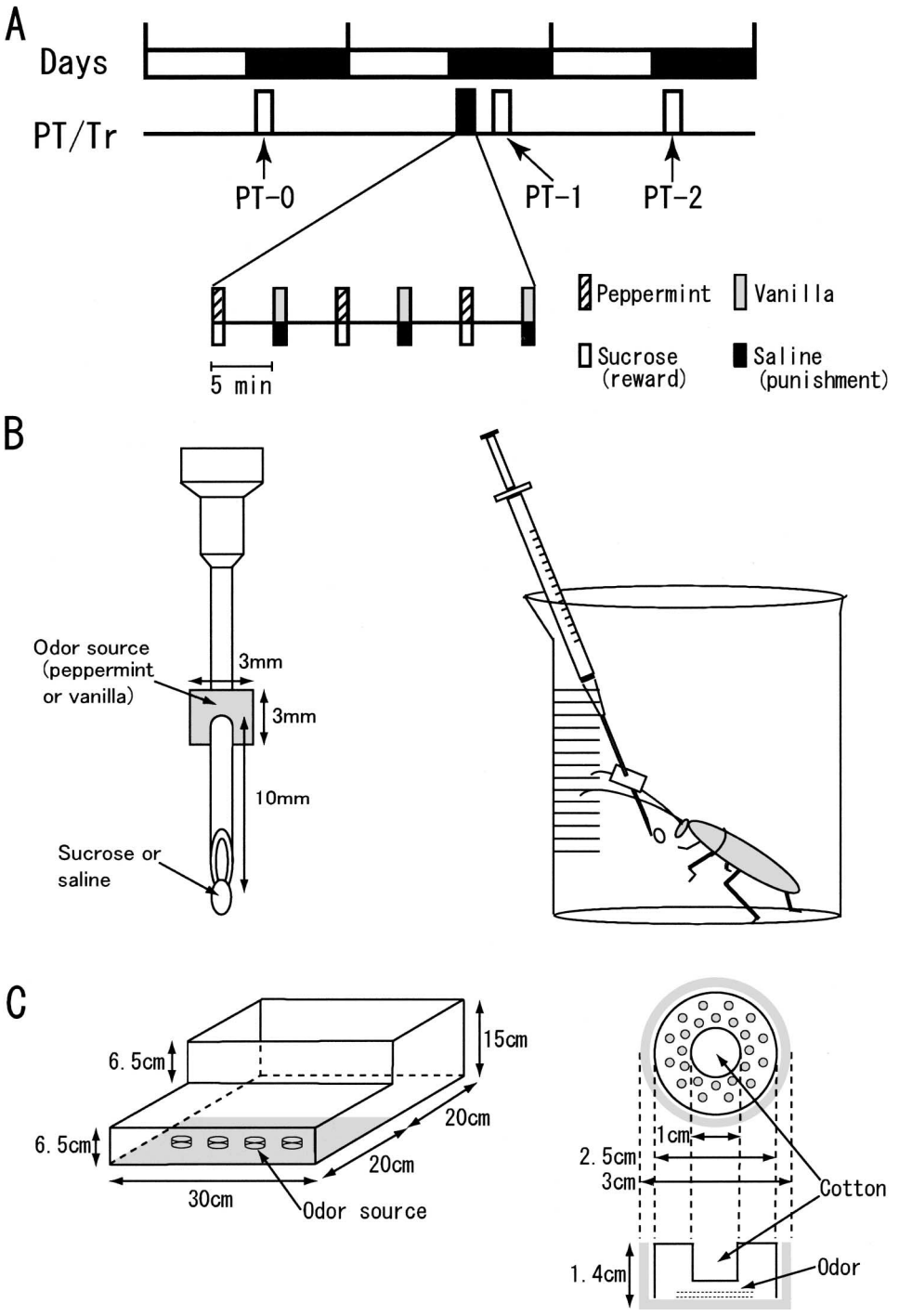


Fig. 1. (A) Typical time schedule for training and testing. Preference tests (PTs, open columns) were performed 1 day before training (PT-0) and 30 min (PT-1) and 1 day (PT-2) after training (Tr, filled column). The white and black parts of the time bar indicate photophase (12 hr) and scotophase (12 hr), respectively. A typical stimulus schedule for training, in which the hatched and shaded bars above the line indicate the presentation of peppermint and vanilla odors and the white and black squares below the line indicate the presentation of sucrose and saline solutions, respectively, is shown. For the appetitive conditioning trial, peppermint odor was associated with sucrose solution (reward), and for the aversive conditioning trial, vanilla odor was associated with saline solution (punishment). (B) Experimental arrangement for conditioning. A syringe containing sucrose or saline solution was used for conditioning. A filter paper soaked with peppermint or vanilla essence was attached to the needle of the syringe (left). The filter paper was placed within 1 cm of the cockroach's head so as to present a particular odor, and sucrose or saline solution was then presented to the mouth (right). (C) Diagrams of the testing chamber and odor source. The white half of the floor of the chamber is the "residence side", in which a refuge is placed and food and water are provided, and the gray half is the "testing side", in which odor source is placed during testing (left). An odor source is illustrated on the right side. The upper figure is a view from above the source, and the lower figure is a view of the vertical section. The gray and solid lines indicate a glass dish and a concave-shaped plastic cover, respectively. The broken double-line indicates filter papers soaked with vanilla or peppermint essence.

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freely visit a pair of odor sources and to drink sucrose solution (reward) at one odor source and to drink saline solution (punishment) at the other odor source. This operant conditioning procedure, however, is of limited use for studying neural mechanisms underlying learning and memory processing, because the experience of individual cockroaches during conditioning could not be controlled by the experimenter. To overcome this difficulty, we have developed a classical conditioning procedure in which the timing of the presentation of olfactory conditioning stimuli and gustatory unconditioned stimuli can be precisely determined by the experimenter. We found that this procedure is applicable even to cockroaches that have been harnessed in plastic tubes. This procedure may be useful for future electrophysiological and pharmacological studies aimed at elucidation of neural mechanisms of olfactory learning.

MATERIALS AND METHODS

Insects

Adult male cockroaches, *Periplaneta americana,* were obtained from a laboratory colony maintained under a light-dark cycle (LD 12:12) at 26–28°C . All experiments were carried out in a room with a reversed light-dark cycle (LD 12:12) (light on from 1:00 to 13:00) at 26–28°C.

One week before the start of experiment, a group of 20-30 cockroaches was placed in a testing chamber to allow them to become familiarized with the environment. The testing chamber was made of an acrylic plate and consisted of two parts, a “resident side” and a “testing side” (Fig. 1-C, left). The wall of the chamber was smeared with liquid paraffin to prevent the cockroaches from escaping, and the floor was covered with black cardboard. In the resident side, there was a wooden refuge and two small cups, one supplying sugar-free yeast extract and the other supplying water. Cockroaches were allowed to drink water and were fed a diet of sugar-free yeast extract *ad libitum* until four days before conditioning and were then deprived of drinking water in order to enhance their motivation to search for sucrose solution. One day before conditioning, each cockroach was placed individually in a beaker.

Classical conditioning procedure

The conditioning procedure was modified from that used for crickets by Matsumoto and Mizunami (2002) and Matsumoto *et al*. (2003). Training and testing were performed during the dark period under dim red light. Cockroaches placed in beakers (Fig. 1-B, right) were subjected to differential conditioning trial consisting of an appetitive conditioning trial in which peppermint odor was associated with 10% sucrose solution followed by an aversive conditioning trial in which vanilla odor was associated with 20% sodium chloride solution. Since cockroaches have an innate preference for vanilla odor over peppermint odor (Sakura and Mizunami, 2001), conditioning was designed to associate peppermint odor with reward and vanilla odor with punishment. Hypodermic syringes (each 1 ml) were used to apply conditioning and unconditioning stimuli. A small filter paper (3 mm×3 mm) was attached to the needle of the syringe at 10 mm from its tip (Fig. 1-B, left). The syringe used for the appetitive conditioning trial was filled with sucrose solution, and the filter paper attached to the needle was soaked with peppermint essence. The syringe used for the aversive conditioning trial was filled with saline solution, and the filter paper attached to the needle was soaked with vanilla essence. For odor presentation, the filter paper was placed within 1 cm of the cockroach’s head. Previous studies in other insects showed that conditioning was achieved when the onset of odor stimulus preceded taste stimulus by 1–5 s in honey bees (Menzel, 1990) and crickets (Matsumoto and Mizunami, 2000) and by 1–3 s in moths (Fan *et al*., 1997). In this study, at 2 sec after the onset of odor presentation, a drop of sucrose solution or saline solution was presented to the mouth of the cockroach for 2 sec. Then the air in the beaker was ventilated. After training, each cockroach was placed in a beaker and fed a diet of yeast extract but was deprived of drinking water until the odor preference test.

In one experiment, cockroaches were subjected to an odor preference test (PT-0) and were then harnessed in plastic tubes with a thin plastic plate between the head and thorax (Fig. 2). They could not move their limbs but could move their antennae and mouths freely. On the next day, they were subjected to differential conditioning. Immediately after the conditioning, each cockroach was released from the tube and placed in a beaker and was fed a diet of yeast extract *ad libitum* but was deprived of water until the next odor preference test.

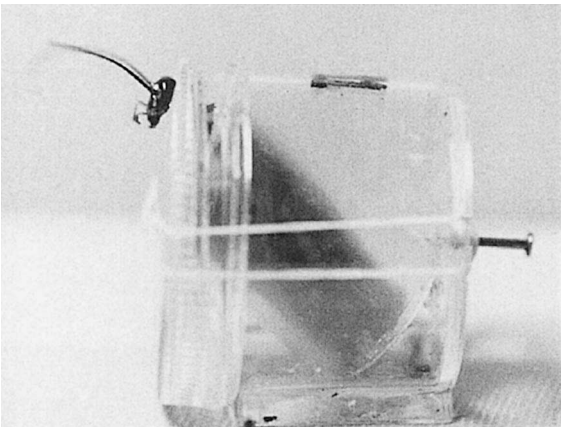


Fig. 2. A cockroach harnessed in a plastic tube. It can move its mouth and antennae freely.

Odor preference test

The testing procedure was modified from that used by Sakura *et al*. (2002) for cockroaches. At the beginning of the odor preference test, two vanilla sources and two peppermint sources were placed in the testing side (Fig. 1-C, right). For each source, two rectangular filter papers (0.5 cm×2.0 cm) that had been soaked with 20 μl of vanilla essence or peppermint essence were put into a glass dish and covered with a concave-shaped plastic cover. The cover had 24 small holes to allow the odor to emanate. Then one cockroach was put into the resident side of the chamber to observe which odor source it visited. It was considered that a cockroach had “visited” an odor source when the cockroach probed the plastic cover of the source with its mouthparts (labrum, labium, mandible, and/or maxilla). Immediately after (<1 sec) the cockroach had visited a source, it was driven away from the source to the residence side, and it was then allowed to walk into the testing side again. The arrangement of odor sources was randomly changed after each visit, and the number of visits to either source during a period of 10 minutes was counted. In the tests before conditioning, about 60% of the cockroaches visited the odor source at least once, and the average number of their visits was 1.5. In the tests after conditioning, about 90% of the cockroaches visited the odor source at least once, and the average number of their visits was 2.4. Cockroaches that visited no odor sources were excluded from data evaluation.

Four-day retention test

In another experiment, cockroaches underwent odor preference tests one day after conditioning and were then kept in the testing chamber as a group. They were allowed to drink water and were

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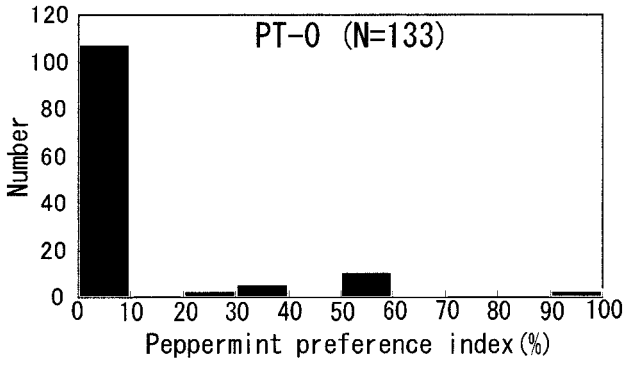


Fig. 3. Odor preference of untrained cockroaches. The histogram shows the distribution of the peppermint preference index for each individual in a group of cockroaches. N is the number of cockroaches. Data from cockroaches that visited no odor sources were excluded from analysis. The average number of visits in untrained cockroach was 1.5, and all cockroaches in the column of 0–10% peppermint preference index visited only vanilla sources.

fed a diet of yeast extract for a few hours, and they were then deprived of drinking water for three days, until the odor preference test at four days after conditioning.

Data analysis

The relative odor preference of each cockroach was determined using a "peppermint preference index (PI)" (%), defined as $100n_p/(n_v+n_p)$, where n_v and n_p are the number of visits to vanilla and peppermint sources during the odor preference test, respectively. Wilcoxon's test (WCX) was used to compare odor preferences between different tests in a given cockroach group, and the Mann-Whitney U-test (M-W) was used to compare odor preferences between different cockroach groups. Z-test was used to evaluate odor preference of a group of untrained cockroaches.

RESULTS

Odor preference of untrained cockroaches

Untrained cockroaches exhibited a significant preference for vanilla over peppermint: The results of Z-test showed that averaged peppermint preference index of this group were significantly less than 50% (P<0.001, T=–22.04, df=132) (Fig. 3). This is in agreement with the results of our previous study in which the odor preference of cockroaches was determined by the time spent at each odor source (Sakura and Mizunami, 2001).

Effect of the number of differential conditioning trials

Cockroaches were subjected to one (group 1 in Fig. 4-A), two (group 2) or three differential conditioning trials (group 3) with an inter-trial interval (ITI) of 5 min. In tests performed at 30 min after conditioning, preferences for peppermint in all groups were significantly greater than that in the untrained group (PT-0 group shown in Fig. 3) (M-W, PT-0-group 1 in Fig. 4-B: P<0.001, df=1, U=69.5; PT-0-group 2: P<0.001, df=1, U=45.5; PT-0-group3: P<0.001, df=1, U=24). The preference for peppermint in the one-trial group (group 1) did not differ from that in the two-trial group (M-W, P>0.05, df=1, U=145.5), and the preference for peppermint

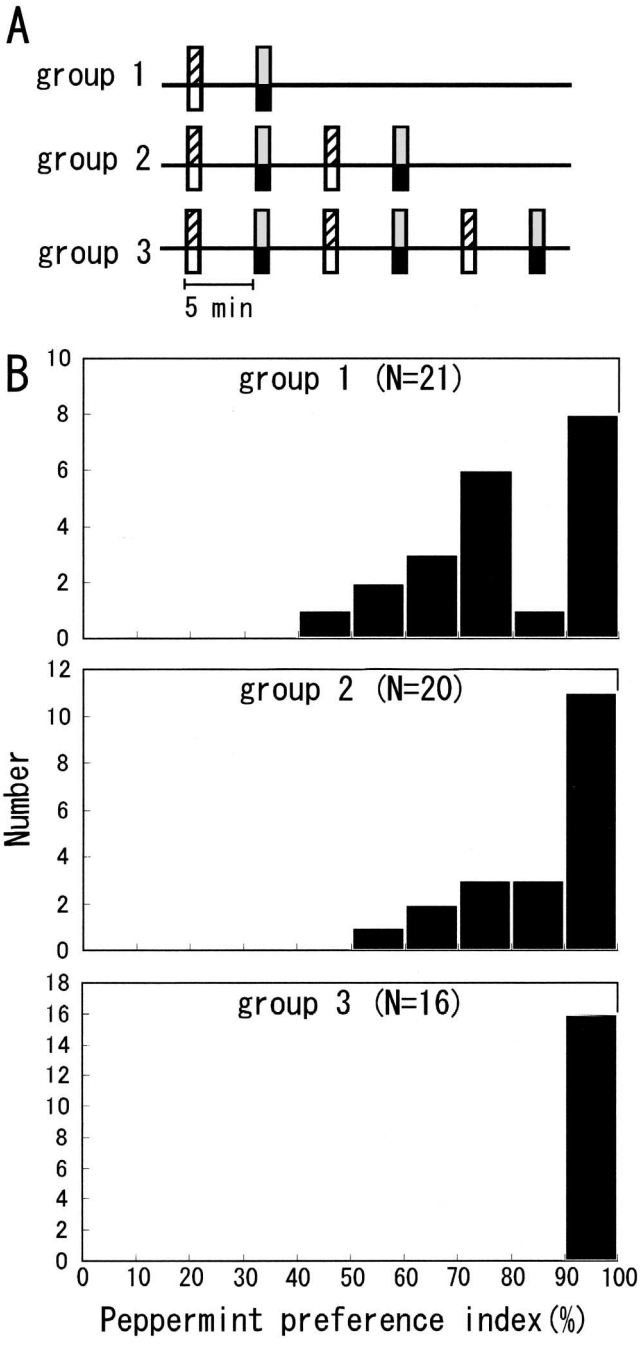


Fig. 4. Effect of the number of differential conditioning trials. (A) Stimulus schedules. Cockroaches in groups 1, 2 and 3 were subjected to one, two and three sets of differential conditioning trials with an ITI of 5 min, respectively. (B) Distributions of the peppermint preference index for each individual in groups 1–3 determined 30 min after conditioning.

in the three-trial group was greater than that of one-trial or two-trial group (M-W, group 1-group 3: P<0.001, df=1, U=56; group 2-group 3: P<0.01, df=1, U=88). Thus, the memory retention measured 30 min after conditioning was highest in the three-trial group.

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Effect of inter-trial interval

Cockroaches were subjected to three differential conditioning trials with inter-trial interval (ITI) of 30 sec (group 1 in Fig. 5-A), 1 min (group 2), 5 min (group 3) or 15 min (group 4). In tests performed at 30 min after conditioning, preferences for peppermint in all groups were significantly greater than that in the untrained group (PT-0 group shown in Fig. 3) (M-W, PT-0-group 1 in Fig. 5-B: $P<0.001$, df=1, U=38; PT-0-group 2: $P<0.001$, df=1, U=19.5; PT-0-group 3: $P<0.001$, df=1, U=24; PT-0-group 4: $P<0.001$, df=1, U=22.5). The preference for peppermint in the 30 sec ITI group did not differ from that in the 1 min ITI group (M-W, $P>0.05$, df=1, U=33), and the preference for peppermint in the 30 sec or 1 min ITI group was significantly less than that in the 5 min ITI group (M-W, $P<0.05$, df=1, U=56). Cockroaches in the 5 min and 15 min ITI groups exhibited saturated levels of retention: All visits during the test session were to peppermint sources. Thus, no significant difference was found between odor preferences in those two groups (M-W,

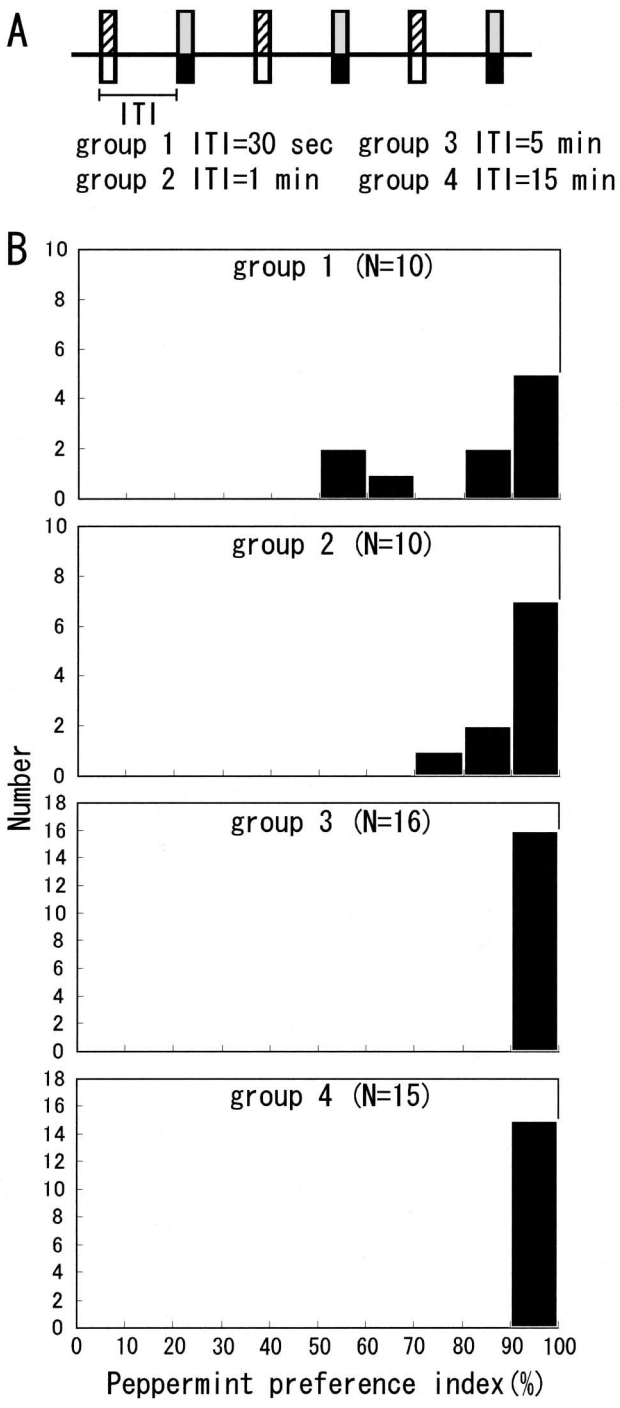


Fig. 5. Effect of inter-trial interval (ITI). (A) Stimulus schedules. Four groups of cockroaches were subjected to three sets of differential conditioning trials with an ITI of 30 sec (group 1), 1 min (group 2), 5 min (group 3) and 15 min (group 4). (B) Distributions of the peppermint preference index for each individual in groups 1–4 determined 30 min after conditioning.

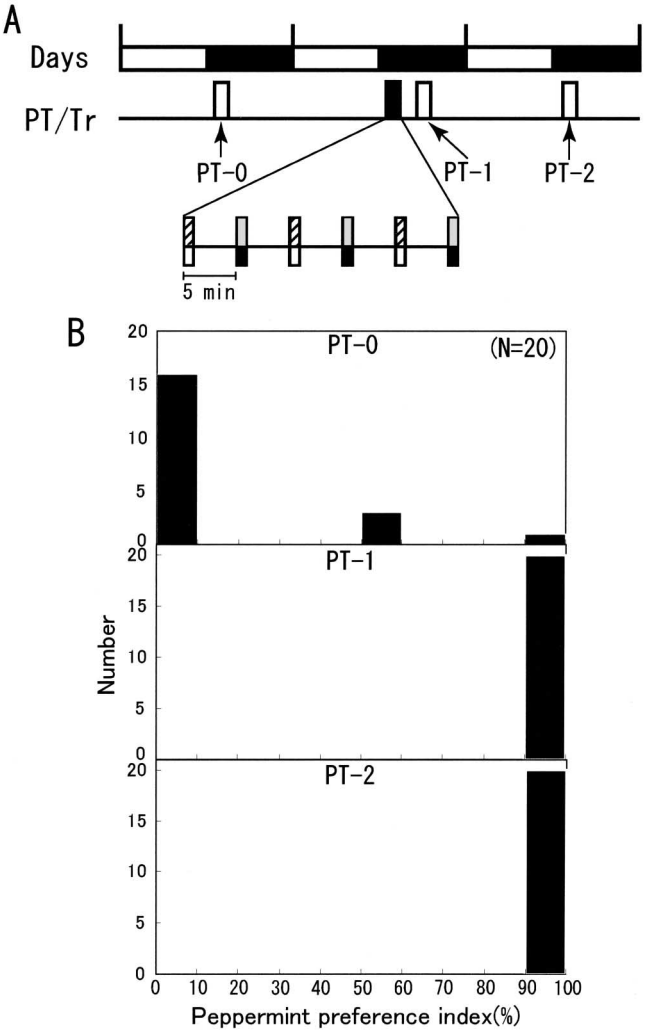


Fig. 6. One-day retention of olfactory memory. (A) Time schedule for the training (Tr, filled column) and odor preference tests (PT, open columns). Cockroaches were subjected to three sets of differential conditioning trials with an ITI of 5 min. Odor preference was tested before (PT-0) and at 30 min (PT-1) and 1 day (PT-2) after conditioning. (B) Distribution of the peppermint preference index for each individual in tests PT-0, PT-1 and PT-2. Data from cockroaches that visited odor sources at least once in all these three tests were used for analysis.

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P>0.05, df=1, U=120). We concluded that three differential conditioning trials with a long (5 min or 15 min) ITI are more effective than trials with a short (30 sec or 1 min) ITI.

Long-term retention of olfactory memory

A group of cockroaches was subjected to an odor preference test (PT-0 in Fig. 6). On the next day, they were subjected to three differential conditioning trials with an ITI of 5 min. The odor preference of cockroaches in the group was tested at 30 min (PT-1) and at 1 day after conditioning (PT-2). The preference for peppermint at 30 min or 1 day after conditioning was significantly greater than that before conditioning (WCX, PT-0-PT-1: *P*<0.01, df=1, J=0; PT-0-PT-2: *P*<0.01, df=1, J=0). At 30 min and 1 day after conditioning, the cockroaches exhibited a saturated level of retention: all visits during the test session were to peppermint sources. Thus, no significant decay of memory was observed during a period from 30 min to 1 day after conditioning.

Another group of cockroaches was subjected to three differential conditioning trials with an ITI of 5 min, and the odor preference of the cockroaches was tested at 1 day (PT-1 in Fig. 7) and at 4 days (PT-2) after conditioning. The preference for peppermint at 1 day or 4 days after conditioning in this group of cockroaches was significantly greater than that of untrained cockroaches (PT-0 group shown in Fig. 3) (M-W, PT-0-PT-1 in Fig. 7-B: *P*<0.001, df=1, U=24; PT-0-PT-2: *P*<0.001, df=1, U=24.5). At 1 day and 4 days after conditioning, this group of cockroaches exhibited an almost saturated level of retention, and no significant decay of memory retention was therefore observed from 1 day to 4 days after conditioning.

Classical conditioning of cockroaches harnessed in plastic tubes

A group of cockroaches was subjected to an odor preference test (PT-0 in Fig. 8), and each cockroach was then harnessed in a plastic tube (Fig. 2). On the next day, they were subjected to three differential conditioning trials with an ITI of 5 min. The odor preference of the cockroaches was tested 1 day after conditioning (PT-1). The preference for peppermint 1 day after conditioning was significantly greater than that before conditioning (WCX, *P*<0.01, df=1, J=0). However, the preference for peppermint of harnessed cockroaches 1 day after conditioning was significantly less than

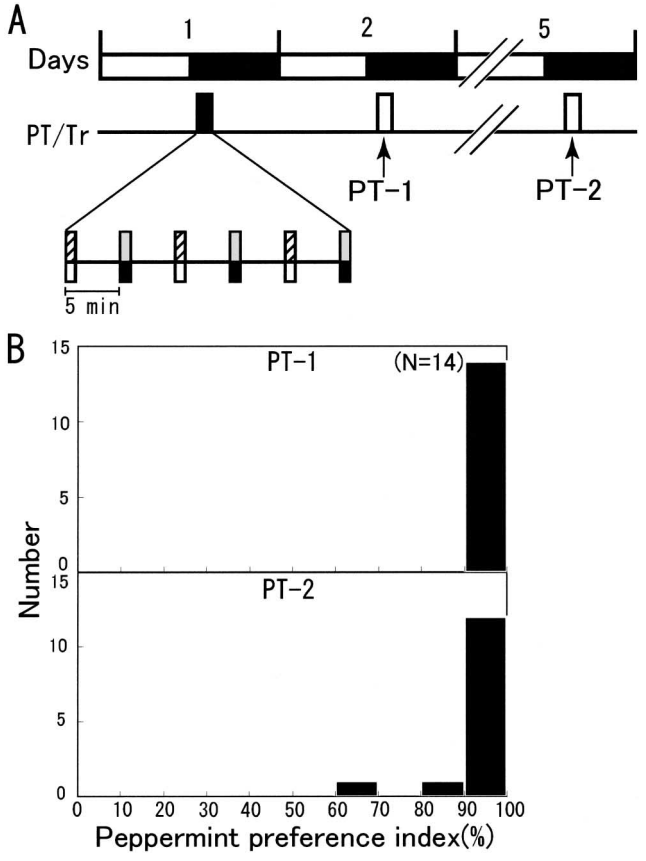


Fig. 7. Four-day retention of olfactory memory. (A) Time schedule for the training (Tr, filled column) and odor preference tests (PT, open columns). Cockroaches were subjected to three sets of differential conditioning trials with an ITI of 5 min. Odor preference was tested 1 day (PT-1) and 4 days (PT-2) after training. (B) Distribution of the peppermint preference index for each individual in tests PT-1 and PT-2. Data from cockroaches that visited odor sources at least once in both tests were used for analysis.

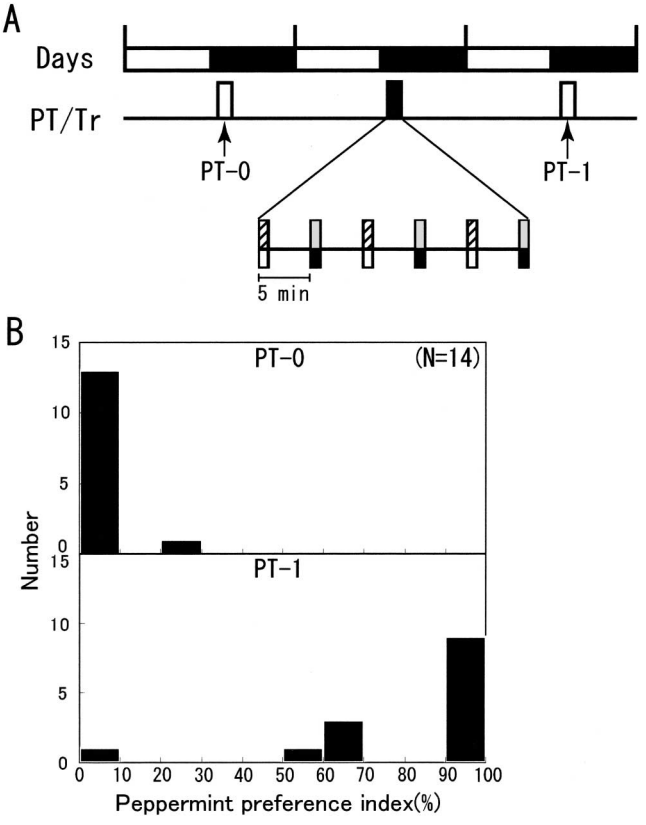


Fig. 8. Effect of conditioning on cockroaches harnessed in plastic tubes. (A) Time schedule for the training (Tr, filled column) and odor preference tests (PT, open columns). Odor preference was tested before (PT-0) and at 1 day (PT-1) after training. (B) Distribution of the peppermint preference index for each individual in tests PT-0 and PT-1. Data from cockroaches that visited odor sources at least once in both tests were used for analysis.

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that of cockroaches moving freely in beakers (M-W, PT-1 in Fig. 7 - PT-1 in Fig. 8: $P<0.05$, df=1, U=63). We conclude that our classical conditioning procedure is effective even for cockroaches harnessed in plastic tubes, although the memory formed was less than that of freely-moving cockroaches.

DISCUSSION

In the present study, we established an effective classical conditioning procedure to associate odors with tastes in cockroaches. One of advantages of this procedure over operant conditioning procedures used previously by Balderrama (1980), Sakura and Mizunami (2001) and Sakura *et al*. (2002) is that sensory experience during conditioning can be precisely controlled by the experimenter and it thus enables the effects of changes in stimulus parameters on conditioning to be determined.

During training, cockroaches were placed individually in beakers, and a pair of odors was presented sequentially, with the first odor paired with a reward and the second odor paired with a punishment. Cockroaches were later placed in a testing chamber and allowed to choose freely between the two odors presented simultaneously and without reinforcement. While there is no *a priori* reason for cockroaches to carry their training experience over to this active test situation, they clearly exhibited a transfer of the training effect. A similar transfer of olfactory memory to a different test situation has also been reported in the fruit fly (Tully and Quinn, 1985), the honey bee (Gerber *et al*., 1996) and the cricket (Matsumoto and Mizunami, 2002).

In most previous studies on cockroach olfactory learning, the odor preference of each cockroach was evaluated by the time spent at each odor source (Balderrama, 1980; Sakura and Mizunami, 2001), whereas it was evaluated by the number of visits to each source in this study. The preference of untrained cockroaches for peppermint determined by the number of visits (shown in Fig. 3) was significantly less than that determined by the time spent at each source in previous studies (Fig. 3 in Sakura and Mizunami, 2001) (M-H, $P<0.001$, df=1, U=3256). Because we have no data to compare the odor preference of trained cockroaches measured by these two methods due to the difference of training procedures used in these studies, it would be interesting to evaluate the odor preference of conditioned cockroaches using both methods and compare the results to determine which method is more appropriate for evaluating the effect of conditioning.

The properties of memory formed by the classical conditioning procedure in cockroaches are similar to those formed by a similar conditioning procedure in crickets reported by Matsumoto and Mizunami (2002) in the following aspects. First, only one differential conditioning trial was sufficient to achieve conditioning in both crickets and cockroaches. Second, a saturated level of olfactory retention was achieved by only two or three differential conditioning trials in both crickets and cockroaches. Third, two or three differential conditioning trials were sufficient for inducing 4-day memory retention in both crickets and cockroaches. Fourth, three differential conditioning trials with an ITI of 2 or 5 min resulted in greater retention than did three differential conditioning trials with an ITI of 30 sec or 1 min in both crickets and cockroaches. Similar results have been attained in honey bees (Gerber *et al*., 1998), fruit-flies *Drosophila* (Tully *et al*., 1994), and the requirement of training sessions with rest intervals to induce long-term memory appears to be a common feature among vertebrates and invertebrates (Dubnau and Tully, 1998). In crickets, two differential conditioning trials with a 10 min ITI were less effective than two differential conditioning trials with a 5 min ITI. In cockroaches, three differential conditioning trials with an ITI of 15 min and an ITI of 5 min resulted in saturated levels of retention, and future studies using a smaller number of trials are therefore needed to compare the effects of ITI on cockroaches and crickets.

This study shows, for the first time in insects except for honey bees (Menzel, 1999) and fruit-flies (Davis, 1996), that both freely-moving and harnessed insects are capable of forming olfactory memory by classical conditioning procedure. The preference for peppermint of harnessed cockroaches 1 day after conditioning was less than that of cockroaches moving freely, probably because the training was less effective due to the stress.

Insects have been shown to be pertinent models for studying the neural basis of olfactory learning and memory. In the honey bee, a mushroom body extrinsic neuron, PE1 neuron, has been suggested to be involved in the processing of short-term olfactory memory (Mauelshagen, 1993), and a neuron which projects from the subesophageal ganglion to the antennal lobes, mushroom bodies and lateral protocerebra (VUMmx1 neuron) possibly mediating the reinforcing function of sucrose reward in classical olfaction conditioning has been identified (Hammer, 1993). However, more electrophysiological and pharmacological studies are needed to clarify the mechanisms underlying memory formation and retention at the levels of neurons and neural networks. We found that our classical conditioning procedure is effective even when cockroaches have been harnessed in plastic tubes. Our next step is to apply this procedure to cockroaches harnessed for electrophysiological and pharmacological experiments and to study neural mechanisms underlying olfactory learning and memory.

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