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Temporal determinants of long-term retention of olfactory memory in the cricket *Gryllus bimaculatus*

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Summary

Temporal determinants of olfactory long-term memory retention in the cricket *Gryllus bimaculatus* were studied. Elementary appetitive and aversive conditioning procedures, as well as a differential conditioning procedure, were applied. In appetitive conditioning, peppermint odour was paired with a water reward. In aversive conditioning, vanilla odour was paired with saline solution. In differential conditioning, an appetitive conditioning trial was followed by an aversive conditioning trial. The odour preference of crickets was tested before and 2 h, 1 day and 4 days after training by allowing the crickets to choose between peppermint or vanilla sources. Differential conditioning or appetitive conditioning alone led to long-lasting memory retention with no significant decay from 2 h to 4 days after training, but retention after aversive conditioning was absent 1 day after training. Studies using differential conditioning have shown (i) that four trials are sufficient to cause a saturated level of acquisition, (ii) that conditioning is successful when the conditioned stimulus is presented immediately or 5 s before the onset of presentation of the unconditioned stimulus, (iii) that the optimal interval between trials is 2–5 min, and (iv) that anaesthetic treatment with CO_2 given immediately after training results in memory disruption but that anaesthetic-resistant memory develops fully 20 min after training. This study demonstrates that a differential conditioning procedure is particularly effective for the formation of long-term memory.

Key words: differential conditioning, memory, olfaction, insect, cricket, *Gryllus bimaculatus*, anaesthetic-resistant memory, long-term memory.

Introduction

Olfactory learning in insects has proved to be a pertinent model in which to study many aspects of learning and memory and their neural mechanisms (Menzel, 1999, 2001). In honeybees (*Apis mellifera*), studies using local cooling of the brain have implicated the participation of the antennal lobe and the mushroom body in olfactory memory processing (Erber et al., 1980), and electrophysiological studies have demonstrated that the activities of some brain neurons change in association with olfactory learning (Mauelshagen, 1993; Grünewald, 1999). In honeybees, the neural pathways that transmit the conditioned stimulus (CS) and the unconditioned stimulus (US) involved in olfactory conditioning have been identified (Hammer, 1993; Menzel, 2001). However, our knowledge of the neural mechanisms subserving olfactory memory processing is still incomplete.

We found in a study using an operant conditioning paradigm that the cricket *Gryllus bimaculatus* can learn an olfactory stimulus (Matsumoto and Mizunami, 2000). Crickets that had been given only one session of training to associate peppermint odour with a water reward and vanilla odour with saline solution exhibited a significantly increased preference for peppermint. Olfactory memory formed by a single training session was retained for at least 24 h, and that formed by three training sessions was retained for at least 7 days.

In the present study, we examined variables that govern olfactory long-term memory retention to determine the basic properties of olfactory memory formation in crickets. We changed our previous operant conditioning paradigm to a classical one because this enabled us to control precisely the timing between the presentation of the unconditioned stimulus (US) and the conditioned stimulus (CS), which has a profound influence on the formation and retention of associative memory in many systems of associative learning (Carew and Sahley, 1986; Rescorla, 1988). We focused on memory retention 2 h, 1 day and 4 days after training, which represent middle- to long-term memory retention in some insects (fruit flies *Drosophila melanogaster*, Tully et al., 1994; honeybees, Gerber et al., 1998; Menzel, 1999), because we wanted to focus our interest on the variables that determine long-term memory in insects. We examined (i) the effects of elementary aversive and appetitive conditioning trials and of differential conditioning trials, (ii) the effects of the interval between the CS and US, (iii) the effects of the interval between CS/US pairing trials, and (iv) the effects of anaesthetic treatment with

CO_2. The results are discussed with reference to data for honeybees and fruit flies.

Materials and methods

Insects

Adult male crickets, *Gryllus bimaculatus* de Geer, reared in a 12 h:12 h light:dark photoperiod (photophase 08:00–20:00 h) at 27±2 °C were fed a diet of insect pellets and water *ad libitum*. Four days before the start of the experiment, a group of 30 crickets was placed in a container and fed a diet of insect pellets *ad libitum*; these crickets were deprived of drinking water to enhance their motivation to search for water. On the day of the experiment, they were placed individually in 100 ml glass beakers. All experiments were carried out 1–2 weeks after the imaginal moult.

Training and testing

For conditioning, peppermint and vanilla odours were used as the conditioned stimuli (CS) and water and 20 % sodium chloride solution as the appetitive and aversive unconditioned stimuli (US), respectively. Because crickets have an innate preference for vanilla odour over peppermint odour (see Results and Matsumoto and Mizunami, 2000), conditioning was designed to associate peppermint odour with the appetitive US (reward) and vanilla odour with the aversive US (punishment). Hypodermic syringes (1 ml) were used for conditioning (Fig. 1A). A small filter paper (3 mm×3 mm) was attached to the needle of the syringe 10 mm from its tip. The syringe used for the appetitive conditioning trial was filled with water, 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 was soaked with vanilla essence. For odour presentation, the filter paper was placed within 1 cm of the cricket's head. At 2 s after the onset of odour presentation, a drop of water or saline solution was placed in front of the mouth of the cricket for 2 s. The air in the beaker was then ventilated for 2 s using a hand-held vacuum cleaner. After training, each cricket was fed a diet of insect pellets *ad libitum* in a beaker until the odour preference test was given.

The apparatus used for the odour preference test was slightly modified from that described previously (Matsumoto and Mizunami, 2000). Briefly, the apparatus consisted of three chambers, a 'test chamber' and two removable 'waiting chambers', one of which was placed at the 'waiting position' and the other at the 'entrance position' (Fig. 1B). There was a sliding door between the waiting chamber at the entrance position and the test chamber. On the floor of the test chamber, there were two circular holes (H in Fig. 1B) that connected the chamber with two of the three sources of odour. Each odour source consisted of a cylindrical plastic container covered with a fine gauze net. The three containers were mounted on a rotatable holder. Two odour sources could be located simultaneously just below the holes at the 'offer position' by rotating the holder.

Before the preference test, a cricket was transferred from the beaker to the waiting chamber at the waiting position and left for approximately 4 min to become accustomed to its surroundings. The waiting chamber was then slid into the entrance position, and the door to the test chamber was opened. When the cricket entered the test chamber, the door was closed and the test started. Two minutes later, the relative positions of the vanilla and peppermint sources were changed by rotating the container holder. An odour source was considered to have been visited when the cricket probed the top net with its mouth. The time spent visiting each odour source was measured cumulatively. If the total time of a visit to either source was less than 20 s, the data were rejected. The preference test lasted for 4 min. At the end of training, the sliding door was opened and the cricket was gently pushed into the waiting chamber and then was transferred to a beaker. Daily testing and training began at 11:00 h and lasted a maximum of 4 h. Following testing, crickets were fed a diet of insect pellets *ad libitum* until the next retention test. When crickets were subjected to retention tests 1 day and 4 days after training, a few drops of water were given after the 1 day retention test and also on the subsequent day, but not thereafter. Each cricket was used only once.

Conditioning procedure

We used five conditioning procedures. In the first, crickets were trained to associate peppermint odour with water, which we refer to as the appetitive conditioning procedure or the P+ (peppermint-rewarded) conditioning trial. In the second procedure, crickets were trained to associate vanilla odour with saline solution, which we refer to as the aversive or the V– (vanilla-punished) conditioning trial. In the third procedure, an appetitive conditioning trial was followed by an aversive conditioning trial, which we refer to as (a set of) differential conditioning trials with peppermint-rewarded and vanilla-punished or P+/V– conditioning trials. In the fourth procedure, an aversive conditioning trial was followed by the presentation of peppermint odour alone, without pairing with the US, which we refer to as (a set of) differential conditioning trials with vanilla-punished and peppermint-unpunished or V–/P^0 conditioning trials. In the fifth procedure, an appetitive conditioning trial was followed by presentation of vanilla odour alone, which we refer to as (a set of) differential conditioning trials with peppermint-rewarded and vanilla-unrewarded or P+/V^0 conditioning trials.

Experiment 1: effects of two-trial conditioning

The effects of two-trial conditioning were tested in three groups of crickets. The first group was subjected to a set of P+/V– conditioning trials (group 1, N=29), the second group was subjected to two V– conditioning trials (group 2, N=15) and the third group was subjected to two P+ conditioning trials (group 3, N=18). The inter-trial interval (ITI) was 5 min for all groups. The odour preference of individual crickets was tested before (PT-0) and 2 h (PT-1), 1 day (PT-2) and 4 days (PT-3) after training; thus, each cricket underwent three cumulative

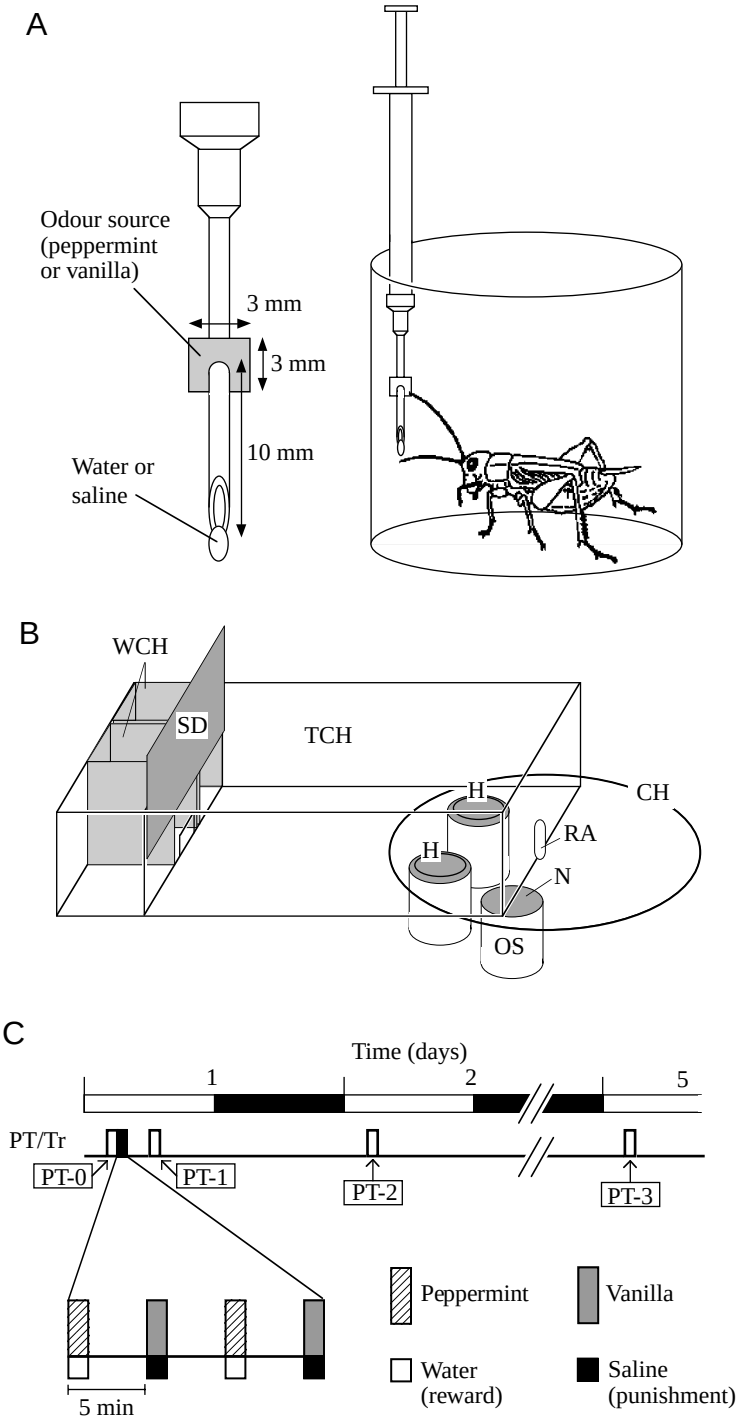

Fig. 1. (A) Experimental arrangement for conditioning. A syringe containing water or saline solution was used for conditioning. A filter paper soaked with peppermint or vanilla essence was attached to the needle of the syringe 10 mm from its tip (see left). The filter paper was placed within 1 cm of the cricket's head so as to present a particular odour, and water or saline was then presented to the mouth (right). (B) The apparatus used for the odour preference test. WCH, waiting chambers; TCH, training chamber; CH, container holder; RA, rotating axle; OS, odour source; N, gauze net; SD, sliding door; H, holes connecting the chamber with two of three odour sources. (C) Typical time schedule for training and testing; preference tests (PTs, open columns) were performed before (PT-0) and 2 h (PT-1), 1 day (PT-2) and 4 days (PT-3) after training (Tr, filled column). The white and black parts of the time bar indicate photophase (12 h) and scotophase (12 h), respectively. A typical stimulus schedule for training is illustrated at the bottom, in which the hatched and shaded bars above the line indicate the presentation of peppermint or vanilla odour, and the white and black squares below the line indicate the presentation of water or saline solution, respectively. For appetitive conditioning, peppermint odour was associated with water (reward); for aversive conditioning, vanilla odour was associated with saline solution (punishment).

retention tests. We have shown that the retention tests, given three times, have no significant extinction effect (Matsumoto and Mizunami, 2000).

Experiment 2: effects of four-trial conditioning

The effects of four-trial conditioning were tested in five groups of crickets. Three groups of crickets were subjected to two sets of P+/V– (group 1, N=45), V–/P^0 (group 4, N=22) or P+/V^0 (group 5, N=23) conditioning trials. The other two groups were subjected to four V– (group 2, N=22) or P+ (group 3, N=26) conditioning trials. The ITI was 5 min for all groups. The odour preference of individual crickets was tested before and 2 h, 1 day and 4 days after training.

Experiment 3: effects of different numbers of differential conditioning trials

To study the effects of different numbers of trials, three groups of crickets were subjected to one (group 1, N=29), two (group 2, N=45) or three (group 3, N=24) sets of P+/V– conditioning trials, and another group was subjected to two P+ conditioning trials followed by two V– conditioning trials (group 4, N=22). The ITI was 5 min. The odour preference of individual crickets was tested before and 2 h, 1 day and 4 days after training.

Experiment 4: non-associative controls and the effects of inter-stimulus interval

Different procedures were used for eight groups of crickets. Non-associative control procedures were used for three groups. The first group received CS presentations without pairing with the US in the sequence peppermint, vanilla, peppermint and then vanilla (CS alone group, N=26); the second group received US presentations without pairing with the CS in the sequence water, saline, water and then saline (US alone group, N=21); and the third group received unpaired presentations of the CS and US, in the sequence peppermint, water, vanilla, saline, peppermint, saline, vanilla and then water (CS/US unpaired group, N=27). The other five groups were subjected to two sets of P+/V– conditioning trials (see Fig. 1C) with different time relationships between the US and CS. In the coincident group, the CS was presented immediately before the

onset of US presentation (N=25). In the backward 4 s group, the CS was presented 4 s after the onset of US presentation (N=25). In the forward conditioning groups, the CS was presented 5, 10 and 20 s before the onset of US presentation (forward 5 s group, N=25; forward 10 s group, N=23; forward 20 s group, N=23). The ITI was 2.5 min for the unpaired group and 5 min for the other groups. The odour preference of individual crickets was tested before and 2 h after training.

Experiment 5: effects of inter-trial intervals

To observe the effects of ITIs, five groups of crickets were subjected to two sets of P+/V– conditioning trials with ITIs of 30 s (group 1, N=39), 1 min (group 2, N=40), 2 min (group 3, N=39), 5 min (group 4, N=45) and 10 min (group 5, N=47). The odour preference of individual crickets was tested before and 2 h, 1 day and 4 days after training.

Experiment 6: effects of anaesthetic treatment with CO_2

To determine the effects of anaesthetic treatment, 12 groups of crickets were anaesthetized with CO_2 for 60 s at different times after two sets of P+/V– conditioning trials (N=21–27). The ITI was 2 min. One group (control group) received training but no anaesthetic treatment (N=38). Another group received anaesthetic treatment 60 min before training (N=17). After treating the crickets with CO_2 for 60 s, the air in the beaker was ventilated for 10 s. Crickets began to move their legs approximately 2 min after the cessation of CO_2 treatment and recovered normal locomotion within 7 min. The odour preference of individual crickets was tested before and 5 h after training.

Data analysis

The initial odour preference of a given cricket group was evaluated by comparing the time that an individual spent visiting the peppermint source (t_p) with the time spent visiting the vanilla source (t_v) using the Mann–Whitney U-test (M-W). To compare initial odour preferences in different cricket groups, the relative odour preference of individuals was measured using the peppermint preference index (PPI) (%), defined as $100t_p/(t_p+t_v)$; PPIs were compared using the Kruskal–Wallis test (K-W). The PPI was also used to evaluate the change in odour preference before and after training of a given cricket group: the preference for peppermint after training (PPI_{after}) was compared with that before (PPI_{before}) training using Wilcoxon's test (WCX). To compare the levels of learning performance in different tests, the learning performance of individuals was measured using the performance index (PI) (%), defined as PPI_{after}–PPI_{before}. Wilcoxon's test (WCX) was used to compare the levels of performance in different tests of a given cricket group, and the Mann–Whitney U-test (M-W) was used to compare those of different cricket groups.

Since initial preferences for peppermint did not differ significantly among groups of crickets used in any experiments (see Results), we also used PPI_{after} to compare the levels of performance of different groups of crickets. Because all statistical comparisons using PPI_{after} resulted in the same conclusions as those using PI, except for slight differences in the levels of significance, we confine our description to the results of comparisons using PI to avoid redundancy.

Results

Initial preference

In the initial preference test, crickets spent significantly more time on the vanilla source than they spent on the peppermint source (t_v=22.1±0.5 s, t_p=10.4±0.3 s, N=941, M-W test, P<0.0001; means ± S.E.M.), indicating that they had a significantly greater initial preference for vanilla over peppermint. No significantly different initial preferences were found among the three groups of crickets used in experiment 1 (Fig. 2B, K-W, P>0.5) nor among groups of crickets used in any of experiments 2–6 (K-W, P>0.5).

Experiment 1: effects of two-trial conditioning procedures

Three groups of crickets were subjected to two trials of P+/V– (group 1 in Fig. 2A), V– (group 2) or P+ (group 3) conditioning (for notation, see Materials and methods). At 2 h after training, all groups exhibited significant levels of conditioning: their preference for peppermint was significantly

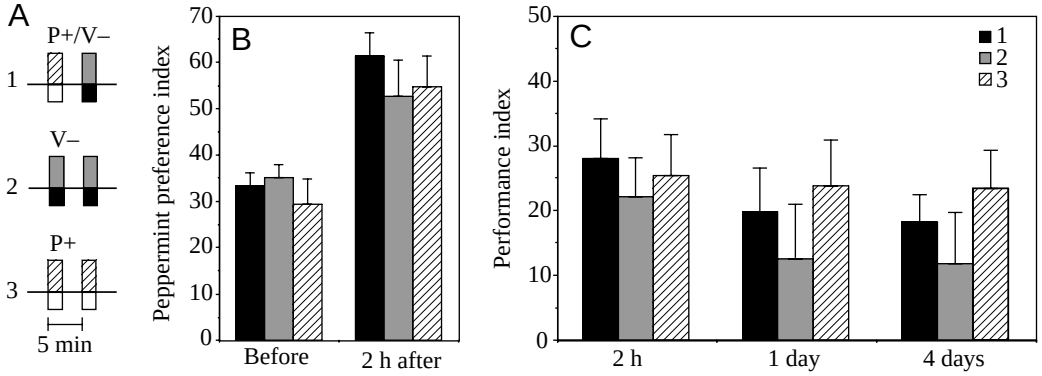

Fig. 2. Effects of two-trial conditioning. (A) Stimulus schedules (see Fig. 1C for key to shading). Groups 1, 2 and 3 were subjected to two trials of differential conditioning (P+/V–) and elementary aversive (V–) and appetitive (P+) conditioning, respectively. (B) The preferences (PPIs) for peppermint of groups 1 (N=29), 2 (N=15) and 3 (N=18) before training and 2 h after training. Values are means + S.E.M. (C) Performance index (PI) of groups 1, 2 and 3 measured 2 h, 1 day and 4 days after training. Values are means + S.E.M.

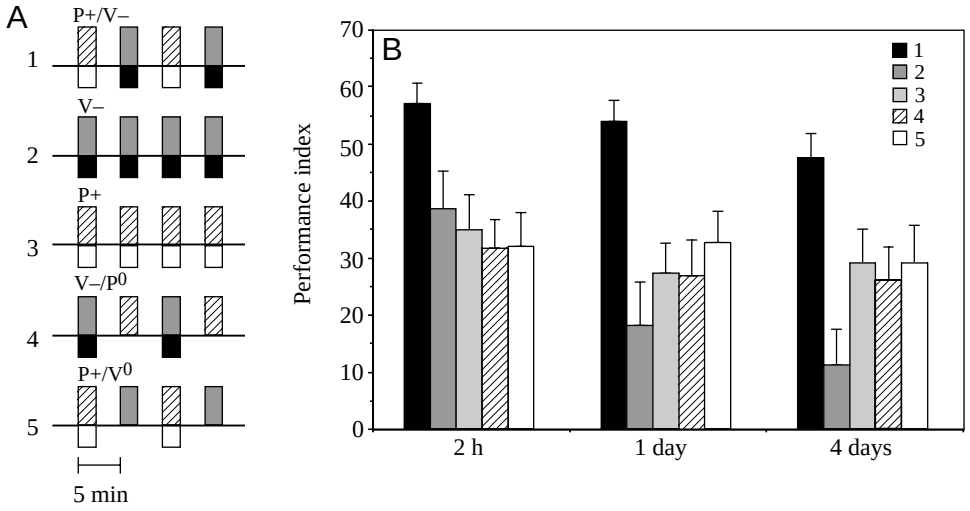

Fig. 3. Effects of four-trial conditioning. (A) Stimulus schedules (see Fig. 1C for key to shading). Groups 1, 4 and 5 were subjected to four differential conditioning peppermint-rewarded and vanilla-punished (P+/V–) trials, vanilla-punished and peppermint-unpunished (V–/P^0) trials, and peppermint-rewarded and vanilla-unrewarded (P+/V^0) trials, respectively. Groups 2 and 3 were subjected to four trials of aversive (V–) and appetitive (P+) conditioning, respectively. (B) Performance indices of groups 1–5 (*N*=45, 22, 26, 22 and 23, respectively) 2 h, 1 day and 4 days after training. Values are means + S.E.M.

greater than that before training (Fig. 2B) (WCX, $P<0.001$, groups 1 and 3; $P<0.01$, group 2). The level of performance (performance index, PI) 2 h after training did not differ significantly among these three groups (Fig. 2C) (M-W, $P>0.05$).

No significant decay in the level of performance from 2 h to 1 day or to 4 days was found in crickets that had received two-trial P+/V– or P+ conditioning (Fig. 2C) (WCX, $P>0.05$). However, no significant level of olfactory retention was found 1 day or 4 days after training in crickets that had received two trials of V– conditioning: their preference for peppermint did not differ significantly from that before training (WCX, $P>0.05$). This appears to reflect a natural decay of retention with time because we have shown previously that the extinction effect of the retention test is very small (Matsumoto and Mizunami, 2000). The results indicate that only two-pairing trials lead to robust retention with no significant decay from 2 h to 4 days after training in differential conditioning or in elementary appetitive conditioning.

Experiment 2: effects of four-trial conditioning procedures

Five groups of crickets were subjected to four trials of V– (group 2 in Fig. 3A), P+ (group 3), P+/V– (group 1), V–/P^0 (group 4) and P+/V^0 (group 5) conditioning. At 2 h after training, all groups exhibited significant levels of conditioning: their preference for peppermint were significantly greater than that before training (WCX, $P<0.001$). At 2 h after training, the level of performance of the P+/V– conditioning group (group 1, in Fig. 3B) was significantly greater than those of the other four groups (M-W, $P<0.05$ compared with group 2; $P<0.01$ compared with group 3; $P<0.001$ compared with groups 4 and 5); and the levels of performance of groups 2–5 were not significantly different (M-W, $P>0.05$).

The V– conditioning group exhibited significant decay in performance from 2 h to 4 days after training (WCX, $P<0.01$) and exhibited no significant olfactory retention 4 days after training: their preference for peppermint did not differ significantly from that before training (WCX, $P>0.05$). All other groups exhibited no significant decay of retention from 2 h to 4 days after training (WCX, $P>0.05$).

The results indicate that differential conditioning with peppermint-rewarded and vanilla-punished stimuli leads to the highest level of olfactory retention. The following experiments were performed using this conditioning procedure.

Experiment 3: effects of different numbers of differential conditioning trials

At 2 h after training, the performance of crickets that had

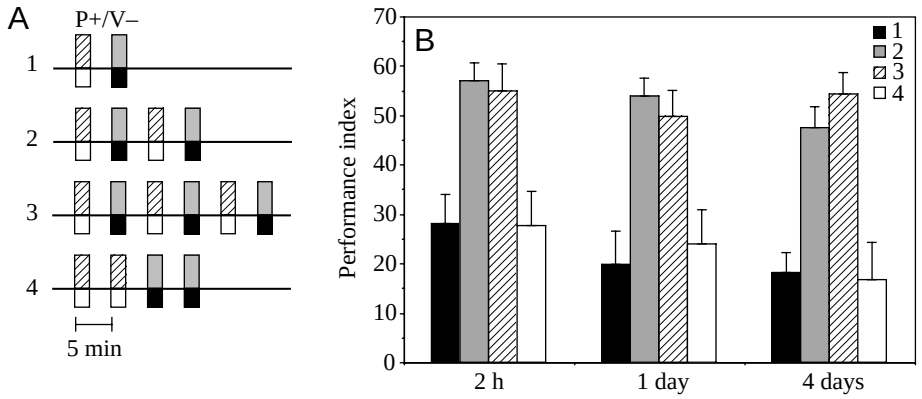

Fig. 4. Effects of different numbers of differential conditioning trials. (A) Stimulus schedules (see Fig. 1C for key to shading). Groups 1, 2 and 3 were subjected to one, two and three sets of P+/V– conditioning trials, respectively. Group 4 crickets were subjected to two elementary appetitive (P+) conditioning trials followed by two aversive (V–) conditioning trials. (B) Performance indices of groups 1–4 (*N*=29, 45, 24 and 22, respectively) 2 h, 1 day and 4 days after training. Values are means + S.E.M.

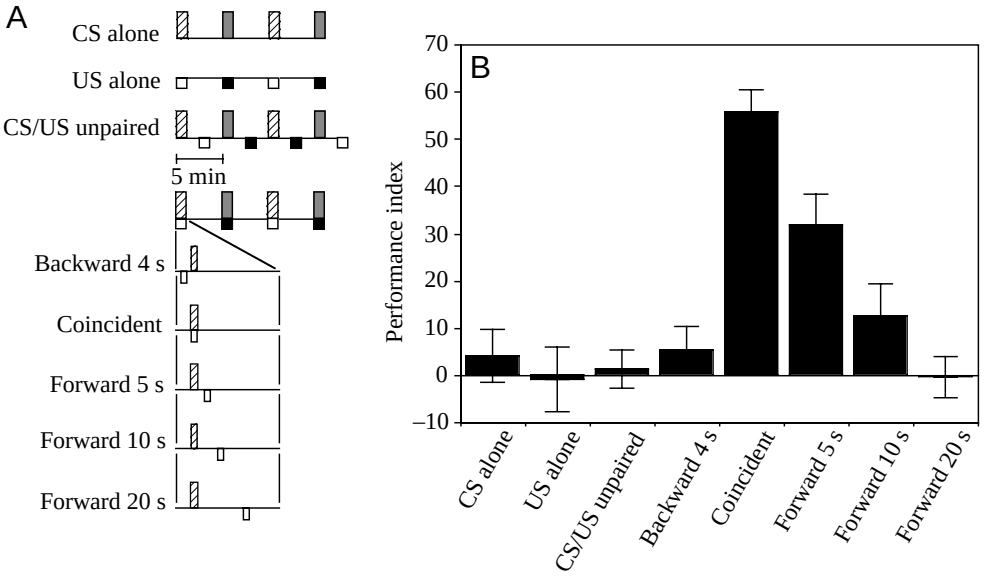

Fig. 5. Non-associative controls and the effects of inter-stimulus interval. (A) Stimulus schedules (see Fig. 1C for key to shading) for three non-associated control groups (CS alone, US alone and CS/US unpaired groups) and for five differential conditioning groups with different relationships between CS and US (backward, coincident and forward 5, 10 and 20 s groups). (B) Performance indices of eight groups at 2 h after training. Values are means + S.E.M. (*N*=26, 21, 27, 25, 25, 23 and 20 from left to right). CS, conditioned stimulus; US, unconditioned stimulus.

been given four-trial P+/V– conditioning (group 2 in Fig. 4) was significantly greater than that of crickets that had been given the two-trial conditioning (group 1) (M-W, $P<0.001$) but did not differ significantly from that of crickets in the six-trial group (group 3). The level of performance at 2 h after training (PI=56±4 %; mean ± S.E.M.) was among the highest observed in the present study (see Figs 2–7). These results indicate that four trials were sufficient to attain a saturated level of olfactory retention 2 h after training.

At 2 h after training, the performance of the group of crickets that had been subjected to two P+ and two V– conditioning trials in block sequence (group 4) was significantly lower (M-W, $P<0.001$) than that of the four-trial P+/V– conditioning group, which had been subjected to two P+ and two V– conditioning trials in alternating sequence (group 2), and did not differ significantly (M-W, $P>0.05$) from that of the two-trial P+/V– conditioning group, which had been subjected to only one P+ and one V– conditioning trial (group 1). This result indicates that the number of alternations of appetitive and aversive conditioning trials, in addition to the number of trials, is an important factor in determining the level of 2 h olfactory retention. In all groups, no significant decay of retention was found from 2 h to 4 days after training (WCX, $P>0.05$).

Experiment 4: non-associative control and the effects of inter-stimulus intervals

No significant change in odour preference was found in the three non-associative training groups, i.e. CS alone, US alone and CS/US unpaired groups (Fig. 5). The preferences (PIs) for peppermint at 2 h after training did not differ significantly from those before training in these groups (WCX, $P>0.05$). The backward 4 s group also exhibited no significant change in odour preference (WCX, $P>0.05$). The coincident and forward 5 s groups exhibited a significantly increased level of conditioning: the preference for peppermint after training was significantly greater than that before training in these two groups (WCX, $P<0.001$). The performance of the forward 5 s group was significantly lower than that of the coincident group (M-W, $P<0.01$). The forward 10 s and 20 s groups exhibited no significant level of conditioning (WCX, $P>0.05$). The CS therefore needs to be presented 0–5 s before the onset of US presentation to achieve conditioning.

Experiment 5: effects of inter-trial interval

At 2 h after training, a significant level of conditioning (WCX, $P<0.001$) was found in groups that had been given four-trial P+/V– conditioning with ITIs of 30 s (group 1), 1 min (group 2), 2 min (group 3), 5 min (group 4) and 10 min (group 5) (Fig. 6). The performance of the 5 min ITI group was significantly greater than that of the 30 s, 1 min or 10 min ITI groups (M-W, $P<0.001$ compared with group 1; $P<0.01$ compared with groups 2 and 5) and did not differ significantly from that of the 2 min ITI group (M-W, $P>0.05$). The level of performance 2 h after training was a non-monotonic function of the ITI: it was highest at an ITI of 2–5 min and was lower at shorter or longer ITIs.

The 1, 2, 5 and 10 min ITI groups exhibited no significant decay in performance from 2 h to 4 days after training (WCX, $P>0.05$). In contrast, the 30 s ITI group exhibited a significant decay of retention from 2 h to 4 days after training (WCX, $P<0.01$). This group exhibited a very low, but significant, level of 4 day olfactory retention (WCX, $P<0.05$).

Experiment 6: effects of anaesthetic treatment with CO_2

In tests performed 5 h after training, the performance of the group that had received CO_2 treatment 60 min before training was not significantly different from that of the control group that had received training but no CO_2 treatment (M-W, $P>0.05$) (Fig. 7). However, groups that had been given CO_2 treatment immediately or 1 min after training exhibited no significant level of conditioning: their preference for

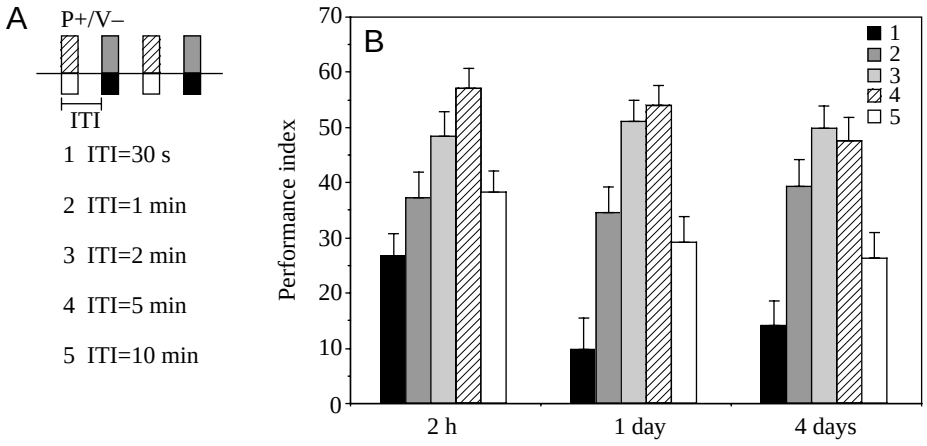

Fig. 6. Effects of inter-trial interval. (A) Stimulus schedules (see Fig. 1C for key to shading). Five groups of crickets were subjected to two sets of differential conditioning trials with inter-trial intervals (ITIs) of 30 s (group 1), 1 min (group 2), 2 min (group 3), 5 min (group 4) and 10 min (group 5). (B) Performance indices of groups 1–5 (*N*=39, 40, 39, 45 and 47, respectively) 2 h, 1 day and 4 days after training. Values are means + S.E.M.

peppermint did not differ significantly from that before training (WCX, $P>0.05$). Apparently, CO_2 treatment induced retrograde amnesia. With an increase in the interval between training and treatment, the effects of the anaesthetic treatment decreased, and anaesthetic-resistant memory developed fully 20 min after training. The level of performance of the 20 min interval group did not differ significantly from that of the control group (M-W, $P>0.05$). This result indicates that olfactory retention after differential conditioning can be divided into two phases, i.e. an early phase sensitive to anaesthetic treatment and a later anaesthetic-resistant phase.

Discussion

In our previous study, we used an operant conditioning paradigm and found that crickets learnt to associate odours with reward or punishment and retain olfactory memory for at least 7 days (Matsumoto and Mizunami, 2000). In the present study, we used classical conditioning and operant testing procedures to examine the conditioning variables that govern olfactory memory retention. During training, the crickets were placed in small beakers, and two odours were presented sequentially. The first odour was paired with the reward and the second with the punishment. The crickets were later placed in an arena and allowed to choose freely between the two odours presented simultaneously and without reinforcement. While there is no *a priori* reason for the crickets 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 *Drosophila melanogaster* (Tully and Quinn, 1985) and in honeybees (Gerber et al., 1996).

The differential conditioning procedure in which one odour was associated with a reward and the other with punishment led to the highest levels of 2 h, 1 day and 4 day olfactory

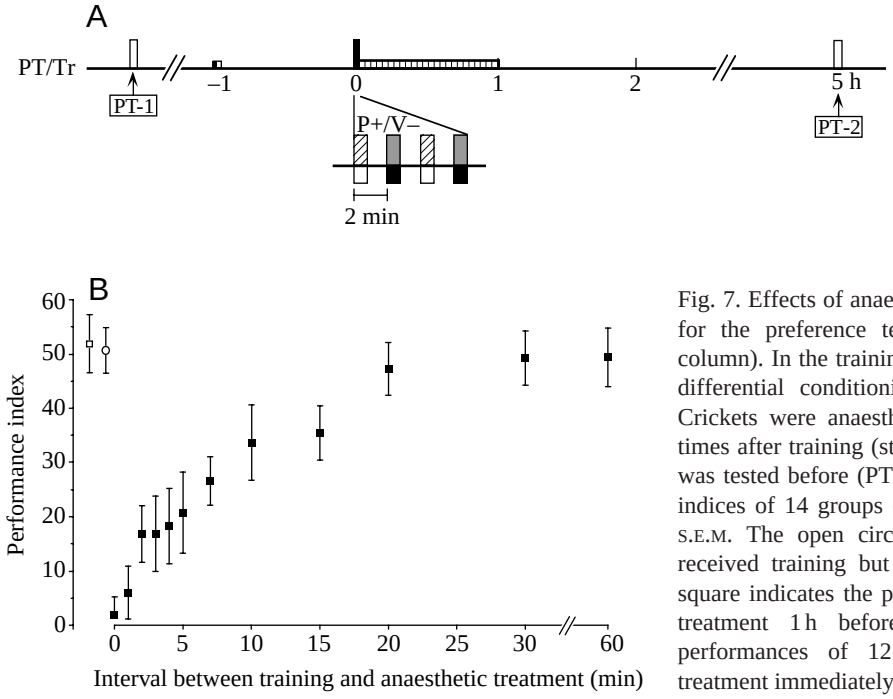

Fig. 7. Effects of anaesthetic treatment with CO_2. (A) The time schedule for the preference test (PT, open column) and training (Tr, filled column). In the training session, crickets were subjected to four trials of differential conditioning with an inter-trial interval (ITI) of 2 min. Crickets were anaesthetized with CO_2 for 60 s before and at various times after training (striped bar on the time schedule). Odour preference was tested before (PT-1) and 5 h after (PT-2) training. (B) Performance indices of 14 groups of crickets 5 h after training. Values are means ± S.E.M. The open circle indicates the performance of the group that received training but no anaesthetic treatment (*N*=38), and the open square indicates the performance of the group that received anaesthetic treatment 1 h before training (*N*=17). Filled squares show the performances of 12 groups (*N*=21–27) that received anaesthetic treatment immediately (0 min) or 1–60 min after training.

memory retention among the five types of conditioning procedures employed (Fig. 3). Notably, many previous studies on insect olfactory learning have been carried out using differential conditioning with one odour stimulus rewarded and a second unrewarded (honeybees, Mauelshagen, 1993; Hammer, 1993), with one odour stimulus punished and the other unpunished (fruit flies, Quinn and Dudai, 1976; Tully and Quinn, 1985) or using elementary appetitive conditioning (honeybees, Menzel, 1990, 1999; Gerber et al., 1998). The use of differential conditioning with one odour rewarded and the other punished is rare. There is one study using fruit flies to determine the effects of differential conditioning training in which one odour was associated with a sucrose reward and the other with an electric shock (Tempel et al., 1983), but olfactory retention was examined only up to 24 h, which represents middle-term rather than long-term memory (Tully et al., 1994).

Four-trial appetitive conditioning resulted in a robust memory with no significant decay from 2 h to 4 days after training, but aversive conditioning resulted in no significant 4 day retention of olfactory memory (Fig. 3). This suggests that different cellular processes occur after appetitive and aversive conditioning trials.

The presentation of appetitive and aversive conditioning procedures in alternating sequence led to higher levels of olfactory memory retention than when presented in block sequence (Fig. 4). Thus, the effect of the differential conditioning procedure in which appetitive and aversive conditioning procedures were presented alternately cannot be explained by simple addition of the effects of elementary appetitive and aversive conditioning procedures. An examination of the cellular processes underlying differential conditioning and a comparison with those underlying elementary conditioning are necessary to reveal the neural mechanisms underlying non-additive summation of the effects of appetitive and aversive conditioning procedures.

Crickets attained a saturated level of conditioning with only four trials of differential conditioning (Fig. 4). Moreover, they had an extremely robust memory with no significant decay from 2 h to 4 days after training with only two trials of differential conditioning or elementary appetitive conditioning (Fig. 2). This excellent olfactory learning capability of crickets is comparable, among insects, only with that of honeybees, in which appetitive conditioning trials performed three times establish a memory that is maintained with no decline for 7 days (Menzel, 1968). The evident ability of crickets to change odour preferences by learning and to retain the altered preferences for a long time may reflect the flexibility and persistency of their feeding behaviour; crickets are omnivores and select what is edible or inedible after testing various organic materials and, in addition, they feed persistently on similar food items from the time of emergence to adulthood, the latter behaviour being typical of hemimetabolous insects. Another example of hemimetabolous omnivores with a high olfactory learning ability is the cockroach *Periplaneta americana*, which can retain olfactory memory for at least 4 weeks (Sakura and Mizunami, 2001).

We showed that the CS/US interval is a sensitive variable for establishing CS/US association: significant conditioning was achieved only when the CS was presented immediately or 5 s prior to the onset of US presentation (Fig. 5). Similar results have been noted in other species of insects. Conditioning was achieved when the onset of the CS preceded the onset of the US by 1–5 s in the honeybee (Menzel, 1990) and by 1–3 s in the moth *Spodoptera littoralis* (Fan et al., 1997). The non-associative control and the backward conditioning groups exhibited no significant levels of conditioning, in accordance with findings in honeybees (Bitterman et al., 1983; Menzel, 1990), fruit flies (Tully et al., 1994) and moths (Fan et al., 1997). Forward pairing appears to be a property common among various systems of associative learning in invertebrates and vertebrates (Carew and Sahley, 1986; Rescorla, 1988).

The highest levels of conditioning 2 h, 1 day and 4 days after training were achieved with ITIs of 2–5 min, with reduced levels of conditioning with shorter (0.5–1 min) or longer (10 min) ITIs (Fig. 6). The finding in the present study that the shortest ITI (30 s) led to only a reduced level of 4 day olfactory memory retention is in agreement with findings in other species of insect. In fruit flies, spaced trials (with rest intervals of 5–15 min), but not massed trials (with no rest interval between pairing trials), established 4 day retention of olfactory memory that represents protein-synthesis-dependent long-term memory *via* the cyclic AMP cascade involving cyclic-AMP-responsive element-binding proteins (Tully et al., 1994). Similarly in honeybees, the level of 4 day olfactory memory retention in the massed training (ITI of 30 s) group was much lower than that in the spaced training (ITIs of 1–20 min) groups (Gerber et al., 1998; Menzel et al., 2001). The finding in the present study that the longest ITI (10 min) led to a reduced level of conditioning, however, differs from previous findings in other insects. In the moth *Spodoptera littoralis*, the level of 2 h olfactory memory retention after appetitive conditioning training with a sucrose reward increased monotonically with an increase in the ITI (Fan et al., 1997). In fruit flies, the level of 4 day olfactory memory retention after differential conditioning training, in which one odour was associated with an electric shock and the other was not, also increased monotonically with an increase in the ITI (Tully et al., 1994). In honeybees, the level of 1 day olfactory memory retention after an appetitive conditioning trial with a sucrose reward increased monotonically with an increase in the ITI, but that of 4 day memory retention depended non-monotonically on the ITI; i.e. the level of memory retention was lower with an ITI of 3 min than with an ITI of 1 or 20 min (Gerber et al., 1998). It remains to be determined whether these differences reflect species-specific features or differences in conditioning procedures.

The olfactory memory of crickets can be divided into two phases: an early short-term memory (STM) that is susceptible to anaesthetic treatment with CO_2 and a later anaesthetic-resistant memory (ARM) (Fig. 7). This is in agreement with findings in honeybees (Menzel et al., 1974; Erber, 1975; Erber et al., 1980) and fruit flies (Quinn and Dudai, 1976). The time

when the anaesthetic-resistant memory developed fully, however, differed among species: it was 20 min after training in crickets (present study), 5 min after training in honeybees (Erber, 1975; Erber et al., 1980) and 30–90 min after training in fruit flies (Quinn and Dudai, 1976; Tempel et al., 1983). In honeybees and fruit flies, a third memory component, i.e. protein-synthesis-dependent long-term memory, was evident (Tully et al., 1994; Wüstenberg et al., 1998; Menzel et al., 2001), and we recently detected this component in crickets: blockade of translation by cycloheximide had little effect on the level of olfactory conditioning 2 h after training, partly blocked 1 day olfactory memory retention and completely blocked 4 day memory retention (Y. Matsumoto and M. Mizunami, unpublished results). The performance of crickets 4 days after training is therefore a good measure of the level of long-term memory.

Our study demonstrates that crickets have a high capacity to form olfactory long-term memory and that a differential conditioning procedure is particularly effective for the formation of associative memory. Crickets have been used as models to study neural mechanisms of behaviour because electrophysiological studies of the activities of brain neurons during walking are possible (Huber, 1990; Böhm and Schildberger, 1992; Kohstall-Schnell and Gras, 1994; Staudacher and Schildberger, 1998). We have recently developed a method of recording the activities of brain neurons during olfactory conditioning training of unrestrained crickets (Y. Matsumoto and M. Mizunami, unpublished results) using chronic extracellular recording techniques originally applied to cockroaches (Mizunami et al., 1998; Okada et al., 1999). Neural correlates of olfactory memory processing in normally behaving crickets will be given attention in future studies.

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